



## USED NASTED-PCR DETECTION OF PHYTOPLASMA CAUSING BIG BUD DISEASE ON TOMATO IN IRAQ

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### ABSTRACT

Article information  
Article history:  
Received:20/7/2022  
Accepted:29/9/2022  
Available:31/12/2022

**Keywords:**

Big-bud, Iraq, Nested PCR, Phylogeny tree, Phytoplasma, Tomato.

DOI:

<https://10.33899/magrj.2022.134252.1179>

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Symptoms similar to those produced by the phytoplasma disease "Big bud" were observed on tomatoes (*Solanum lycopersicum* L.) grown in the field in Mosul city, Iraq. Diseased plants were characterized by twisting, corrugated, yellowing or reddening of leaves. The sepals of the flowers acquired hypertrophied form, were fused together and created a bell-shaped sterile bud (phylloidy) of green or anthocyanin color. The stems of the plants were lignified, and phloem necrosis was observed on the stem. In mid-September 2020, samples of 30 diseased and 2 healthy (control) tomato plants were collected from the fields. Phytoplasmas were detected by PCR and Nested-PCR in 10 diseased samples, use universal and specific primers. The results of a phylogenetic tree consisting of 27 genetic sequences of the 16 S ribosomal RNA gene of *Candidatus* Phytoplasma trifolii, locally isolated from Iraq/Mosul, showed a sequence identity with a high percentage of genetic similarity of 99% with *Candidatus* Phytoplasma trifolii 16Sr member VI-A. Clover proliferation group (16Sr VI), with various isolates from around the world.

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### INTRODUCTION

Tomato *Solanum lycopersicum* L., is one of most important crops of Solanaceae family, as its ranks first in global production, statistics of Food and Agriculture Organization (FAO) indicate that the global production of tomato crop has reached 373 million tons. America, China and Italy are among the most tomato producing countries (FAO, 2017). Nineveh Province is famous for the cultivation of tomato crop, as the percentage of the area planted with this crop reached (11.087) hectares, according to the statistics of the Central Agricultural Statistical Organization/Nineveh 2019. Tomatoes are infected with spectrum pathogens, fungi, bacteria, nematodes and viruses. Recently, phytoplasma diseases occupied a broad position among those causes, as they began to pose a broad threat to crops. (Hemmati *et al.*, 2021) Phytoplasma diseases were recorded with up to 600 diseases affecting many trees, ornamental plants, crops and vegetables (Moghaddam *et al.*, 2017). Tomatoes are infected with Stolubor disease caused by the pathogen *Candidatus* Phytoplasma solani (Sertkaya *et al.*,2007) This disease spreads in many warm regions, where it affects tomatoes, eggplant, peppers, potatoes, and many wild weeds (Sertkaya *et al.*,2007). Many phytoplasma diseases were also recorded on tomatoes globally, especially in the European countries bordering the Mediterranean, North Africa, Asia and the Near

East, and were also found in Ukraine and western Russia (Torres *et al.*, 2014). In some Arab countries, phytoplasma diseases have been recorded on many plants such as Jordan, Lebanon, Syria, Morocco and Iraq (Choueiri *et al.*, 2007; Anfoka *et al.*, 2003; Khalil *et al.*, 2019). Phytoplasma is transmitted by grafting, dodder and many leafhoppers such as *Macrostelei quadripunctulatus*, *Amacratagallia ribanti*. (Trivellone *et al.*, 2005).

In view of the absence of any study on the phytoplasma that specifically affects the tomato crop in Iraq, in addition to the observation of many symptoms suggestive of infection of phytoplasma, as well as the losses caused by these causes, we decided to conduct this research, which aims to identify the phytoplasma causing these symptoms on tomatoes using molecular detection methods.

## **MATERIALS AND METHODS**

### **Samples collection:**

Samples were collected from four fields planted with tomato the object of studies included two cultivars of tomato plant (*Lycopersicon esculentum* Mill.) important for the commercial production in Nineveh Province : (GC) and (Nora), as these fields were distributed in the areas of (Mahlabieh), (Namrud), (Balwat) and (Tal Zalat) during the stage of bud formation and the beginning of flowering from In 2021 (about 40 samples of tomato plants were collected, showing symptoms of phyllodey, as well as yellowing and symptoms of big bud , or what is known as the large bud (Fig. 1-a), The samples were placed in perforated transparent plastic bags, and an identification card was placed with them on which the place of collection, symptoms and variety were written, and they were left in the refrigerator for the purpose of conducting a molecular diagnosis on them later.

**Molecular diagnosis of Phytoplasma:** this diagnosis was conducted over many consecutive steps as follows:

### **Extracting DNA phytoplasma from the Tissue plant :**

The DNA was extracted from the Tissue plant samples containing the symptoms according to the methodology of FAVORGEN Biotechnology, COMPANY which is the supplier of the extraction set (FavorPrep Plant Genomic DNA Extraction Mini kit), using the Spin Column technique. Through which the negatively charged nuclear material is absorbed by the positive silica gel as a result of the charge difference and in the presence of salts that help adhesion and have  $P^H$  is more than 7, followed by the stage of washing the impurities by means of washing solutions, then the stage of obtaining the nuclear material by means of elution solutions containing ions with different charges and an  $P^H$  less than 7, causing repulsion and in this way 90-95% of the nuclear material is obtained. Impurities, which are inclusion bodies and other plant proteins, make up about 5-10%, as shown in (Fig.1-b). And then store the DNA on  $-18\text{ }^{\circ}\text{C}$  for later uses, the forward and reverse primers were designed by the American company (Integrated DNA Technology IDT ) for both ( table 1 ):



Figure (1): a, yellowing symptoms on tomato plant b, big flower bud

Table (1): Primers for amplification of the 16Sr DNA of Phytoplasma

Primer	Specificity 16Sr group	Primer sequence(5'-3')	Position	References
P1	Universal	5' AAGAATTTGATCCTGGCTCAGGATT 3'	16S rDNA	Dickinson Hodgetts & '2013
P7	Universal	5' CGTCCTTCATCGGCTCTT 3'	23S rDNA	Wei et al, 2008
R16F2n	VI-A	5' GAAACGACTGCTAAGACTGG 3'	16S rDNA	Lorenz et al, 1995
R16R2	VI-A	5' TGACGGGCGGTGTGTACAAACCCCG 3'	16S rDNA	Lorenz et al, 1995

**Prepare the PCR master mix :** The Mix Master one step was prepared by the company for the whole polymerization interaction by using the Korean equipment GeNetBio according to the required sizes of the interaction contents for each sample as shown in table (2) .

Table (2): Components of the master-mix for Nested-PCR.

Reagents	Volumes ( μl)
2x Master Mix ( the main solution )	10
PCR grade water ( distilled water free from Enzymes	5
Forward primer 20 μM	1
Reserve primer 20 μM	1
DNA Template ( DNA extracted from the Sample)	3
Total	20

**Amplification reaction:**

The Eppendorf ,PCR tubes are placed in Thermal Cycler , Bio –Rad, USA T100TM Thermocycler device, then the program specialized in PCR was used as

shown in table 3 , and then the tubes were removed from the device and placed in the refrigerator at 4-8 °C until electrophoresis to detect the results of the DNA amplification process .

Table (3): steps of the amplification reaction program.

Step	Temperature °C	Required duration	Number of cycles
Initial Denaturation DNA	95	10 minutes	1
Denaturation DNA	95	45 seconds	35 X
Annealing DNA	*	45 seconds	
Extension	72	1 minutes	
Final Extension	72	5	1
Cooling	4	∞	-

\* The optimum temperature of Annealing depend on primers(p1 = 64 °C/p7= 56 °C, R16F2n = 58°C / R16R=72°C)

### **Agarose gel electrophoresis:**

Agarose gel was prepared with 2% concentration by dissolving 1,2 gram of Agarose powder in 100 ml of Tris-Boric EDTA buffer ( TBE ) with a power 1X by using 250 ml glass flask , then the flask was shaken well to ensure that the solution was mixed with Tris –Boric EDTA ( TBE ) , after that the flask was placed in microwave for two minutes until boiling , then left to cool, and before hardening , 3 ul of Gel Red was added and mixed well , then it was poured into the mold of electrophoresis device which contains 14 holes . The gel was left to harden for 15- 20 minutes , then the comb was carefully pulled from the hardened Agarose making an empty and ready holes , then the Agarose gel was place in electrophoresis container , and 500 ml from TBE with a power 1X till the Agarose gel was completely submerged then 7ul from the results of DNA amplifying process that resulting from the serial polymerization interaction was placed in specific holes , while the indicator, 3ul of ( 300bp) DNA ladder was placed in the first hole existing in Agarose gel . The voltage of 80 volts and 300 mA was connected for a period of sixty minutes and after completing the electrophoresis process, the gel was extracted and placed in an imaging device Gel Doc EZ Gel Documentation System ( Bio Rad , USA ),which supplies ultraviolet rays to detect the amplifying results and then the results were saved to analyze them later .

### **Comparison of the sequences of the nucleotides (Contigs) with the global sequences strain of Phytoplasma:**

For the purpose of finding areas of similarity and difference between the sequences of nucleotides, was used the program BLAST (Basic Local Alignment Search Tool) (Yang, *et al.*, 2009). Which compares the sequences of nucleotides or proteins between the local Phytoplasma strain with all the global sequences preserved in the GenBank (NCBI).

### **phylogenetic analysis:**

Consensus sequences similar to those of the reference genomes were used in the BLAST program, in order to determine the percentage of similarity with the global phytoplasma sequences and also to determine the genetic relationship between them, the identical sequences were downloaded and comparison was made using program

(Clustal W) software included in the MEGA platform (10th edition) . It was run in order to build the genetic tree that is applied according to the method of neighbor-joining (Kumar *et al.*, 2016).

## RESULTS AND DISCUSSION

The studied plants showed symptoms of leaf curling with yellow or red discoloration, in addition to the enlargement of the flower calyx, the fusion of its bell-shaped petals, the sterility and foliage of flowers and the appearance of anthocyanic pigments on them, and these symptoms are typical of the disease of the large bud on the tomato (Fig. 1-a), After conducting a direct polymerase chain reaction (PCR) test on the DNA isolated from samples 7-10, a complete product with a molecular weight of 1.7 kb was obtained (Fig.2-a). While the presence of phytoplasma was not detected in samples 4,5 and 6, which were showing clear symptoms of infection, and the reason may be due to tomato plants containing non-phytoplasmic symptoms the samples 1,2 and 3 that did not show any symptoms of the disease. When using the specialized primers R16F2h/R16R2 in the Nested-PCR test, a product with a molecular weight of 1.2 kilobases (Fig.2-b) was obtained on the samples on which the normal PCR test was conducted. This result is consistent with the results of several studies that direct PCR using universal primers that amplify an inner portion of the phytoplasma genome with a size of ~1.8 kb of the 16 Sr DNA gene (Lee *et al.*, 1998; Choueiri *et al.*, 2007; Salehi *et al.*, 2014 ; Dilger *et al.*, 2020).

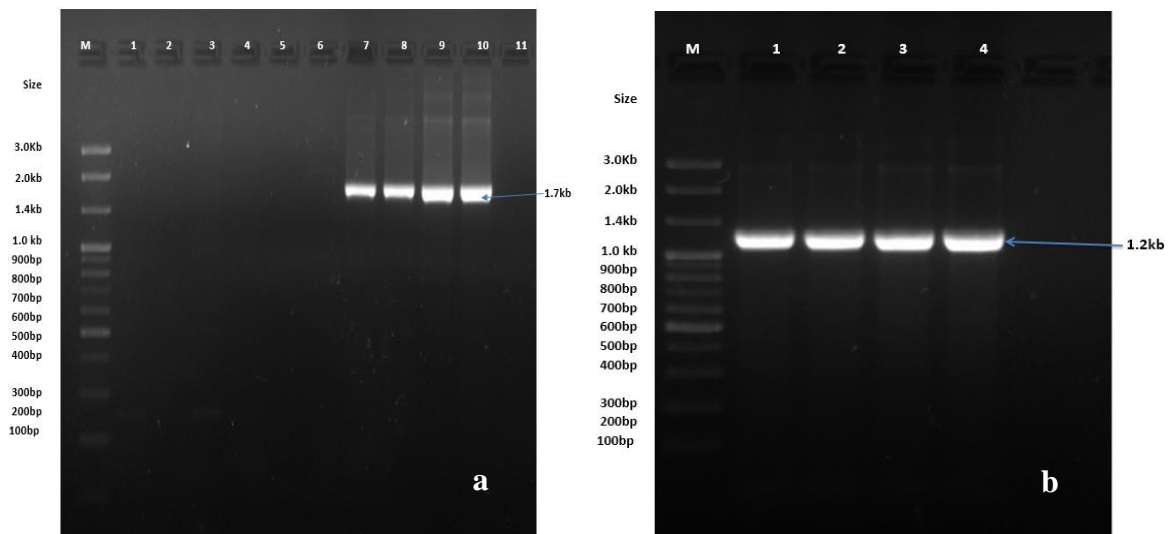


Figure (2):PCR amplification of the DNA from -infected and healthy samples using universal primers P1:16S rDNA /P7: 23S rDNA. Tracks 7-10 are samples of tomato plants with big bud symptoms, Hole 1- samples from healthy tomato plant (control), holes 2,3,4,5 and 6 It must be samples from infected plants. (a) . Nested- PCR amplification from product PCR using primersR16F2n/R16R2 (b), M= Molecular markers ladder of segments 3000-100 bp.

The results of a phylogenetic tree consisting of 27 genetic sequences of the 16 S ribosomal RNA gene of *Candidatus* Phytoplasma trifolii, locally isolated from Iraq/Mosul, showed a sequence identity with a high percentage of genetic similarity of 99% with *Candidatus* Phytoplasma trifolii 16Sr member VI-A. Clover proliferation group (16Sr VI), with various isolates from around the world.

Importantly, the genetic sequence of the conductor isolate previously recorded in GenBank under Accession number (ON204171) has a common genetic match of <98.9% similarity with *Phytoplasma* species (Columbia Basin potato purple top phytoplasma strain WA , Chile pepper phytoplasma strain Brote Gran, Iranian cabbage yellows phytoplasma , Cucumber phyllody phytoplasma strain Cuph, Willow proliferation phytoplasma isolate XS , 'Brassica juncea' phyllody phytoplasma clone OM2, 'Cucurbita pepo' phytoplasma clone Neyshabur , Behshahr(Iran) periwinkle phyllody phytoplasma , Willow proliferation phytoplasma isolate XS, Cucumber phyllody phytoplasma strain Cuph1). The local isolate had a high percentage of affinity with the Turkish isolate (MN224665.1) and the Iranian isolate (JF508516.1), with a genetic matching rate of 99.2%. Where isolates in similar regions tend to be more closely related (Fig.4). Thus, the detected phytoplasma could represent a new type of phytoplasma and to the best of our knowledge, this is the first report on such phytoplasma infecting tomato in Iraq. The absence of phytoplasma following other groups such as Stolubor 16Sr XII group, yellow ester 16SrI group and others in the studied samples may be attributed to the narrow and limited geographical area from which the samples were taken, as the aim was only to prove the presence of the pathogen in Mosul. According to our information, this is the first registration of the disease in Iraq.

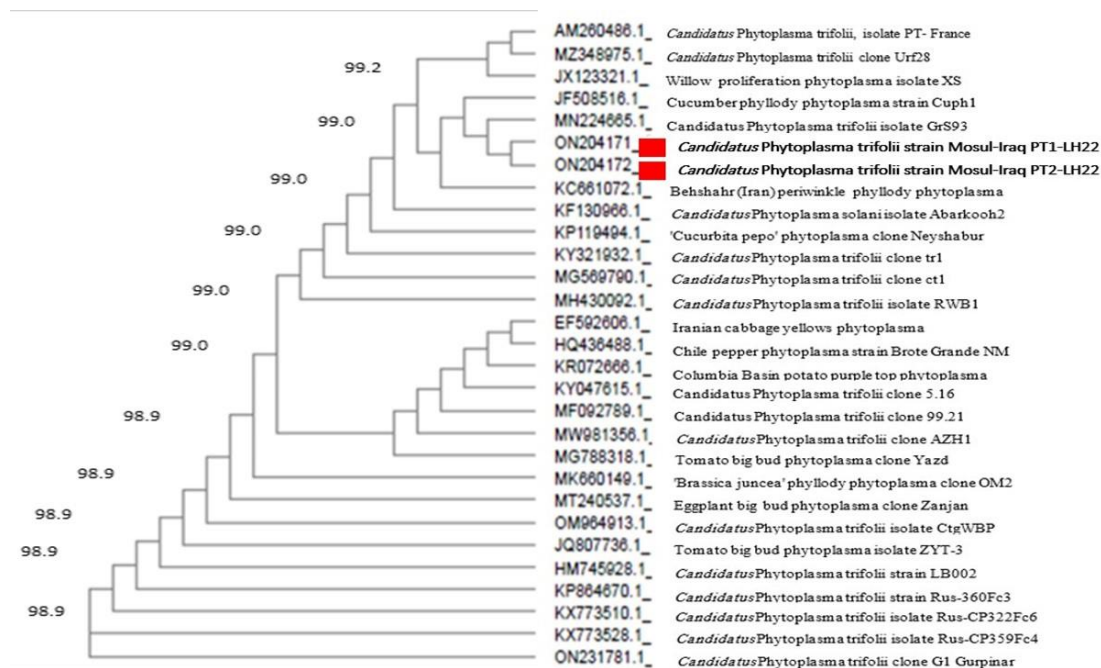


Figure (3): Phylogeny tree constructed by the neighbour-joining method, comparing the 16S rDNA sequence of the phytoplasma with 27 of other phytoplasmas, the red dots refer to strain of Mosul, Iraq

## CONCLUSION

The presence of phytoplasma in tomato samples containing symptoms of twirling, Phyllody and big bud. Phytoplasmas were detected by PCR and Nested-PCR in diseased samples, use universal and specific primers. The genetic sequence of the conductor isolate previously recorded in GenBank under accession number (ON204171). The results of a phylogenetic tree consisting of 27 genetic sequences of the 16 S ribosomal RNA gene of *Candidatus Phytoplasma trifolii*, locally isolated

from Iraq/Mosul, showed a sequence identity with a high percentage of genetic similarity of 99% with *Ca. Phyto. trifolii* 16Sr member VI-A. Clover proliferation group (16Sr VI), with various isolates from around the world. This research is the first record this type of phytoplasma in Mosul city and Iraq that infects the tomato crop.

#### ACKNOWLEDGMENT

This study was supported by the college of Agriculture and Forestry /Mosul University-Iraq.

#### CONFLICT OF INTEREST

Authors declare no conflict of interest regarding the publication of this study.

استخدام طريقة تضاعف البلمرة المتسلسل -المتداخل Nested-PCR للكشف عن الفايوتوبلازما المسببة  
لمرض تضخم البراعم على الطماطة في العراق

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#### الخلاصة

تمت ملاحظة اعراض شبيهة بالأعراض الفايوتوبلازمية على محصول الطماطة *Solanium lycopersicum* المزروعة في حقول مختارة من مدينة الموصل/العراق، في مرحلة تكوين البراعم وبداية التزهير وتمثلت تلك الاعراض بالتفاف وتقرم وتجعد الأوراق، تشوه البراعم الزهرية، تورق للأزهار، بالإضافة الى تضخم كأس الزهرة والتحام البتلات على شكل جرس، تم جمع بحدود 30 عينة ممثلة لتلك الاعراض وعينيتين سليميتين للمقارنة تم اثبات وجود الفايوتوبلازما خلال فحص PCR و Nested-PCR بعشرة عينات وباستخدام بادئات عامة ومتخصصة بأنواع P1/P7 الفايوتوبلازما تعود الى مجموعة تورق البرسيم (الجت) Clover proliferation وتحت المجموعة *Candidatus phytoplasma trifolii* (16Sr VI-A). وكشف التسلسل والتحليل الوراثي Phylogeny tree أن العزلة المحلية من مدينة الموصل لها هوية تسلسلية بنسبة > 99.2% مع *Candidatus Phytoplasma trifolii* 16Sr VI-A وهي عضو في مجموعة فرط النمو الورقي للبرسيم (16Sr VI). مع 27 تسلسل وراثي تابع لنفس المجموعة من حول العالم. ويعد هذا البحث التسجيل الاول في الموصل لمثل هذا النوع من الفايوتوبلازما التي تصيب الطماطة في العراق.

**الكلمات المفتاحية:** الشجرة الوراثية، الطماطة العراق، الفايوتوبلازما، تضاعف البلمرة المتسلسل - المتداخل، مرض تضخم البراعم

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