



# The First recorder of slow degrading nematode on citrus fruits caused by *Tylenchulus semi-penetrans*

Wurood J A Jabbar and Estabraq M Abedulridah  
Department of Plant Protection, College of Agriculture, University of Karbala, Iraq  
E-mail: [wurood.j@s.uokerbala.edu.iq](mailto:wurood.j@s.uokerbala.edu.iq)

## Abstract

Molecular diagnosis by polymerase chain reaction technology after morphological diagnosis of citrus trees samples in Holy Karbala confirmed that the trees are infected with this genus *Tylenchulus semipenetrans*, and this is the first molecular diagnosis of this genus at the country level

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## INTRODUCTION

Citrus growers abound in the tropics and subtropics between latitudes 40 north and south of the equator. Citrus fruits ranked third after grapes and apples in terms of quantitative production. Where oranges occupy the first place in the world among citrus species (Hosni, Muhammad, 2008,). Iraq's production of oranges in 2020 amounted to 142717 tons, with an average productivity of 22.4 kg per tree for the winter season. The highest production rate was in the governorates of Salah al-Din, Baghdad and Diyala, respectively (Annual Statistical Group, 2020)

Trees infested with citrus nematodes in high density are weak and small in size, yellowish leaves and fall off early, and the terminal

branches usually showing Die-Back symptoms . The roots appear lesioned in a dark brown color due to the adhesion of the soil granules to the gelatinous egg masses. The adhesion of the clay to the roots increases with increase of infections and egg masses, and washing with a slight stream of water may lead to the separation of root cortex from the vascular cylinder. The infection may be accompanied by some pathogenic fungi, and bacteria and fungi causing root rot. On stained infected roots, the nematode female appears clear with their posterior part prominent on the surface of the roots. Usually, symptoms of slow decline appear on the affected trees after 3-5 years post infection. The branches may become completely bare of leaves,



which greatly affects the quantity and size of the resulting fruits. (Mohamed, 2021). Because Karbala is also famous for its citrus cultivation in its various regions, and for the lack of studies on the slow decline citrus nematodes in Karbala, this survey was conducted to investigate the causes of citrus trees decline, assess the rate and severity of infection in the affected orchards, and to determine the causes and factors that involved in spread of infection

Citrus trees are affected by viral, fungal, bacterial, and parasitic plant nematodes, including slow decline disease on citrus caused by the citrus nematode *Tylenchulus semipenetrans* Cobb, 1993. Citrus nematodes are one of the most important types of nematodes specialized on citrus, infecting more than 80 species and varieties of citrus. Yet, no any citrus cultivar reported to be immune or nob host for the citrus nematodes. However, the ability of different citrus cultivars varies in the extent of their susceptibility to infection and the degree ranging from a very susceptible, moderate host to a low susceptible (resistant) (Al-Hazmi, 2017). This nematode infects 19 species and 21 hybrids of citrus in addition to persimmon, olive, grape and lilac. The slow decline on citrus was recorded in Iraq in 1965. In the last two decades, the slow decline was widely spread in Iraq's orchards and nurseries by more than 95% and the severity of infection reached 250,000 juveniles / kg of soil, with a root size reduction by 80% compared to healthy roots. Al-Hakim, 2013). The slow decline of citrus fruits in Iraq was first recorded by Natour et al. (1965) and during the years 1966-1969, the results of the survey conducted by the Abu Ghraib Department of Plant Diseases showed that these nematodes are widespread on citrus in all regions of Iraq.

## **MATERIAL OF THE METHODIES**

### **1-Preparation of Nematode vaccine slow dia-back on citrus *Tylenchulus semi-penetrans* used in experiments.**

The method of Hussey and Bakker (1973) to extract nematode inoculum was followed by slow decline. Capillary roots infected with citrus nematodes were brought from one of the orchards in Al-Hussainiya / Karbala district after noticing the symptoms of infection on the vegetative system with death and stiffness of the limbs. The samples were transferred to the laboratory, the affected roots were washed with a light stream of water to remove the suspended dust, and they were cut with sterile scissors into small pieces 2-3 cm and placed in a glass beaker of 1000 ml of 1% sodium hypochlorite solution. It was placed in the electric mixer for 30 seconds, then the solution was passed through a series of sieves of varying diameters (100, 150, 250, 400) mesh. The contents of the sieves were washed several times with a light stream of water to get rid of traces of sodium hypochlorite, where the first and second were to isolate roots and impurities, and the last sieve was to collect eggs. Second stage juveniles and after collecting the vaccine in a flask, 1 ml of the suspension was taken by a sterile pipette to the counting slide and the number of eggs was calculated using the compound light microscope under magnification and the same method was followed when preparing each vaccine in field experiments.

### **2-Morphological diagnosis of slow dia-back on citrus *Tylenchulus semi-penetrans***

The morphological diagnosis of the sexes was based on the physical characteristics of the adults, as the nematodes extracted either from the soil or the plant parts were examined by picking them up with a fine needle and transferring them to a clean glass slide containing a drop of glycerine after placing the slide cover. The Optika micro scope equipped with the Optika B5



Digital Camera and the OptikalSview version 3.9.0.605 driver has a magnification of 400 and 1000 times. The genera was diagnosed according to the taxonomic key developed by ( alhialy, 2021)

### 3 - Molecular diagnosis of nematodes slow degradation on citrus fruits

After 60 days of preparing the pure culture, paragraph 3, and upon completion of the slow decline nematode life cycle, samples were taken from the culture and subjected to molecular diagnosis to confirm or support the morphological diagnosis, and the diagnosis was made in the Scientific Progress Laboratory / Baghdad, according to the following work steps:

Isolation of DNA from slow-degrading nematodes

1. Grind 50 mg of wet weight .
2. Add 400 µl of FAPG1 Buffer and 8 µl of RNase A stock solution (50 mg/ml) and vortex vigorously. Incubate the mixture at room temperature for 2 minutes then at 65°C for 10~20 minutes and invert 2-3 times during incubation.
3. Add 130 µl of FAPG2 Buffer to the mixture. Vortex to mix well and incubate the mixture on ice for 5 min.
4. Place a Filter Column to a Collection Tube and transfer the entire mixture from previous step to the Filter Column. Centrifuge the Filter Column at full speed 14,000 rpm for 3 min.
5. Transfer the clarified lysate (supernatant) from the Collection Tube to a new microcentrifuge tube (not provided). Discard used **Filter Column and Collection Tube. And measure the volume of clarified lysate.**
6. Add 1.5 volume of FAPG3 Buffer (ethanol added) to the clarified lysate and mix well by pipetting. Make sure that ethanol (96~100%) has been added to FAPG3 Buffer when first use.
7. Place a FAPG Column to a new Collection Tube and transfer up to 750 µl of the sample mixture carefully to the FAPG Column. Centrifuge at full speed 14,000 rpm for 1 minute. Discard the flow-through and place the FAPG Column back to the Collection Tube. Repeat step 7 for the rest of the sample mixture.
8. Add 400 µl of W1 Buffer (ethanol added) to the FAPG Column. Centrifuge at full speed 14,000 rpm for 30 seconds. Discard the flow-through and place the FAPG Column back to the Collection Tube. Make sure that ethanol (96~100%) has been added into W1 Buffer when first use.
9. Add 650 µl of Wash Buffer (ethanol added) to FAPG Column. Centrifuge at full speed 14,000 rpm) for 30 seconds. Discard the flow-through and place the FAPG Column back to the Collection Tube. Make sure that ethanol (96~100%) has been added into Wash Buffer when first use. Repeat step 9 for one more washing.
10. Centrifuge at full speed 14,000 rpm for an additional 3 minutes to dry the FAPG column completely.-Important step! This step will avoid the residual liquid to inhibit subsequent enzymatic reactions.
11. Combine the FAPG Column with a Elution Tube, Add 100 µl of preheated Elution Buffer to the membrane center of FAPG Column. Stand the FAPG Column for 1 minute at room temperature. Important step! For effective elution, make sure that the Elution Buffer is dispensed onto the membrane center and is absorbed completely.
12. Centrifuge at full speed 14,000 rpm for 1 minute to elute purified DNA.



### Polymerase Chain Reactions

The polymerase chain reaction (PCR) was carried out with a reaction mixture consisting of 2 µl of the extracted DNA with 1 µl each of the forward and reverse primer,

5 µl of Pri mix, in addition to 16 µl of free water, with a total volume of 25 microliters and using a reaction tube of 50 microliters, then amplification using a Thermocycler type Mini - MultiGene produced by Labnet according .

**Table (1): The sequence of primers that used this study.**

Primer	Sequence	Primer sequence	Tm (°C)	GC%	Size of Product (bp)
ITS	F	5'- TCCGTAGGTGAACCTGCGG -3'	60.3	50 %	550-600
	R	5' TCCTCCGCTTATTGATATGC-3'	57.8	41 %	

\*Distilled water was added to each starter separately and according to the instructions of the producing company to obtain a concentration of 100 picomoles / ml (the base solution), then the primers were

diluted to obtain a concentration of 10 picomoles / ml by adding 10 microliters of the base solution to 90 microliters of sterile distilled water and kept in 20-PM until use.

Table (2) Optimal conditions for PCR for primers used in the diagnosis of nematodes  
 5 µl of the PCR product was migrated on a 1.5% agarose gel for 1.5 hours under a voltage of 5 V/cm using Electrophoresis DNA Gel device.

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	5 min	1 cycle
2-	Denaturation -2	95°C	sec45	35 cycle
3-	Annealing	52°C	1 min	
4-	Extension-1	72°C	1 min	
5-	Extension -2	72°C	5 min.	1 cycle

### Electrophoresis

Electrophoresis was performed to detect the nucleic acid amplification product by polymerase chain reaction as follows:

Acarose gel numbers (determination of DNA concentration)

Gel electrophoresis for DNA quality analysis-:

- 1- An agarose solution was prepared by dissolve 1g of agarose powder in 100

ml of 1x TBE in the (100) ml flask, agarose was melted in hot block until the solution became clear.

- 2- The agarose solution was made cool to about (50- 55°C), swirling the flask occasionally to cool evenly.
- 3- Red stain (3 µl) was added to the warm gel then sealed the ends of the casting tray with two layer of tape.
- 4- The combs were placed in the gel-casting tray.



- 5- Melted agarose solution was poured into the casting tray.
- 6- The agarose was allowed to solidify at room temperature, the comb pulled out carefully and the tape was removed. The gel was placed onto the electrophoresis chamber that was filled with TBE (1x) buffer.
- 7- DNA samples (5µl) were mixed with (3µl) DNA loading buffer and loaded in agarose gel wells.
- 8- The agarose gel electrophoresis was completed at 70V, 65Amp for 1hour. The DNA was observed by viewed under UVtrans illuminator.(Maniatis.et.1982)

#### **Determine the sequences of nitrogenous bases**

The sequences of the nitrogenous bases of the PCR results of the slow degradation nematode gene were determined using primers. Only the front strip of the initiator was sent to the Korean company Macrogen for the purpose of sequencing the DNA sequences.

#### **Analysis of the nitrogenous base sequence**



Figure(1):

Second: Molecular diagnosis of the slow-degrading nematode *Tylenchulus Spp* based on molecular techniques.

#### **data of the slow-deterioration nematode genome**

The nucleotide sequences obtained from the Korean company Macrogen from the National Center for Biotechnology Information (NCBI) were analyzed and an phylogenetic tree was drawn based on the nucleotide sequences of global isolates registered in the Gene-Bank

#### **Results and discussion**

First:

The results of the microscopic examination of the roots of citrus and orange plants contaminated with citrus nematodes showed the appearance of clear females and their posterior part prominent on the surface of the root with a lemon shape, as well as observing the egg masses on the root system and soil particles attached to gelatinous egg masses, which are among the clear symptoms at the beginning of infection with citrus nematodes, as well as the characteristics that Females are characterized by it, and this is one of the most important signs of infection and evidence of the disease (Al-Hazmi, 2009, Abu Gharbia et al., 2010).

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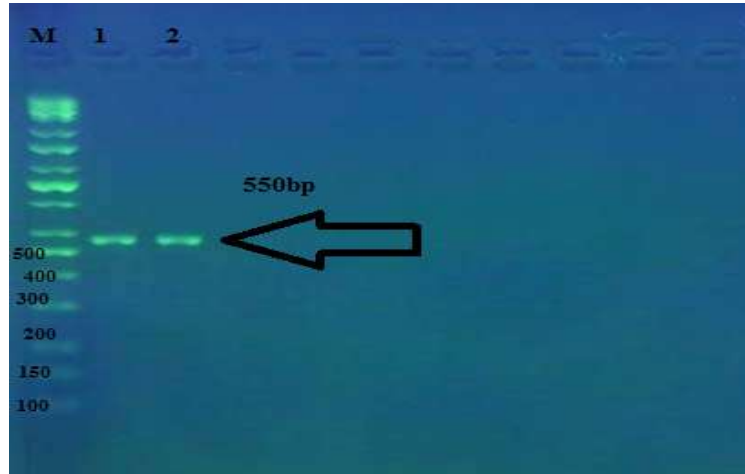


between 1,5.8S-18S genes of the type *Tylenchulus-semipenetrans* using the primer D2A\D3B. These results are in agreement with the results of the study conducted by Rashidifard Shokoohi, Hoseinipour, Jamali (2015), which indicated the efficacy of the D2A/D3B initiator in diagnosing slow deterioration of the species level nematode. The results show the emergence of a bundle

of size (550 bp) nitrogen base.

Figure (2): shows the results of the appearance of a bundle of size (bp) 550 nitrogen base

Analysis of the nucleotide sequencing results of the deoxyribonucleic acid of the slow-deterioration nematode genome.



Table(3):

ترقيم المختبر	ترقيم الطالب	Dna extraction	PCR Prod
1	<i>Tylenchulus semipenetrans</i>	+	+

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The results of the nucleotide sequences from the Korean company Macrogen showed that they matched the sequences of global isolates of slow-deteriorating nematodes spp. *Tylenchulus* and registered in the database of the National Center for Biotechnology Information (NCBI) and determination of the type of nematode through the official website of the World Organization <https://blast.ncbi.nlm.nih.gov/Blast.cgi> The results indicated the presence of the type - *Tylenchulus semipenetrans* and appeared in

a percentage of 99% match with the sequences of the Chinese isolate ON140588.1 and the Turkish isolate by the presence of some variations in the sequence of the nitrogenous bases of the Iraqi isolate KX461936.1 with the sequences of the nitrogenous base of the Chinese isolate, as shown in the figure) G rule. These variations shown in the figure apply to the rest of the global isolates corresponding to the Iraqi isolat



**Tylenchulus semipenetrans isolate HNYZ1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence**

Sequence ID: [KX461936.1](#) Length: 804 Number of Matches: 1

Range 1: 71 to 770 [GenBankGraphics](#) [Next Match](#) [Previous Match](#)

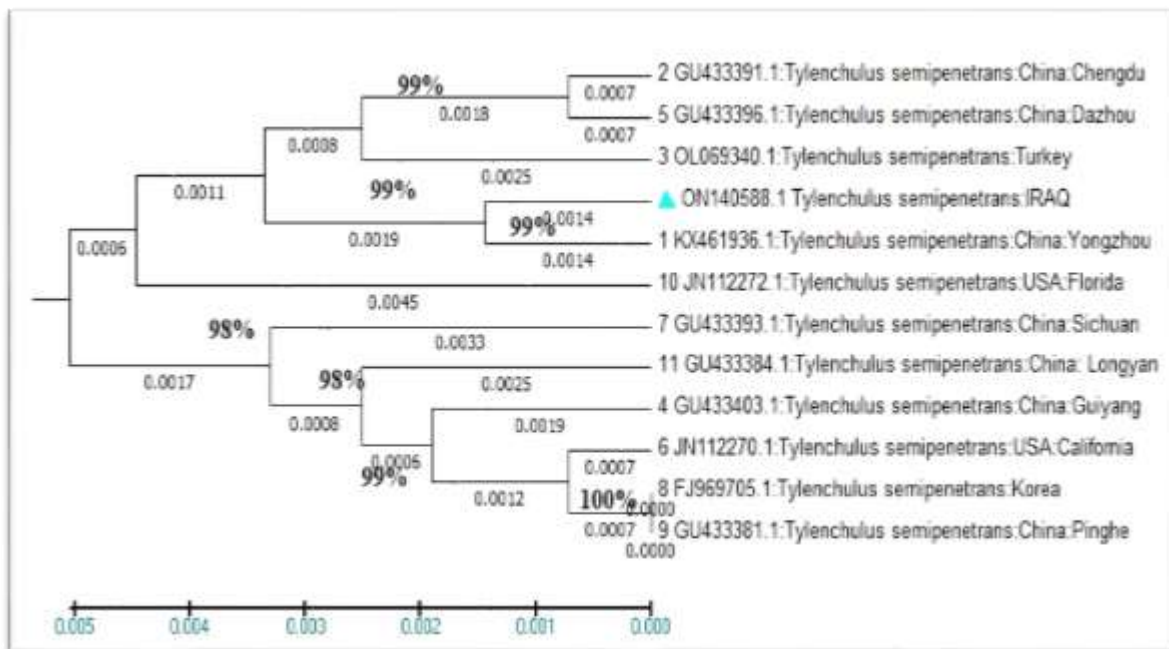
Score	Expect	Identities	Gaps	Strand
1254 bits(1390)	0.0	698/700(99%)	0/700(0%)	Plus/Plus
Query 1	AAGCTTCTACCAGGTTGAGCAGAGTCCTTGACTGCCGGCACATCGGGTGGAGAGGACAAG	60		
Sbjct 71	.....	130		
Query 61	CCAGTTTGTGGGAGCTGTGGCTGCTTCTGGCACTGGGAGTCTGTGGTCATACTTCCT	120		
Sbjct 131	.....	190		
Query 121	CTGCCGCTGAGGAAGGGATACTGAGCTTTTGTCCCTCTCGGCTCTTACCTGGCCTCGACT	180		
Sbjct 191	.....	250		
Query 181	ACAGGCGAAACCGGCTGTGCTGGATCCTATGTACGCTGAGCGACTGTTGAATAAAGTC	240		
Sbjct 251	.....A.....	310		
Query 241	CGTGGTCTGCAATGAGGTGTTACGATAGCCTTCTCCACATGTCGCTGTGGAGGGTAGGA	300		
Sbjct 311	.....	370		
Query 301	TTAATGAGTCCAGATTCGGTCCGSCCAGCAGATCCTTTCTTTTCACtttttttttCTG	360		
Sbjct 371	.....A.....	430		
Query 361	AAAACAAAAATACTAGTCTTGGCGGTGGATCACTTGGCTCGTAGGTCGATAAAGATCG	420		
Sbjct 431	.....	490		
Query 421	CAGCCAAACGCGATAGTTGGTGCGAAGTGCAGATATTCTGAGCACTAAAGTATCGAATGC	480		
Sbjct 491	.....	550		

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Phylogenetic tree was drawn using Mega6 program for the Iraqi isolate KX461936.1 of the species *Tylenchulus semipenetrans* to clarify the relationship between it and the global isolates in the World Bank for Biotechnology NCBI based on the genetic region 18s rRNA present in all nematodes belonging to the genus *Tylenchulus semipenetrans* and all the simple sequences

of nematodes except Figure () Diagram of the genetic tree between the Iraqi isolate and the global isolates, and these results are consistent with the results of the study conducted by Rashidifard, Shokoohi, Hoseinipour, Jamali (2015), which indicated the efficiency of the D2A/D3B initiator in diagnosing slow deterioration of the species level nematoda.



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Figure (4) phylogenetic tree that shows the kinship relationship between the sequences of the Iraqi isolate and the global isolates is based on the nucleotide sequences registered in the NCBI World Bank according to the Mega6 program..

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