

New horizons in the Biodiversity of *Ralstonia solanacearum* Associated with Tomato through Biochemical and Molecular Analysis

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Abstract

Current study aimed to detect the spread of new strains of bacterial wilt disease on tomato in Iraq particularly some areas of Najaf province for the agricultural season 2024. Results of the pathogenicity test of *Ralstonia solanacearum* on tomato for the 22 studied isolates showed that the two isolates, *R. solanacearum* 1 and *R. solanacearum* 4, outperformed the other isolates in the severity of the disease and reached 46.67% and 42.74% respectively. Based on the diagnostic keys, the results confirmed that the bacterial isolates belong to *R. solanacearum*. Results of microscopic and morphological examination indicated that the bacteria were Gram-negative, rod-shaped, and had the appearance of white colonies with red centers on the (TZC) medium, while in the SPA medium, transparent colonies appeared. Biochemical tests also indicated that *R. solanacearum* gave positive results for some tests, including Catalase test, Oxidase test, and Pectolytic test. The tests that gave negative results included KOH, Livan, Hydrolysis of Gelatin, Arginine, Starch Hydrolysis, Aesculin Hydrolysis, and Indol test. Biovar test results showed that it was from Race 3, which infects the Solanaceae family, including tomatoes. The molecular diagnosis, which was based on the 16 SrRNA gene, showed that new strains were obtained for the first time and were registered in (NCBI) with the accession numbers (PP473560 and PP473561).

Keywords: *Ralstonia solanacearum*, tomato, biochemical tests, molecular diagnosis.



Introduction

Tomato (*Solanum lycopersicum*) is an important vegetable crop grown worldwide and belongs to the Solanaceae family, it contains many antioxidants, minerals, fats, proteins, carbohydrates, and some vitamins (8).

Tomato plants are affected by many bacterial diseases that lead to low crop production, among these diseases is bacterial wilt caused by *Ralstonia solanacearum*, which is considered one of the important pathogens that cause severe wilting of this important crop. Due to its scientific and economic importance, *R. solanacearum* is ranked second among the ten most important bacterial pathogens that infect plants worldwide (18).

Symptoms appear as a general wilting of the plant while it remains green, symptoms on stem are the appearance of light-colored lines, tissues turn brown and curl, and the leaves decompose and contain white secretions (13).

R. solanacearum is a pathogen that possesses a set of virulence factors that make it a subject of interest to researchers due to the damage it causes to plants. The virulence factors of *R. solanacearum* consist of a set of enzymes that degrade the plant cell wall (20 and 3).

The wilt-causing bacteria colonize the vascular bundles of the tomato plant, and then *R. solanacearum* releases the virulence factor, which is polysaccharide (EPS), outside the cell, leading to an

increase in the viscosity of the xylem fluid and blockage of the xylem vessels, it also causes obstruction in water transport, which leads to the plant surrendering to bacterial wilt. Controlling this bacterium is very complex due to its complex mechanism in causing plant diseases, its persistence in the soil, the nature of internal growth, dispersal through water, and its association with some weeds (13, 14).

When the pathogen enters the plant tissues, it faces unfavorable environmental conditions in addition to the defensive reactions produced by the plant, especially when the number of bacteria is low. Despite this, bacteria possess many enzymes that work to analyze the plant's defense compounds (30).

The family range of bacteria consists of 5 species and includes more than 200 species of tropical and subtropical plants worldwide (Species 1 (Race 1), it has a broad spectrum, as it infects plants of the Solanaceae family and other families, but the rest of the species each have a limited host range (9).



Materials and methods

Isolation of *R. solanacearum*

bacteria causing bacterial wilt of tomato

The pathogen of bacterial wilt on tomatoes in some areas of Najaf Ashraf (Al-Haidariya, Al-Abbasiya, Al-Qazwiniya, and Al-Mahajar) was surveyed, the infected stems were collected randomly and placed in bags with the data of the collection area and the variety written on them. Then the samples were transferred to the laboratory for isolation and diagnosis of the pathogen after it was isolated from infected tomato stems. The samples were washed with water to remove dirt and impurities, then the stems were cut into small pieces of 1 cm in size. Then, it was superficially sterilized with a 3% sodium hypochlorite solution from the commercial preparation for two minutes, washed with sterile water to remove the sterilizing solution and left on Whatman No. 1 filter paper to dry. The small pieces were transferred to Petri plates containing N.A. medium, and the plates were incubated at a temperature of $(28 + 2)$ (17). The infected part was cut off and its end was immersed in a glass cup containing water. After 5-10 minutes, bacterial oozing was observed coming out of the end of the cut part into the water, which indicates the presence of a bacterial infection. A portion of the resulting suspension was also taken using a knotted inoculation loop (after heat sterilization) and inoculated Petri plates containing NA culture medium using the planning method, plates were incubated at $28 + 2^{\circ}\text{C}$ for 48 hours (16, 23).

Pathogenicity test on tomato plants

Pathogenicity test was done to distinguish between pathogenic isolates, as a reliable method for distinguishing between biovars, of *R. solanacearum* the causative agent of tomato wilt disease by applying Koch's postulates to prove the responsibility of the pathogen for causing the disease. Tomato seeds of the Al-Ala variety were planted under controlled conditions, then the bacterial vaccine was prepared by inoculating the N.A medium with 22 bacterial isolates using the planning method, each one individually, plates were incubated at $28 + 2^{\circ}\text{C}$ for 48 hours. Bacterial growth was harvested using a glass diffuser by adding 5 ml of sterile physiological saline solution. A series of dilutions of 10^{-1} – 10^{-8} of the bacterial suspension of the isolated bacteria were prepared in test tubes containing sterile physiological saline. 1 ml of each of the last three dilutions was placed into Petri plates using a sterile pipette specific for each dilution (three sterile Petri plates). 20 ml of the culture medium was poured at 45°C into each plate then plates gently shaken to homogenize the bacterial suspension with the culture medium, plates were incubated at $28 + 2^{\circ}\text{C}$ for three days (12). The number of colony-forming units/ml (CFU/mL) of bacterial suspension was estimated according to Clark, (10), 15-day-old tomato plants were inoculated with the bacterial inoculum prepared at a $10^8 \times 1$ (CFU/ml) concentration for the purpose of testing the pathogenicity of the isolates under study. This was done by injecting the stems in several locations using a sterile medical syringe for the 22 isolates.



Four plants were inoculated for each isolate with the control treatment in which sterile water was used in the injection process and placed in the greenhouse. Results were recorded 21 days after the inoculation process (19). The disease was calculated based on the severity of the infection according to the pathological index (26) as follows: 1 = No symptoms on the leaves 2 = One wilted leaf 3 = Two or three wilted leaves 4 = Four or more wilted leaves 5 = Entire plant wilted. The percentage of infection severity was calculated according to the following equation:

$$\text{Disease severity\%} =$$

$$\frac{\text{Total (number of infected plants per degree} \times \text{degree number)}}{\text{Total number of plants} \times \text{highest degree}}$$

$$\times 100$$

Anatomical variations of tomato plants infected with bacterial wilt

A necropsy of infected tomato plants with bacterial wilt was done, and the hand-cutting method was used to prepare the slices, according to what was stated in (1). A stem was taken from the healthy plant and a stem from infected plant with the bacteria *R. solanacearum*, which is the most pathogenic on tomato plants, and they were preserved in flasks with FAA (Acid Acetic Formalin) solution which consists of 40% formalin, glacial acetic acid, and 70% ethanol alcohol. Tomato stems were cut into transverse sections and samples were placed directly into glass flasks. Data for each sample were recorded on each bottle. Transverse sections were prepared by removing fresh samples from

the flasks and removing traces of the solution by immersing them in hot water for 5 minutes. The samples were then washed twice with ethyl alcohol and xylene for another 5 minutes. They were then examined microscopically.

Microscopic and morphological diagnosis of *R. solanacearum*

A portion of the pure colonies growing on the selective medium Tetrazolium Chloride Agar (TZC) was transferred to a clean glass slide containing a drop of normal saline solution, mixed well and spread on the slide. It was stained with Gram stain and examined under a light microscope using an oil lens (X1000). The cells' reaction was observed with the shape of the cells and their clusters (7).

Nature of growth on solid nutrient medium Nutrient Agar (NA)

Nutrient Agar was inoculated with bacterial isolates, the plates were incubated at 28 + 2°C for 48 hours, growth was examined and phenotypic characteristics were recorded (25, 7).

The nature of growth on the selective medium (TZC)

Tetrazolium Chloride Agar (TZC) medium poured into sterile plates was inoculated with bacterial isolates and incubated at 28 + 2°C for 48 hours as mentioned (7).

Nature of growth on SPA medium

R. solanacearum was cultured on semi-selective culture medium (SPA) Sucrose Peptone Agar Sucrose 2% and Peptone (27).

Biochemical tests for *R. solanacearum*



The species was diagnosed through some biochemical tests, taking into consideration the study of the characteristics through several biochemical tests as follows: Catalase test, Hydrolysis of Arginine, KOH Solubility, Hydrolysis Starch, Aesculin, Hydrolysis Oxidase test, Hydrolysis Gelatin, Pectolytic, Levan (22), biovar testing was also performed based on the use of carbon in disaccharides and hexose alcohols and for the sugars lactose, cellobios and maltose (15, 5).

Molecular diagnosis of *R. solanacearum*

Molecular identification of the bacteria causing bacterial wilt disease on tomato was carried out, as *R. Solanacearum* isolate 1 and *R. Solanacearum* isolate 4 from Al-Haidariyah area were selected and isolated from the stems, which were more pathogenic than the rest of the studied isolates on tomato plants, which were diagnosed based on the 16S rRNA gene using the Favour prep TM Genomic DNA Minikit kit prepared by Interon Company, originating from Korea (29). Using the forward primer '3-AGAGTTTGATCCTGGCTCA -5' and the reverse primer '3-GGTTACCTTGTTACGACTT -5'. The amplified gene products were subjected to electrophoresis using agarose gel at a voltage of 7 V for 2 hours. Agarose gel was prepared by dissolving 1.5 g of agarose in 100 ml of TBE buffer. Then, they were photographed under ultraviolet

light, and the amplified products of the 16S rRNA gene, along with the forward and reverse primers, were sent to the Korean company Macrogen for the purpose of analyzing the nitrogenous base sequences of the duplicated nucleic acids from the bacterial isolates.

To identify the isolated bacteria, the DNA sequence of each isolate was put into the US National Center for Biotechnology Information data base (DNA Sequence). The Alignment Search Tool (BLAST Basic Local) website was used to compare the sequence results with the standard sequences available on the NCBI website.

Results and Discussion

Isolation of *R. solanacearum*

used in the study

The pathogenic bacteria causing bacterial wilt of tomato plants were isolated from infected stems from some areas of Najaf province, where the highest percentage of



infection was recorded in Al-Haidariyah and Al-Qazwiniyah, reaching 37.4% and 25.2% respectively, as shown in Figure (1). The reason for this is that the pathogen *R. solanacearum* spreads more under conditions of high humidity and high temperatures. It was discovered that in the summer, bacterial wilt cases occur, with the infection rate reaching 10-100% in greenhouses (28). High soil moisture has an effect on the survival and reproduction of the pathogen in many plant crops, and the infection rate as the wilt rate is lower in dry soil conditions (31 and 6).

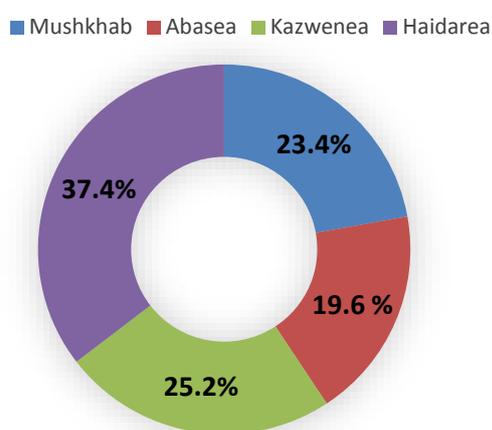


Figure 1. Shows the percentage of infection in some fields of Najaf province.

Pathogenicity test for *R. solanacearum*

The pathogenicity was tested on 22 isolates from the infected plant stems. Results of Table (1) showed that 12 isolates were the most severe isolates in causing wilt disease on tomato plants of the Al-Ala variety. While in the rest of the isolates, the bacterial infection appeared on

the stems by injecting them with bacterial suspension 108 (CFU/ml). Symptoms began to appear on the plant 21 days after the injection as general wilting of the plant, the appearance of lines on the stems, and their brown discoloration. The infection progresses plant completely wilts and the inside of the stem becomes empty, as shown in Figure (2). Results obtained from the pathogenicity test of *R. Solanacearum* on tomato plants showed that the two isolates 1 and 4 were more pathogenic than the other isolates, which reached 46.67% and 42.74%, while the isolate 21 was less pathogenic, reaching 26.87%, whereas, other isolates had a graded disease severity compared to the control treatment to which only water was added. Bacterial wilt disease begins with the entry of bacteria through wounds or natural openings in plant roots. These bacteria then flourish in the xylem vessels and produce extracellular polysaccharides that hinder water supplies and cause the plant to wilt. They are also transmitted through the soil and have a high ability to survive in soil, water and weeds (32) as in the anatomical Figure (2). This is due to the ability of bacteria to infect plants and their susceptibility to toxicity, invasion and transmission as a result of the encoding of pathogens through genes carried on chromosomes and plasmids (21 and 2). The genome of *R. solanacearum* contains 20 cell membrane-bound receptors called chemotaxis proteins. The pathogen then moves towards plant roots through the flagellum, as the flagellum and pilus are two essential factors for plant colonization and disease development (11 and 3).

Table 1. The pathogenicity of *R. solanacearum* isolates on tomato plants (Al-Ala variety) 21 days after inoculation

Isolate No.	Disease severity %	Isolate No.	Disease severity %
Control	0.00	<i>R. solanacearum</i> 12	0.00
<i>R. solanacearum</i> 1	46.67	<i>R. solanacearum</i> 13	36.21
<i>R. solanacearum</i> 2	0.00	<i>R. solanacearum</i> 14	29.65
<i>R. solanacearum</i> 3	0.00	<i>R. solanacearum</i> 15	0.00
<i>R. solanacearum</i> 4	42.74	<i>R. solanacearum</i> 16	30.87
<i>R. solanacearum</i> 5	35.81	<i>R. solanacearum</i> 17	28.54
<i>R. solanacearum</i> 6	30.44	<i>R. solanacearum</i> 18	0.00
<i>R. solanacearum</i> 7	40.45	<i>R. solanacearum</i> 19	37.12
<i>R. solanacearum</i> 8	0.00	<i>R. solanacearum</i> 20	0.00
<i>R. solanacearum</i> 9	37.62	<i>R. solanacearum</i> 21	26.87
<i>R. solanacearum</i> 10	0.00	<i>R. solanacearum</i> 22	0.00
<i>R. solanacearum</i> 11	0.00		
L.S.D	1.36		

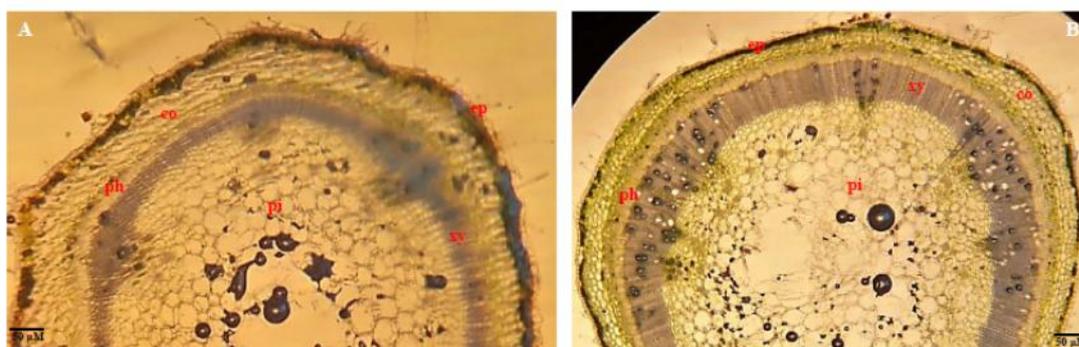
Microscopic anatomical variations in tomato plant tissue infected with bacteria and healthy tissue

The results of anatomical and microscopic changes in tomato plant tissue, as shown in Table (2) and Figure (2), showed that the percentage of change in the shape and size of the tissues infected with the pathogen *R. Solanacearum* was clear compared to the healthy tissue, as the thickness of the epidermis and cortex in the infected tissue reached 8.27 μM and 22.47 μM . In healthy tissue, the volume reached 10.32 μM and 24.38 μM . The effect was clear in the decrease in the volume of xylem and bark tissue to 11.61 μM and 14.58 μM

compared to their normal volume in healthy tissue, which reached 23.64 μM and 47.85. In addition to the small size of the pulp, which reached 264.51 μM in the affected tissue, while its volume in healthy tissue was 352.71 μM . This is because *R. solanacearum* colonizes xylem vessels with few nutrients, where the tracheal elements are dead and where the osmotic pressure is low, which leads to easy penetration by pathogens and their accumulation in large quantities in the plant wood tissues, increasing the blockage of the vessels. This prevents the plant sap from reaching the leaves, causing them to wilt. As the infection progresses, the pulp turns brown, and then the bark turns brown near the soil line (24).

Table 2. Microscopic anatomical variations in tomato plant tissue

Tissue name	Infected (μM)	Not Infected (μM)
Epidermis (ep)	8.27	10.32
Cortex (co)	22.47	24.38
Phloem tissue (ph)	11.61	23.64
Xylem tissue (xy)	14.58	47.85
Pith (pi)	264.51	352.71

**Figure 2. Cross section of tomato stem (A) infected tissue (B) Healthy tissue.**

SPA, while the colonies growing on the N.A medium appeared white and circular

Diagnosis of *Ralstonia* genus

The microscopic and morphological characteristics of 12 bacterial isolates were studied and their ability to cause disease on tomato stems was proven. Results indicated that the bacteria were Gram-negative and rod-shaped. The morphological diagnosis results of the bacteria growing on TZC media, SPA media and N.A media indicated, as shown in Table (3) and Figure (3) shows the nature of bacterial growth after 24 hours of incubation at 28 ± 2 C. The colonies were characterized by their transparent appearance on the semi-selective medium

in shape, while the colonies growing on the specialized medium TZC, the results showed that the virulent isolates produced red colonies with white edges, while the non-virulent isolates produced smaller, white, and non-liquid colonies (7).



N.A. medium

T.Z.C. medium

S.P..A. medium

Figure 3. Illustrates the growth pattern of *R. solanacearum* on different media.

Diagnosis of *R. solanacearum* based on biochemical tests

Biochemical tests were conducted to diagnose 12 isolates of *R. solanacearum* as shown in Table (3). Results showed the isolates that gave positive results for some tests based on the classification keys, including Catalase test, Oxidase test, and Pectolytic test. The tests that gave negative results are KOH, Lavan, Hydrolysis of Gelatin, Arginine test, Starch Hydrolysis, Aesculin Hydrolysis, Indol test. (22 and 4).

Results of the Biovar test also showed that the *R. solanacearum* isolates obtained from tomato plants are able to decompose

the three sugars (Lactose, Cellobios, (Maltose) and alcohols within 3-5 days by producing acid and gas. The acid production in the sugar decomposition test by the bacterial isolates is demonstrated by changing the color of the medium from yellow to red. Gas production was observed through the appearance of gas bubbles in inverted tubes and oxidation of mannitol sugar by the indicated bacterial isolates with the production of yellow to red color. This indicates that all *R. solanacearum* isolates belong to Biovar 3 (III) which infects the Solanaceae family including tomato (33).

Table 3. Biochemical tests to diagnose *R. solanacearum* bacteria

Biochemical tests		<i>R. solanacearum</i>
Biovar3	Lactose	+
	Cellobios	+
	Maltose	+
KOH		-
Catalase test		+
Oxidase test		+
Hydrolysis of Gelatin		-
Pectolytic		+
Levan		-
Arginine test		-
Starch Hydrolysis		-
Aesculin Hydrolysis		-
Indol test		-

Molecular diagnosis of *R. solanacearum* using PCR

Polymerase chain reaction(PCR) technique was used to confirm the biochemical diagnosis of *R. solanacearum* bacteria. The diagnosis was made for two isolates which were the most pathogenic on tomato plants, namely *R. solanacearum* 1 and *R. solanacearum* 4, which were isolated from tomato stems from Al-Haidariya area in Najaf Al-Ashraf. The diagnosis was made based on the 16S rRNA gene. The results of electrophoresis on agarose gel of the gene amplification products showed the appearance of a band with a molecular size

of 300 base pairs (Figure 4), indicating that the isolation is due to *R. solanacearum*. Through the results obtained from the analysis of the sequence of the nitrogenous bases of the double DNA bands of the bacteria, which were compared with the data available at the American National Center for Biotechnology Information (NCBI). A new strain of *R. solanacearum* was obtained and registered in NCBI with Accession Numbers (PP473560 and PP473561).

Table 4. New strains of *R. solanacearum* registered in GenBank.

Isolate name	Isolate source	Bacteria name	Accession number
NhSb1	Tomato stem tissue	<i>R. solanacearum</i>	PP473560
NhSb2	Tomato stem tissue	<i>R. solanacearum</i>	PP473561



Conclusion

Bacterial wilt disease of tomato plants is one of the diseases that poses a danger and causes great losses in greenhouses. Due to the lack of studies on this disease in Najaf region, current study focused on the occurrence of bacterial wilt disease by conducting several biochemical and molecular tests were conducted to confirm the results of this disease. These results helped solve the difficulties facing the crop, enhance its growth, and improve its productivity.

Conflict of interest

The authors declare no conflict of interest.

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