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Article

In Vitro, Pathogenicity of Pseudomonas savasana Isolated from Olive Trees in Iraq on Fruits of Various Plant Species and its Detection by Polymerase Chain Reaction (BCR)

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Abstract

In this study, after a field survey of olive trees in some northern areas in the Kurdistan region of Iraq and the province of Dohuk, samples were collected for bacteria *Pseudomonas Savastoni*from olive trees infected with olive knot—the period from the beginning of March until the end of May for the diagnosis of Pseudomonas Savastoni. After activating the bacteria, we started inoculating the bacteria on the fruits of other plants, such as carrots, lemons, beans, local apples and hidden apples. These fruits were subjected to the same environmental conditions of humidity, temperature and incubation period. However, no symptoms of infection of the fruits with this bacteria appeared. Only the carrots were found to be infected with this bacteria after 13 days of incubation, but after 20 days, the bacteria had infected all the carrots. We isolated the bacteria again from the infected carrot. We performed all microscopic, phenotypic and biochemical tests and subjected the bacterial isolates to a device determined using the VITEK2 system to identify the type of bacteria. Bacteria we conducted molecular tests on them, such as the test PCR and the test RT-PCR for the gene 16S r RNA. After the results appeared, the isolates were subjected to genetic sequencing to ensure the occurrence of genetic mutations of the gene 16S r RNA.

Keywords: *Pseudomonas Savastoni*, 16S r RNA, RT-PCR, VITEK2, Sequencing

Introduction

The diversity of plants makes it possible to get infected by gram-negative plant pathogenic bacteria such as *Pseudomonas savastanoi*[1]. Before DNA-relatedness studies, it was thought to be a set of strains of *Pseudomonas syringae*, but it has since been recognized as a distinct species. It is called after Savastano, a worker who demonstrated between 1887 and 1898 that bacteria was the reason for the occurrence of olive knots[2]. *Pseudomonas savastanoi* pv savastanoi, the pathogen that roots the illness caused by bacteria in olives, is of the most significant economic value[3]. On infected trees, galls can grow as a symptom; this is analogous to how the well-known crown gall pathogen, *Agrobacterium tumefaciens*, induces tumor formation by metabolizing indoleacetic acid. Both the soil and the air contain these microorganisms. It also contaminates the roots; the germs are spread by wind to trees[4]. Every region that grows olives has olive knot disease. *P. savastanoi*

v. Savastanoi grows and spreads more readily in olive orchards in climates with warm temperatures and rainy autumn and spring[5]. Oleander knot is a common condition brought on by *P. savastanoi* pv. nerii. Olive tuberculosis would manifest when olive trees become infected with olive tuberculosis bacteria[5,6]. The infection first manifests as numerous clusters and clumps on the terminal branches[4]. It then worsens when the major skeletal branches of the tree become affected. Lumps are visible to the unaided eye; they resemble stones or pebbles hanging from a tree[6,7]. If the tree is not cared for, the infestation will have a horrifying aspect[7]. This disease significantly impacts olive tree productivity and can occasionally cause the tree to die completely. *P. savastanoi* pv. Savastnoi Savastnoi is the reason for the majority of plant infections. Wounds produce it, particularly cracks from a late-frost event, hailstones, solifluction dust, agriculture actions (including harvesting a crop and trimming), and flower, leaf and blooming[2]. When the knot disease is growing (a few millimeters in diameter), with a pale, more prominent excrescence that grows gradually, occasionally reaches the diameter of multiple centimeters, and eventually turns brown or the color of greenish-brown[8].

Materials and Methods

On stems and branches of pomegranate trees in the primary growing zones, soft juvenile knots with smoother surfaces were randomly selected for the samples between 2021 and 2022. Fresh olive knots collected from orchards contained three isolates of olive in the Dohuk Governorate of the Iraqi Kurdistan region. Every knot was handled separately. Young knots were cleaned with rushing water to remove the soil, and dust particles were cleaned by dipping them for two to three minutes in 25% (v/v) sodium hypochlorite. It is continuously disinfected after being dried on sterile filter paper and rinsed with sterile distilled water (SDW) with ethanol-soaked cotton. Low portions (5-10 mm) of every knot were sterilized slicely using a scalpel. For 10 minutes, they were placed in a sterile Eppendorf tube with 1 ml of sterile saline (085%NaCl). Tenfold serial dilutions were applied on plates containing PVF-1 agar and King's medium B (K.B.), and the plates were cultured at 26°C for three days. Re-streaked onto fresh K.B. surfaces were single fluorescent or nonfluorescent representative colonies of the major morphological types of bacterial isolates, and PVF-1 plates were grown for three days at 26 °C. Pure single colonies were grown on K.B. slants and kept at 4°C in 20% glycerol at -80°C pending further Identification. Two dangerous Psvisolates were produced from olive trees in the same area and a reference isolate (Hav-ran2b) used in an earlier study[9] was also offered as a basis of comparison.

Morphological examination.

Cetrimide agar swabs were cultured. The isolate was identified based on culture features, biochemical tests, and microscopic appearance following gram staining.

Microscope examination.

After staining with Gram stain, the bacterial isolates were studied microscopically using a bacterial smear to determine cell morphology and aggregation and their interaction with Gram stain.

Biochemical test.

P. Savastanoi was diagnosed using the following biochemical tests:

Catalase test

Some part of the bacterial plant was moved to a clean glass slide and placed on the MacConkey Agar medium. Using sterile wooden sticks, place a 3% drop of hydrogen peroxide. When gas bubbles were formed, it was confirmed that the results were positive[10].

Oxidase test

A portion of the bacterial plant was transferred to a clean glass slide and placed on MacConkey Agar media. Using sterile wooden sticks and two drops of oxidase detector over the filter paper, the appearance of dark violet signals a positive examination[10].

Molecular study of the genotype content of bacterial isolates.

The solutions were prepared as the company (Korea) Bioneer instructed the Primers. To obtain a concentration of 100/microliter, each primer solution was separately applied at a concentration of 10/microliter by taking 10/microliters of each stock buffer solution and adding 90/microliters. Deionized sterile distilled water Mix with Vortex and store the solutions at a temperature of - 20°C, taking into account mixing the primer solution after exhaling it from the ice using Vortex for homogenization.

Source	Temperature of Annealing (°C)	primer sequence(5-3)		Gene
Primers study		5`-GACACTGAGGTGCGAAAGCG-3`	R	S16rRNA
		5`-GACACTGAGGTGCGAAAGCG-3`	F	

Table. 1. Primers were utilized in this study.

Results

The samples were acquired randomly by collecting young soft knots with smoother surfaces on stems and branches of pomegranate trees in the main growing zones between 2021 and 2022. Three olive isolates were identified from fresh olive knots from orchards in the Dohuk Governorate of Iraqi Kurdistan regions. Separate approaches were taken to each knot, according to the following diagram. Young knots were first cleaned in running water to remove any soil or dust that had adhered to them. The surface was then disinfected in 25% (v/v) sodium hypochlorite for a few minutes.

They were also sterilized by being washed in sterile distilled water (SDW), dried on sterile filter paper, and finally disinfected using cotton soaked in ethanol. A sterile scalpel was used to aseptically cut small fragments (5–10 mm) from each knot, which was then put in a sterile

Eppendorf tube with 1 ml of sterile saline (085%NaCl) and left to sit for 10 minutes. On plates containing King's medium B(K.B.) and PVF-1 agar, tenfold serial dilutions were applied, and the plates were cultured at 26°C for three days. On fresh K.B. and PVF-1 plates, single fluorescent or nonfluorescent representative colonies of the most common morphological kinds of bacterial isolates were streaked.

Figure 1 Organism-infected plant tissue byPseudomonas savastanoi pv. Savastanoi.

Bacterial infection of fruits.

The cells inoculate the bacteria to a group of fruits to determine whether the bacteria infect the fruits. All of the following outcomes were obtained.

The use of bacteria on lemon fruits.

Inoculated lemon fruits were stored for 18 days at a humidity and temperature of 20°C. Where the outcome was negative, the bacteria cells did not infect the lemon fruits.

Infection of bacteria on carrot fruits.

Based on the results up to the ninth day, no illness symptoms were shown when the carrot pieces had been injected with bacteria under the same environmental conditions, including temperature and humidity. This is depicted in the figure below.

As illustrated in the following image, a carrot-sized piece of mucous material was present where the infection occurred after the thirteenth day but on the twentieth day.

Figure 2. (A) Using the bacteria *Pseudomonas savastanoi* pv. Savastanoi carrots were sliced after a 13-day inoculation (A). I am following a 20-day *Pseudomonas savastanoi* pv. Savastanoi inoculation, carrots are shown chopped in (B).

Infection of bacteria on Apples' local and imported apple fruits.

Despite exposing two different apple fruit types to the bacteria *Pseudomonas savastanoi* pv. Savastanoi and subjecting them to the same humidity and temperature conditions, there was no bacterial infection of the apple fruits even after twenty days. The following figure illustrates this.

Bacterial contamination of beans.

As seen in the following image, after 20 days of infection with the bacterium used to infect the bean fruits under the same settings as the other fruits, no signs were visible on the fruits.

Molecular study.

One of the most significant sources of injury in orchards and fields worldwide is *Pseudomonas savastanoi* pv. savastanoi strains, many of which are pathogenic FOR plants such as olive and carrot. The results of the PCR& RT PCR gene test revealed that the bacteria to which the carrot fruit was exposed is *Pseudomonas savastanoi* pv. savastanoi, as illustrated in Figures 3, 4, and Table 2**.**

Three *Pseudomonas savastanoi* pv. savastanoi isolates with the 16S ribosomal RNA gene were chosen for this study from the infected cut carrots. These isolates demonstrated that they could infect the under-researched carrot fruit.

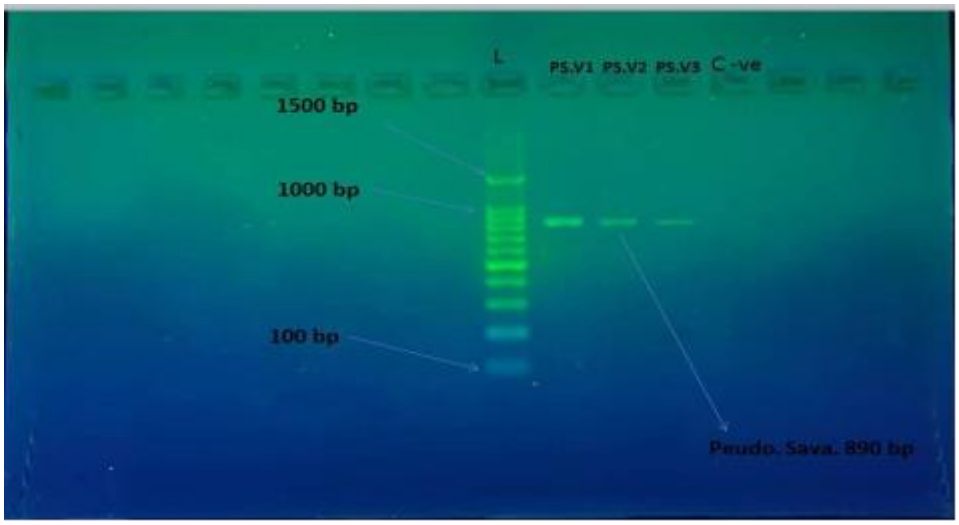

Figure 3. Displays the PCR on gel electrophoresis results.

Well	Color	Sample Name	Cq	Efficiency	Efficiency R^2	Result
1		PS.V1	22.01	0.97	0.99200	POSITIVE
2		PS.V2	24.19	0.88	0.99110	POSITIVE
3		PS.V3	22.14	0.87	0.99445	POSITIVE

Table 2. Results of RT-PCR *16S ribosomal RNA* gene isolation in 3.

Using RT-qPCR, the ratios of the 16S ribosomal RNA gene to the housekeeping gene were determined. After the real-time PCR runs, the findings showed amplification of the examined genes as curves that represented the actual amplification status. The Real-time PCR findings are displayed in Figure 4.

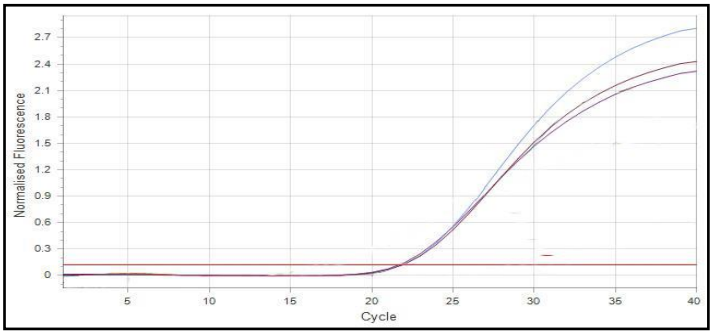

Figure 4. Results of RT-PCR *16S ribosomal RNA* gene isolation in 3.

Alignment and Identification for *16S ribosomal RNA* genes of *Pseudomonas-savastanoi pv. savastanoi* Local Isolates Using the NCBI Database.

The 16S ribosomal RNA genes were amplified using the PCR technique and delivered to Macrogen Company Korea for sequencing, as the ensuing figure illustrates—use of the world's website https://blast.ncbi.nlm.nih.gov/. Likewise, identify the variations between the nitrogenous base locations in the gene as illustrated in the following figure.

It was discovered that mutations occur in some sections of the gene by using the BLAST® site Search and gene sequencing**.** Gene's alignment with the NCBI Gene Bank. The sample is represented by Query, while the National Center for Biotechnology Information database is represented by Subject (NCBI).

Pseudomonas savastanoi pv. savastanoi has the two transition G>C coding -GGCCT, according to the *16S ribosomal* RNA gene sequencing results. Additionally, the nitrogenous base sequences of the genes T>G, GA> A.G., and A>C have changed. According to the Gene Bank, the subject of the *16S ribosomal* RNA gene in NCBI under sequencing has a 98.54% compatibility with a portion of the *16S ribosomal* RNA gene.

Discussion

It has been proven through the current work that it is possible to infect carrot plants with Pseudomonas savastanoi bacteria, and the inability of this bacteria to infect other types of plants that were selected in this study, such as lemons, peas and apples, as shown by the results. P.s isolates kept in the same environmental conditions of heat and humidity showed symptoms on the ninth day of inoculation. The first infection of carrot slices appeared on the thirteenth day in a small way, but on the twentieth day of inoculation, the infection appeared in an obvious way and was in the form of a mucous patch the size of a carrot slice[5]. Various host plants, and among these plants was the carrot plant. The bacteria under study was isolated from the nodes formed in the inoculated sites on the carrot slices. Biochemical and physiological tests diagnosed the isolated bacteria, and the results confirmed that Pseudomonas savastanoi bacteria caused the infection. In the province of Hatay and Turkey, P.S. bacteria is a crucial problem affecting olive, pomegranate, myrtle and jasmine plants[11]. In severe infection cases, the bacterial infection causes the death of young seedlings. To confirm the infection with P.S. bacteria (for the mentioned plants)[12], the diagnostic tests take a long time, which has benefited from the carrot plant. In developing a rapid method for bacterial growth (and this is indeed what was shown by the current study, as the carrot plant was an effective medium for the growth of P.s bacteria),

swabs were taken from the mentioned and infected plants and carrot slices were inoculated from the isolated swabs. The growth of the first typical node was observed within a week. After 14 days of luck, the injury became clear and obvious[7]. To confirm the pathogenicity of the isolated and developing bacteria on the carrot slices, isolates were taken from the carrot nodule, and the olive plant (Gemlik variety) was inoculated, where an apparent nodule growth was observed in the stabbing site two months after the operation[13]. P.S. Savastano, using PCR& RT PCR gene tests and molecular methods, the results showed that the carrot slice method is a straightforward and rapid technique to test the pathogenicity of P. S. pv. Savastano.

Conclusion

In this experiment, there were no negative results; all results were positive, as the results showed injury to the traction pieces only, and there was no injury to the remaining fruits. Furthermore, this is what was found to be the reason for discussing the results. The stages of the experiment began with collecting samples from northern Iraq and determining from the governorate of Dohuk, and then exposing some fruits to bacteria and exposing these fruits inoculated with bacteria to the same environmental conditions.

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