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Similarity and distance indices among *Pseudomonas savastanoi* strains isolated from *Olea europaea* trees in Mosul city

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ABSTRACT

Pseudomonas savastanoi is considered the main cause of knot disease in olive trees (*Olea europaea*), which is an important economic and food source in the Mediterranean region, including Iraq. In this study, there were several parts. The first part was about isolating Pseudomonas savastanoi bacteria from Olea europaea olive tumors, which can be found on infected olive trees in different parts of Nineveh Governorate and olive farms. The bacteria were then grown on different media, including King-B, MaCconky, Nutrient Sucrose Agar, and PVF-1 Agar. The white and pale colonies showed up as yellow, circular, raised colonies with smooth, airy edges. The study also did reinfections to see how strong the bacteria were at spreading the disease. PS3 isolate was more virulent, reaching an infection rate of 82% and conducting biochemical tests. The second part of the study involved the extraction of bacterial DNA. To find genetic links between isolates from different places, the RAPD-PCR method used five primers: OPR1, OPR2, R1, R2, and OPA1. We then selected five of these primers. The isolates PS1 and PS2 formed one group, while the isolates PS3 and PS4 formed another group through genetic analysis using a dendritic diagram and genetic similarity.

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Introduction

Pseudomonas sp. is a group of bacteria including more than 60 known species, most of them pathogenic (Gomila et al., 2017). Most of them belong to the *Pseudomonas syringae* species, which causes plant diseases, as they were discovered by studying tumors formed on agricultural plants (Morris et al., 2022). The bacterium *P. savastanoi* is a pathogenic species that belongs to the *P. syringae* multifaceted. Research has proven that it infects a wide variety of wild and cultivated plants, including herbaceous and woody plants. Cancerous growths on olive trees, oleander (*Nerium oleander* L.), and ash (*Fraxinus excelsior* L.), along with other plants, are caused by the bacterium *Pseudomonas* sp. These symptoms show up on olive plants because bacteria can produce plant hormones that change the hormonal balance in the hurt tissues. This makes the hurt areas grow, which makes the cells nearby the poison bigger. *Pseudomonas syringae* is the most economically important plant disease-causing species in all parts of the world (Mansfield et al., 2012).

The olive (*Olea europaea* L.) is considered one of the most important economic fruit trees grown in the world, especially in the Arab world, as the number of olive trees in Iraq is about 250 thousand trees, of which Nineveh Governorate ranks first with about 65 thousand trees. The economic importance of olives is represented in the production of oil from their fruits, as well as in the manufacture of black and green pickles, oil for cooking, and soap, in addition to the use of the remains of the fruits as animal feed. Wood is also used in the

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manufacture of furniture due to its strength, and the trees themselves are planted as wind catchers.

The multipart is too separated at the sub specific equal into 65 disease species clear by their houseplant varieties, Bull and Koike (2015). Wide host variation of *P. syringae* can be observed thru most isolates specializing for cultivated crops, and conversely environmental isolates show a broader host range, Berge et al., (2014) and Monteil et al., (2016). Furthermore, a new study displayed that *P. syringae* straining form an overlying range of throng specificity and that Pathovar species are incompatible with *P. syringae*, Morris, et al., (2019). Accepting this difficulty at altered levels needs understanding the bacterial pathogenicity systems. And facilitating the combination of phenotypic, genomic, and evolutionary documents. Although there is much research to show a variety of fingerprints based on the polymerase chain reaction used to study the genetic variety of bacterial populations and determine their relationships through the use of PCR in plant bacteriology for the purposes of detection and diagnosis using PCR protocols for detection. And identifying plant pathogenic bacteria with the aim of facilitating their access and preserving data that can be greatly utilized in the diagnosis of pathogenic bacterial species in laboratories. Thus, populations of *P. syringae* appear as one of the best related models for studying host specificity in microbial plant pathogens, Vinatzer, et al., (2019) and Moreno-Perez, et al., (2020).

One of the molecular techniques used to investigate *Pseudomonas savastanoi* is restriction fragment length polymorphism (RFLP). This technique was used to detect a collection of bacterial straining isolated from Italian olive trees to study the variance between these straining, Sisto, et al., (2007), and in another study use this technology used to study relationship between species of Rhizobial bacteria in deferent location in Iraq using specific primers, Al-Shakarchi (2023). random amplification and PCR were also used. Polymorphic DNA (RAPD) to estimate relatives between *Pseudomonas savastanoi* pushovers strains and their geographic spreading, Krid et al., (2009). This research aims to investigate the characteristics and genetic relationships of *Pseudomonas savastanoi*, the primary pathogen causing knot disease in olive trees (*Olea europaea*), which are a critical economic and food resource in the Mediterranean region, including Iraq. The study focuses on isolating the bacteria from olive tumors in Nineveh Governorate, conducting phenotypic and biochemical analyses, and exploring genetic similarities among bacterial isolates using the RAPD-PCR technique.

Materials and Methods

Isolation of bacteria from the tumors.

We collected samples from olive tree tumors from various locations in Nineveh Governorate, a region known for its widespread olive tree cultivation. We selected seven locations: Al-Rashidiya, Al-Qubba District, Hawi Knessa, Al-Arabi District, Al-Fadhiliya, Bashiqa, and Nineveh Horticulture Station, each with five replicates. We kept a few of them in sterile containers specifically designated for this purpose and transported them to the laboratory, where they grew on agricultural media. The knots on the olive stems were removed without any cuts or wounds occurring in the samples after they were washed with water to remove any dust stuck to them. The mouthpiece was surface sterilized by immersing it twice in 96% alcohol used for 2 minutes, washing it three times with sanitary purified water, immersing it in 3% NaClO for 15 minutes, and honeycombing it three times with sterile distilled water (Abdel-Azeem & Salem 2012). The sterile mouthpiece was dried. After incubation in Nutrient Agar medium, we took unstained samples and crushed them in saline using a sterile glass rod. We took 1 ml of the solution and grew it on Nutrient Agar medium. It was then kept at 28°C for 24 hours and diluted to get single colonies that grew on King-B Media, King et al. (1954). We used an oil lens to look at the colonies under a microscope to make sure they were pure after being stained with Gram stain. We also tested their ability to grow tumors by putting bacteria into plant parts and waiting 7 days. This showed that the bacteria isolates could indeed infect the parts and grow tumors on them (Schaad et al. 2001).

Identification by biochemical tests

We conducted several biochemical tests on the bacteria isolated from the tumors. As part of the Lennet et al. (1995) method, the bacteria were grown in media that contained different types of carbohydrates, such as xylose, glucose, mannose, sucrose, galactose, fructose, lactose, maltose, starch, and rhamnose. The goal was to find out how well the bacteria could ferment the target sugar. The researchers used the oxidase enzyme production method, as described by Holt et al. (1994), to ascertain the bacteria's ability to produce the cytochrome oxidase enzyme. Catalase test by placing a loop campaign from a pure colony of *P. savastanoi* with a drop of saline solution on a slide with the addition of 3% of H_2O_2 (Strattan & Tang 2006). The motility test and an aerobic test using the Staabing

method, growing the bacteria on a motility test medium in accordance with Moore et al. (1988).

To do the fluorescence test, you need to grow small groups of bacteria on King B Medium for 24 to 72 hours and then check to see if the bacteria can glow when they are exposed to 320 nm ultraviolet light (Singh et al. 2008). For arginine di-hydrolase production, recovered bacteria were added to Thornley's medium, and four days after the inoculation, the medium turned red, which meant that the test was positive by releasing NH_0 (Goussous & Al-Gharaibeh 2008).

DNA extraction

According to Mazzaglia et al. (2011), the Bacterial DNA was extracted by using Bacterial Genomic DNA Isolation Kits from fresh colonies. Nano Drop was used to get a 50 ng/µl DNA concentration and check the purity of the DNA. Tris EDTA buffer (Tris-HCl and EDTA) was used and kept at -80°C (pH 8.0). To perform the RAPD-PCR reaction, we selected the primers OPR1 (ACTGG (ACTGG GTCGG), OPR2 (CCAGTCCCAA), R1 (GTTTCGCTCC), R2 (CGGCCCCTGT), and OPA1 (CTATGGAGTA). After the last 25 µl of the reaction was done, 12.5 µl of 2× Taq PCR Master Mix (Aid lab Biotechnologies Co., Ltd., Beijing, China) was added. 1 µl of double-purified water, 1 µl of each primer (10 nmol/µl), and 1 µl of template DNA. We carried out the Rep PCR amplification for seven minutes at 95°C. This was tracked by 35 cycles of denaturation at 94°C for 60 seconds, annealing at each primer's temperature for 60 seconds, and extension at 72°C for eight minutes. We carried out the final addition step at 72°C for fifteen minutes. Agreeing to the program: first denaturation round at 95°C for 7 minutes; forty cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and addition at 72°C for five min; a final addition step of 72°C for 15 minutes (Sambrook & Russell 2001).

Statistical analysis

All the recorded data was analysis by MVSP 22.3 that the results of all treatments were compared using group analysis was supplied by similarity matrices generated according to Kovach (1999).

Results

We used the different selective media mentioned in Table 1 to examine the morphological criteria of the recovered bacteria isolated from the olive plants under investigation. After 6 days of incubation, round, smooth, grayish-white, and pale blue colonies that were 2-3 mm in size on these solid media appeared. The biochemical and physiological tests showed that all the isolates were both negative for Gram stain and the pathogenicity test (Table 3).

Table 1 Growth of *Pseudomonas savastanoi* isolates on different selective media.

Selective Media	Bacterial isolates			
	Ps1	Ps2	Ps3	Ps4
King	+	+	+	+
MaCconky Agar	+	+	+	+
Nutrient sucrose Agar	+	+	+	+
PVF-1 Agar	+	+	+	+

Table 2 Virulence of recovered bacteria.

	Bacterial isolates			
	Ps1	Ps2	Ps3	Ps4
Virulence	+	+	+	+
Period (Week)	6	4	5	7
Incidence rate %	61	72	82	75

Virulence of bacteria When inoculated with the isolated bacterial inoculum, tumors formed on all the tested plants, and their duration varied depending on the place of isolation, as shown in Table 1. This indicates a difference in the tested plants' reaction and the bacteria's ability to cause infection. The difference in the time period for tumor formation is shown in Figure (1) and Table (2).

Fig 1. Infected branched showed tumor due to bacterial infection.

The results presented in Table (3) indicate that the isolates, except for isolate Ps1, did not form fluorescent dyes when grown on KB media. They also formed white and pale yellow, circular, raised colonies with smooth,

airy edges, and demonstrated the ability to grow at temperatures ranging from 25-30°C. Motility tests, oxidase and catalase tests all came back positive for the isolates. All but isolate PS2 fermented sucrose, galactose, maltose, fructose, glucose, and sucrose; all but isolate PS4 fermented xylose; and none of the isolates fermented ramose, lactose, or starch.

Table 3 Biochemical tests for bacterial isolates under investigation.

Biochemical test		Isolates Bacteria		
		Ps2	Ps3	Ps4
Motility		+	+	+
Oxidase		+	+	+
Catalase		+	+	+
Aerobic		+	+	+
Fluoresce		+	+	+
Acid form mannose		+	+	+
Acid form Glucose		-	+	+
Acid form rhamnose		-	-	-
Acid form xylose		+	+	-
Acid form galactose		+	+	+
Acid form fructose		+	+	+
Acid form maltose		+	+	+
Acid form lactose		-	-	-
Acid form sucrose		+	+	+
Acid form starch		-	-	-
25	Temperature Growth	+	+	+
26		+	+	+
27		+	+	+
28		+	+	+
29		+	+	+
30		+	+	+
Levan		+	+	+
Arginine		+	+	-
Potato soft rot		-	-	-
Gram reaction		-	-	-

To find out how much Levant was being made, pure cultures of bacterial isolates were inoculated on SNA medium, to detect the presence or absence of mucus. Three isolates formed mucus sheath in comparison with other. We tested the bacteria's ability to infect plants (age of inoculated plants). Looking at the same table, the Ps2 isolate did better than the other isolates at infecting plants faster, by a rate of 72% after 4 weeks, while the Ps4 isolate did better still, reaching a rate of 75% after seven weeks. Based on the same table, isolate Ps3 had the highest infection rate, at 82%. The severity of the infection and how long it lasts depends on how well the bacteria cause it and how well the plant resists the bacteria's virulence and resistance, as well as the presence of special genes that cause the infection.

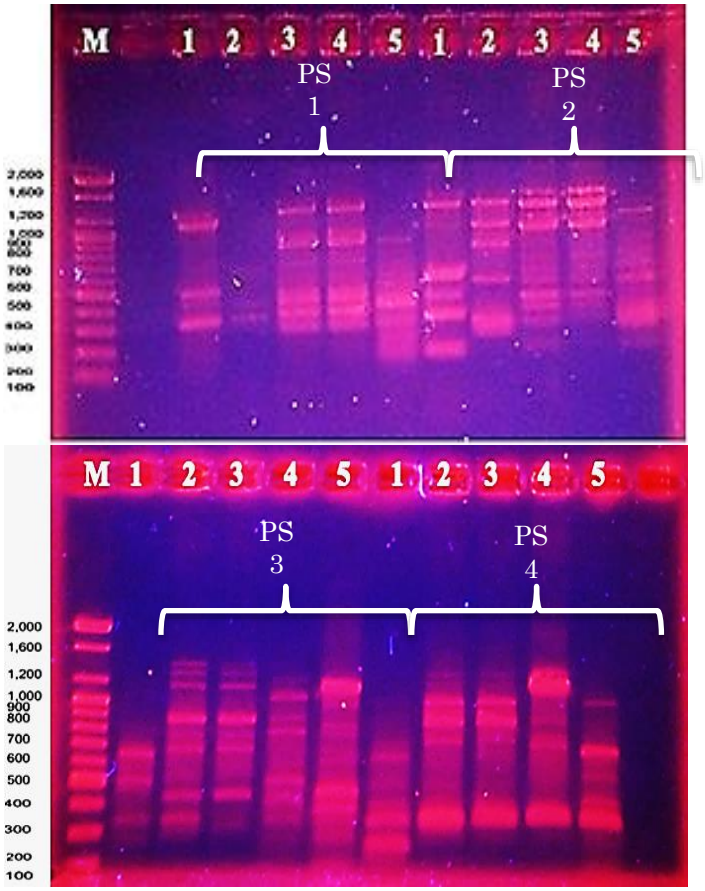

Fig 2. RAPD-PCR amplification of genomic DNA from 4 bacterial isolates of *Pseudomonas savastanoi* with five selected primers (1) OPR1, (2) OPR2, (3) R1, (4) R2, (5) OPA1.

By performing electrophoresis in 2% agarose gel for the PCR products figure (2), table (4) it was observed that there were 154 bands of DNA fragments that ranged from 200 to 1600 bp, for all reaction RFLP-PCR of DNA were purification from 4 isolation bacteria as shown in the figure (4).The another hand It was found that there was a

difference between the primers, as R1 showed the best efficiency and discrimination compared to the rest of the primers, reaching 24.7 and 24.4, respectively, while OPR1 was the lowest, as its results ranged between 14.9 and 15.5, respectively.

Table 4 The efficiency and discriminatory of the primers used in RFLP-PCR reaction.

No.	Name of Primer	Sequence	Number of packages	Bands Hetero	Efficiency of primers	Discrimination of primers
1	OPR1	ACTGG GTCGG	23	21	14.9	15.5
2	OPR2	CCAGTCCCAA	27	24	17.5	17.8
3	R1	GTTTCGCTCC	38	33	24.7	24.4
4	R2	CGGCCCCTGT	32	29	20.8	21.5
5	OPA1	CTATGGAGTA	34	28	22.1	20.7
	Total of bands		154	135		

The table (5) shows the percentage of similarity between the isolates of bacteria C and D isolated from olive trees that were eaten reached 62.745%, while the percentage of other isolates reached 54.545% for isolates A and B. In the same table, it can be noted that the percentage of similarity between the first and second groups reached 47.382% for trees cultivated in the study areas. It is possible to use the UPGMA cluster analysis method to find out how genetically similar isolates of the same species and different species are and then use that information to make a tree diagram (Figure 3). We input the genetic distance matrix values into a computer using the MVSP program, specifically version 3.44 of the cluster load diagram program. This shows three nodes (Table 5). They include isolates of Pseudomonas aeruginosa of varying numbers and possessing varying values of genetic variation. Bacterial isolates can be classified into two groups: the first includes bacteria A and B, and the second includes isolates C and D.

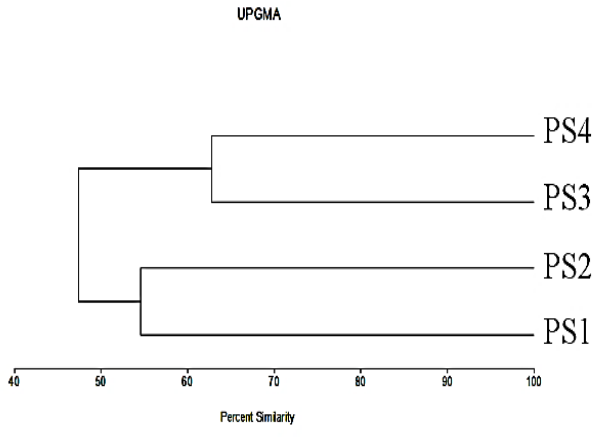

Fig 3. Dendrogram and genetic similarity of four bacterial isolates from olive tree tumors.

Table 5 The node of similarly between Isolate

Node	Group 1	Group 2		
1	PS3	PS4	62.745	2
2	PS1	PC2	54.545	2
3	Node 2	Node 1	47.382	4

Discussion

Kozik et al. (2006) observed when studying the bacteria that causes bacterial spot disease, Pseudomonas syringae pv. Researchers in Poland determined the genetic relationships between four Polish P. syringae subspecies in tomatoes. We determined these genetic relationships using PCR-RFLP technology. Using 33 primers to amplify bacterial DNA showed that the isolates that were studied by Kozik et al. (2006) and Moreno-Pérez et al. (2020) were very similar to each other. The results showed that there is genetic variation between these isolates and that they belong to two separate groups, 1 and 2. This suggests that there may be genetic variation between strains of the same type, which is in line with the results.

This supports the current study when using PCR and random amplification of polymorphic DNA (RAPD) to evaluate the relation between Pseudomonas savastanoi pushovers isolates and their diversity within the area locations studied, as this study agrees with Krid et al., (2009) and Thakur et al., (2021) when he studied the genetic diversity of Pseudomonas savastanoi pv farms. savastanoi and the percentages of difference and similarity among isolates and according to geographical locations using (RAPD) to distinguish for 58 Tunisian isolates and twenty-one isolates from different other area of P. savastanoi pv. Which causes tumors in olive trees, and as a result, tumors form on their branches. The obtained isolates were placed into three groups using cluster analysis and coordinated principal analysis of RAPD results using primers (OPR-12, OPX-7, and

OPX-14), and the group of isolates varied between southeastern Tunisia and European isolates. The group also included two isolates from northern Tunis, while another group included the majority of the isolates obtained from five locations in central Tunisia. It was found that isolates of *P. savastanoi* pv. *savastanoi* had a lot of genetic diversity, which depended on the differences in the study area (Scortichini et al., 2004; Dillon et al., 2022).

Many various studies using the analysis of amplified fragment length polymorphism (f-AFLP) technique to study the genetic variation of groups of bacterial isolates from different olive regions around the world. The genetic diversity of *Pseudomonas savastanoi* pv. Was carried by Lopez et al. (2008) in Spain. As Eltalbany et al., (2012) was able to study the genomic similarity between French and German *P. savastanoi* isolates, The BOX-PCR fingerprint obtained from the *P. savastanoi* bacterial isolates was similar and also very similar to that obtained from other *P. savastanoi* isolates that were isolated from oleander, olive, jasmine, and silk plants.

Studies have also shown that *P. savastanoi* bacterial isolates showed identical DNA hybrid patterns by plasmid isolation using the RIBA probe, which indicates that their plasmid content is similar, consisting of at least three plasmids. Their weights ranged between 10-42 kbp and 73-88 kbp exclusively. The plasmid type of the French, Slovenian, and Spanish bacterial isolates was identical, but it showed slight differences compared to the German and American isolates. After extensive research, we classified the studied isolates into groups based on their genetic similarity. Moretti et al. (2017) studied 124 bacterial isolates of the P. savastanoi type, which were divided into two main groups and 4 subgroups. Tsuji et al. (2017) and Nowell et al. (2023) also studied 19 Japanese isolates, differentiating them into five groups using PCR technology.

Conclusion

This research was conducted in the laboratories of the College of Education for Pure Sciences at the University of Mosul. It focused on isolating and characterizing Pseudomonas savastanoi bacteria from olive tree tumors collected from various farms in different locations across Nineveh Governorate. Advanced microbiological and molecular methods were used in the study to look at phenotypic traits, virulence factors, and genetic diversity among the isolates. These results help us learn more about the genetic relationships and harmful effects of P. savastanoi, which is very important for creating good ways to control it. The outcomes of this research underline the importance of continued investment in agricultural and environmental studies to mitigate plant diseases that impact economically significant crops such as olives.

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Conflict of interest

The authors declare that they have no conflict of interest.

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