T.R.
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL SCIENCE AND TECHNOLOGIES

IN VITRO PATHOGENICITY OF *Pseudomonas savastanoi* ISOLATED FROM OLIVE TREES IN IRAQ ON FRUITS OF VARIOUS PLANT SPECIES AND THEIR MOLECULAR CHARACTERISATION

Prepared By
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M.Sc. Thesis

January 2023
KAYSERİ

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SCIENTIFIC ETHICS CONFORMITY

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Ghaith Adil ABDULHAMEED

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The MSc thesis entitled **"IN VITRO PATHOGENICITY OF *Pseudomonas savastanoi* ISOLATED FROM OLIVE TREES IN IRAQ ON FRUITS OF VARIOUS PLANT SPECIES AND THEIR MOLECULAR CHARACTERISATION"** has been composed in accordance with the Postgraduate Thesis Proposal and Thesis Writing Directions of Erciyes University.

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This study entitled "**IN VITRO PATHOGENICITY OF *Pseudomonas savastanoi* ISOLATED FROM OLIVE TREES IN IRAQ ON FRUITS OF VARIOUS PLANT SPECIES AND THEIR MOLECULAR CHARACTERISATION"** has been accepted by our juries as a Master Thesis in the Department of Agricultural Science and Technologies, Graduate School of Natural And Applied Sciences, Erciyes University.

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ABSTRACT

The present study was carried out to determine the pathogenicity of *Pseudomonas savastanoi* on carrots and some other fruits, and to characterize the isolates using molecular methods. Pathogenic *Pseudomonas savastanoi* bacterial samples were collected from olive trees with olive knot after a field survey of olive trees from around Duhok province in Iraq. The study period was determined to include the beginning of March until the end of May, when after collecting the samples and transferring the samples to the laboratory and growing them on culture media. The samples were cultured on Blood agar, MacConkey agar and Cetrimide agar mediums for the diagnosis of *Pseudomonas savastanoi* and further studies. After activation, bacteria were inoculated on the carrots and other fruits of plants such as, lemons, beans, local apples and commercial apples. These fruits were subjected to the same environmental conditions of humidity, temperature and incubation period.

Results revealed that infection symptoms not appeared on the fruits after bacterial exposure. However, only some of the carrots were found to be infected after 13 days of incubation. On the other hand, when the incubation period was prolonged to 20 days, all the carrots samples were infected. Bacteria were re-isolated from infected carrots and some basic microscopic, phenotypic and biochemical tests including oxidase, catalase and urease were performed on them. In addition, VITEK2 system were used for determining the isolates in species level. Also, PCR and RT-PCR and sequencing of 16S rRNA were performed to detect possible mutations .

Key words: *Pseudomonas savastanoi*, 16S r RNA,, RT-PCR, VITEK2, Sequencing**.**

IRAK'TA ZEYTİN AĞAÇLARINDAN İZOLE EDİLEN *Pseudomonas Savastanoi* BAKTERİSİNİN ÇEŞİTLİ BİTKİ TÜRLERİNİN MEYVELERİ ÜZERİNDE İN VİTRO PATOJENİSİ VE MOLEKÜLER KARAKTERİZASYONU

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ÖZET

Bu çalışma, *Pseudomonas savastanoi* bakterisinin havuç ve bazı bitkisel meyveler üzerindeki patojenitesinin belirlenmesi ve moleküler karakterizasyonu amacıyla yapılmıştır. Patojenik *Pseudomonas savastanoi* bakteri örnekleri, Irak'ın kuzey bölgesinde yer alan Dohuk ili çevresindeki zeytin dal kanseri bakterisi ile enfekte olmuş zeytin ağaçlarından toplanmıştır. Mart-Mayıs dönemi numunelerin alınıp laboratuvara nakledilmesi ve kültür ortamında yetiştirilmesi dönemdir. Örnekler, *Pseudomonas savastanoi* tanısı için Blood agar, MacConkey agar ve Cetrimide agarlarda kültüre alınmıştır. Bakteriler aktive edildikten sonra havuç ve limon, fasulye, yerel elma ve ticari elma bitkilerin meyvelerine bakteri aşılaması yapılmıştır. Bu meyveler nem, sıcaklık ve inokülasyon süresi bakımından aynı çevresel koşullara tabi tutulmuştur.

Çalışmanın bu kısmı sonucunda, meyvelerde bu bakteri ile herhangi bir enfeksiyon belirtisi görülmediği, ancak 13 günlük inkübasyondan sonra sadece bazı havuçların bu bakteri ile enfekte olduğu tespit edilmiştir. Inkubasyon süresi 20 güne kadar uzatıldığında ise bakterilerin tüm havuç örneklerini enfekte ettiği gözlenmiştir. Enfekte olan havuçlardan bakteriler tekrar izole edilmiş ve bu izolantlar üzerinde oksidaz, katalaz ve üreaz dahil olmak üzere tüm önemli bazı biyokimyasal, mikroskobik, fenotipik testler gerçekleştirilmiştir. Ayrıca, bakteri izolatları, bakteri türünü belirlemek için VITEK2 sisteminde kullanılan bir cihazla analize tabi tutulmuştur. Daha sonra bu bakteriler üzerinde PCR, RT-PCR ve 16S rRNA dizilemesi yapılarak mutasyonlar belirlenmeye çalışılmıştır.

Anahtar Kelimeler: *Pseudomonas savastanoi*, 16S rRNA, RT-PCR, VITEK 2, Sequencing.

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CHAPTER I

INTRODUCTION

1.1. Background of Study

A diversity of plants is possible to get infected by the gram-negative plant pathogenic bacteria such as *Pseudomonas savastanoi* [1]. Prior to DNA-relatedness studies, it was thought to be a set of strain 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 root the illness caused by bacterial in olive, 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. Additionally, it also contaminate 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. Manifestation of olive tuberculosis would happen when olive trees become infected with the olive tuberculosis bacteria[6][5]. 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[7]. Lumps are visible to the unaided eye; they resemble stones or pebbles hanging from a tree[7][6]. If the tree is not cared for, the infestation will have a really horrifying aspect[7]. This disease has a significant

impact on olive tree productivity and can occasionally cause the tree to die completely[1].

P. savastanoi pv. Savastnoi Savastnoi is the reason to cause majority of plant infections. It is produced by wounds, particularly cracks from a late-frost event, hailstones, solifluction dust, actions of agriculture (including harvest a crop and trimming), and flower, leaf and blooming[2]. When the knot disease is growing (a few millimeters in diameter), with a pale larger excrescences that grows gradually, occasionally outreach the diameter with multiple centimeters, and eventually turn into brown or the colour of greenish-brown[8]. Beneath the surface, the knots are composed of dense, the space would be filled with flabby tissue where bacterial cells along with lysogenic cavity[8]. Within 6 to 8 months of their creation, the knots degenerate, become partially necrotized, crack, and eventually die. They may persist longer and grow larger under certain conditions[2]. When knots partially or totally encircle branches in cases of severe infections, the affected limbs are stunted, defoliated, or even died. Some parts are girdling the limbs that make up the tree's framework, knots on young seedlings in the field could weaken the plant[9].

Due to the pathogen's global spread over the past 200 years, the classification of *P. savastanoi* pv. *savastanoi* as an organism that needs to be taken care in isolated place in the European Union. This organisms falls under phytosanitary laws, and lately, it belongs to the 10 of bacteria that is pathogenic in the Molecular Plant Pathology journal[5]. *P. savastanoi* pv. *savastanoi* has a multiple of virulence factors, including motility, exopolysaccharide (EPS), biofilm formation, Type III secretion system (T3SS)[10]. P. *savastanoi* pv. *savastanoi* used three different system such as Quorum sensing, two-component signal transduction, and bis-(3′-5′)-cyclic di-GMP (c-di-GMP) to synchronize the production of virulence components at different stages in order to establish an infection successfully. The most recent research *P. savastanoi* pv. *savastanoi* on aspects of virulence of is presented using this review, emphasizing their involvement in bacterial pathogenesis and the areas that require additional study[11].

The prophylactic use of antibiotics (such as oxytetracycline or streptomycin) throughout the spring time noticiable to be the most significant of the few control methods now available[12]. However, the prospects of the use of antiobiotic in the long run are

severely constrained by regulatory constraints, public health issues, and the emergence of bacterial resistance[13]. Promising solutions to reduce or replace antibiotic effects and prevent the development of defiance may be provided by biological control techniques. Many Enterobacteriaceae, including *P. savastanoi* pv. *savastanoi*, produce curli, a complex extra-cellular matrix acts as a primary component of proteinaceous. It is known that curli fibers have a role in biofilm development, agglomeration of cells, and surface adherence. Curli acts as an host mediator cell adhesion and encroaching. In addition to triggering inflammatory reactions in the host[5]. Among the bacterial fibers belonging to a growing that have been described so far, curli's structure and biogenesis are distinctive[14].

1.2. Research Objective

The objectives of this study:

1. *P. savastanoi* was isolated and identified from olive trees in the Duhok Governorate in northern Iraq.
2. Concerning bacteria grown in nutrient-rich broth and detected by sophisticated methods like VITEK2.
3. Fruits exposed to the bacterium *P. savastanoi* pv. *savastanoi*
4. Genetic sequencing for the same gene, test PCR for the *16S ribosomal* RNA gene, and isolation and verification of bacteria recovered from infected fruits.

CHAPTER II

LITERATURE REVIEW

2.1. General Characteristics of *P. Savastanoi*

A gram-negative plant pathogenic bacterium called *Pseudomonas savastanoi* affects a wide range of plants. It was previously thought to be a strain of bacteria of *Pseudomonas syringae*. However, similarity DNA investigations led to its establishment as a distinct species [9]. Savastano was used to name this organism, as he demonstrated this bacteria as the reason for olive knots occurred between 1887 and 1898[15].

The pathogen that causes the illness olive knot, *Pseudomonas savastanoi* pv. *savastanoi*, has the greatest economic importance[4]. It causes numerous symptoms, including the production of galls on infected trees and tumor formation. These symptoms are driven by the bacteria's indoleacetic acid biosynthesis, similar to crown gall pathogen study, *Agrobacterium tumefaciens* [7]. These microbes can be found in the soil or the air. These germs are also able to infect trees through both roots and the wind [12].

Figure 1.1. Olive branch knots (younger greenish galls on the figure's left side, and larger knots on the right) (Photo: I. Dminić)[16].

The scientific categorization of this bacteria, according to Jawetz et al, (2010)[17]. is as follows:

- Domain: Bacteria
- Phylum: Proteobacteria
- Class: Gammaproteobacteria
- Order: Pseudomonadales
- Family: Pseudomonadaceae
- Genus: *Pseudomonas*
- Species: *Pseudomonas savastanoi* pv. *savastanoi*

The olive (*Olea europaea L.*) and oleander (*Nerium oleander L.*) are classified as knot disease are caused by the phytopathogenic bacterium *Pseudomonas savastanoi*[18]. The classification and taxonomy of this species has long been contentious, including the on was isolated from Ash (Fraxinus excelsior L.) [8]. *P. savastanoi* pv. *savastanoi* (Psv), pv. fraxini (Psf), and pv. nerii (Psn) are recognized as distinct pathovars based on a variety of phenotypic, physiological, biochemical, and molecular parameters. They were discovered in olive, oleander, and ash[5].

In fact, based on experimental study, the hosts are vary from these strains, and it was proven as greater than the concept of a pathovar would be assumed. For instance, the infection of *P. savastanoi* was found on both olive and ash [1].

However, various host contribution and the prevalence of nonsocial infection in natural contexts are still unclear [12]. Contrarily, it was already determined that the amount and diffusion of *P. savastanoi* populations were firmly connected and the number of the disease. It naturally exists on healthy epiphytes and/or plants that are asymptomatic. Also, it is living inside the illness juvenile knots and plants that are symptomatic [19].

2.2. Historical and Clinical Significance *P. savastanoi* pv. Savastanoi

In the fourth century BC, the Greek philosopher Theophrastus gave the earliest description of olive knot illness. Savastano, L. 1886, who was first successfully isolated this bacteria and replicating symptoms of illness by artificially inoculating healthy olive plants. This discovery was also validated by G. Arcangeli's suspicion that a bacterial etiology for these irregular growths existed in 1885 [20]. Smith then named the pathogen bacterium *savastanoi* in Savastano's honor in 1908 after recognizing the importance of his research. Steven later classified this bacteria to the genus of *Pseudomonas* as *P. savastanoi*. Following that, similar infections caused by two distinct but related bacteria known as *P. savastanoi* var. fraxini were reported in Fraxinus (ash) and Oleander [21].

Followed the study of pathovar from variety of hosts, *P. savastanoi* was considered to be recognizeas of three strains of of *P. syringae* subsp. *savastanoi*. These name of this strain at first was invalid when proposed and were only finally validated and recognized to be a species *P. savastanoi*[5]. The three strains were studied: parenchymatous galls were found on various Oleaceae species and other hosts due to pv. *savastanoi*, wart-like excrescences were found on both Nerium oleander and Oleaceae species due to pv. nerii, meanwhile excrescences of wart-like was found on Fraxin due to pv. fraxini [21].

Work by Mugnai [22]. also supported the pathogenic differences. The pathovar nomenclature was formalized in this classification, which also considers the pathogenic characteristics and other distinguishing qualities of strains isolated from different host plants. It includes details on the pathogens *savastanoi*, particularly gives negative effects on olives, and pv. nerii with ash, oleander, and olives [21, 23-24].

2.3. Pathogenic of *Pseudomonas savastanoi* pv. *savastanoi*

The classification of olive knot by developing tissue of knots and galls, primarily on limbs and stems that are young. This correlates with the ability *P. savastanoi* pv. *savastanoi* to occasionally infecst roots, leaves and other organs [10][19]. Most plant infections originate through wounds, notably cracks brought on by agricultural, hail, wind-blown sand, and late spring frost procedures (such as crop pruning and harvesting), leaf, flower, and raceme fall [25].

The effects would be notices when the knots start as little (diameter of several millimeters), pale-green excrescences that eventually grow to multiple centimeters in diameter before slowlyturning into greenish-brown or colour of brown. The knots are spongy inside, dense tissues where bacterial cells affect lysogenic cavities fill the space. Within six to eight months of forming, the knots begin to break, become partially necrotized, degrade, and eventually die with age [26].

Studying the anatomy of infected twig tissues, the formation of knots, and the plant's defense responses allowed researchers to better understand how the olive knot disease spreads [27]. This starts when bacterial inoculum invaded the cortical parenchyma at the inoculation site, weakening the primary cell walls by slowly reaching into the intercellular spaces[27-28]. The development of the knot is caused by the parenchyma cells' hyperplastic activity, which is accompanied by the pathogen's proliferation into the twig's various tissues [29].

It happens simultaneously when the knot was composed of many cambia, xylem components from various origins, and hyper-plastic cells. Bacterial cavities also would be surrounded by cells with lignified walls, and fresh periderm covered the knots' outer surface [30]. At later stages of knot development, the periderm at the knot's surface is pressured by the hyperplastic activities of new tissue mass, which caused fissures that cracked open outside and exposed bacterial cavities[28].

In fact, every region that grows olives has olive disease. *P. savastanoi* pv. *savastanoi* thrives in olive trees in climates with warm temperatures, spring seasons and rainy autumn [31]. The indications and symptoms olive knot occurs as rough swellings or galls on twigs, trunks, roots, branches, leaves, or peduncles that range in diameter from 0.5 to 2 inches (fruit stems). It is possible to girdle, defoliate, and destroy little sprouts. Additionally, lesions on the trunk or limbs might develop galls [32].

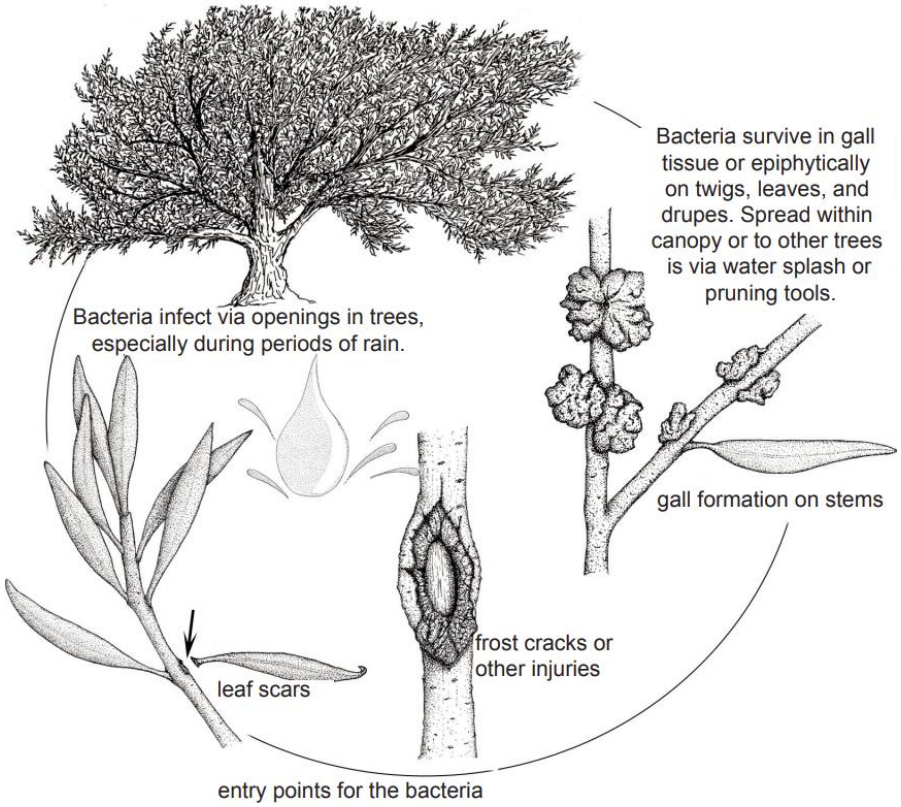


Figure 1.2. Life cycle of Olive knot disease[32].

If infections develop on the trunks of young trees, olive knot can girdle and the tree will then die. It slowly causes decreasing in yield on older trees by branches and girdling twigs. A fruit's bad flavour is also linked to infection to this infection[6]. Olive knot incidence often correlates with annual precipitation, which is more severe in regions with higher annual precipitation or during years of high annual precipitation. At any time of the year, they are easily dispersed by water. This occurs as bacteria lives in galls and on leaves and twigs as epiphytes. They could colonize asymptomatic plant tissue as an epiphyte during fall, winter, and spring[33] .

Furthermore, when the knots begin to form in the late spring, the trees begin to grow again and continue to form throughout the summer. Therefore, order for bacteria to enter a plant, openings must exist [34]. These openings can be found in bark cracks caused by freezing, leaf scars or pruning wounds[34]. It occurs because the subsequent defoliation and split bark which typically happens in the winter when it rains and might transmit the disease as this freeze injury can create disease epidemics[35].

Olive knot is yet challenging to eradicate and demands frequent applications of bactericide [11]. Essential treatments are required as it will lower the number of pathogens on the plant's surface then will reduce the risk of infection. In order to get rid of galls on twigs and branches, it is also beneficial to carefully prune during the dry season[36]. If trimming at other times of the year, make sure to frequently clean the pruning shears as the germs may be carried on them [37].

By partially girdling the branches that make up the trees' structure, knots on young plants in the field could weaken the plant[38]. Although uncommon, fruit infections can take the appearance of numerous roughly circular, the brown spots that are 0.5-2.5 mm in diameter will be immediately formed[1]. Then it is subsequently depressed at the lenticels, or neoplastic change of the peduncle's monocarp prevents the growth of fruit and causes alteration[35]. Although the disease's impact on olive crop has not been well examined, heavy contamination has negatively impacted the tree structure that will lead to low amount of crops. These are the signs when severe infection has affected the quality of olives[25].

2.4. Characteristics of Disease caused *by P. savastanoi*

Research from the past has shown that olive will associate *P. savastanoi* pv. *savastanoi* and oleander with *P. savastanoi* pv. nerii would be thriven on the leaves surface, twigs, and fruits of host plants if they are in their saprophytic phase[39][40][41]. In addition, In the spring and fall, *P. savastanoi* pv. *savastanoi* population densities on phylloplanes host-plant were at the maximum points[12].

Infection mostly happened during these seasons when the plants are actively growing and particularly sensitive. There seems to be a relationship between phylloplane colonization in olive and leaf age, cultivar, and agronomic techniques[42]. Inoculum which acts as essential sources involve *P. savastanoi* pv. *savastanoi* epiphytic populations and releases active knots, although additional research is required. Other sources mentioned that of inoculum process correlated with scars from invasion, hail damage, frost damage, and agronomic practices[42].

When it rains and aerosol is produced by wind-driven rain, the grove contains the plants that are contaminated by the bacteria are seeping from knots and/or living on the

phylloplane would be spread[43]. Additionally, pathogens that live on healthy plants' phylloplanes or because subclinical contaminations could be transferred on propagation of buds, plants, and cuttings. The primary method of long-distance distribution of *P. savastanoi* is transferred via planting material[4].

Although it has never been proven, earlier research has suggested the possibility of latent contaminations, particularly the migration system of *P. savastanoi* in olive cells [44]. When leaves fall off, cuttings are being readied for rooted, or the plants are being grafted, latent infections in olives may appear. Additional in-depth research is still required on the epidemiological significance and phytosanitary effects of latent infections [45].

The disease cycle for *P. savastanoi* pv. nerii is comparable to the pathogen of olive knot, nonetheless, oleander is a decorative bush and is not subjected to the ongoing harmful the growth of olive[44]. In this disease, the bacterium that causes oleander knots travels into the laticifer vessels and causes secondary knots[46]. When it rains and aerosol is produced by wind-driven rain, the grove contains the plants that are contaminated that are seeping from knots and/or living on the phylloplane can be spread. However, field isolations show that only strains of olive and oleander can be isolated from their respective hosts, indicating that each strain is unique to that host in the wild [14].

2.5. Genomic Insights into P. *Savastanoi* pv. *Savastanoi* Pathogenicity and Virulence

P. savastanoi pv. *savastanoi* NCPPB 3335 is the recent sequencing draft genome where it is also the three-plasmid complement has a completed sequence that has some negative effects. The study discovered that the virule complement of gene from woody hosts which has pathogen of tumour-inducing [5][9].

Plasmic called pIAA in pathogen in oleander strain were carried by the genes where it has two types of enzymes, iaaM and iaaH. Meanwhile in olive strains, the genes are found on the chromosome[47]. When transformed with pIAA, mutants that have been cured of pIAA are returned to full virulence on oleander[47]. Additionally, iaaM has been cloned, and research has shown how it contributes to virulence. There was no any

modification on genetic of host tissue that was caused by *P. savastanoi*. On the other hand, *Agrobacterium tumefaciens* where it affected the crown gall disease where DNA (T-DNA) is stable to integrate with the tumor-inducing (Ti) into the nuclear genome[48]. In two mechanisms in *P. Savastanoi*, tryptophan is transformed into IAA by the enzymes made by the genes iaaM (Tryptophan monooxygenase) and iaaH (indoleacetamide hydrolase)[49]. Furthermore, the majority of *P. syringae* complex pathovars possess the iaaL gene, which is utilized by *P. savastanoi* pv. nerii (oleander isolates) to transform IAA into IAA-lysine[50].Despite having two iaaL alleles, *P. savastanoi* pv. *savastanoi* pathovar that was separated from olives did not show any production of IAA-lysine in their culture filtrates[51]. iaaM, iaaH, and iaaL alleles which are encoded were also appear in the *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome. On the other hand, it appeared that the iaaM-2 and iaaH-1 alleles were pseudogenes[9].

Recent sequencing that were reformed of these two loci revealed that iaaM-2 is a pseudogene while iaaH-1 encodes the entire coding sequence (CDS). Inside the *P. syringae* complex, gene iaaL is broadly distributed[52].However, horizontal transfer may be found in the clumping of iaaL from *P. syringae* pv. oryzae[52]. This is expected given that plasmids frequently include several copies of iaaL[19]. Despite the fact that the *P. syringae* complex seems to be the preferred location for the translocation. However, some pathovars include CDSs whose offspring have very little in common with iaaMH[53]. It shares a high degree of homology with putative monooxygenase and amidase genes that are shared by *P. syringae* but whose function in IAA production is unknown.[53]. In contrast, highly retained of iaaMH alleles are contained in just a small number of *P. syringae*[54].

In *P. savastanoi* pv. *savastanoi*, genes for phytohormone biosynthesis are dispersed over the genome with genes for cytokinin (CK) biosynthesis preferentially found in plasmids of the pPT23A family[19]. A putative genomic island was discovered in plasmid pPsv48A of *P. savastanoi* pv. *savastanoi* NCPPB 3335 contained the ptz gene, which encodes an isopentenyl transferase and has a low G + C content (43.4% G + C)[4].

Olive knots are produced in olive plants by *P. savastanoi* free from ptz-containing plasmids are lower than those produced by random strains [4][54]. They also exhibit a spiral vasculature. The plasmid pPsv48C of *P. savastanoi* pv. *savastanoi* NCPPB 3335 contained the gene ipt, which encodes a putative isopentenyl-diphosphate delta-isomerase and is thought to be involved in biosynthesis of CKs However, this gene's potential role in virulence has not been investigated[6].

2.6. Virulence factors of *P. savastanoi* pv. *savastanoi*

The pathogenicity of *P. savastanoi* pv. *savastanoi*'s in olive is significantly influenced by quorum sensing (QS) regulation. *P. savastanoi* pv. *savastanoi* strain DAPP-PG 722 QS system, contains luxI and luxR homologous (pssI and pssR)[55]. In planta, it has been shown that the presence of wild-type *E. toletana*, a nonpathogenic bacterium usually found in conjunction with the olive knot pathogen, complements the absence of pathogen that produced signal by the pssI mutant[56].

Erwinia toletana and *P. savastanoi* pv. *savastanoi* both produce the same molecules of N-acyl-homoserine lactone. Additionally, *E. toletana* population drastically diminish in a certain time after being injected into olive tissues but grow when paired with a strain of *P. savastanoi* pv. *savastanoi* [2]. Also, the of *P. savastanoi* pv. *savastanoi* population also increases effectively since the co-innoculation of *E. toletana*. The size of knot is also growing, indicating a rise in pathogenicity[57]. Although the exact mechanism underpinning this link is not well understood, it occurs to be brought on at least partly by the sharing of QS signals, which is mediated by N-acyl-homoserine lactones[57].

According to Höfte, M., and Vos, P. D. (2007), additional virulence factors in plant-pathogenic *Pseudomonas* includes phytotoxins, adhesion, cell wall-degrading hydrolytic enzymes, extracellular polysaccharides, absorption of iron systems, against to antibiotics of plant-derived, general motility, and chemotaxis mechanisms[58]. 551 genes from *P. savastanoi* pv. *savastanoi* NCPPB 3335 draft genome were found to be annotated, and the majority of these genes are conserved in *P. syringae* pv. phaseolicola 1448A [58]. These genes may be involved in several processes that could improve virulence [58].

The subset of *P. savastanoi* pv. *savastanoi* NCPPB 3335-specific genes include genes absent from 1448A, including cellulase, pectate lyase, and a putative filamentous haemagglutinin [59]. The number of levansucrase genes varies from three to one in all sequenced *P. syringae* strains[59]. For the exopolysaccharide levan to be produced, this enzyme is required. There is only one levansucrase-coding gene in *P. savastanoi* pv. *savastanoi* NCPPB 3335 (PSA3335 2033) (Rodríguez-Palenzuela, P., et al. 2010). This fact is assumed as *P. savastanoi* pv. *savastanoi* strains are frequently levan negative, unlike the *P. syringae* pathovars [59].

2.6.1. Type III Secretion System (T3SS) and Effectors

Based on clumping analysis of the HrpS protein sequences, group I was found to be classified for *P. savastanoi* pv. *savastanoi* NCPPB 3335which only includes proteins from *P. syringae* pathovars from genomospecies 2[60]. The HrpA2 gene in *P. savastanoi* pv. *savastanoi* NCPPB 3335 is very similar to the HrpA genes in *P. syringae* pathovars phaseolicola, glycinea, and tabaci [60].

An earlier study looked into how a T3SS mutant of strain NCPPB 3335 might not only produce knot development in woody olive trees but also multiply in olive tissues[61]. The necrosis and the gaping spaces inside seen in knots On immature micropropagated olive plants, tumors caused by the T3SS mutant did not produce any of the wild-type strain's products, which is an exciting finding [61].

Hop gene prediction using 19 probable T3SS effectors that share 65%–80% of their amino acid sequences with known effectors, was made possible by *P. savastanoi* pv. *savastanoi* NCPPB 3335 genomic sequence bioinformatic analysis[6]. An additional 11 potential genes also have no sequence similarity to recognized effectors[61]. Four novel potential effectors, AvrPto1, HopAT1′, HopAZ1, and HopF4, were discovered after this genome sequence underwent modification[6][61]. Two of the T3SS effector genes, hopAF1 (plasmid pPsv48A) and hopAO1 (plasmid pPsv48B), are also plasmid encoded, according to the sequencing of this strain's three plasmid complements [61].

2.7. Disease Association Olive Knot With Bacteria P. Savastanoi pv. Savastanoi

Epiphytic bacteria on host plants or bacteria seeping through knot cracks are the principal sources of *P. savastanoi* inoculum[62]. Wind-driven rain is the primary mechanism via which inoculum spreads over short distances, however, additional farming practices including trimming, grafting, fruit harvesting, and contaminated propagation material all help with local spread[43]. Although the evidence is either inconclusive or conflicting, insects may have a crucial role in spreading the virus[62].

The olive fly *(Bactrocera oleae*), which the bacterium was assumed to be symbiotically associated with, was formerly thought to be significant in the spread of *P. savastanoi* pv. *savastanoi*[36]. On the other hand, previous study has proved that there is no symbiotic relationship with *P. savastanoi* pv. *savastanoi*. It is simple to identify and reject plant components (such as cuttings, buds, etc.) that exhibit knots[36][62]. Allowing plants that have passed inspection to be moved to disease-free zones is a common practice. Phylloplane, latent infections, and *P. savastanoi* pv. *savastanoi* bacterial populations, however, have raised major concerns[46][62].

Many regions where olives are grown are endemic to olive knot disease, but its economic effects are poorly known and are often underestimated[63]. The disease's effects on olive tree production levels are unclear. However, other research has shown that medium infections (0.5-1 knots/ft, or roughly 30 cm of fruitwood) are linked to smaller fruits and 28% lower production than mild infections (0.1-0.3 knots/ft of fruitwood), which has an adverse effect on farm profitability[63][64][65]. Despite the fact that various authors have indicated it, The disease's effects on oil production and quality have not received much attention. However, it's been discovered that green table olives from diseased trees have an unusual flavor (bitter, salty, sour or rancid)[64]. The infection affects on fruit quality and production need to be better defined through additional in-depth research[36].

Due to older knots are frequently invaded by secondary bacteria with rapid growth rates that can stymie the pathogen's ability to establish colonies, it is preferable to isolate at the earliest stages of infection. On nutritional agar or King's medium B surface-dried plates, tiny tissue fragments are scraped off the edges of lesions, comminute in sterile water, and streaked. These useful all-purpose separation media discourage fungal

contamination.. It usually takes pathogenic pseudomonads 2-3 days before small colonies start to form, and they grow more slowly than saprophytic bacteria[40][57].

A presumed indicator of the pathogen's isolation is the emergence of mostly unmixed colonies. When *P. savastanoi* pv. *savastanoi* is derived from several sources such as oleander knots, olive and the phylloplane of sick olive, yellow bacterial colonies typically formed by *Pantoea agglomerans*. This phenomenon is commonly occuring but not always observed[46]. Suppose separation from old cankers or the phylloplane is required, semi-selective medium for *P. savastanoi* pv. *savastanoi* are likely to be effective. ANS-S, PVF-1, and OKA are the three available selective media[66]. Although PVF-1 was formerly thought to be a suitable substrate for the isolation of *P. savastanoi* pv. *savastanoi* from knots and the phylloplane of olive or oleander, recent study has shown that each of these media has limits in terms of selectivity, recovery, or colony morphology[66].

The majority of the time, all that is necessary to determine this pathogen is confirmation of the bacteria from knot symptoms on *P. savastanoi* LOPAT Group 1 hosts[46]. Negative results were obtained for the following assays for *P. savastanoi* pv. *savastanoi*: levan generation, tobacco hypersensitivity reaction, oxidase activity, pectate lyase and polygalacturonase (potato rot) activity, and arginine dihydrolase activity. However, these tests provide a positive result for this organism. When tested for levan (exopolysaccharide) synthesis, several *P. savastanoi* pv. *savastanoi* strains respond well[62]. The reactions in these conclusive tests are the same for *P. syringae* pv. Syringae. Therefore, it can be used to distinguish these pathogen [66].

Pathogenicity tests serve as confirmation. On the stems of olive and oleander plants that are actively developing, bacterial suspensions containing no more than 10,000,000 CFU/ml are injected. According on the plant's response and the environment, Even though virulence assays should continue at least 60 days, swellings at the injection site start to occur 10–20 days after inoculation. Oleander strains (*P. savastanoi* pv. nerii) will knot both oleander and olive plants, but olive strains (*P. savastanoi* pv. *savastanoi*) exclusively induce knots in olive plants[40].

As they come from the same genus, it's quite challengeing to tell the difference between *P. savastanoi* pv. *savastanoi* and *P. savastanoi* pv. nerii. The knot-forming pathogens of Fraxinus (*P. savastanoi* pv. fraxini) may be distinguished from *P. savastanoi* strains found in olive and oleander using changes in the concentration of fatty acids, research has shown. However, it proved difficult to distinguish between pv. *savastanoi* and pv. nerii strains because of the similarities in their fatty acid content. Through the use of molecular techniques like DNA restriction fingerprinting, it may be possible to distinguish between olive and oleander strains [5][14][40].

This has been successfully identified by using molecular techniques, such as DNA restriction fingerprinting, Recent research has produced a nested-PCR detection technique [67][68]. The iaaL as specific primers have been developed to detect *P. savastanoi* pv. *savastanoi* using PCR. Both internal and external primers-IAALF/IAALR and IAALN1/IAALN2—were applied. These bacteria were confirmed to be present as epiphytic and/or endophytic in asymptomatic olive plants utilizing the newly developed nested-PCR approach, using the PVF-1 medium, colorimetric detection, and a pre-enrichment step. The technique may be helpful for epidemiological research as well as programs for selecting and certifying olive plants that are free of *P. savastanoi* pv. *savastanoi*[40][68].

These techniques would only be employed in research studies where it was essential to distinguish between the two strains or where a elevated degree of investigation certainty was required to prop up eradication programs or isolation limitations involving compensation. An ELISA-DAS test was used to determine whether the organism was *P. savastanoi* pv. phaseolicola[69].

The International Seed Federation's 2006 method for identifying *P. savastanoi* pv. phaseolicola is effectively can be derived from bean seed through isolation on a semiselective media. Once the identification of the resultant isolates has been established, ELISA and PCR can be utilized. For the isolation of *P. savastanoi* pv. phaseolicola, Milk Tween Agar (MTA) and Modified Sucrose Peptone Agar (MSPA) are both effective media to be used[70]. When cultivated on MSPA, *P. savastanoi* pv. phaseolicola colonies were described as being pale yellow, convex, and lustrous, when isolated on MTA, it is creamy-white, flat, and round[71]. The TaqMan real-time PCR-

based pathogen identification method has been proposed as a potential tool to rapidly identify *P. savastanoi* pv. phaseolicola[72].

2.8. Prevention and Control

In order to ascertain whether products are legitimately permitted for use in the country when contemplating material control, reference must be made to the list of approved pesticides or the relevant authorities due to the varied restrictions surrounding the (de)registration of pesticides. The use of pesticides must always follow the product label and be legal[12].

2.8.1. Phytosanitary Measures

In European nations, *P. savastanoi* pv. *savastanoi* is a part of the certification process for olives since olive knots can cause serious harm in most olive-growing regions[12]. Planting pathogen-free nursery stock should be used for creating new olive groves from areas and nations where the knot pathogen is non-existent. Oleander bushes nearby (within 500 m) should be removed as a precaution until the host specificity of olive and oleander strains is fully recognized, to eradicate potential sources of harmful bacteria that could otherwise endanger the new grove[73]. According to standard nursery practices, Copper sprays should always be applied to nursery plants. Copper oxychloride or copper hydroxide may be used, as well as the Bordeaux mixture (1 kg copper sulphate; 2 kg lime; 100 liters water). Until it is demonstrated that the application won't result in leaf harm in the local environment, caution is initially suggested[31][73].

An established method of introducing the pathogen to damaged tissue is the technique of harvesting olive branches by pounding them with sticks[73]. Using fabric-wrapped, periodically spaced sticks dipped in a copper solution (like Kocide), especially when traveling between trees, may help to reduce the spread of the disease and illness. The techniques, including pruning, are the most significant control measures. The condition cannot be eliminated entirely from trees or orchards, as has already been mentioned, although careful pruning might lessen its worst effects (Young, J. M. 2004) [2][74].

The effective way to reduce the damage is to consider the growth of the tree during its formative years. To prevent the time of spring susceptibility, yearly pruning should be finished before the late winter [74].Young trees should only have healthy branches left after pruning to serve as the mature tree's support structure. A guide for removing particular weaker branches should come from an individual assessment. Young sick trees do not necessarily need to be removed unless they are extraordinarily infected and have little chance of developing into fruit-bearing tree. Avoid pruning before a predicted rainstorm [74].The base of the traditional truncated cone with a sharp upturn can be changed to upright truncated cones with a downwardly tapering base to enhance the symmetry of the crowns of very old trees that have been severely injured by followed by bacterial knot infection from hail [74].

2.8.2. Chemical Control

Spraying of Chemicals Efficacy tests of copper spray compounds has been documented in several papers (e.g. copper hydroxide). They don't always work, and there's little to no chance they will be cost-effective[23]. Sprays is essentially to be used consistently to continue providing year-round bactericidal protection while avoiding harm from phytotoxic copper spray levels. It's challenging and possibly not economical to do this. Recent research by Lavermicocca, P., et al. 2002 suggests the potential for employing a *Pseudomonas syringae* pv. ciccaronei bacteriocin to manage olive knot. The defense against infection niches and lowering the solidity of *P. savastanoi* pv. *savastanoi* on the phylloplane are two things this bacterium's bacteriocin preparations are good at[64].

2.9. Polymerase Chain Reaction (PCR)

Whether DNA is a component of the genome or a byproduct of the process, the Polymerase Chain Reaction (PCR) involves the amplification of DNA. Taq DNA polymerase, which is thermally stable and has two par primers, is used to carry out this operation with a DNA template containing tens of thousands of nucleotides. In addition to buffer solution (dNTPs), it is a component made up of many nucleotides [41] . Using this method, high- and niche-sensitive microorganisms are found and controlled. In order to find specific pathogens in environmental samples, it is a quantity and analytical tool. The microbiological quality of food and water is evaluated using this method [75].

Kerry Mullis (PCR) technology was first introduced to the globe in 1983. These were the biotechnology disciplines' swift advancements[76]. The absence of reliance on the biological system, along with the ability to regulate the quantity of DNA and the rate of production, is the main factor in this technique's success[76].. Due to their high accuracy and sensitivity, PCR is a molecular diagnostic method that is crucial in the diagnosis of bacteria and the identification of resistant strains and mutants, as well as giving preference to the diagnosis and detection of genes resistant to antibiotics or responsible for infection and spreading prevention[77]. The current DNA strand, which is complementary to the desired template strand, is created using this method's primary mediated enzymatic DNA amplification. Due to the elevated sensitivity and specificity of many targeted genes, such as ecfX, oprL, and gyrB, Ps. aeruginosa has been identified on clinical samples using these genes. According to[77][78].

There are enough time to distinguish between bacterial organisms and learn about their antibiotic resistance thanks to the (PCR) test, which is based on the inspection and evaluation of the quality of bacterial strains[79]. There are numerous pathogenic isolates in microbiology, which are a vital substitute for conventional methods of classification like an antibiotic and other traits, which have grown ineffective at separating bacterial isolates, especially those close to one another and require time and effort to accomplish. Due to its affordability and capacity to manage many models, the (PCR) technique has been widely employed in experimental and practical research[80][81].

2.10. Real-time PCR (RT-PCR)

The DNA amplification mechanism behind the Real-Time PCR methodology is the same as that of conventional PCR methods, although it differs from them in several ways[82]. Electrolytes were utilized in traditional PCR to measure PCR DNA amplification during the reaction's final stage. Because of its high sensitivity, high productivity, greater specialization, and reliance on the exponential phase, real-time PCR can measure amplification in the early stages of the reaction. This method can also measure quantity accurately and quantitatively because it depends on the exponential phase[83].

With different yet distinct procedures, real-time PCR and qPCR are sometimes confounded. Reverse transcriptase is used to clone an RNA DNA strand, and cDNA divides the reaction to do RT-PCR, which is used to determine gene expression[83][84]. The reverse transcriptase enzyme and dNTPs form the first tape's reaction's nucleus, the first instance of the mRNA being copied into DNA. The second phase, polymerization, necessitates DNA and polymerase and is followed by the conversion of the single-strand cDNA into a double bar and then amplified using the RNAase H enzyme. The RNAase H enzyme encapsulates the RNA molecules and so decomposes the RNA from the RNA-cDNA (Mackay, A., et al. 2007)[84].

qPCR and PCR reverse transcriptase were coupled to create a method known as QRT-PCR or RT-qPCR that was used to evaluate the quality of gene expression[85]. After each cycle, the DNA will grow in number and the length of the DNA cassettes. Due to the SYBR Green dye's complex molecular composition and association with DNA molecules, more light is released. This method has a drawback in that the dye binds to all dsDNA double tape templates, including undesired Primer-dimers, in the solution. If the emitter is nearby, the receiver quencher will absorb the energy it emits[13]. Due to the divergence between the emitter and the receiver, which prevents the receiver from absorbing the energy emitted, the light emitted is detected when the polymerase enzyme separates the fluorescent reporter from the end 5' and the receiver quencher at the end 3'[86].

CHAPTER III

METHODOLOGY

3.1. Materials and Apparatus

All chemicals used were ABIOpureTM Total DNA (ABIOpure, USA), absolute ethanol (Bioneer, Korea), Agarose (Bio Basic INC, Canada), Crystal violet stain 1% (Promega, USA), Ethanol 70% (BHD, England), Glyserol (BDH, England), GoTag qPCR Master Mix, Nuclease-Free Water, QuantifierdsDNA System (Promega, USA), Iodine, Aceto and Safranin (Institut of sera and vaccine, Iraq), Kovacs reagent (Vac and sealant, Iraq), methyl red (Fluka, Switzerland), N,N,N,N(tetramethyl-p- phenylenediamine dihydrochloride (Difco, England), normal saline (Schuchard, German) and Primers (Macrogen, Korea). Instruments and major apparatus were used in this study including autoclave (Hirayama, Japan), balance (Kevn, Jordan), centrifuge (Fisher Scientific, USA), Compound Light Microscope (Olympus, Japan), cooler incubator (Binder, England), distillatory (G.F.L., Germany), electric oven (Binder, U.K.), MIC qPCR Cycler (BioMolecular, Australia), Quantus Fluorometer (Promega, USA), refrigerator (Fiocchetti, Italia), Vitek2 (Bio mexieux, France), Vortex (Biocote, U.K.), and water bath (Gallenkamp, England). For culture media several sources were used including Blood agar base, Brain-Heart infusion broth, Cetrimide agar, MacConkey agar, Methyl red-Voges-Proskauer broth, Muller-Hinton agar, Nutrient agar which all were purchased from Oxoid (England) and Cetrimide agar that was purchased from Himedia (India).

3.2. Experimental Procedure

3.2.1. Reagent preparation

3.2.1.1. Catalase Reagent

Bacterial isolates were tested for their capacity to generate catalase using 3% hydrogen peroxide in a dark container as the test solution (Tadesse and Alem., et al. 2006).

3.2.1.2. Oxidase Reagent

The reagent was made by dissolving 0.1 g of N-N-N-N- Tetra methyl para-phenylene diamine dihydrochloride in 10 mL of distilled water in a dark vial. It was then used to test bacteria for their propensity to produce oxidase [87].

3.2.1.3. Gram Stain Crystal Violet

Crystal violet stain for 0.1 g was dissolved in 10ml of distilled water to create this stain. Millipore filters were used to filter 0.1 g of Crystal violet added to 10 ml of distilled water. The solution was stored at 4^0C until bacterial isolates used [88].

3.2.2. Culture Media Preparation

3.2.2.1. Blood Agar

Agar base was prepared and dissolved followed the company's instructions. It was then sterilized and was let to cool down to 50^0C. The blood was mixed in a 7% concentration and plates were allowed to harden in the hood [89].

3.2.2.2. MacConkey Agar Medium

Both the lactose fermentation test and the detection of Gram-negative bacteria were performed using this media, which was prepared in accordance with directions provided by the Himedia firm [89].

3.2.2.3. Nutrient broth

It was made in accordance with the company's processed instructions (Oxoid) for the development and preservation of the isolates [89].

3.2.2.4. Agar medium of Mueller-Hinton

This media was made according to the instructions of the business equipped (Rashmi) and used to examine the antibiotic sensitivity of microorganisms [89].

3.2.2.5. Cetrimide Agar

45.3 grams of Cetrimide agar medium was dissolved in 1000ml of distilled water and an autoclave was use to sterilize with the temperature of 121°C for 1.5br to 15min. It was then cooled to 45^0C before adding Nalidixic acid of 15mg. Mix with sterile dishes, allow to solidify, then use for bacteria isolation and diagnosis [89].

3.2.2.6. Brain heart infusion broth

The Brain heart infusion broth was performed based on the company's instructions (Rashmi) and utilized for the activation and preservation of bacteria isolate [89].

3.2.2.7. Voges-Proskauer reagent

It was prepared up of two solutions:

Solution 1 was made by dissolving 5 gram of naphthol in 100ml of pure ethyl alcohol.

Solution 2 was prepared by making KOH solution (potassium hydroxide), dissolve 40gm of this ingredient in 90ml of distilled water. This is used to detect glucose fermentation and bacterial isolate generation by acetone [89].

3.2.2.8. Urea Agar Medium

95 mL distilled water was used to dissolve agar with 2.4 g of basic Urea. Continue with the medium that was sterilized in an autoclave and allowed to cool down up to 45°C. Using 0.45 µm fine filters and piping medium, add 5 ml of sterile urea solution at 40% concentration. It was then cut diagonally and stored at 4 ° C as it was used to test bacteria's susceptibility to urease production. Forbes and colleagues [89].

3.2.2.9. Indole Test Medium

20 g of peptone combined with 5 g of sodium chloride was dissolved in 20 mL of distilled water, then the amount increased to 1 liter and sterilizedwith autoclave. Then distributed into tubes and keept at 4 °C. By isolating microorganisms, you may analyze nature's production.

3.2.2.10. Vitek 2

This system, supplied by Biomerieuy USA, was employed as a confirmatory diagnostic test after all traditional procedures, including culture characteristics and biochemical assays, had been used.

3.2.3. Sterilization method

3.2.3.1. Autoclaving

All autoclavable culture mediums and solutions were in an autoclave for sterilization at the temperature of 121°C and 1.5 br for 15 minutes.

3.2.3.2. Dry sterilization

For 2 hours, every glass was sterilized in an oven set to 160-180 ºC.

3.2.3.3. Sterilization by filtration

All solutions that were heated by high temperatures were sterilized using filtering, 0.4 μm millipore filters.

3.2.4. Sample collection

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 around the city of Duhok in the Northern Iraq region. Every knot was handled separately. Young knots were cleaned with rushing water to get soil off of them and dust particles are cleaned by dipping them for two to three minutes in 25% (v/v) sodium hypochlorite. It continuously with disinfected after being dried on sterile filter paper and rinsing with sterile distilled water (SDW) with ethanol-soaked cotton. Low portions which was 5-10 mm) of every knot were sterilized slicely using a sterilized scalpel. For 10 minutes, they

were placed in a sterile Eppendorf tube with 1 ml of sterile saline (085%NaCl). On plates containing PVF-1 agar and King's medium B (KB), tenfold serial dilutions were applied, and the plates were cultured at 26°C for three days. Re-streaked onto fresh KB 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 KB slants and kept at 4°C in 20% glycerol at -80°C pending further identification. Two dangerous *P. savastanoi* isolates were produced from olive trees in the same area and a reference isolate (Hav-ran2b) used in an earlier study (Mirik and Aysan 2011) also offered for a basis of comparison.

3.2.5. Bacterial Isolated Identification

3.2.5.1. Morphological examination

Cetrimide agar swabs were cultured. The isolate was identified based on culture features, biochemical tests, and microscopic appearance following gram staining [90].

3.2.5.2. Microscope examination

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

3.2.5.3. Biochemical test

P. Savastanoi was diagnosed using the following biochemical tests:

1. Catalase test

Some part of the bacterial plant was moved to a clean slide of glass 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 [91].

2. 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 were added of oxidase detector over the filter paper, the appearance of dark violet signals a positive examination [91].

3. Indol Production Test

The peptone water medium was cleaned with a bacterial colony and incubated at 37C° for 24 hours before adding 5 drops of COFACS to the wall reagent and mixing as the emergence of a red ring signals a positive test and the ability of the bacteria to manufacture the tryptophan amino acid [91].

4. Methyl red test (MR)

Bacteria suspension was added to Medium MR-VP and incubated at 37C° for 24 hours, then 3-4 drops of red-haired reagent were gently added to the medium. The color turned red within two minutes of positive tests. This means that the bacteria have the ability to ferment glucose and acid generation [92].

5. Kliglar iron agar test

This medium's tubes were injected on the slanting surface and poked in the bottom, and at 37°C for 24 hours, all tubes were incubated. The presence of yellow color shows the fermentation of sugars, and the appearance of black color at the bottom indicates the susceptibility of bacteria to H_2S gas production, indicating a positive result [92].

3.2.6. Bacterial Isolates Preservation

3.2.6.1. Preservation of Short Term

Pseudomonas savastanoi pv. *savastanoi* bacterial isolation was cleared on the nutritive medium of the slanted surface and then incubated the tubing for 24 hours, recorded at 12C°, and saved at 4C°. Month after month, the uncertainty persisted [93].

3.2.6.2. Long Term Preservation

In addition to glycerol and 15% autoclave, brain-infusion broth was used. After incubating the tubes containing 5 ml of the bacterial implant at 37°C for 24 hours, they were frozen at -20°C [54].

3.2.7. Determination of *Ps. savastanoi* using the Vitek-2 compact system

We used a device Vitek-2 compact system to determine the type of bacteria using the card to identify the types of bacteria, which contains almost special holes that contain biochemical reactions that occur inside the card and after 24 hours the result of these chemical reactions we can determine the type of bacteria [94].

The Working Method:

1. All bacterial isolates were disseminated on nutrient agar and cultured for 24 hours at 37^0 C.
2. The Kan tube was used to test the sensitivity and add 3 mL of standard saline solution before removing the Loop from a single colony in the Kan tube. For the work of the tubes for sensitivity testing and measuring turbidity using the VITEK Densichek device, as well as suspension turbidity (0.63-0.50)
3. The Card transfer tube unit (AST Card) was inserted into the Kan tube and secured to a specific holder.
4. To transfer the sample from the Kan tube to the AST Card, the sample was inserted into the filling door. After that, the samples are manually transported to the loading door and incubated at 37 ° C. The antibiotic sensitivity test results are provided between 3.2.8 The genotype content of bacterial isolates.
5. The BioMerieux instructions were followed and the antibiotic sensitivity test result for each AST card identified in the incubator was printed.

3.2.8. Molecular Study of the Genotype Content of Bacterial Isolates

3.2.8.1. Primers Solution

The solutions were prepared as instructed by the company (Korea) Bioneer to the Primers. To obtain a concentration of 100 µl, each primer solution was separately applied at a concentration of 10 µl by taking 10 µl of each stock buffer solution and adding 90 µl . 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.

Table 3.1. Primers and properties used in the study

Source	**Temperature of Annealing (°C)**	**Primer Sequence (5-3)**	**Gene**
Primers	60	5`-GACACTGAGGTGCGAAAGCG-3` R	16S rRNA
		5`-GACACTGAGGTGCGAAAGCG-3` F	

3.2.8.2. Extraction of genomic DNA

The following steps were taken to isolate genomic DNA from bacterial growth:

1. Overnight culture was applied to 1 ml of pellet calls for 2 minutes at 13000 rpm. After that, the supernatant is discarded.
2. For gram-positive bacteria, 100 µl Nuclease-free water plus 100 µl the pellet was mixed with lysozyme solution and vortexed.
3. Then it was incubated for 30 minutes at the temperature of 37 °C in water bath.
4. It continued with centrifugation of samples for 2 minutes with the speed of 13000 rpm to eliminate the supernatant.
5. The digestion of cell lysis and protein, 20 mg/mL of Proteinase K solution was used and 200µl of Buffer BL were added to the sample, then the tube was forcefully agitated with a vortex meanwhile incubating at the temperature of 56°C for 30 minutes, then at 70 °C for 30 minutes.
6. To fully combine the substance, 200µl of 100% ethanol were added to the sample.

7. After carefully transferring each combination to the mini-column, the mixtures were centrifuged for one minute at 6,000 x g above (more than 8,000 rpm), and the collecting tube was replaced with a new one.
8. Buffer BW 600 µl was added to the mini-column, which was then centrifuged for one minute at 6,000 x g above (more than 8,000 rpm) using a new collecting tube.
9. Buffer TW 700µl was utilized, followed by 1 minute of centrifugation at 6,000 x g above (more than 8,000 rpm). The mini-column was put back into the collection tube after the pass-through was taken out.
10. To get rid of any leftover wash buffer, the mini-column was centrifuged at its highest setting (>13,000 x g) for one minute. It was then deposited in a fresh 1.5 ml tube.
11. 100µl of Buffer AE was added, and the mixture was then incubated for 1 minute at room temperature before being centrifuged for 5 minutes at 5,000 rpm.

3.2.8.3. DNA quantitation

The concentration of extracted DNA was measured using a Quantus Fluorometer to assess the quality of samples for subsequent applications. 1 µl of DNA was combined with 199 µl of diluted Quantity Flour dye. DNA concentrations were measured after 5 minutes of incubation at room temperature.

3.2.8.4. Setup of Reaction and Thermal Cycling

Table 3.2. 16S r RNA gene with number of PCR cycles

Gene: 16S r RNA gene				
No. of Reaction	12	RXN	No. of PCR Cycles	60
Reaction Volume /run	10	µl		
Safety Margin	5	%		

Table 3.3. Materials and quantities utilized in the genetic research

Master mix components	Stock	Unit	Final	Unit	Volume	
					1 sample	3 Sample
Master Mix	2	X	L	x	5	30.0
Forward primer	10	µM	L	µM	0.5	3.05
Reverse primer	10	µM	L	µM	0.5	3.075
Nuclease-Free Water					3	9.3
DNA	10	ng/µL	10	ng/µl	1	
Total volume					10	
Aliquot per single reaction	9 µl of Master mix per tube and add 1 µL of template					

Table 3.4. Thermal Cycling program for 16S rRNA region

Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00.30	60
Annealing	55	00.30	
Extension	72	00.30	1

CHAPTER IV

RESULTS AND DISCUSSION

4.1. Sample Collection

The samples were acquired at random by collecting young soft knots with smoother surfaces on stems and branches of pomegranate trees in main growing zones between 2021 and 2022. Three olive isolates were identified from fresh olive knots gathered from orchards around the city of Duhok in the Northern Iraq region. Separate approaches were taken to each knot. According to the following diagram

Figure 4.1. Organism-infected plant tissue by *Pseudomonas savastanoi* pv. *savastanoi*

Young knots were first cleaned in running water to remove any soil or dust that had adhered to it. The surface was then disinfected by dipping it 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 that had been 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(KB) (King et al. 1954) and PVF-1 agar (Surico and La-

vermicocca 1989), tenfold serial dilutions were applied, and the plates were cultured at 26°C for three days.On fresh KB and PVF-1 plates, single fluorescent or nonfluorescent representative colonies of the most common morphological kinds of bacterial isolates were streaked. These plates were then incubated at 26°C for three days. The isolated pure single colonies were cultivated on KB slants, then kept at 4°C in 20% glycerol at 80°C pending further identification. For comparison, two virulent Psvisolates (HZP2 and Hav-ran2b), which were isolated from olive trees growing in the same area, and a reference isolate (Mirik and Aysan 2011) were also added.4.2 Identification of bacterial isolates.

4.2. Microbial Identification

4.2.1. Morphological Investigation

As shown in the following picture, bacteria colonies form when the bacteria swab from the infected plant tissue is cultured on the nutrient medium of the bacteria *Pseudomonas savastanoi* pv. *savastanoi* and kept in the incubator at 20° for a period of 3–7 days.

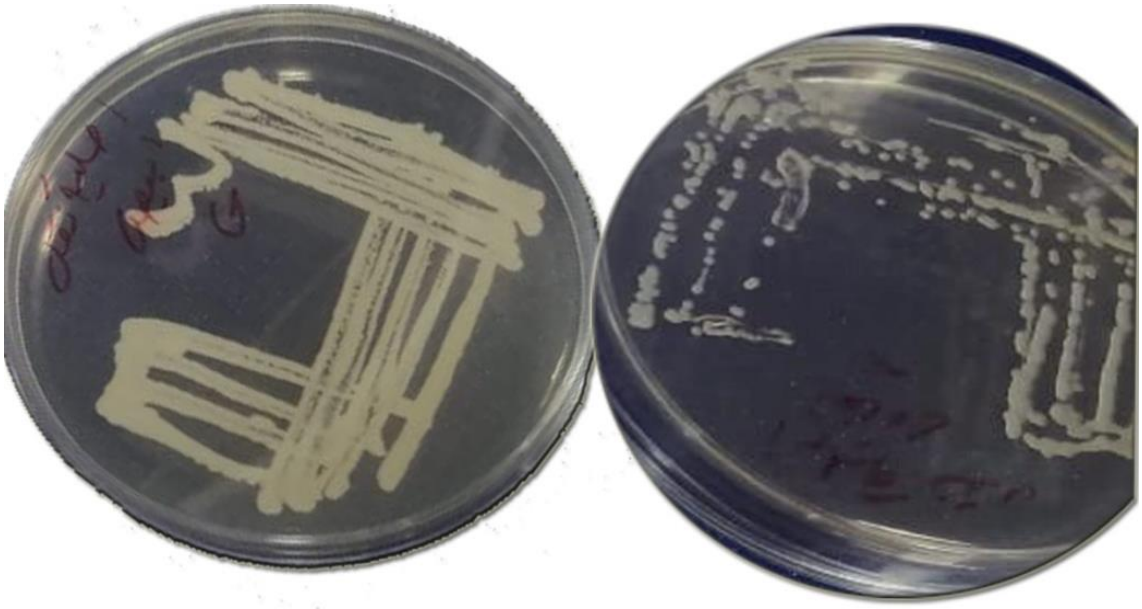


Figure 4.2. Demonstrating the hue and development of bacterial colonies on the bacteria-containing culture media. pv. *savastanoi*, also known as *Pseudomonas*

4.2.2. Microscope Investigation

After removing the bacteria colonies from the media and using Gram stain to stain them, which revealed that the bacteria were negative for Gram stain, we looked at the bacteria under a microscope to determine the types of bacterium cells that were present.

4.2.3. Biochemical Tests

The colonies of bacteria were subjected to biochemical examinations to determine the characteristics of the bacteria, including the Catalase, Oxidase, Indol generation, Methyl red (MR), and Kliglar iron agar tests. The outcomes are shown in the following table

Table 4.1. Different physical and chemical tests used to identify *Pseudomonas savastanoi* pv. *savastanoi* and how bacteria respond to these tests

Test	**Results**	**Test**	**Results**
Gram stain	Negative	Urease	Positive \ Negative
Shape cells	Rods shape	Indole	Negative
Blood agar	β –hemolysis	Methyle red	Negative
Cetrimide agar	Positive	Voges proskauer	Negative
MacConkey agar	Non Lactose fermented	Citrate utilization	Positive
Oxidase	Positive	Kligler iron agar	K\K
Catalase	Positive	Growth at 4C°	Negative

4.3. The Vitek-2 Compact System

In order to recognize bacterial patterns, use the Vitek- 2 compact system. Because only bacteria pathogenic to people are neutralized by the Vitek-2 compact system, no results were produced in this device when bacteria pathogenic to plants were tested.

4.4. Bacterial Infection of Fruits

The bacteria are inoculated by the cells to a group of fruits in order to determine whether the bacteria infect the fruits. All of the following outcomes were obtained.

4.4.1. 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 lemon fruits did not become infected by the bacteria cells, as indicated in the following figure.

Figure 4.3. Shows the fruits of lemon when exposed to bacteria and the absence of infection

4.4.2. Infection of Bacteria on Carrot Fruits

Based to the results up to the ninth day, no illness symptoms had 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.

Figure 4.4. Demonstrating sliced carrots following bacterial inoculation *Pseudomonas savastanoi* pv. *savastanoi*

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

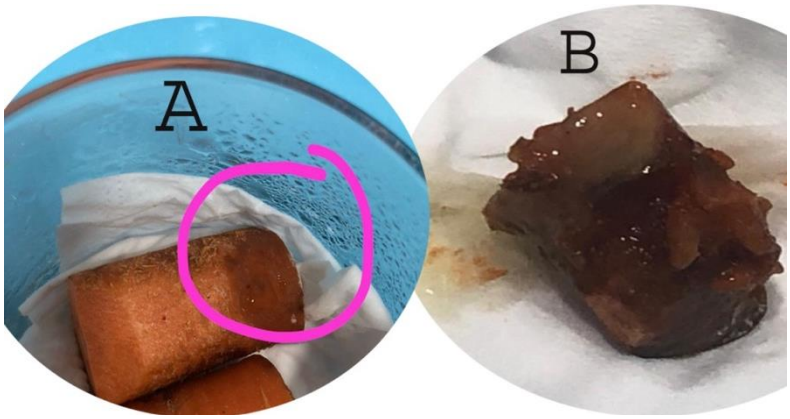


Figure 4.5. (A) Using the bacteria *Pseudomonas savastanoi* pv. *savastanoi*, carrots were sliced after a 13-day inoculation (A). Following a 20-day *Pseudomonas savastanoi* pv. *savastanoi* inoculation, carrots are shown chopped in (B).

4.4.3. Infection of Bacteria on Apples Local and Imported Apples Fruits

Despite exposing two different types of apple fruit 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.

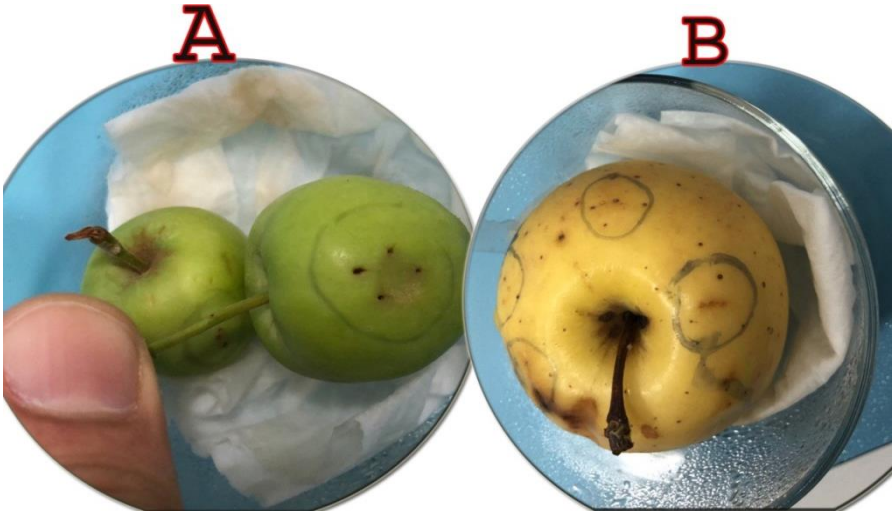


Figure 4.6. (A) displaying an apple from local Iraq after 20 days of *Pseudomonas savastanoi* pv. *savastanoi* inoculation. (B) displaying an apple that was brought into Iraq from outside the country after being exposed for 20 days to the bacteria *Pseudomonas savastanoi* pv. *savastanoi*.

4.4.4. Bacterial Contamination. Bacterial Contamination of Beans.

As can be 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.

Figure 4.7. *Pseudomonas savastanoi* pv. *savastanoi* bacteria have been injected into bean fruits, causing an infection.

4.5. Molecular Study

4.5.1. DNA from *Pseudomonas savastanoi* pv. *savastanoi* isolates was extracted, and the purity and concentration of the DNA were determined

Three samples of bacteria that had been obtained following fruit infection with carrots were each given a DNA extraction. In bacterial isolates, the DNA concentration was examined. According to the findings, isolate PS. V3 had a concentration of 15 ng/l while isolate PS. V2 had a value of 26 ng/l. in accordance with table (4.2).

Table 4.2. Shows the concentration DNA of three bacterial isolates

Number of isolates	Concentration (ng/µl)
PS. V1	20
PS. V2	22
PS. V3	15

4.5.2. The use of PCR and RT PCR to Detect the Presence of Certain Genes in *Pseudomonas savastanoi* pv. *savastanoi*.

One of the most significant sources of injury in the orchards and fields around the world is *Pseudomonas savastanoi* pv. *savastanoi* strains, many of which are pathogenic FOR plant such as olive and carrot. When using the PCR& RT PCR gene test, the results revealed that the bacteria to which the carrot fruit was exposed is a bacterium *Pseudomonas savastanoi* pv. *savastanoi*, as illustrated in the Figures 4.8, 4.9 and Table. 4.3.

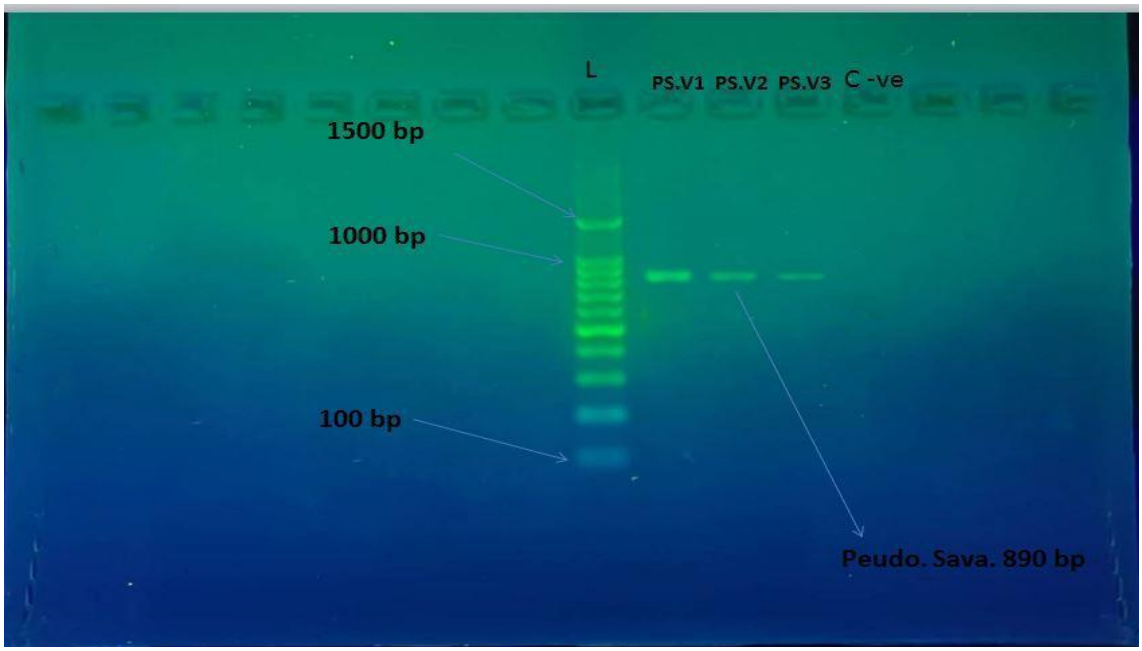


Figure 4.8. Gel electrophoresis result of 16S rRNA region .

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

Using RT-qPCR, the ratios of the 16S ribosomal RNA gene to the housekeeping gene were determined. After the real-time PCR runs were finished, 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 4.9

Table 4.3. Results of RT-PCR for *16S ribosomal RNA* gene in isolates.

Well	Color	Sample Name	Cq	Efficiency	Efficiency R²	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

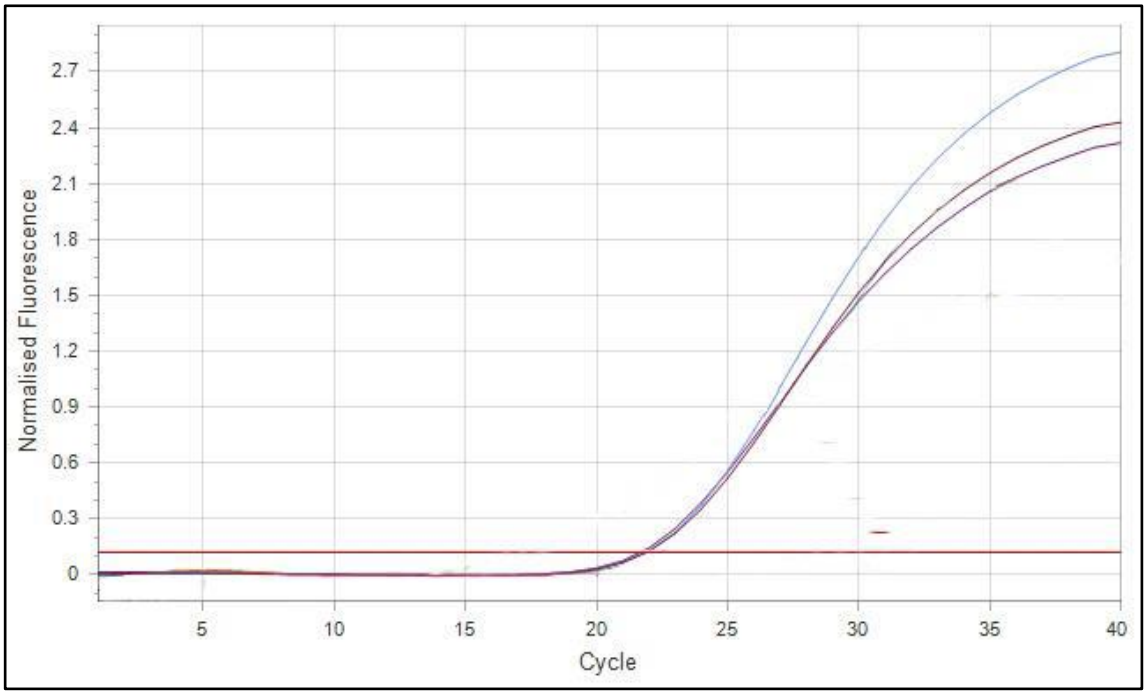
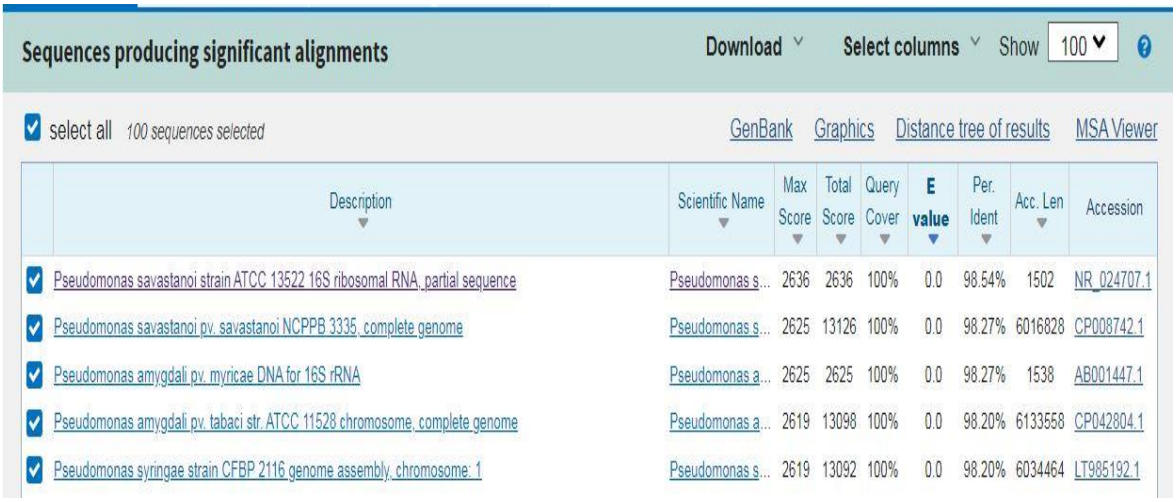


Figure 4.9. Results of RT-PCR for *16S ribosomal RNA* gene region in isolates

4.5.3. 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. And 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.



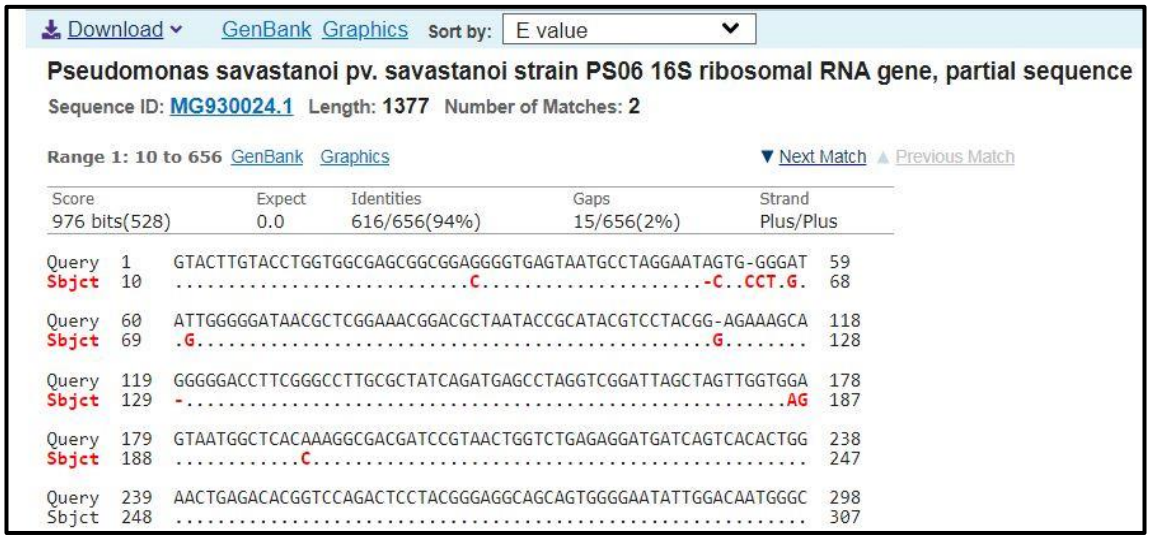
Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas savastanoi strain ATCC 13522 16S ribosomal RNA, partial sequence	Pseudomonas s...	2636	2636	100%	0.0	98.54%	1502	NR_024707.1
Pseudomonas savastanoi pv. savastanoi NCPPB 3335, complete genome	Pseudomonas s...	2625	13126	100%	0.0	98.27%	6016828	CP008742.1
Pseudomonas amygdali pv. myricae DNA for 16S rRNA	Pseudomonas a...	2625	2625	100%	0.0	98.27%	1538	AB001447.1
Pseudomonas amygdali pv. tabaci str. ATCC 11528 chromosome, complete genome	Pseudomonas a...	2619	13098	100%	0.0	98.20%	6133558	CP042804.1
Pseudomonas syringae strain CFBP 2116 genome assembly, chromosome: 1	Pseudomonas s...	2619	13092	100%	0.0	98.20%	6034464	LT985192.1

Figure 4.10. Results of sequencing *Pseudomonas savastanoi* pv. *savastanoi*'s *16S ribosomal* RNA gene

It was discovered that mutations occur in some sections of the gene by the use of the BLAST® site Search and the use of gene sequencing.



Download — GenBank Graphics — Sort by: E value

Pseudomonas savastanoi pv. savastanoi strain PS06 16S ribosomal RNA gene, partial sequence

Sequence ID: MG930024.1 Length: 1377 Number of Matches: 2

Range 1: 10 to 656 GenBank Graphics — Next Match — Previous Match

Score	Expect	Identities	Gaps	Strand
976 bits(528)	0.0	616/656(94%)	15/656(2%)	Plus/Plus

```
Query  1    GTACTTGTACCTGGTGGCGAGCGGCGGAGGGGTGAGTAATGCCTAGGAATAGTG-GGGAT  59
Sbjct  10   ............................C.....................-C..CCT.G.  68

Query  60   ATTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCCTACGG-AGAAAGCA  118
Sbjct  69   .G.................................................G........  128

Query  119  GGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGA  178
Sbjct  129  -.....................................................AG  187

Query  179  GTAATGGCTCACAAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGG  238
Sbjct  188  ............C.............................................  247

Query  239  AACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGC  298
Sbjct  248  ............................................................  307
```

Figure 4.11. Analysis of the *Pseudomonas savastanoi* pv. *savastanoi* 16S ribosomal RNA gene's alignment with the NCBI Gene Bank. Sample is represented by Query, while a 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>AG, 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* pv. *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. With *Pseudomonas savastanoi* pv. *savastanoi* isolates and keeping them in the same environmental conditions of heat and humidity, no symptoms appeared until 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 noticeably and clearly and was in the form of a mucous patch the size of a carrot piece, and these results are consistent with what was stated in the literature.

(Penyalver 2000)[40] was able to isolate *Pseudomonas savastanoi* pv. *savastanoi* bacteria from different 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, where the isolated bacteria were diagnosed by biochemical and physiological tests, and the results confirmed that the infection was by bacteria *Pseudomonas savastanoi*. In the province of Hatay and Turkey, *Pseudomonas savastanoi* pv. *savastanoi* bacteria is one of the important problems that affect olive, pomegranate, myrtle and jasmine plants. In severe infection cases, the bacterial infection causes the death of young seedlings. To confirm the infection with *Pseudomonas savastanoi* pv. *savastanoi* bacteria for the mentioned plants, diagnostic tests take a long time, which has been used in the development of the carrot plant. A quick 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 *Pseudomonas savastanoi* pv. *savastanoi* bacteria. Swabs were taken from the mentioned and infected

plants and carrot slices were inoculated from the isolated swabs, and the growth of the first typical node was observed within a week and after 14 days

Fortunately, the infection became clear, and 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 was inoculated.

Gemlik cultivar, where a clear nodule growth was observed at the site of the stabbing two months after the operation, and for the diagnosis process, bacterial isolates were taken from carrot slices and their identity was confirmed as *Pseudomonas savastanoi* pv. *savastanoi*. using Sequencing, PCR & RT-PCR tests and molecular methods, and the results showed that the carrot slice method is a very simple and rapid technique for testing the pathogenicity of *Pseudomonas savastanoi* pv. *savastanoi*, and this technique can be described as sensitive and fast as it gives correct results within 14 days (Filiz 2020)[95]

In the Marche city in central Italy, olive trees spread in abundance, and in the recent period, an increase in bacterial infections was observed, especially at the end of the winter, when there was a lot of frost, which prompted researchers to investigate this noticeable increase in injuries, as ten varieties of olives known locally were selected and studied under field conditions in 6 different orchards in the Marche region, and in all orchards the incidence of olive knot was Its severity is positively related to the damaged organs due to frost, which leads to the penetration of this bacteria and thus all varieties were damaged by frost in late winter and the symptoms of olive knot disease appeared after 6 months, and the infection was confirmed after conducting diagnostic tests, which confirmed that all isolated infections were caused by *Pseudomonas savastanoi* pv. *savastanoi* bacteria, and this indicates that this bacteria can be more virulent to damaged (wounded) trees, and this damage is not necessarily due to frost, but even tree pruning can prepare An ideal condition to infect trees with this bacteria (Valverde 2020)[96].

This is one of the things that must be taken into consideration for those interested in such a study, as the growth of bacteria nodes in olive trees was observed, the focus of the study, after being stabbed with isolates taken from carrot strips, which led to the growth of this species of bacteria significantly, which is consistent with the study

conducted in the Marche region. It is also consistent with what was confirmed in the Mediterranean basin, where olive cultivation is widespread and infected with a type of bacteria, which necessitated a diagnostic study of the harmful bacteria that infect these trees, especially newly pruned trees. of *Pseudomonas savastanoi* pv. *savastanoi* type, and to confirm the diagnosis, laboratory inoculation was carried out on oleander and oak plants, and after the infection appeared on the taken plants, the smears were isolated and diagnosed, and the diagnosis confirmed that the bacteria are the same as *Pseudomonas savastanoi* pv. *savastanoi* that the test The used in the diagnosis was End Point and Real-Time PCR (Tegli 2010)[14]. Through the study conducted, it was found that the bean plant is not easily infected with this bacteria, because the effect of salicylic acid, which is the main hormonal regulator of the immune system in the plant, which has an antibacterial effect Especially *Pseudomonas savastanoi* pv. *savastanoi*, the bacterial pathogen of beans and bean, where tests confirmed a decrease in gene production and an increase in cellular methylglyoxal concentrations after treating an infected plant with salicylic acid, culture assays showed that salicylic acid alters bacterial growth curves more than other benzoic acid isomers. These data revealed that salicylic acid is an antibiotic and that *Pseudomonas savastanoi* pv. *savastanoi* bacteria significantly alter its protein.

In response to salicylic acid in vitro similar changes occur in the bacterial protein in Beans during a complex immune reaction when salicylic acid increases at the site of infection and so in beans where salicylic acid prevents bacterial infection by altering the bacterial protein in a way reverse.

(Halimi 2019, Cooper 2022.)[97][98] The negative results of the growth of *Pseudomonas savastanoi* pv. *savastanoi* bacteria were not limited to the bean plant, but the fruits of lemon and apple had the same results, as laboratory tests conducted on these fruits showed the resistance of the fruits to the growth of *Pseudomonas savastanoi* pv. *savastanoi* bacteria, and we note that the anti-effect of these fruits was higher than what is It is found in the olive plant, and this is one of the promising things in the biological control of plant diseases

As (Michavila 2017, Mikiciński 2016)[99][100] was able to observe a biological activity by lemon and apple trees to control bacterial pathogens that infect their above-

ground organs, and the ability of plants to resist bacterial infections through the production of lipopolysaccharide, which in turn works to stimulate defenses against The wilting of these trees after microbial infection (Mina 2020, Patel 2020)[43][101] and through the results that were presented, we note a great agreement between the current study and what has been studied by many researchers. One of the things that called for a study on olive knot disease, as well as a study of its behavior

The growth of this bacteria on more than one plant is the virulence of this bacteria and the speed of its spread on plants, especially olives, and in many areas that have not previously recorded any infections with this disease, where we notice in Nepal and between 2006-2008, an examination of plant bacteria was conducted on olive plants in commercial olive groves Makanpur area, where it was observed the formation of knots with a diameter of (0.5 - 3 cm) and these knots were observed on the branches in olive trees that are 12 years old.

Then, a laboratory study was conducted in the greenhouses, where 10 olive trees of two years old were injected and injected by puncturing two sites for each needle tree under the skin with a bacterial suspension containing (108 colony-forming units per ml), and the characteristic knot symptoms were observed on all inoculated plants. Within six months after inoculation, bacterial cultures with characteristics similar to the original strain used for inoculation were re-isolated.

Of all the olive knots that have been developed. Molecular identification was achieved by 16S rRNA sequencing and the results were 100% identical to similar sequences for *Pseudomonas savastanoi* pv. *savastanoi,* which is available in genetic databases, and this is the first record of olive knot disease on olive trees (Olea europea) in Nepal (Balestra 2009)[102]. During the field surveys conducted in 2007 in the areas of Syrian olive cultivation

The main symptoms of bacterial *streptococcus* were observed on olive branches, and the highest percentage (70%) was in the coastal region (Lattakia and Tartous). Ten representative bacterial strains were subjected to identification tests and all strains were identical to the results of identification tests for *Pseudomonas savastanoi* pv. *savastanoi* bacteria and to ensure work was inoculated. One-year-old olive plants (Nabali and Glot

cultivars) by introducing bacterial suspensions of 108 colonies/ml) into the wounds made in the bark with a sterile scalpel and then re-isolation of bacteria with similar characteristics to the original strains of the inoculated plants based on morphological, biochemical, physiological and pathogenesis tests As well as the molecular analyzes, it was concluded that the Syrian strains belong to *Pseudomonas savastanoi* pv. *savastanoi* and this is believed to be the first reliable report on the symptoms of olive knot disease in Syria caused by *Pseudomonas savastanoi* pv. *savastanoi* (Alabdalla 2009)[103]

Olive knots caused by pathogens or pathogens are described within the group Bacterial pathogens currently known as *Pseudomonas savastanoi* pv. *savastanoi*, the ecology, transmission and methods of controlling pathogens are being discussed, and strategies are recommended to reduce the effects of infection and these depend on attention to specific details in pruning programmers, irrigation and fertilizer application. There is no cost-effective way to control the knot in olive orchards using currently available sprays of germicides, the use of known disease-resistant varieties and future breeding programs to improve resistance will lead to effective control on the. The long term pathogen distribution in Australia is now being re-examined in light of recent investigations of host privacy In Turkey, bacterial colonies were isolated from young nodules in pomegranate trees (Young 2004)[104]. on plates containing selective nutrient media. Biochemical assays, fatty acid analysis and PCR were performed to identify the nodule pathogen. The analyzed isolates were described as *Pseudomonas savastanoi* pv. *savastanoi* using the previous tests, and to confirm the result, the pomegranate plants (hicaz variety) were inoculated with bacterial suspensions, as 25 bacterial isolates out of 54 caused a typical knot at the inoculation site, and the polymerase chain reaction (PCR) analysis proved, using Specific basis for *Pseudomonas savastanoi* pv. *savastanoi* With a sequence similarity of the Turkish pomegranate isolate with 99% to the corresponding genetic sequence of *Pseudomonas savastanoi* pv. *savastanoi* in the databases and based on symptoms, biochemical, molecular and pathogenesis tests and sequencing analyses, the knot disease factor observed on pomegranate trees is *Pseudomonas savastanoi* pv. *savastanoi*. Pomegranate can act as a natural host for *Pseudomonas savastanoi* pv. *savastanoi* which expands the list of host plant species affected by pathogens in the world and Turkey (Bozkurt 2014).[104] Conducting such a study on more than one plant, as well as an explanation of what researchers have

reached about this disease, which should not be overlooked because of its rapid spread and its great impact on plants and its adaptation to more than one plant to prevent it from spreading in countries that have not recorded its infection, as well as how to address it in case Injury, methods of diagnosis and treatment

CONCLUSION

In this study, during a field survey in olive orchards in northern Iraq, specifically in Dohuk Governorate. After collecting the samples and using them on a group of fruits designated for this experiment 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 rest of the fruits, and this is what was found to be the reason for discussing the results. . The stages of the experiment began with the process of collecting samples from northern Iraq and identifying them from Dohuk Governorate, then exposing some fruits to bacteria and exposing these fruits inoculated with bacteria to the same environmental conditions. After infecting the fruits, bacteria were isolated from the infected fruits and biochemical tests were applied to them, then molecular tests were performed on these isolates such as PCR &RT-PCR and we also conducted a test on the DNA of the bacteria on the gene 16SrRNA.

Recommendations

1. Continuing to conduct laboratory tests for all olive farms containing this infestation in Iraq for permanent discoveries of *Pseudomonas savastanoi* disease and its treatment.

2. Molecular study of antibiotic resistance and virulence factors of *Pseudomonas savastanoi* using polymerase chain reaction.

3. Further studies should be conducted to discover new effective antibiotics against *Pseudomonas savastanoi*. The study of environmental isolates and their role in transferring virulence factors to environmental isolates.

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LANGUAGE

Arabic: Native language

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EXPERIENCES

Good experience in computer software, Excel, SPSS

Good experience in agriculture, good experience in field analysis

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