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Molecular characterization of genetically related isolates of Pseudomonas savastanoi pv. savastanoi recovered from olive and athel trees using specific PCR

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التعريف الجزيئي لعزلات *Pseudomonas savastanoi pv. savastanoi* القريبة وراثيا والمعزولة من نباتي الزيتون والاثل باستخدام *PCR* محدد

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Abstract:
Olive knot is one of the most important bacterial diseases of olive orchards. Athel (Tamarix aphylla) has recently been reported as a new host of Pseudomonas savastanoi pv. savastanoi (Pss) in Libya, raising questions about the genetic relatedness and virulence of isolates from both hosts. This study characterized 23 Pss isolates recovered from olive (Olea europaea) and athel using primers designed from the 16S rRNA and ptz genes and related the molecular data to hypersensitive reaction (HR) and pathogenicity tests. Most isolates induced a necrotic HR on pepper leaves, whereas a few did not. Pathogenicity tests showed that several olive and athel isolates produced typical knots on olive seedlings, while no clear knots developed on athel seedlings, despite recovery of the bacterium from this host. All isolates yielded the expected amplicon of the 16S rRNA gene, confirming their affiliation to the genus Pseudomonas, whereas the plasmid-borne ptz gene was detected only in eight isolates from both hosts. Combining HR, pathogenicity and PCR results allowed the isolates to be classified into four groups reflecting differences in genetic load and virulence, and indicated a genetic relatedness between olive and athel isolates. These findings contribute to improving diagnosis and management of olive knot disease under local conditions.

Keywords: Olive knot disease; Pseudomonas savastanoi pv. savastanoi; Olea europaea; Tamarix.

الملخص:
يُعَدُّ مرض تعقّد أغصان الزيتون أحد أهم الأمراض البكتيرية في بساتين الزيتون. وقد سُجِّلت شجرة الأثل (Tamarix aphylla) حديثًا كعائل جديد لبكتيريا Pseudomonas savastanoi pv. savastanoi (Pss) في ليبيا، مما يثير تساؤلات حول العلاقة الوراثية ودرجة الضراوة للعزلات من كلا العائلين. هدفت هذه الدراسة إلى توصيف 23 عزلة من Pss معزولة من الزيتون (Olea europaea) والأثل باستخدام بادئات مصمَّمة من الجينين 16S rRNA وptz، وربط البيانات الجزيئية باختبار فرط الحساسية (HR) واختبارات الإمراضية. سببت معظم العزلات استجابة نخرية (HR) على أوراق الفلفل، في حين لم تُحدِث بعض العزلات هذه الاستجابة. أظهرت اختبارات الإمراضية أن عددًا من

عزلات الزيتون والأثل سببت تعقّدات نمو نموذجية على شتلات الزيتون، بينما لم تتكوّن تعقّدات واضحة على شتلات الأثل رغم إعادة عزل البكتيريا من هذا العائل. أعطت جميع العزلات حزمة مضخَّمة بالحجم المتوقع لجين 16S rRNA، مما يؤكد انتماءها لجنس Pseudomonas، في حين كُشف عن الجين البلازميدي ptz في ثمانٍ فقط من العزلات من كلا العائلين. أتاح دمج نتائج HR والإمراضية و PCRتقسيم العزلات إلى أربع مجموعات تعكس اختلافات في الحمولة الجينية والضراوة، وأشار إلى وجود علاقة وراثية بين عزلات الزيتون والأثل. تسهم هذه النتائج في تحسين تشخيص وإدارة مرض تعقّد أغصان الزيتون في الظروف المحلية.

الكلمات المفتاحية: مرض تعقّد أغصان الزيتون؛ Pseudomonas savastanoi pv. savastanoi؛ Olea europaea؛ Tamarix aphylla

Introduction:

The olive tree (*Olea europaea*) is one of the most important crops in the Mediterranean Basin due to the high nutritional and health value of its oil, which is attributed to its high content of antioxidants (Moretti et al., 2017; Košćak et al., 2023). This importance is one of the main drivers for protecting olive trees from pathogenic diseases that affect productivity and the quality of oil and table olives. Olive knot disease is considered one of the most serious bacterial diseases affecting this tree, as it leads to the formation of tumors and knots on branches, twigs, stems and sometimes on leaves and fruits, which reduces vegetative growth, weakens olive production and causes significant economic losses in most olive-growing regions worldwide (Moretti et al., 2008; Krid et al., 2009; Kenyon et al., 2013; Nguyen et al., 2018; Košćak et al., 2023).

The main causal agent of this disease is the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (Pss), which attacks a number of woody and herbaceous hosts such as oleander (*Nerium oleander*), broom (*Retama sphaerocarpa*), ash (*Fraxinus excelsior*), jasmine (*Jasminum* spp.), pomegranate (*Punica granatum*), and myrtle (*Myrtus communis*) (Krid et al., 2009; Tsuji et al., 2017; Nguyen et al., 2018; Hamed et al., 2022). More recently, athel (*Tamarix aphylla*) has been recorded as a new host for the knot bacterium in Libya (Al-Majdoub et al., 2018). Athel is a tree tolerant to heat, drought and salinity, used as a windbreak and for soil stabilization, and has multiple timber and medicinal uses (Orwa et al., 2009; Venturella et al., 2018). This environmental and economic importance of athel makes its discovery as a host for the knot bacterium an agricultural and ecological issue worthy of in-depth study.

The bacterium usually enters the plant host through wounds caused by mechanical factors or environmental conditions such as frost, triggering a series of physiological and molecular reactions in plant tissues that lead to the formation of bacterial knots through activation of type III secretion system genes, *hrp* genes and the production of plant hormones, especially auxins and cytokinins such as indole-3-acetic acid (IAA) and cytokinins (Krid et al., 2009; Zucchini et al., 2023). It has been shown that *P. savastanoi* strains carry plasmids of the pPT23A family containing important virulence genes such as *iaaM*, *iaaH*, *iaaL* responsible for IAA synthesis, and the *ptz* gene responsible for the synthesis of the cytokinin trans-zeatin, which directly contributes to tumor formation and increased virulence (Pérez-Martínez et al., 2008; Caballo-Ponce et al., 2017; Añorga et al., 2020; Alloush et al., 2018; Hamed et al., 2022).

Traditional methods for identification and diagnosis of the bacterium causing olive knot disease rely on physiological and biochemical tests such as the LOPAT system and pathogenicity tests on susceptible hosts. However, these methods are often slow, costly and require high expertise (Krid et al., 2009). Therefore, recent studies have turned to molecular techniques, foremost among them polymerase chain reaction (PCR), which enables rapid and accurate identification of bacterial isolates and comparison of genetic relatedness among isolates on the same host or different hosts using techniques such as RAPD-PCR, rep-PCR and AFLP-PCR (Palacio-Bielsa et al., 2009; Tsuji et al., 2017; Srinivasa et al., 2012). The *16S rRNA* gene is one of the most widely used genes in bacterial identification and in studying evolutionary relationships due to its high conservation and presence in all prokaryotic bacteria, with variable regions that can be exploited to differentiate between genera and species (Srinivasa et al., 2012; Sune et al., 2020; Al-Sukni et al., 2018). In light of the recording of athel as a new host for olive knot bacterium in Libya, and the need for reliable molecular tools to diagnose Pss isolates from olive and athel and compare their pathogenic capabilities, the importance of employing primers designed from the *16S rRNA* and *ptz* genes in PCR reactions becomes evident, along with linking molecular results to the pathogenic phenotype on the plant host. Therefore, this research aims to use primers designed from the *16S rRNA* gene and the *ptz* gene to identify *Pseudomonas savastanoi* pv. *savastanoi* isolates recovered from olive and athel; to compare the results obtained from PCR reactions with the different primers and evaluate the effectiveness of each in identifying the isolates under study and differentiating among them; and to test the pathogenicity of some selected isolates on olive and athel plants to determine their ability to cause infection and knot

formation, then compare the results obtained from the molecular study with the results of pathogenicity tests on the plant hosts, with the aim of clarifying the relationship between molecular markers and the actual virulence level of the bacterial isolates and establishing more accurate bases for control and biological monitoring programs of the disease in local agricultural systems.

Materials and Methods:

Bacterial isolates, culture media and laboratory conditions:

In this study, twenty-three (23) previously identified bacterial isolates of *Pseudomonas savastanoi* pv. *savastanoi*, isolated from olive (*Olea europaea*) and athel (*Tamarix aphylla*), were used. These isolates had been preserved in glycerol medium at the Department of Plant Protection, Faculty of Agriculture, University of Tripoli (Al-Majdoub et al., 2018).

The following culture media were used: Nutrient Agar (NA), Nutrient Broth (NB), and Pseudomonas Agar Base (PAB).

The media were prepared and sterilized according to standard laboratory procedures, then the bacterial isolates were reactivated by inoculating each isolate separately into NB medium and placing it on a shaker for 24 hours to prepare it for subsequent tests. After that, colonies were transferred using a loop needle to plates containing PAB medium, and plates were incubated at 25 °C.

A hypersensitive reaction test was carried out on the isolates under study to verify their activity and to confirm their pathogenic capability. The isolates were then preserved in slant tubes containing solid nutrient medium, as well as in 40% glycerol medium (prepared by adding 40 ml glycerol to 100 ml distilled water and sterilizing it), and then stored at 4 °C and −20 °C.

Table (1): *Pseudomonas savastanoi* pv. *savastanoi* isolates used in the study.

Isolate code	Host (scientific name)	Host (common name)
OS11w	*Olea europaea*	Olive
OS15w	*O. europaea*	Olive
OS20w	*O. europaea*	Olive
OS26w	*O. europaea*	Olive
OS31w	*O. europaea*	Olive
OS32w	*O. europaea*	Olive
OS35w	*O. europaea*	Olive
OS49w	*O. europaea*	Olive
OS51w	*O. europaea*	Olive
OS52y	*O. europaea*	Olive
OS54w	*O. europaea*	Olive
Ta1w	*Tamarix aphylla*	Athel
Ta2w	*T. aphylla*	Athel
Ta3w	*T. aphylla*	Athel
Ta3y	*T. aphylla*	Athel
Ta4w	*T. aphylla*	Athel
Ta10w	*T. aphylla*	Athel
Ta11y	*T. aphylla*	Athel
Ta12w	*T. aphylla*	Athel
Ta13w	*T. aphylla*	Athel
Ta15y	*T. aphylla*	Athel
Ta20w	*T. aphylla*	Athel
Ta22w	*T. aphylla*	Athel

All isolates were obtained from the laboratory of the Department of Plant Protection (Al-Majdoub et al., 2018).

Hypersensitive reaction (HR):

The hypersensitive reaction test was conducted on pepper seedlings. A bacterial suspension of the pathogenic isolates was prepared by adding sterile distilled water to plates containing 24-hour-old bacterial colonies. This suspension was injected with suitable syringes into the intercellular spaces on the lower surface of pepper leaves, and the isolate code and injection date were recorded around the injected areas of the leaf.

Plants were examined 24 hours after injection, and the resulting symptoms were recorded to evaluate the occurrence of hypersensitive reaction.

Pathogenicity tests:

Olive and athel seedlings were used to apply Koch's postulates and verify the pathogenic capability of the studied P. savastanoi pv. savastanoi isolates. Seedlings were prepared for treatments at three replicates per isolate.

Wounds were made on stems or branches, 1 cm in length and 3 mm in width, using a sharp sterilized scalpel, and the wounds were injected with bacterial isolates grown in liquid NB medium (24 hours on a shaker) using sterile fine syringes. **Two inoculation methods were applied:**

1. Injection in stem and branch regions.
2. Removal of leaves and direct injection in leaf scars.

Injection sites were covered with Parafilm tape, and the seedlings were covered with black plastic bags to prevent light penetration and to maintain humidity, in order to increase bacterial activity for 24 hours.

Control seedlings injected with sterile distilled water in the same way were used. All inoculated seedlings were kept in the laboratory under regular irrigation and maintained at room temperature for four months, after which disease symptoms were evaluated and results recorded.

Molecular tests:

DNA extraction:

Bacterial colonies grown on prepared plates for the experiments were collected and transferred to a test tube containing 1 ml of distilled water. The tube was heated to boiling for 10 minutes in a water bath, then centrifuged for 5 minutes at 1000 rpm.

After centrifugation, the supernatant was discarded and the pellet, representing the target DNA, was retained. The concentration of extracted DNA from all bacterial isolates was measured using a NanoDrop device, and this DNA was used in PCR reactions.

Polymerase chain reaction:

PCR was applied to all bacterial samples. A volume of 5 µl of template DNA was mixed with 45 µl of Reddy-Load PCR ready mix, **consisting of:**

Buffer, dNTPs, forward primer, reverse primer, template DNA, $MgCl_2$, Taq polymerase, and PCR H_2O. In this study, specific PCR was performed based on the primers shown in Table 2.

Table (2): Sequences of primers used in the PCR reaction.

Primer	Sequence (5'–3')	Source
16S rRNA Forward (F)	5'-TGGGGAGCAAACAGGATTAG-3'	Seqence 2 Company, Tunisia
16S rRNA Reverse (R)	5'-TAAGGTTCTTCGCGTTGCTT-3'	Seqence 2 Company, Tunisia
Ptz Forward (F)	5'-GATTAGGAGGTGCGGATGAA-3'	Seqence 2 Company, Tunisia
Ptz Reverse (R)	5'-ACTCGTCTAACAACCCCGTG-3'	Seqence 2 Company, Tunisia

The annealing temperature of the 16S rRNA primer was 53 °C, whereas the annealing temperature of the Ptz primer was 69 °C.

Mixture, thermal cycling and analysis of PCR products:

The PCR reaction passed through three basic successive stages: denaturation, annealing, and elongation. All stages of the reaction were carried out in a thermal cycler.

PCR products were detected using agarose gel electrophoresis containing ethidium bromide stain. The reaction products were loaded after mixing with loading dye into the wells of the agarose gel, and electrophoresis was carried out.

At the end of electrophoresis, the gel was transferred and DNA bands were visualized using a UV light source; then photographs of the gel were taken with a dedicated camera.

Bands obtained for each pair of primers used were compared to evaluate their capacity to discriminate among *Pseudomonas savastanoi* pv. *savastanoi* (Psv) isolates. DNA concentration of the studied isolates was measured using a NanoDrop device, then unified so that all samples had a concentration of 20 ng, at the Scientific Research Unit of the Misurata Central Laboratory.

Results and discussion:

Hypersensitive Reaction:

A hypersensitive reaction test was carried out on 23 isolates of *Pseudomonas savastanoi* pv. *savastanoi* isolated from olive (*Olea europaea*) and athel (*Tamarix aphylla*) and preserved in glycerol medium (Table 1). Most of the tested isolates showed localized necrotic lesions on pepper seedling leaves 24–48 hours after injection, indicating a positive hypersensitive response, whereas four isolates, Ta4w, Ta10w, Ta13w and Os49w, showed no clear response in this test (Figure 1).

Figure (1): Hypersensitive reaction test on pepper leaves: a, control; b, 24 hours after injection; c, 48 hours after injection.

These results agree with Al-Majdoub et al. (2018) regarding the efficiency of the hypersensitive reaction test in differentiating pathogenic Pss isolates, and the negativity of some isolates in this test is consistent with Taghavi and Hasani (2012), who attributed this to differences in the ability of isolates to trigger immune responses in different plant hosts or to variation in the expression level of genes responsible for the hypersensitive reaction in plant tissues.

Pathogenicity Tests:

Results of the pathogenicity test on olive and athel seedlings using the isolates shown in Table 1 indicated that olive isolates Os11w, Os15w, Os26w, Os31w and Os32w induced clear growth knots on olive seedling branches two months after inoculation; these knots were similar to those observed on naturally infected olive trees. In contrast, no knots were recorded on athel seedlings inoculated with the same isolates. Athel isolates Ta2w, Ta4w, Ta12w, Ta13w and Ta22w induced knot formation on olive seedlings, whereas athel seedlings inoculated with these isolates showed no knot symptoms even after repeating the experiment a second time (Figure 2A, B).

A **B** **C**

Figure (2A): Pathogenicity test on athel: A, control plant; B, plant inoculated with Pss isolated from olive; C, plant inoculated with Pss isolated from athel.

D **E** **F**

Figure (2B): Pathogenicity test on olive: D, control plant; E, plant inoculated with Pss isolated from olive; F, plant inoculated with Pss isolated from athel.

These results agree with Al-Sukni et al. (2018) regarding the appearance of knots on olive after several weeks of inoculation and support Al-Majdoub et al. (2018) who recorded athel as a new host for olive knot bacterium in Libya. The absence of knots on athel seedlings, despite isolation of the bacterium from this host, may be attributed to the young age of seedlings, the need for a longer period to develop infection, or a certain level of resistance in the cultivars used. On the other hand, the ability of athel isolates to induce knots on olive suggests a functional and genetic relatedness between olive and athel isolates, in contrast to Sisto et al. (2004), who showed a different genotypic pattern for isolates from olive and oleander and reported that oleander isolates do not infect olive. This difference may be due to variation in environmental or technical conditions or to different propagation and experimental methods among studies.

Molecular Tests:

Amplification of the 16S rRNA gene:

All isolates under study yielded a single bright band of the expected size (about 212 bp) when the target region of the *16S rRNA* gene was amplified using primers 16SrRNAF/16SrRNAR on agarose gel (Figure 3). This confirms that the isolates belong to the genus *Pseudomonas* and supports their preliminary identification as Pss isolated from olive and athel. It was observed that bands of some isolates such as Ta1w, Ta10w and Os49w were relatively faint, which could be attributed to low DNA concentration used in the reaction or partial degradation of DNA during extraction or storage, as reported by previous studies (Arslan et al., 2021).

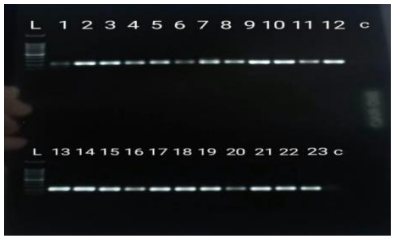

Figure (3): Genomic DNA amplification of Pss isolates using primers 16SrRNAF/16SrRNAR.

These results agree with Al-Sukni et al. (2018) regarding the success of using the *16S rRNA* gene to confirm the bacterial identity of Pss isolates on olive, and are consistent with the wide use of this gene in studies of genetic relationships among plant pathogenic bacterial isolates (Tsuji et al., 2017), reinforcing its reliability as a diagnostic tool in this study.

Amplification of the *Ptz* gene:

Using primers specific to the *Ptz* gene (PTZF/PTZR) produced bands of the expected size (about 481 bp) in eight isolates: Os15w, Os32w, Ta2w, Ta4w, Ta12w, Ta13w, Ta15y and Ta22w, while no visible bands appeared in the remaining isolates on the agarose gel (Figures 4, 5, 6). This indicates the presence of the *Ptz* gene in a defined subset of isolates recovered from both olive and athel, and its absence or loss in the other isolates.

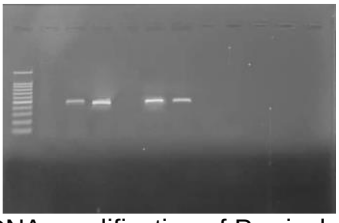

Figure (4): Genomic DNA amplification of Pss isolates using PTZR/PTZF.

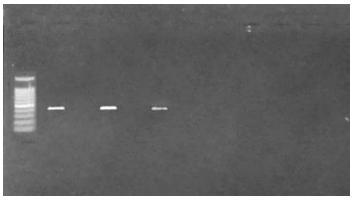

Figure (5): Genomic DNA amplification of Pss isolates using PTZR/PTZF.

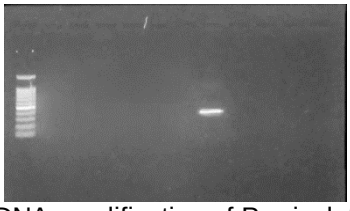

Figure (6): Genomic DNA amplification of Pss isolates using PTZR/PTZF.

The absence of bands in a number of isolates, despite confirmation of their belonging to Pss, is likely due to loss of the plasmid-borne gene as a result of mutations, plasmid instability, or environmental changes related to repeated sub-culturing and transfer under laboratory conditions, as indicated by Bardaji et al. (2011) and Moffatt et al. (2015) concerning the tendency of plasmids carrying virulence genes to be lost or rearranged. These results support the idea that the presence of *Ptz* is not fixed in all isolates and that its plasmid-borne nature makes its stability subject to environmental conditions and to how isolates are maintained in the laboratory.

Classification of isolates into groups according to test results (A–D):

Based on the results of the hypersensitive reaction, pathogenicity tests, and molecular tests (*16S rRNA* and *Ptz*), the isolates were divided into four main groups as shown in Table 3.

- **Group A:** Includes isolates that gave positive results in all tests and comprises Ta2w, Ta4w, Ta12w, Ta22w, Ta15y, Os15w and Os32w. These isolates showed a positive hypersensitive reaction, the ability to induce knots (where tested), and the presence of both *16S rRNA* and *Ptz* genes.

- **Group B:** Includes isolates that were negative in all previous tests (hypersensitive reaction and *Ptz*), except for *16S rRNA* which was positive. These isolates are Ta3y, Ta10w, Os49w, Os52y and Os54w, noting that pathogenicity tests were not conducted for all isolates.
- **Group C:** Includes isolates that were positive in the hypersensitive reaction and *16S rRNA*, but negative in *Ptz* and not tested for pathogenicity. This group includes Ta1w, Ta3w, Ta11y, Ta20w, Os11w, Os20w, Os26w, Os31w, Os35w and Os51w.
- **Group D:** Includes a single isolate, Ta13w, which was negative in the hypersensitive reaction but positive in molecular tests (*16S rRNA* and *Ptz*) and showed the ability to cause disease in the pathogenicity test (Table 3).

The results for Group A indicate that these isolates are identical or genetically close and carry genes responsible for hypersensitive reaction and pathogenicity, whether plasmid- or chromosome-borne, which is in line with Kenyon (2013) regarding the association of a positive hypersensitive reaction with the presence of pathogenicity genes.

In contrast, Group B isolates show a different pattern; they produced positive results only in *16S rRNA*, with no reaction in hypersensitive and *Ptz* tests and without pathogenicity testing in all cases. This can be explained by possible loss of the gene responsible for disease symptoms if plasmid-borne, or its transfer to the chromosome with reduced expression levels, which agrees with Kenyon (2013). Based on their similar response patterns, these isolates are considered genetically related (Cinelli et al., 2014).

Group C isolates show a positive hypersensitive reaction but are negative in the *Ptz* test and were not tested for pathogenicity. This may reflect specific loss of the *Ptz* gene or reliance on other pathogenic genes responsible for knot formation or symptom expression, as suggested by Cinelli et al. (2014), while maintaining the ability to trigger immune responses in pepper plants.

As for Group D, represented by isolate Ta13w, it is characterized by a negative hypersensitive reaction but positive pathogenicity and molecular tests. This pattern indicates the presence of pathogenicity genes with weak or delayed expression in the hypersensitive test or that the bacterial inoculum concentration was too low to trigger localized cell death, despite its ability to cause disease in the pathogenicity test, which agrees with Jones and Dangl (2006), Schwessinger and Ryan (2012), McCann et al. (2017), and what Ma and Ma (2016) indicated regarding the possibility that the effect of some pathogenicity genes is weak or conditional on environmental factors. Other studies also confirm that absence of a hypersensitive reaction does not necessarily mean absence of pathogenicity genes (Kenyon, 2013), making this isolate genetically unique.

Table (3): Classification of test results into four groups

Group	Hypersensitive response test	Pathogenicity test	PCR (16S rRNA) result	PCR (ptz) result	Plasmid profile for comparison (Al-Majdoub et al., 2018)	Isolate code	Host (scientific name)	Host (common name)
A	+	+	+	+	4+	Ta4w	*Tamarix aphylla*	Athel
A	+	+	+	+	NP	Ta2w	*T. aphylla*	Athel
A	+	+	+	+	NP	Ta12w	*T. aphylla*	Athel
A	+	+	+	+	3+	Ta22w	*T. aphylla*	Athel
A	+	NT	+	+	1+	Ta15y	*T. aphylla*	Athel
A	+	+	+	+	4+	Os15w	*Olea europaea*	Olive
A	+	+	+	+	4+	Os32w	*Olea europaea*	Olive
B	−	NT	+	−	4+	Ta3y	*T. aphylla*	Athel
B	−	NT	+	−	NP	Ta10w	*T. aphylla*	Athel
B	−	NT	+	−	2+	Os49w	*Olea europaea*	Olive
B	−	NT	+	−	4+	Os52y	*Olea europaea*	Olive
B	−	NT	+	−	4+	Os54w	*Olea europaea*	Olive
C	+	NT	+	−	NP	Ta1w	*T. aphylla*	Athel
C	+	NT	+	−	NP	Ta3w	*T. aphylla*	Athel
C	+	NT	+	−	3+	Ta11y	*T. aphylla*	Athel
C	+	NT	+	−	NP	Ta20w	*T. aphylla*	Athel
C	+	+	+	−	4+	OS11w	*Olea europaea*	Olive
C	+	NT	+	−	4+	OS20w	*Olea europaea*	Olive
C	+	+	+	−	4+	OS26w	*Olea europaea*	Olive

C	+	+	+	−	3+	Os31w	*Olea europaea*	Olive
C	+	NT	+	−	NP	Os35w	*Olea europaea*	Olive
C	+	NT	+	−	4+	Os51w	*Olea europaea*	Olive
D	−	+	+	+	3+	Ta13w	*T. aphylla*	Athel

- Hypersensitive response test: **+** = positive; **−** = negative.
- Pathogenicity test: **+** = successful pathogenicity test; **NT** = pathogenicity test not performed.
- PCR (16S rRNA) result: **+** = positive reaction.
- PCR (ptz) result: **+** = positive reaction; **−** = negative reaction.
- Numbers from 1–23 refer to isolate numbers in the 16S rRNA PCR results.
- **NP** = plasmidless isolate; **1+, 2+, 3+, 4+** = number of plasmids per isolate as reported by Al-Majdoub et al. (2018).

Integration of hypersensitive reaction, pathogenicity and molecular test results:

Combining the results of hypersensitive reaction, pathogenicity tests and molecular tests (*16S rRNA* and *Ptz*) shows that genes responsible for pathogenicity in Pss isolates may be located either on plasmids or on chromosomes, with clear variation in expression level among different isolates. Isolates that showed consistently positive results in hypersensitive reaction, pathogenicity tests and *Ptz* gene are strong candidates to represent highly virulent strains, whereas isolates negative in some of these tests suggest possible loss, mutation, or reduced expression of pathogenicity genes under environmental or technical conditions (Bardaji et al., 2011; Moffatt et al., 2015; Smith et al., 2016; Leung and Francisco, 2010; Cinelli et al., 2014).

The results also support a genetic relatedness between isolates recovered from olive and athel, particularly those carrying the *Ptz* gene and showing similar plasmid patterns, as reported by Al-Majdoub et al. (2018), in addition to the possibility of genetic exchange between different strains via plasmids or through ecological relationships between olive and athel in fields where athel is used as windbreaks in olive orchards in some regions of Libya.

Conclusion:

This study combined hypersensitive reaction, pathogenicity, and PCR analysis of 16S rRNA and ptz genes to clarify diversity of *Pseudomonas savastanoi* pv. *savastanoi* isolates from olive and athel in Libya. 16S rRNA amplification confirmed that all isolates are closely related and belong to *Pseudomonas*, whereas *ptz* was detected only in a subset, indicating instability of plasmid-borne virulence genes. Isolates carrying *ptz*, showing positive HR, and inducing knots on olive behaved as highly virulent strains, while *ptz*-negative or HR-negative isolates (e.g. Ta13w) revealed distinct virulence patterns that may depend on other genes. Athel isolates induced knots on olive but not on athel seedlings, highlighting athel as a potential reservoir and stressing the need for integrated molecular–biological diagnostics for olive knot management.

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