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Isolation, Identification, and In Vitro Biological Control of Olive Knot Disease Agent With Antagonist Bacillus spp.

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ABSTRACT

In this study, 18 olive orchards in the provinces of Mersin and Osmaniye in Türkiye, where olives are grown, were visited, and 26 bacterial isolates were obtained from six trees showing symptoms of olive knot disease. The bacterial isolates were identified by morphological and biochemical tests (Gram reaction and LOPAT characteristics) and by MALDI-TOF MS analysis at the species level. Based on pathogenicity tests, all bacterial isolates were identified as Pseudomonas savastanoi pv. savastanoi. In biocontrol studies, the antibacterial effects of 19 epiphytic biocontrol agent (BCAs) bacterial isolates were investigated using in vitro dual culture tests in Petri dishes. In the dual culture test, all antagonistic BCA bacterial isolates formed an inhibition zone of 2.0 to 9.3 mm and thus inhibited the growth of the pathogen. Diagnostic tests for BCA bacterial isolates performed with MALDI-TOF identified all effective antagonist isolates as Bacillus spp. Five antagonist bacterial isolates belonging to the same statistical group (*Bacillus subtilis* KSY9, *Bacillus subtilis* ABK-FY-2-5, *Bacillus amyloliquefaciens* ssp. *plantarum* MTHM.3, *Bacillus amyloliquefaciens* ssp. *plantarum* FT2.1 and *Bacillus amyloliquefaciens* ssp. *plantarum* MTHM2) were identified as the most successful in inhibiting pathogen growth. The isolate *Bacillus subtilis* KSY9 formed an average inhibition zone of 9.33 mm and was thus the most successful antagonist. This study once again emphasises the importance of biological control as an environmentally friendly method of organic farming to combat diseases.

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Zeytin Dal Kanseri Hastalık Etmeninin İzolasyonu, Tanısı ve Antagonist *Bacillus* spp. *İn vitro* Biyolojik Mücadelesi

ÖZET

Bu çalışmada, Mersin ve Osmaniye illerinde zeytin üretimi yapılan 18 zeytin bahçesi ziyaret edilerek, dal kanseri hastalık belirtileri gösteren 6 adet urlu ağaçtan 26 adet bakteri izolatı elde edilmiştir. Bakteriyel izolatlar morfolojik, biyokimyasal (Gram Reaksiyon, LOPAT karakterleri) ve MALDI-TOF MS testleriyle tür düzeyinde tanılanmıştır. İzolatların tamamı patojenite testi sonucunda *Pseudomonas savastanoi* pv. *savastanoi* olarak belirlenmiştir. Hastalık etmeni ile biyolojik mücadele çalışmalarında, 19 adet epifitik biyolojik mücadele etmeni antagonist bakteri izolatlarının antibakteriyel etkisi *in vitro* ikili kültür petri denemeleriyle araştırılmıştır. İkili kültür testinde, tüm antagonist BCA izolatları, patojenin gelişimini 2.0-9.3 mm aralığında değişen engelleme bölgesi (inhibisyon zonu) oluşturmuşlardır. Etkili antagonistlerin MALDI-TOF ile yapılan tanı testlerinde tümünün *Bacillus* cinsine ait izolatlar olduğu saptanmıştır. Aynı istatistiki grupta yer alan beş antagonist (*Bacillus subtilis* KSY9, *Bacillus subtilis* ABK-FY-2-5, *Bacillus amyloliquefaciens* ssp. *plantarum* MTHM.3, *Bacillus amyloliquefaciens* ssp. *plantarum* FT2.1 ve *Bacillus amyloliquefaciens* ssp. *plantarum* MTHM2) patojen gelişimini engellemede en başarılı izolatlar olarak belirlenmiştir. *Bacillus subtilis* KSY9 izolatı ortalama 9.33 mm engelleme zonu oluşturarak en başarılı antagonistik izolat

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olarak belirlenmiştir. Yapılan bu çalışmayla, hastalıkların baskılanmasında çevre dostu ve organik tarımda kullanılan mücadele yöntemlerinden biri olan biyolojik mücadelenin önemi bir kez daha ortaya konulmuştur.

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INTRODUCTION

Olive knot disease is caused by a gram-negative aerobic bacterium known as *Pseudomonas savastanoi* pv. *savastanoi* (Smith) Gardan, Bollet, Abu Ghorrah & Grimont, 1992). This bacterium causes knots in olive branches. Olive Knot Disease caused by *Pseudomonas savastanoi* pv. *savastanoi* also leads to knot formation in plants belonging to the *Oleaceae* family, such as Oleander (*Nerium oleander*) (*Pseudomonas savastanoi* pv. *nerii*), Ash (*Fraxinus excelsior* L.) (*Pseudomonas savastanoi* pv. *fraxini*), and Broom (*Retama sphaerocarpa*) (*Pseudomonas savastanoi* pv. *retacarpa*) (Garcia De Los Rios, 1999). Moreover, other hosts include Lilac (*Syringae* spp.), Privet (*Phillyreaspp.*), Japanese Privet (*Ligustrum japonicum*), Jasmine (*Jasminum* spp.) (Janse, 1981), Forsythia (*Forsythia* spp.) (Iacobellis et al., 1998), Buckthorn (*Rhamnus alaternus*) (Saad & Hanna, 2002), Myrtle (*Myrtus* spp.) (Mirik et al., 2004), Italian Buckthorn (*Rhamnus alaternus*), Phillyrea (*Fontanesia phillyreoides*) (Mirik et al., 2011), Rocktrumpet (*Mandevilla sanderi*) (Eltlbany et al., 2012), Chinese Fringeflower (*Loropetalum chinense*) (Conner et al., 2013), and Pomegranate trees (Bozkurt et al., 2014; Sancar, 2018).

The formation of knots induced by *Pseudomonas savastanoi* pv. *savastanoi* is primarily due to the bacterial synthesis of indole-3-acetic acid (IAA) and cytokinins, which cause hypertrophy and subsequent abnormal cell division (hyperplasia) in plant tissue (Rodríguez-Moreno et al., 2009; Quesada et al., 2012; Saygılı et al., 2006). Knots caused by *Pseudomonas savastanoi* pv. *savastanoi* can also occasionally be observed on leaves and fruits (Trapero-Casas & Ramos, 2009). *Pseudomonas savastanoi* pv. *savastanoi* mainly lives as an epiphyte (Quesada et al., 2012) and/or endophyte in the phyllosphere of olive trees (Buonaurio et al., 2015). This disease is seen in the Marmara (Mirik et al., 2007), Aegean (Azeri, 1993; Servi & Baştaş, 2012), and Mediterranean Regions in Türkiye (Basım & Ersoy, 2000; Tatlı & Benlioğlu, 2004; Mirik et al., 2004; Mirik et al., 2008a).

Pathogens, particularly those caused by late spring frosts, hail, and agricultural practices (such as pruning and crop harvesting), enter the plant through wounds and cracks in the slender branches. This pathogen cannot enter the plant through natural openings (stomata, hydatodes, and lenticels). It can also be spread by rain, water splashes, wind, insects, birds, and human activities (pruning and harvesting) (Quesada et al., 2010). The most suitable temperature for the development of this pathogen is between 20 and 25°C, with a minimum of 5 to 10°C and a maximum of 32°C. Infections occur mainly in spring and autumn when climatic conditions are ideal for disease development (Lavermicocca et al., 2002). Infections generally lead to leaf drop, branch drying, and a reduction in tree vigour, ultimately resulting in reduced yield, fruit size, and oil quality. Preventing the entry and spread of *Pseudomonas savastanoi* pv. *savastanoi* into the plant is the most promising strategy in the fight against olive knot disease. However, because bacteria can survive epiphytically, even in parts of the plant where there are no visible symptoms of disease, it is very difficult to control (Penyalver et al., 2006). In addition, failure to take appropriate hygiene measures when equipment comes into contact with infected trees can lead to the spread of the pathogen to healthy trees. Elimination of the pathogen from a tree or orchard after infection is extremely difficult (Quesada et al., 2010). If the disease agent is left uncontrolled, the increasing population of the pathogenic bacteria from year to year can significantly reduce the quality and quantity of olives and lead to significant economic losses (Quesada et al., 2010). The fight against pathogens is primarily based on preventive measures. These preventive measures often include the use of resistant varieties, clean production materials, pruning practices, and chemical control, or a combination of these (Trapero-Casas et al., 2009; Abuamsha et al., 2024). Chemical control is mainly based on the use of copper-containing compounds, usually copper hydroxide and copper sulphate, during the dormant season (Trapero-Casas et al., 2009). Extensive and intensive use of copper preparations may lead to the development of copper resistance in *Pseudomonas savastanoi pv. savastanoi* populations (Nguyen, 2017). Furthermore, the negative effects of fungicides on human health and the environment cannot be ignored. Therefore, strategies that contribute to the sustainable management of this disease have become one of the priority issues in the scientific community (Al-Khatib et al., 2010; Krid et al., 2012; Bozkurt et al., 2020; Soylu et al., 2022; Şahin et al., 2022; Soylu et al., 2024). Biological control of plant pathogens using antagonists, which have the potential to eliminate or reduce pathogen populations in plant tissues, has gained importance in recent years as an alternative to chemical control in sustainable agriculture (Cook, 1993; Di Francesco et al., 2016; Filiz-Doksöz & Bozkurt, 2022b). Although there is currently no effective biological control of the disease, some researchers have reported promising results. It has been reported that strains of *Pseudomonas fluorescens* and *Bacillus subtilis* obtained from the roots and leaves of olive trees are effective against *Pseudomonas savastanoi* pv. *savastanoi* under *in vitro* conditions (Krid et al., 2010).

The lack of an effective bactericide against pathogenic bacterial diseases such as olive knot disease and the inadequacy of chemical control necessitate studies on biological control. The aim of this work is to carry out field visits to olive groves in our region, to strain *Pseudomonas savastanoi* pv. *savastanoi* from branches of olive trees affected by the disease, to perform morphological and biochemical tests on the strains obtained (Gram reaction, Levan, oxidase, pectolytic activity, arginine dehydrogenase, tobacco hypersensitive reaction), to diagnose them using MALDI-TOF MS, and to study their biological control using antagonistic bacteria under İn vitro conditions.

MATERYAL and METOD

Material

Pathogenic Bacterial Strains: In this study, pathogenic bacterial strains were obtained from soft cream-colored knots on the branches and trunks of olive trees in a residential garden in Erdemli district of Mersin province, and in the olive production areas of Merkez and Kadirli districts of Osmaniye province, where knot symptoms were reported. The strains used were Cmm 3/1-A coded *Clavibacter michiganensis* subsp. *michiganensis*, YA-223 coded *Erwinia amylovora*, YA-888 coded *Acidovorax citrulli*, YA-703 coded *Pectobacterium carotovorum*, YA-10 coded *Pseudomonas syringae* pv. *tomato*, and YA-647 (PD 704) coded *Pseudomonas corrugata*, obtained from the culture collection of the Bacteriology Laboratory within the Department of Plant Protection, University of Çukurova, serving as control strains for the biochemical diagnosis of pathogenic bacterial strains.

Candidate Antagonist Bacterial Strains: Nineteen epiphytic bacterial strains from the culture collection of the Organic Agriculture Management Laboratory at Osmaniye Korkut Ata University were used as candidate antagonists.

Methods

Collection of Knotted Branches from Host Plants

Areas showing characteristic symptoms of the disease on olive trees in the provinces of Mersin and Osmaniye were surveyed between March and May in 2022. Following the submission of information by a producer specialising in the cultivation of olive saplings in the central district of Osmaniye province, a visit was conducted to the nursery with the aim of implementing disease control measures. During the inspection, knots were detected in the pruning sites and branches of the sapling. The inspections proceeded with a visit to an olive orchard in the Kadirli district of Osmaniye. A survey was conducted by agricultural engineers from the Kadirli District Directorate of Agriculture in Yukarıbozkuyu and İlbistanlı villages, where symptoms of a disease were detected. Olive groves were examined as part of this survey. In the course of the investigations conducted in these areas, knots were detected on the branches and trunks of the trees. Creamy soft knots of various sizes were found on the branches and trunks of olive trees. Knotted branches were cut off at about 30 cm from the diseased part and placed in pet bottles containing tap water for transport to the laboratory (Mirik & Aysan, 2011).

Isolation and Diagnosis of Pathogenic Bacteria

On arrival at the laboratory, the surfaces of the samples were disinfected with 70% alcohol, and then approximately two millimetres of bark tissue was peeled off from the knotted area using a scalpel. The collected pieces were further surface disinfected with alcohol. After crushing the samples in a sterile mortar, they were homogenised using a saline buffer (0.85% NaCl) solution (Öztürk & Soylu, 2022). Using a sterile loop, the prepared suspension was placed on Petri dishes containing Tryptic Soy Agar (TSA) medium and then incubated at 25°C. Colonies with a cream-coloured morphology were purified. Pathogenic strains obtained from the isolation process were stored at +4°C in Yeast extract-dextrose-CaCO3 Agar (YDCA) medium for further studies (Filiz-Doksöz & Bozkurt, 2020). Morphological and biochemical tests were carried out to diagnose bacterial strains suspected of being *Pseudomonas savastanoi* pv. *savastanoi*, following the methods introduced by Lelliot & Stead (1987). These methods included examination of bacterial strains for fluorescent pigment formation, determination of the Gram reaction with the KOH (potassium hydroxide) test, and LOPAT tests (levan-negative (L-), oxidase-negative (O−), pectolytic activity on potato-negative (P−), arginine dihydrolase-negative (A−), and tobacco hypersensitivity-positive (T+)).

MALDI-TOF MS: Diagnosis of bacterial strains was performed by Matriks assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) technology based on peptide mass fingerprint analysis of their proteins at the Research and Application Centre of Plant Health Clinic of Mustafa Kemal University (Soylu et al., 2020; Soylu et al., 2021). A MALDI-TOF MS score ≥2 indicates species-level identification, while scores between 1.7 and 1.9 indicate genus-level identification, and scores <1.7 are considered unreliable (Pavlovic et al., 2012; Uysal et al., 2019; Kara & Soylu, 2022)

Pathogenicity Tests

Pathogenicity tests were conducted on carrot slices and olive seedlings.

Carrot slice pathogenicity test: Carrots to be used in the pathogenicity test were surface sterilised with 70% alcohol, sliced to a thickness of 1 cm with a sterile scalpel, placed in 9 cm glass Petri dishes containing sterile filter paper, and moistened with sterile distilled water. The carrot slices were inoculated with 50 µl of a bacterial suspension prepared using the spectrophotometrically standardised solution of strains from knot-ridden plants (10^8 cells/ml) and then incubated at 25°C for 14 days. The experiment was performed in triplicate. The virulence levels of the strains were determined by measuring knot weights after disease symptoms developed in the positive control (Küsek, 2007; Filiz-Doksöz & Bozkurt, 2020; Geylani Yüzbaşıoğlu & Aysan, 2021).

Olive seedling pathogenicity test: Seedlings of the Gemlik variety, commonly used in Türkiye and obtained from a commercial nursery, were used for the pathogenicity test. Wounds were made on the branches of the seedlings using a sterile scalpel, and then 10 µl of a bacterial suspension prepared at a concentration of 10^8 cells/ml was inoculated using an automatic pipette. Inoculation sites were moistened with sterile distilled water, covered with cotton, and sealed with parafilm. Three branches were used for each strain. The virulence of the strains was determined by measuring knot weights after disease symptoms appeared in the positive control. The strain with the highest virulence was stored at +4°C in YDCA medium for use in dual culture tests (Filiz-Doksöz & Bozkurt, 2020).

Determining of *In vitro* Antagonistic Effects of Antagonists

Nineteen previously obtained epiphytic bacterial strains were inoculated evenly at three points on TSA medium plates (Bitgen & Mirik, 2021) and incubated at 25°C for two days. A suspension of the pathogen strain was prepared at a density of 10^8 cells/ml, adjusted to a wavelength of 600 nm using a spectrophotometer. This pathogen solution was then sprayed onto the developed candidate antagonists using a hand sprayer after 24 hours of incubation on the plates. Inhibition zones formed after 48 hours of incubation at 25°C were measured and recorded in millimetres. Data obtained based on inhibition zones formed by effective strains were analysed using CoStat statistical software (CoHort Software, Pacific Grove, CA, U.S.A. version 6.4). One-way ANOVA was performed, and differences between treatments were determined using the LSD multiple comparison test at a significance level of $p \leq 0.05$

RESULTS and DISCUSSION

Collection of Knotted Branches from Affected Plants

In the present study, 18 olive production areas in the provinces of Mersin and Osmaniye were surveyed for knot disease. Olive knot disease caused by *Pseudomonas savastanoi* pv *savastanoi* was detected in six olive production areas. Cream-coloured, soft-knotted symptoms were observed in this region in March. During knot disease surveys, a total of 9 knotted branches were taken to the laboratory for strain collection. In the Kadirli district of Osmaniye, one out of four olive orchards in the village of İlbistanlı and three out of twelve olive orchards in the village of Yukarıbozkuyu showed symptoms of knot disease. In the Erdemli district of Mersin, one out of four trees in a residential garden was found to be affected. In a survey of a nursery in the central district of Osmaniye, only one seedling was found to be affected. The detection of knotting symptoms in nurseries represents a significant risk for the spread of the disease to both production areas and other regions. Early detection of disease symptoms in nurseries and effective management strategies can help prevent production losses and contribute to sustainable agriculture.

Isolation of Pathogenic Bacteria and LOPAT Characterization

In studies conducted in olive orchards in Mersin and Osmaniye provinces between March and May 2022, a total of 26 bacterial strains were purified from knotted branches collected from olive trees aged between 2 and 15 years. The strains exhibited viscous, mucoid streaming in the potassium hydroxide (KOH) test and were all found to be gram-negative. The strains did not form Levan-type colonies in Sakkaroz Nutrient Agar (SNA) medium, and oxidase, arginine, and pectolytic activity tests on potato were all negative. All strains inoculated on tobacco leaves induced necrosis in the inoculated area and were positive in the tobacco hypersensitivity test (Table 1). The strain causing knot disease in olive, *Pseudomonas savastanoi* pv *savastanoi*, exhibited gram-negative properties and similar LOPAT (----+) properties as previously reported by researchers such as Tatlı & Benlioğlu (2004), Servi & Baştaş (2012), Mirik & Aysan (2011), Sivri (2012), Sancar (2018), Basım et al. (2019), Filiz-Doksöz & Bozkurt (2020), and Bitgen & Mirik (2021).

Table 1. LOPAT characters of pathogenic bacterial isolates
Çizelge 1. Patojenik bakteri izolatların LOPAT karakterleri.

No	Bacterial isolates	Location	L*	O	P	A	T
1	YKB1-1	Kadirli-Osmaniye	-	-	-	-	+
2	YKB1-2	Kadirli-Osmaniye	-	-	-	-	+
3	YKB1-3	Kadirli-Osmaniye	-	-	-	-	+
4	YKB1-4	Kadirli-Osmaniye	-	-	-	-	+
5	YKB2-1	Kadirli-Osmaniye	-	-	-	-	+
6	YKB2-2	Kadirli-Osmaniye	-	-	-	-	+
7	YKB2-3	Kadirli-Osmaniye	-	-	-	-	+
8	YKB2-4	Kadirli-Osmaniye	-	-	-	-	+
9	YKB3-1	Kadirli-Osmaniye	-	-	-	-	+
10	YKB3-2	Kadirli-Osmaniye	-	-	-	-	+
11	YKB3-3	Kadirli-Osmaniye	-	-	-	-	+
12	YKB3-4	Kadirli-Osmaniye	-	-	-	-	+
13	YKB4-1	Kadirli-Osmaniye	-	-	-	-	+
14	YKB4-2	Kadirli-Osmaniye	-	-	-	-	+
15	YKB4-3	Kadirli-Osmaniye	-	-	-	-	+
16	YKB4-4	Kadirli-Osmaniye	-	-	-	-	+
17	ZAE1	Erdemli-Mersin	-	-	-	-	+
18	ZAE2-1	Erdemli-Mersin	-	-	-	-	+
19	ZAE3	Erdemli-Mersin	-	-	-	-	+
20	ZFO1-1	Merkez-Osmaniye	-	-	-	-	+
21	ZFO1-2	Merkez-Osmaniye	-	-	-	-	+
22	ZFO1-3	Merkez-Osmaniye	-	-	-	-	+
23	ZFO1-4	Merkez-Osmaniye	-	-	-	-	+
24	ZFO1-5	Merkez-Osmaniye	-	-	-	-	+
25	ZFO1-6	Merkez-Osmaniye	-	-	-	-	+
26	ZFO1-7	Merkez-Osmaniye	-	-	-	-	+
27	YA-223	Gevye/SAKARYA	+				+
28	YA-888	Yüreğir/ADANA		+			
29	YA-703	Erdemli/MERSIN			+		
30	YA-647 (PD 704)	HOLLAND				+	
31	YA-10	Karatas, ADANA					+

*: L: Levan, O: Oxidase, P: Pectinolytic activity, A: Arginine dihydrolase, T: Excessive sensitivity in tobacco. +: positive reaction of strain in this test; -: negative reaction of strain in this test

Pathogenicity Tests

Pathogenicity test on carrot slices: The formation of knots on carrot discs was observed 10 days after inoculation. After 20 days, the knots had reached their maximum size. All pathogen strains induced knot formation on carrot discs. When the knot sizes were statistically grouped, strains YKB1-1 and ZFO1-6, which produced knots weighing 0.8 and 0.7 g, respectively, were identified as the most virulent strains, producing the largest knots. Strains ZFO1-3, ZAE2-1, YKB2-1, and YKB2-4, which produced knots weighing 0.5 and 0.4 g, were identified as other virulent strains. In addition, 10 strains formed knots weighing 0. 3 g, 8 strains formed knots weighing 0.2 g, and 2 strains formed knots weighing 0.1 g (Table 3). Six strains (YKB1-1, ZFO1-6, ZFO1-3, ZAE2-1, YKB2-1, and YKB2-4), which were found to be the most virulent, were selected to induce pathogenicity on olive seedlings. A one-way analysis of variance (ANOVA) was conducted to evaluate the pathogenicity of different strains of *Pseudomonas savastanoi* pv. *savastanoi*. The results revealed a statistically significant difference among strains, $F(25, 52) = 3.02$, $p = 0.0004$. The effect size, calculated using eta squared (η^2), was 0.592, indicating a moderate to large effect *(Table 2).*

In their study, Filiz-Doksöz & Bozkurt (2020) determined the pathogenicity of 14 olive strains on carrot discs using knot weights and found that the knots ranged in weight from 0.14 g to 1.76 g. In some cases, pathogenicity tests for plant pathogens can be performed using alternative hosts in addition to the natural host plant. In the case of annual plants such as vegetables, it is easier to perform pathogenicity tests on the original host than on perennial hosts, and results are obtained more quickly. Carrot discs have been used in recent years in studies of the pathogenicity of *Pseudomonas savastanoi* pv. *savastanoi* (Filiz-Doksöz & Bozkurt, 2022a).

Table 2. Analysis of variance table for the effects on carrot slices of pathogenic bacterial strains
Çizelge 2 Patojenik bakteriyel izolatların havuç dilimlerinde etkilerine ait varyans analiz tablosu

Soure of variation	df	SS	MS	F	P
Pathogenic bacterial strains	25	1.85	0.07	3.02	.0004
Error	52	1.27	0.02<-		
Total	77	3.12			

Table 3. Potential of pathogenic bacterial isolates to cause disease symptoms (knot) on carrot slices
Çizelge 3. Patojen bakteri izolatların havuç dilimi üzerinde hastalık belirtilerini (ur) oluşturma potansiyelleri

Bacterial isolates	Average Weight of Knot (g)	Bacterial isolates	Average Weight of Knot (g)
YKB1-1	0.8 a*	ZAE1	0.3 cde
ZFO1-6	0.7 ab	YKB4-3	0.3 cde
ZFO1-3	0.5 bc	ZFO1-4	0.3 cde
ZAE2-1	0.4 bcd	YKB1-3	0.2 cde
YKB2-1	0.4 bcd	YKB3-2	0.2 cde
YKB2-4	0.4 bcd	YKB2-2	0.2 cde
YKB4-4	0.3 cde	YKB1-4	0.2 cde
YKB3-1	0.3 cde	YKB4-2	0.2 cde
YKB4-1	0.3 cde	ZAE3	0.2 cde
ZFO1-7	0.3 cde	YKB3-4	0.2 cde
ZFO1-5	0.3 cde	YKB2-3	0.2 cde
YKB3-3	0.3 cde	ZFO1-1	0.1 de
YKB1-2	0.3 cde	ZFO1-2	0.1 e

*: Post-hoc comparisons using the LSD test ($\alpha = 0.05$) showed that means sharing the same letter in the same column are not significantly different from each other ($p > 0.05$).

Pathogenicity test on olive seedlings: Suspensions prepared at a density of 10^8 cells/ml of the six strains with the highest knot weights (YKB1-1, ZFO1-6, ZFO1-3, ZAE2-1, YKB2-1, and YKB2-4) were inoculated onto fresh shoots of one-year-old olive seedlings. The virulence levels of the strains were determined by weighing knots after the appearance of knot symptoms on positive control plants at 4 months after inoculation. Knot weights induced in olive seedlings by the strains ranged from 0.2 to 0.9 g (Table 5). Statistically, the strain coded YKB1-1 was identified as the most virulent strain with a knot weight of 0.9 g. The strains ZFO1-6 and ZFO1-3 were identified as other virulent strains, inducing knots of 0.6 g and 0.5 g, respectively. Although the strains YKB2-4 and YKB2-1 induced knots of 0.3 g, the strain ZAE2-1 induced knots of 0.2 g. The strain YKB1-1, which induced knots of 0.9 g, was identified as the most virulent strain and will be further investigated in the subsequent study. A one-way analysis of variance (ANOVA) was conducted to evaluate the pathogenicity of different strains of *Pseudomonas savastanoi* pv. *savastanoi*. The results revealed a statistically significant difference among strains, $F(5, 12) = 6.45$, $p < 0.001$. The effect size, calculated using eta squared (η^2), was 0.75, indicating a large effect (Table 4).

Table 4. Analysis of variance table for the effects in olive seedlings of pathogenic bacterial strains
Çizelge 4 Patojenik bakteriyel izolatların zeytin fidanlarındaki etkilerine ait varyans analiz tablosu

Soure of variation	df	SS	MS	F	P
Pathogenic bacterial strains	5	0.96	0.19	4.78	.0123
Error	12	0.48	0.04<-		
Total	17	1.44			

Similar to the present study, Mirik et al. (2008a) reported that all 33 strains they tested for pathogenicity in olive seedlings formed knots. In a separate study, the same researchers found that 88 out of 208 bacterial strains obtained from knotted branches caused knots on olive seedlings (Mirik and Aysan, 2012). Filiz-Doksöz & Bozkurt (2020) indicated that 14 strains they tested caused knot formation with weights ranging from 0.28 g to 0.98 g. Bitgen & Mirik (2021) obtained and identified 52 pathogen strains in their study. They reported that all pathogen strains obtained and identified as *Pseudomonas savastanoi* pv. *savastanoi* caused knot formation in olive seedlings used for pathogenicity testing. In the present study, all strains tested for pathogenicity on olive seedlings (YKB1-1, ZFO1-6, ZFO1-3, ZAE2-1, YKB2-1, and YKB2-4) produced knots and were identified as *Pseudomonas savastanoi* pv. *savastanoi* at the pathovar level.

Table 5. Potential of pathogenic bacterial isolates to cause disease symptoms (knot) on olive seedlings
Çizelge 5. Patojen bakteri izolatların zeytin fidanları üzerinde hastalık belirtilerini (ur) oluşturma potansiyelleri.

Bacterial isolates	Knot Weight (g)
YKB1-1	0.9 a
ZFO1-6	0.6 ab
ZFO1-3	0.5 ab
YKB2-4	0.3 b
YKB2-1	0.3 b
ZAE2-1	0.2 b

*: Post-hoc comparisons using the LSD test ($\alpha = 0.05$) showed that means sharing the same letter in the same column are not significantly different from each other ($p > 0.05$).

Diagnosis of Pathogenic Bacterial Strains by MALDI-TOF MS

In the diagnosis of olive strains by MALDI-TOF MS, the similarity index values were determined as shown in Table 6, and the species-level agreement was achieved as *Pseudomonas savastanoi*. Many studies in recent years have reported the MALDI-TOF diagnostic system as a next-generation system for species identification of plant pathogenic and BCA bacterial isolates (Duman & Soylu, 2019; Soylu et al., 2020; Soylu et al., 2021; Kara & Soylu,2022). Koščak et al. (2023) characterised *Pseudomonas savastanoi* pv *savastanoi* strains obtained from infected olive trees in Croatia, Slovenia, and Portugal using colony morphology, physiological and biochemical characteristics. The researchers used real-time PCR for molecular identification of *Pseudomonas savastanoi* pv *savastanoi* and MALDI-TOF for species identification by proteomic analysis. The researchers found that 46.6% of the strains were *Pseudomonas savastanoi*.

Table 6. Similarity index values of pathogenic bacterial strains identified by MALDI-TOF
Çizelge 6. MALDI-TOF ile tanılanan patojenik bakteriyel izolatların benzerlik indeksleri.

Strain Code	Bacterial Species	Similarity Index
ZAE2-1	*Pseudomonas savastanoi*	2.43
ZFO1-3	*Pseudomonas savastanoi*	2.35
ZFO1-6	*Pseudomonas savastanoi*	2.32
YKB2-4	*Pseudomonas savastanoi*	2.23
YKB1-1	*Pseudomonas savastanoi*	2.21
YKB2-1	*Pseudomonas savastanoi*	2.14

Determining of *in vitro* Antagonistic Effects of Antagonists

In vitro dual culture tests were carried out using the three-point inoculation method to determine the antagonistic activity of 19 candidate antagonist bacterial strains obtained from different crops. Considering the results of the dual culture tests shown in Table 8, all antagonists suppressed the development of the pathogen and formed inhibition zones ranging from 2.00 mm to 9.33 mm. Among the antagonists in the same statistical group, five strains (designated as KSY9, ABK-FY-2-5, MTHM3, FT2.1, MTHM2) were identified as the most successful strains in inhibiting pathogen development. The strain designated KSY9 was found to be the most successful antagonist strain, producing an average inhibition zone of 9.33 mm. The strain designated ABK-FY-2-5, which produced an inhibition zone of 9.00 mm, was considered to be another successful strain. The antagonistic strains designated MTHM3, FT2.1, and MTHM2 formed inhibition zones of 8.67, 7.33, and 7.00 mm, respectively, and were therefore among the successful antagonists. Fourteen strains, considered effective antagonists, produced inhibition zones ranging from 2.00 mm to 6.67 mm, were classified into eight different groups based on statistical analysis, and were determined to be capable of suppressing the pathogen. A one-way analysis of variance (ANOVA) was conducted to evaluate the inhibitory effect of different antagonistic strains on *Pseudomonas savastanoi* pv. *savastanoi*. The results revealed a statistically significant difference among strains, $F(18,38) = 6.45$, $p < 0.001$. The effect size, calculated using eta squared (η^2), was 0.75, indicating a large effect (Table 7).

Table 7. Analysis of variance table for the effects of Antagonistic strains
Çizelge 7 Antagonist izolatların etkilerine ait varyans analiz tablosu

Soure of variation	df	SS	MS	F	P
Antagonistic strains	18	299.58	16.64	6.45	0000
Error	38	98.00	2.58<-		
Total	56	397.58			

Table 8. Inhibitory effect of antagonistic bacterial isolates on the growth of *Pseudomonas savastanoi* pv. *savastanoi'* under *in vitro* conditions.

Çizelge 8. Antagonistik bakteri izolatların in vitro koşullarda Pseudomonas savastanoi pv. savastanoi gelişimini engelleyici etkisi.

Antagonist bacterial isolates	Inhibition Zone (mm) on Petri plates			Mean
	1	2	3	
KSY9	10	10	8	9.33±1.51 a*
ABK.FY.2-5	9	9	9	9.00±1.39 ab
MTHM3	9	9	8	8.67±1.36 abc
FT2.1	7	7	8	7.33±1.15 a-d
MTHM2	8	6	7	7.00±1.14 a-e
TOROS6-1	7	6	7	6.67±1.05 b-f
ABK.FK.2-6	3	11	5	6.33±2.08 c-f
ABK.FT.2-5	5	6	5	5.33±0.85 d-g
ABK.FY.2-4	6	4	6	5.33±0.93 d-g
ABK.FY.2-6	6	2	6	4.67±1.28 e-h
ABK.FK.2-5	2	7	5	4.67±1.20 e-h
ABK.FT.1-5	2	6	5	4.33±1.09 f-i
ABK.FK.1-5	4	3	4	3.67±0.61 ghi
ABK.FT.2-4	3	3	4	3.33±0.56 ghi
ABK.FY.1-5	4	3	2	3.00±0.61 ghi
ABK.FT.1-6	3	2	3	2.67±0.47 hi
ABK.FY.1-6	1	4	2	2.33±0.76 hi
ABK.FK.1-6	5	1	1	2.33±1.22 hi
ABK.FK.2-4	3	1	2	2.00±0.52 i

*: Post-hoc comparisons using the LSD test (α = 0.05) showed that means sharing the same letter in the same column are not significantly different from each other.

Bouaichi et al. (2019) found that 18 antagonists used *in vitro* tests for biological control of bacterial strains from olive trees in Morocco formed inhibition zones ranging between 8 and 23 mm. Bitgen & Mirik (2021) found that İn vitro tests of 110 antagonistic bacterial strains obtained from the green parts and soil of olive trees suppressed pathogen development, with inhibition zones ranging from 3.5 to 28.00 mm for 23 antagonistic strains. In a study investigating the effect of antagonistic *Pseudomonas* strains obtained from different plants on the development of *Pseudomonas savastanoi* pv. *savastanoi*, Zadeh et al. (2008) reported that antagonistic strains showed a suppressive effect on pathogen development by forming inhibition zones ranging between 5 and 20 mm in dual culture plates. Filiz-Doksöz & Bozkurt (2022b) investigated the possibilities of biological control against Pseudomonas savastanoi pv. savastanoi using epiphytic and endophytic bacterial strains from healthy olive tree roots and shoots. They reported that 81 epiphytic and endophytic bacterial strains inhibited pathogen development to varying degrees. As a result of their study, it was highlighted that *Bacillus subtilis* HZEN1 (78.72%), *Bacillus megaterium* HZEP7 (60.87%), *Pseudomonas koreensis* HZEN27 (46.09%), and *Bacillus pumilus* HZEP29 (44.87%) strains could be considered as biological control agents against olive knot disease.

Diagnosis of Antagonistic Strains by Using MALDI-TOF MS

The most effective strains identified through MALDI-TOF MS diagnostic analysis were all found to belong to the genus *Bacillus*. According to the MALDI-TOF MS results, strain KSY9 (score: 1.761) and strain ABK.FY.2-5 (score: 1.768) were identified as *Bacillus subtilis*. Strains MTHM3, MTHM2, and FT2.1, with scores of 1.753, 1.722, and 1.702, respectively, were identified as *Bacillus amyloliquefaciens* subsp. *plantarum*.

In previous studies, it was observed that *Bacillus subtilis* strains are effective against the pathogen *Pseudomonas savastanoi* pv. *savastanoi*. This efficacy was associated with mechanisms such as antimicrobial activity, metabolite production, and systemic resistance of *Bacillus subtilis*, suppressing pathogen development (Shoda, 2000; Whipps, 2001). Bouaichi et al. (2018) determined that *Rahnella aquatilis*, *Enterobacter ludwigii*, *Paenibacillus brasiliensis*, and *Bacillus subtilis* strains selected from the Regional Agricultural Research Center collection were effective against *Pseudomonas savastanoi* pv. *savastanoi* strains under *in vitro* conditions. Inhibition zones ranged between 8 and 23 mm, with *Bacillus subtilis* strains notably forming wider inhibition zones. Similarly, Al-Khatib et al. (2010) demonstrated the antifungal activity of different bacterial strains (*Bacillus subtilis*, *Bacillus megaterium*, and *Bacillus cereus*) from air and water against *Venturia oleaginea*. *Bacillus* species secrete various extracellular metabolites such as antimicrobial substances, enzymes, pigments, and toxins, which trigger plant growth and enhance plant tolerance to biotic and abiotic stresses. Some of these species are commercially available as phytostimulants, biopesticides, and biostimulants. With this functional aspect, *Bacillus* spp. is one of the most commonly used species in the agricultural biotechnology industry (Etesami et al., 2023).

Studies also showed that different species of *Bacillus* have antimicrobial effects against various pathogenic bacteria. For instance, Aktepe & Aysan (2023) showed the effectiveness of *Bacillus* species in the biological control of Fire Blight Disease caused by *Erwinia amylovora*. Similarly, Güldoğan et al. (2022) used different *Bacillus* species in the biological control of Bacterial Speck Disease caused by *Pseudomonas syringae* pv. *tomato*. Tümen et al. (2022) and Mirik et al. (2008b) used *Bacillus* species in the biological control of bacterial spot disease in peppers. Antagonist bacterial isolates do not display antibacterial activities but also antifungal activities against various plant pathogenic fungal disease agents. Soylu et al. (2020) investigated the antimicrobial effectiveness of bacterial strains from vermicompost against soilborne fungal disease agents *Sclerotinia sclerotiorum, Macrophomina phaseolina, Botrytis cinerea*, and *Verticillium dahliae* under *in vitro* conditions, and reported that most of the strains exhibiting antagonistic effects belonged to the genus *Bacillus*. *Bacillus* species can persist in various ecosystems and provide natural protection against harmful organisms and promote plant growth, producing several traits (Soylu et al., 2016; Aktan & Soylu, 2020).

CONCLUSION

Makale A total of 26 pathogenic bacterial strains were isolated from nine olive branches exhibiting knot symptoms as part of the investigation into olive knot disease. All isolates exhibited characteristic features of Pseudomonas savastanoi pv. savastanoi, as indicated by their LOPAT profile (----+). Identification of the strains through MALDI-TOF MS analysis yielded high similarity index values, confirming species-level identification as Pseudomonas savastanoi. The antagonistic activities of 19 epiphytic and endophytic bacterial strains, previously archived in culture collections, were evaluated against the pathogen. Among these, strains KSY9, ABK-FY-2-5, MTHM3, FT2.1, and MTHM2 demonstrated the strongest inhibitory effects, with inhibition zones measuring 9.33 mm, 9.00 mm, 8.67 mm, 7.33 mm, and 7.00 mm, respectively. MALDI-TOF MS analysis identified all effective antagonists as members of the genus Bacillus. These findings suggest that these Bacillus strains have strong potential as biological control agents against *Pseudomonas savastanoi* pv. *savastanoi*, the causal agent of olive knot disease. Future research should focus on evaluating their efficacy under in vivo conditions in naturally infected olive orchards, with particular attention to elucidating their mechanisms of action. Furthermore, the use of these naturally occurring bacterial antagonists aligns with the principles of sustainable agriculture and highlights their potential as environmentally friendly tools within organic olive production systems.

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The authors declare that they have contributed equally to the article.

Conflict of Interest

The authors of the articles declare that they have no conflict of interest.

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All data generated or analyzed during this study are included in this published article.

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This article does not contain any studies with human participants or animals performed by the author.

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