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The Detection of Crown Gall Disease Caused by *Rhizobium vitis* in Kahramanmaras and Surrounding Provinces

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ABSTRACT
The study aims to isolate the bacterial disease agent *Rhizobium vitis* causing crown gall of grapevine in Kahramanmaraş and its surrounding provinces, determine its prevalence in the region, and conduct its identification. Between June and September of 2017-2019, strains obtained from the shoots and galls of vines showing symptoms of galls in vineyards in Kahramanmaraş, Adıyaman, Kilis, Gaziantep, and Şanlıurfa provinces were subjected to pathogenicity tests, classical, molecular, and MALDI-TOF MS identification. In this study, the prevalence of crown gall disease in 46 vineyards showing disease symptoms was determined as follows: Kahramanmaraş 76.47%, Adıyaman 75%, Gaziantep 50%, Kilis 33.33%, and Şanlıurfa 20%. From the 46 contaminated vineyards in the study area, 24 pathogenic *Rhizobium vitis* strains were obtained. In the pathogenicity test conducted with *R. Vitis* strains on the Hatun Parmağı grape variety under greenhouse conditions, the most virulent strains were determined based on the weights of the galls formed, and they were identified as CU52-3/2, CU39-11/5, and CU39-8/1. According to classical tests, 13 strains showed 100% similarity to *R. vitis*, while molecular characterization with *R. vitis*-specific primers revealed that 17 strains had the *virA* gene, 13 strains had the *pehA* gene, 10 strains had the *virF* gene, and 6 strains had the octopine-type plasmid. Ten strains, representing the sample, were identified as *R. vitis* through MALDI-TOF MS analysis. This study revealed the impact of pathogenic strains with different virulences on the same grape variety. In line with this, control methods against highly virulent bacterial agents in reducing losses in plants can be investigated for their applicability in field and orchard areas in subsequent studies. Research on different grape varieties is essential to develop varieties that are resistant or tolerant to *R. vitis*, the pathogen causing this prevalent disease in Turkish viticulture.

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Kahramanmaraş ve Çevre İllerdeki *Rhizobium vitis* Tarafından Neden Olunan Asma Uru Hastalığının Tespiti

ÖZET
Çalışmanın amacı, Kahramanmaraş ve çevre illerde asma ur hastalığına neden olan bakteriyel etmeni *Rhizobium vitis*'i izole etmek, bölgedeki yaygınlığını belirlemek ve teşhisini yapmaktır. 2017-2019 Haziran-Eylül aylarında Kahramanmaraş, Adıyaman, Kilis, Gaziantep ve Şanlıurfa illerindeki bağ alanlarından ur belirtisi gösteren asmanın sürgün ve urlarında izolayon sonucunda elde edilen izolatlar patojenite testi, klasik, moleküler ve MALDI –TOF MS tanılaması yapılmıştır. Bu çalışmada hastalık belirtisi gösteren 46 bağ alanında, asma ur hastalığı yaygınlığı Kahramanmaraş %76,47, Adıyaman %75, Gaziantep %50, Kilis %33,33 ve Şanlıurfa %20 olarak belirlenmiştir. Çalışma bölgesindeki bulaşık 46 bağ alanından 24 hastalık etmeni *Rhizobium vitis* izolatı izole edilmiştir. Sera koşullarında Hatun Parmağı asma

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çeşidinde *R. vitis* izolatları ile yapılan patojenite testi sonucunda oluşan urların ağırlıklarına göre en virülent izolatlar; CU52-3/2, CU39-11/5 ve CU39-8/1 olarak belirlenmiştir. Klasik testler sonucunda 13 izolatın *R. vitis*'e %100 benzer olduğunu; *R. vitis*'e özelleşmiş primerler ile yapılan moleküler karakterizasyon sonucunda 17 izolatın *virA* geni, 13 izolatın *pehA* geni, 10 izolatın *virF* geni, 6 izolatın ise oktopin tipi plazmide sahip olduğu belirlenmiştir. Temsilen seçilen 10 izolat MALDI-TOF MS analizi sonucunda *R. vitis* olarak teşhis edilmiştir. Bu çalışma ile aynı asma çeşidi üzerinde farklı virülenslikteki patojen izolatların etkisi ortaya konmuştur. Bu doğrultuda, yüksek virülens bakteriyel etmenlere karşı kontrol yöntemleri, bitkilerdeki kayıpları azaltmak için tarla ve bahçe alanlarında uygulanabilirliğinin araştırılması sağlanacaktır. Türkiye bağcılığında sorun olan bu hastalık etmeni için farklı asma çeşitleri üzerinde çalışmalar yapılarak *R. vitis*'e dayanıklı veya tolerant çeşitlerin geliştirilmesi sağlanabilecektir.

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INTRODUCTION

Anatolia lands, including Türkiye, is the homeland of the grapevine (*Vitis vinifera* L.). Grape production in Türkiye has a significant place in the world with 4,165,000 tonnes in an area of 3,845,365 decares in 2022 (excluding 2021) by increasing in the last five years in terms of variety richness, large vineyard areas and yield (Anonim, 2022). In the region of Kahramanmaras and its surrounding provinces, viticulture predominantly takes place in calcareous and arid areas, primarily utilizing low-structured, unsupported vineyard configurations such as goblet-style, and focuses on the cultivation of seeded table grapes and seeded drying grapes (Küsek, 2007). Today, many diseases and pests threaten the development of viticulture. These diseases and pests cause significant losses in growth and yield with the formation of favorable climatic conditions (Çelik et al., 1998). *Rhizobium* (*Agrobacterium*) *Vitis* ((Ophel & Kerr 1990) Young et al. 2001) is a bacterial agent carried by sap in grapevines and causes crown gall of grapevine disease (Young et al., 2001). The typical symptom of the disease is the formation of galls on the root collar, trunk, and shoots older than one year. It is not possible to determine the infection with the pathogen until the galls in grapevines become apparent. Fresh galls on the shoots and trunk are initially light in color and irregularly clustered, gradually darkening and developing a rough texture over time until they crack (Aysan & Küsek, 2018). The crown gall disease was first identified in vineyards in our country by Öktem (1978). In recent years, studies have reported crown disease in grape production areas in Central Anatolia, Mersin, Adana, Osmaniye, Hatay, Gaziantep, Adıyaman, Kahramanmaras, Tokat, and the Aegean and Thrace regions of Turkey (Benlioglu and Özakman, 1998; Argun, 2001; Küsek, 2007; Altınparmak, 2009; Orel, 2013; Durak et al., 2017; Akgül et al., 2018; Sivri, 2020). Burr and Katz (1983) suggested that the most suitable period for isolating the pathogen from the soil and gall is from June to September, while the period from April to May is recommended for isolation from plant sap. The entry of the pathogen through wounds and its ability to persist in plant tissues and soil for extended periods, coupled with the absence of an appropriate pesticide to prevent the disease, highlights the significance of the condition. Cultural measures, the use of biological control agents, and the planting of disease-resistant grape varieties are methods that can be applied in the control of the disease. In our country, Demir et al. (1998) determined the sensitivities of 24 different *V. vinifera* varieties and 20 different American grape rootstocks to various *R. Vitis* strains. The study concluded that the Cardinal grape variety had the least gall weight and exhibited the highest resistance. Additionally, varieties such as Hatun Parmağı, Pink Seedless, Sultana Seedless, Royal, Hamburg Misketi, Perlette, İtalya, and Alphonse Lavallee were identified as susceptible grape varieties to *R. Vitis* strains.

The physiological and biochemical tests proposed by Moore et al. (2001) are employed for the species-level identification of the *Rhizobium* genus. The molecular characterization of *Rhizobium vitis* strains obtained in our country was conducted using primers specialized for different plasmid types. Argun (2001) reported that all 8 *R. Vitis* strains obtained from the

Central Anatolian region produced octopine/cucumopine, while Akgül et al. (2018) determined, through PCR tests using specialized primer sets targeting the *pehA*, *virF*, *octopine*, *nopaline*, and *virD2* gene regions, that 16 *R. Vitis* strains from vineyards in the Aegean region produced octopine/cucumopine type opines. Küsek et al. (2007) identified 47 *R. Vitis* strains through PCR testing using specialized primers for the *virA* and *tms2a* gene regions.

The study aims to isolate the bacterial agent causing grape tumor disease in Kahramanmaraş and its surrounding provinces, determine its prevalence in the region, and conduct its identification. In line with this, control methods against highly virulent bacterial agents in reducing losses in plants can be investigated for their applicability in field and orchard areas in subsequent studies.

MATERIAL and METOD

The primary plant material for the study consisted of the diseased grapevine tissues collected from vineyards in the provinces of Kahramanmaras, Adıyaman, Kilis, Gaziantep, and Sanlıurfa from June to September in the years 2017-2019.

Sampling Method

The prevalence and incidence of the disease were calculated as percentages for each vineyard exhibiting symptoms in the study area. To determine the disease incidence in a vineyard, 50-200 plants were randomly selected based on the vineyard's size, beginning from different sections. These selected plants were examined for disease symptoms, and the number of plants displaying symptoms was recorded. The incidence was then calculated by multiplying the ratio of symptomatic plants to the total number of plants by 100 (Bora and Karaca, 1970). To assess the disease prevalence in vineyards, representative vineyards from each province were surveyed. The incidence was calculated by dividing the number of infected vineyards by the total number of surveyed vineyards and multiplying the result by 100.

Isolation of Disease Agent *Rhizobium* spp. Strains

Following the surveys in the study area, the skin tissue of freshly collected galls was incised using a sterile scalpel, and small fragments were excised from the underlying fresh living tissue. The gall tissue underwent superficial sterilization by immersion in 70% alcohol-soaked cotton. Subsequently, within a sterile mortar, the grape tissues were homogenized in sterile saline buffer (8.5% NaCl) and left to stand for one hour. A portion of this suspension was inoculated onto glass Petri dishes containing King B agar medium by streaking with a loop. Petri dishes were incubated at 25 ± 1°C for two days, and the typical colonies of *Rhizobium* spp. (cream-colored, partially transparent, flat, round, odorless, and convex) that developed on the petri dishes were subcultured until pure cultures were obtained by streaking on an NGA medium. Pure cultures were then transferred to Nutrient Broth (NB) agar plates containing +4°C and stored at -20°C with 20% glycerol for subsequent use in further studies.

Identification of Disease Agent *Rhizobium* spp. Strains

Pathogenicity test: Purified pathogenic strains obtained from grapevine tissues were carried out pathogenicity tests on Mother of Thousands (*Bryophyllum daigremontianum*), tomato (*Solanum esculentum* Mill.), and grapevine (*Vitis vinifera* L. cv. Hatun Parmağı) plants. After culturing the bacteria strains on Nutrient Glucose Agar (NGA) medium, they were inoculated using a sterile toothpick onto Mother of Thousands and tomato 2-3 weeks old seedlings plants using a sterile toothpick. For the Hatun Parmağı grapevine variety, which was rooted from 3-4-buds vineyard bud, were created three holes (5 mm wide, 5 mm deep) using a hand drill. Into these holes, 100 µl of a bacterial suspension containing 10^8 cfu/ml was inoculated using a sterile pipette. The holes were then sealed with moist cotton and wrapped with parafilm. After 24 hours of incubation, the cotton and parafilm were removed, and three months later, the formation of galls from the holes was assessed. The dimensions of the galls, including width, length, and height, were measured using calipers.

Classical identification: The classical identification of *Rhizobium* spp. strains demonstrating gall formation in pathogenicity tests were evaluated through the criteria specified by Moore et al. (2001). This involved assessing Gram reaction, acid clearing on PDA+$CaCO_3$ medium, growth on medium containing 2% NaCl, acid production from sucrose and melezitose, reaction in litmus milk, alkali production from malonic acid, oxidase test, KOH test, growth at 35°C, and citrate utilization.

Molecular identification: DNA isolation was performed for the 24 *Rhizobium* spp. strains with positive pathogenicity tests, following the protocol outlined by De Boer and Ward (1995). Polymerase Chain Reaction (PCR) studies utilized seven different primer pairs for specific genes: PGF/PGR for the *pehA* gene specific to *R. vitis* (Szegedi and Bottka, 2002), virA1/virA2 for the *virA* gene (Eastwell et al., 1995), virFF1/virFR2 for the *virF* gene (Burr et al., 1997), OCTF/OCTR for the *octopine* synthesis gene (Burr et al., 1997), NOPF/NOPR for the *nopalin* synthesis gene (Burr et al., 1997), virD2S4F/virD2S4R for the

virD2 gene (Burr et al., 1997), and Tms2A/Tms2B for the *Tms2* gene specific to *R. radiobacter* (Pulawska and Sobiczewski, 2005). For the PCR mix of *Rhizobium* spp. strains, 2 µl of DNA sample was combined with 38 µl of PCR Mix (4 µl Buffer, 1 µl DNTP mix, 1 µl Forward primer, 1 µl Reverse primer, 0.5 µl DNA Taq polymerase, 30.5 µl ddH_2O). Amplification in the PCR machine included an initial denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 10 seconds, annealing at 56°C for 15 seconds for *R. vitis*-specific PGF/PGR, virFF1/virFR2, and OCTF/OCTR primers, 52°C for 15 seconds for NOPF/NOPR primers, 58°C for 15 seconds for virD2S4F/virD2S4R primers, 50°C for 30 seconds for virA1/virA2 primers, 63°C for 30 seconds for *R. radiobacter*-specific tms2A/tms2B primers, and extension at 72°C for 15 seconds. Finally, a 5-minute extension at 72°C concluded the amplification. PCR products were analyzed on a 1.5% agarose gel electrophoresis following the protocol of Sambrook et al. (1989). For gel loading, 2 µl loading buffer and 6 µl of the PCR product mixture were added to the wells. Electrophoresis was run at 80 volts and 350 mA for approximately 2 hours. To determine band weights, a 100 bp molecular weight marker (Fermentas SM 0632) was used. Bands were visualized under UV light after staining the gels with Ethidium bromide, and the bands on the gels were examined and photographed.

Identification with MALDI-TOF MS: The 24 *Rhizobium* spp. strains, isolated from diseased plant tissues and determined to be pathogens based on pathogenicity studies, were also identified at the species level using MALDI-TOF MS (Bruker Daltonics GmbH, Bremen, Germany) for diagnosis (Pavlovic et al., 2012; Soylu et al., 2022). The identification service was obtained through service procurement at the Plant Health Clinic Application and Research Center of Hatay Mustafa Kemal University (https://www. mku.edu.tr/ departments.aspx?birim=218).

RESULTS and DISCUSSION

In the vineyards inspected in Kahramanmaraş and surrounding provinces, it has been determined that out of 74 vineyards, 46 vineyards are infected with crown gall disease. In Kahramanmaraş province, out of 34 vineyards inspected, 26 vineyards are infected with crown gall disease. In Adıyaman province, out of 12 vineyards inspected, 9 are infected. In Gaziantep province, out of 14 vineyards inspected, 7 are infected. In Kilis province, out of 6 vineyards inspected, 2 are infected. In Şanlıurfa province, out of 10 vineyards inspected, 2 are infected.

Referencing Table 1, it is evident that Kahramanmaras province exhibits the highest disease prevalence at 76.47%, whereas Şanlıurfa province demonstrates the lowest prevalence, standing at 20%. Analyzing the frequency of disease occurrence, Kahramanmaras emerges as the province with the highest incidence, recording a rate of 4.05%, whereas Sanlıurfa exhibits the lowest incidence at 0.73%. In the study by Altınparmak (2009), 280 *R. Vitis* strains were derived from grapevine samples in Konya province, demonstrating a prevalence rate of 90.61%. Similarly, Durak et al. (2017) isolated 34 *R. Vitis* strains from vineyards in Tokat province, reporting an infection rate of 75.65%. Sivri (2020), in an investigation on *R. Vitis* prevalence in the Thrace region, identified rates of 88.86% in Kirklareli, 80.21% in Edirne, and 65.91% in Tekirdag.

Table 1. The incidence and prevalence of crown gall disease by province.
Çizelge 1. Asma ur hastalığının illere göre rastlanma sıklığı ve yaygınlığı

Province	Incidence (%)	Prevalence (%)
Kahramanmaras	4.05	76.47
Adıyaman	3.87	75.00
Gaziantep	1.84	50.00
Kilis	1.00	33.33
Sanlıurfa	0.73	20.00

A total of 107 *Rhizobium* spp. strains were examined, and 24 strains demonstrated gall or hairy root formation on *Bryophyllum daigremontianum*, tomato (*Lycopersicum esculentum* Mill.), and the Hatun Parmağı grapevine cultivar were identified (Table 2). Küsek (2007) obtained 47 *R. Vitis* strains from grapevine gall samples collected in the Central Mediterranean Region. Akgül et al. (2018) obtained 16 *R. Vitis* strains from 31 gall samples collected from vineyards in the Aegean Region.

In the pathogenicity test on the Hatun Parmağı (susceptible) grapevine cultivar, 21 strains showed gall formation, and the strains with the highest gall dry weight were evaluated as CU52-3/2, CU39-11/5, and CU39-8/1, respectively. The CU38-1/2 strain could not be evaluated due to environmental adversities in the grapevine plant, and the CU37-1/4 and CU39-11/4 strains did not show gall formation in the grapevine. This situation is thought to arise from differences in host plant sensitivity, pathogen virulence levels, or the interaction between both (Anderson and Moore, 1979; Küsek, 2007; Altıparmak and Baştaş, 2011).

The classical diagnostic of the 24 candidate *Rhizobium* spp. strains showing gall/root hair formation in the pathogenicity test were determined through biochemical and physiological tests, as recommended by Moore et al. (2001). Similarity ratios to *Rhizobium vitis* are presented in Table 3. Among the pathogen strains obtained from pathogenicity

tests and classical tests, 13 strains exhibited 100% similarity to *Rhizobium vitis* in 12 tests specified by Moore et al. (2001). The remaining isolates showed similarities to *R. vitis* ranging from 58% to 92% in 7-11 tests. These findings align with the results of the studies conducted by Argun (2001) and Küsek (2007), supporting that the dominant species causing galls in vineyards is *R. vitis*.

Table 2. Strains that formed gall as a result of pathogenicity tests
Çizelge 2. Patojenite testi sonucunda ur oluşturan izolatlar

Isolate no	Pathogenicity Test			Sampling location	Coordinate
	Tomato	Mother of thousands	Grapevine		
CU5-4/4	+	+	+	Gölbaşı/Adıyaman	37°43'17"K; 37°33'22"D
CU5-4/10	+	+	+	Gölbaşı/Adıyaman	37°43'17"K; 37°33'22"D
CU14-3/1	+	+	+	İslahiye/Gaziantep	36°53'37"K; 36°34'16"D
CU14-3/2	+	+	+	İslahiye/Gaziantep	36°53'37"K; 36°34'16"D
CU14-3/7	+	+	+	İslahiye/Gaziantep	36°53'37"K; 36°34'16"D
CU25-2/1	+	+	+	Hilvan/Şanlıurfa	37°32'8"K; 38°46'50"D
CU37-1/4	+	+	-	Kılağlı/Kahramanmaraş	37°41'51"K; 37°3'48"D
CU38-1/2	+	+	+	Bahçeli/Kahramanmaraş	37°42'30"K; 37°6'12"D
CU38-1/11	+	+	+	Bahçeli/Kahramanmaraş	37°42'30"K; 37°6'12"D
CU39-11/2	+	+	+	Başdervişli/Kahramanmaraş	37°43'24"K; 37°9'4"D
CU39-11/3	+	+	+	Başdervişli/Kahramanmaraş	37°43'24"K; 37°9'4"D
CU39-11/4	+	+	-	Başdervişli/Kahramanmaraş	37°43'24"K; 37°9'4"D
CU39-11/5	+	+	+	Başdervişli/Kahramanmaraş	37°43'24"K; 37°9'4"D
CU39-13/2	+	+	+	Başdervişli/Kahramanmaraş	37°43'24"K; 37°9'4"D
CU39-8/1	+	+	+	Başdervişli/Kahramanmaraş	37°43'24"K; 37°9'4"D
CU39-8/2	+	+	+	Başdervişli/Kahramanmaraş	37°43'24"K; 37°9'4"D
CU52-2/2	+	+	+	Bulutoğlu/Kahramanmaraş	37°39'46"K; 36°48'34"D
CU52-2/4	+	+	+	Bulutoğlu/Kahramanmaraş	37°39'46"K; 36°48'34"D
CU52-3/1	+	+	+	Bulutoğlu/Kahramanmaraş	37°39'46"K; 36°48'34"D
CU52-3/11	+	+	+	Bulutoğlu/Kahramanmaraş	37°39'46"K; 36°48'34"D
CU52-3/2	+	+	+	Bulutoğlu/Kahramanmaraş	37°39'46"K; 36°48'34"D
CU52-3/4	+	+	+	Bulutoğlu/Kahramanmaraş	37°39'46"K; 36°48'34"D
CU52-3/5	+	+	+	Bulutoğlu/Kahramanmaraş	37°39'46"K; 36°48'34"D
CU52-3/6	+	+	+	Bulutoğlu/Kahramanmaraş	37°39'46"K; 36°48'34"D

+: Pathogenicity test positive; -: Pathogenicity test negative

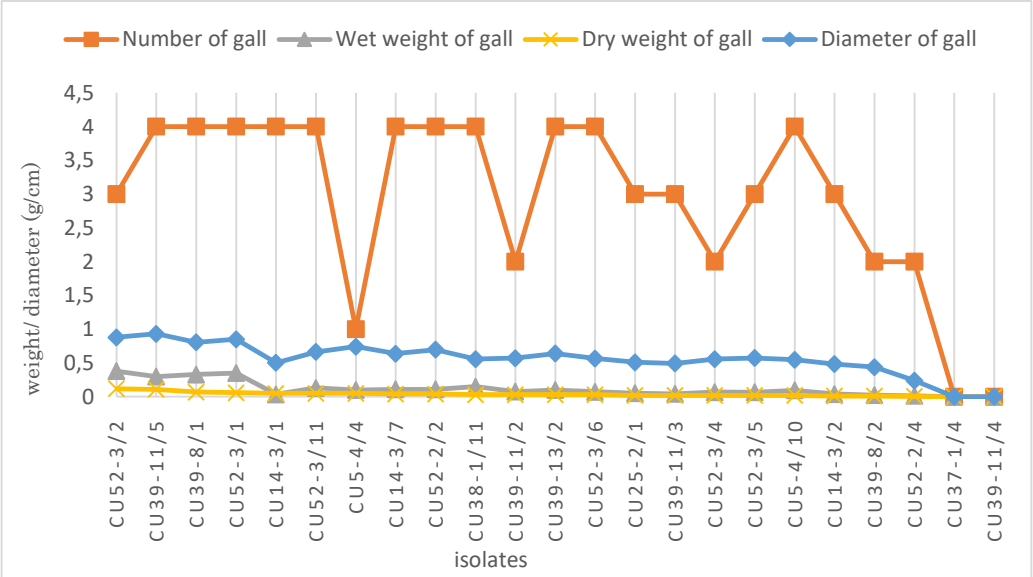

Figure 1. Pathogen strains occorred number, weight, and diameter of the gall of in Hatun Parmağı grapevine cultivar
Şekil 1. Hatun Parmağı asma çeşidinde izolatların ur sayısı, ağırlık ve çapları

As a result of pathogenicity and classical tests, molecular identification of 24 strains thought to be *R. Vitis* was performed using different primers, and electrophoresis gel images were taken (Figure 2). As a result of the PCR test with Tms2A/Tms2B, NOPF/NOPR, and virD2S4F/ virD2S4R primer pairs, none of the strain formed bands. According to the PCR test results with the PGF/PGR primer pair, which amplifies a fragment of 466 bp; CU38-1/2, CU39-8/1, CU39-8/2, CU39-11/3, CU39-11/5, CU39-13/2, CU52-2/2, CU52-2/4, CU52-3/1, CU52-3/4, CU52-3/5, CU52-3/6 and CU5-4/4 strains, according to the PCR test results with virA1/virA2 primer pair, which amplifies a fragment of 480 bp; CU14-3/2, CU39-8/1, CU39-8/2, CU39-11/2, CU39-11/3, CU39-11/4, CU39-11/5, CU39-13/2, CU52-2/2, CU52-2/4, CU52-3/1, CU52-3/2, CU52-3/4, CU52-3/5, CU52-3/6, CU52-3/11, and CU5-4/4 strains, according to the PCR test results with virFF1/virFR2 primer pair, which amplifies a fragment of 382 bp; CU39-8/2, CU39-11/4, CU39-11/5, CU39-13/2, CU52-2/4, CU52-3/1, CU52-3/2, CU52-3/5, CU52-3/6 and CU52-3/11 strains and according to the PCR test results with OCTF/OCTR primer pair, which amplifies a fragment of 475 bp; CU39-8/2, CU39-11/5, CU52-2/2, CU52-3/2, CU52-3/2, CU52-3/5 and CU52-3/11 strains formed bands. Additionally, strain CU14-3/1, CU14-3/7, CU25-2/1, CU37-1/4, CU38-1/2, CU38-1/11, and CU5-4/10 did not produce bands in the gel electrophoresis in the PCR test with the specific primer pairs (Table 4).

Table 3. The biochemical test used to differentiate Rhizobium spp. strains results.
Çizelge 3. Rhizobium spp. türlerini ayırmak için kullanılan biyokimyasal test sonuçları

Isolate No	Gram reaction	KOH Test	Oxidase Test	Litmus milk	Growth in % 2 NaCl	Growth in % 4 NaCl	Acid formation from sucrose	Acid formation from melioidosis	Alkali formation from malonic acid	Citrate use	Growth in 35 °C	Acid cleaning in PDA+$CaCO_3$	Similarity rate (%)
CU5-4/4	-	+	+	ALK	+	+	-	-	+	+	+	-	100
CU5-4/10	-	+	+	ALK	+	+	-	-	+	+	+	-	100
CU14-3/1	-	+	-	ALK	+	+	-	-	+	+	+	-	100
CU14-3/7	-	+	+	ALK	+	+	-	-	+	+	-	-	100
CU38-1/2	-	+	Z+	ALK	+	+	-	-	+	+	+	-	100
CU39-8/1	-	+	+	ALK	+	+	-	-	-	+	+	-	100
CU39-8/2	-	+	Z+	ALK	+	+	-	-	-	+	+	-	100
CU39-11/5	-	+	+	ALK	+	+	-	-	-	+	+	-	100
CU39-13/2	-	+	+	ALK	+	+	-	-	-	+	+	-	100
CU52-2/2	-	+	Z+	ALK	+	+	-	-	-	+	+	-	100
CU52-3/1	-	+	+	ALK	+	+	-	-	-	+	+	-	100
CU52-3/2	-	+	Z+	ALK	+	+	-	-	-	+	+	-	100
CU52-3/5	-	+	+	ALK	+	+	-	-	-	+	+	-	100
CU52-3/11	-	+	+	ALK	+	+	-	-	-	+	+	-	100
CU39-11/3	-	+	Z+	ALK	+	+	-	-	+	-	+	-	92
CU39-11/2	-	+	+	AC	+	+	-	-	+	+	-	-	83
CU14-3/2	-	+	-	ALK	+	+	+	+	+	-	+	-	75
CU38-1/11	-	+	-	ALK	-	-	-	-	+	-	+	-	75
CU37-1/4	-	+	+	ALK	-	-	-	+	+	-	-	-	67
CU39-11/4	-	+	-	AC	+	+	+	-	NC	-	-	-	67
CU52-2/4	-	+	-	AC	+	+	+	-	-	NC	-	-	67
CU52-3/4	-	+	-	ALK	-	-	+	-	-	+	+	-	67
CU52-3/6	-	+	Z+	ALK	+	+	+	+	+	+	-	-	67
CU25-2/1	-	+	Z+	AC	+	+	-	+	NC	-	-	-	58

AC: Acid; ALK: Alkaline; NC: No colour; +: 80% or more isolates positive; -: 80% or more isolates negative; M+ : Mild positivite

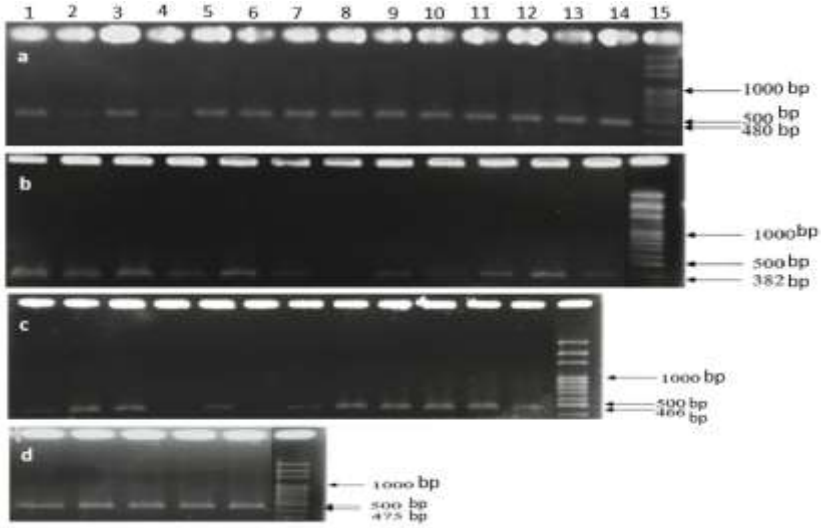

Figure 2. Primer gel images; strains forming bands for a) the VirA gene, b) the PehA gene, c) the virF gene, and d) the Octopine gene.

Şekil 2. Primer jel görüntüleri; a) VirA geni bant oluşturan izolatlar; b) PehA geni bant oluşturan izolatlar; c) virF geni bant oluşturan izolatlar; d) Oktopin geni bant oluşturan izolatlar

Table 4. The band formation status of Rhizobium spp. strains with different primers

Çizelge 4. Rhizobium spp. izolatlarının farklı primerlerde bant oluşturma durumları

	Primers						
Isolate no	virA1 virA2	PGF PGR	virFF1 virFR2	OCTF OCTR	NOPF NOPR	virD2S4F virD2S4R	Tms2A Tms2B
CU5-4/4	+	+	-	-	-	-	-
CU5-4/10	-	-	-	-	-	-	-
CU14-3/1	-	-	-	-	-	-	-
CU14-3/2	+	-	-	-	-	-	-
CU14-3/7	-	-	-	-	-	-	-
CU25-2/1	-	-	-	-	-	-	-
CU37-1/4	-	-	-	-	-	-	-
CU38-1/2	-	+	-	-	-	-	-
CU38-1/11	-	-	-	-	-	-	-
CU39-11/2	+	-	-	-	-	-	-
CU39-11/3	+	+	-	-	-	-	-
CU39-11/4	+	-	+	-	-	-	-
CU39-11/5	+	+	+	+	-	-	-
CU39-13/2	+	+	+	-	-	-	-
CU39-8/1	+	+	-	-	-	-	-
CU39-8/2	+	+	+	+	-	-	-
CU52-2/2	+	+	-	+	-	-	-
CU52-2/4	+	+	+	-	-	-	-
CU52-3/1	+	+	+	-	-	-	-
CU52-3/2	+	-	+	+	-	-	-
CU52-3/4	+	+	-	-	-	-	-
CU52-3/5	+	+	+	+	-	-	-
CU52-3/6	+	+	+	-	-	-	-
CU52-3/11	+	-	+	+	-	-	-

Genov et al. (2015) reported that most of the strains isolated from grapevines were *A. vitis* and some were *A. tumefaciens*. Considering the opine utilization of these strains, they reported that most *A. vitis* and some *A. tumefaciens* strains contained octopine/cucumopin plasmids, but nopaline-type plasmid was detected only in one *A. tumefaciens* strain.

The primers used in the study are synthesized from the plasmid regions of the bacterium, and mutations

that may occur on the plasmid can affect the PCR product formation (Küsek, 2007). In the PCR test conducted by amplifying *R. vitis*-specific primer sets, 17 strains were identified as *R. Vitis* with genes in different plasmid regions (*pehA*, *virA*, *virF*, *octopine*).

Eastwell et al. (1995) stated that the use of the pehA primer is more suitable for all *R. Vitis* strains causing galls in grapevines. Altıparmak and Baştaş (2011) identified 280 *R. vitis* strains using pehA and virA primers. In a study by Rouhrazi and Raiminan (2012), where vir gene sequences of 99 strains belonging to *R. radiobacter* and *R. vitis* species were determined, 49 strains were found to have octopine, 35 strains vitopine, and 8 strains nopaline-type opine synthesis genes, while the opine type of 7 strains could not be determined. In the primer set specific to *R. radiobacter*, it was observed that none of the strains produced a band. The results of molecular identification were found to be consistent with the results obtained from classical tests. Lamovsek et al. (2014) identified 80 *Agrobacterium* spp. strains from Slovenian vineyards as *A. vitis* and *A. tumefaciens* using pehA and multiplex PCR methods. Orel et al. (2017) revealed the presence of nopaline-type plasmids in 18 strains and vitamin-type plasmids in three strains among 82 *R. Vitis* strains obtained from Turkish vineyards using PCR and opine-specific primers. Akgül et al. (2018) determined that 16 *R. Vitis* strains obtained from the Aegean region were responsible for the synthesis of octopine/cucumopine-type opines The pathogenicity, classical tests, and molecular characterization of the 24 *Rhizobium* spp. strains were determined, and their identification was performed through service procurement using MALDI-TOF MS (Bruker Daltonics GmbH, Bremen, Germany) at the Plant Health Clinic Application and Research Center of Hatay Mustafa Kemal University.

Table 5. The species identification of Rhizobium spp. strains by MALDI-TOF MS
Çizelge 5. Rhizobium spp. izolatlarının MALDI-TOF MS ile tür tanılaması

Isolate No	Matching Results	Reliability Level
CU14-3/7	CFBP-5523-*Rhizobium vitis*_20170306	2,215
CU39-8/1	CFBP-5523-*Rhizobium vitis*_20170306	2,149
CU39-11/2	CFBP-5523-*Rhizobium vitis*_20170306	2,325
CU39-11/5	CFBP-5523-*Rhizobium vitis*_20170306	2,291
CU39-13/2	CFBP-5523-*Rhizobium vitis*_20170306	2,336
CU52-2/2	CFBP-5523-*Rhizobium vitis*_20170306	2,211
CU52-3/1	CFBP-5523-*Rhizobium vitis*_20170306	2,110
CU52-3/2	CFBP-5523-*Rhizobium vitis*_20170306	2,331
CU52-3/5	CFBP-5523-*Rhizobium vitis*_20170306	2,071
CU52-3/11	CFBP-5523-*Rhizobium vitis*_20170306	2,153

2.30-3.0: Highly reliable at the species level; 2.00-2.299: Reliable at the genus level, highly reliable at the species level; 1.70-1.999: Reliable at the genus level, probable at the species level; <1.7: Unreliable identification

After MALDI-TOF analysis, the similarity status of 10 strains with an index value of 2.00 and above to *Rhizobium vitis* species is presented in Table 5. CU39-13/2, CU39-11/2, and CU52-3/2 strains showed a high similarity to *R. Vitis* at the species level, while CU14-3/7, CU39-11/5, CU39-8/1, CU52-3/11, CU52-2/2, CU52-3/1, and CU52-3/5 strains exhibited a definite resemblance to *R. Vitis* at the genus level and a high similarity at the species level. MALDI-TOF MS is a rapid, inexpensive, and reproducible method for the identification of plant pathogenic and saprophytic microorganisms (Uysal et al., 2019; Weis et al., 2020). It allows the differential identification of filamentous fungi, endophytic, and epiphytic bacterial species from a variety of environments using the distinct protein and peptide profiles of microbial cells. (Aktan and Soylu, 2020; Kara et al., 2020; Soylu et al., 2021; Tarfeen et al., 2022; Uysal et al., 2022; Soylu et al., 2022; Bozkurt et al., 2023).

CONCLUSION and RECOMMENDATIONS

The prevalence of crown gall disease in vineyard production areas in Kahramanmaraş, Adıyaman, Gaziantep, Kilis, and Şanlıurfa provinces was determined as follows: Kahramanmaraş 76.47%, Adıyaman 75.00%, Gaziantep 50.00%, Kilis 33.33%, and Şanlıurfa 20.00%. As a result of surveys conducted in the study area, a total of 24 pathogenic strains were obtained from galls collected from grapevines in the July-August period. These strains were tested on *Bryophyllum daigremontianum* (kalanchoe) and tomato (*Lycopersicum esculentum* Mill.) plants, resulting in the formation of galls and/or hairy roots. In the pathogenicity test conducted on the Hatun Parmağı grapevine variety, strains with virulence were determined based on gall dry weights as CU52-3/2, CU39-11/5, and CU39-8/1. The strains CU38-1/2 could not be evaluated due to developmental issues observed in the plant in the pathogenicity test. In the biochemical tests, 13 of the

24 pathogenic strains showed 100% similarity to *Rhizobium vitis*, as specified by Moore et al. (2001), while 11 strains exhibited similarity ranging from 66.6% to 75% with *R. vitis*. As a result of the molecular characterization of pathogenic strains, it was determined that 17 strains possessed the *virA* gene, 13 strains had the *pehA* gene, 10 strains contained the *first* gene, and 6 strains carried the *octopine* synthesis gene. None of the strains was found to have plasmids containing the nopaline, virD2, and Tms2 genes. Using primer sets specific to *R. Vitis*, 18 strains (CU14-3/2, CU38-1/2, CU39-8/1, CU39-8/2, CU39-11/2, CU39-11/3, CU39-11/4, CU39-11/5, CU39-13/2, CU52-2/2, CU52-2/4, CU52-3/1, CU52-3/2, CU52-3/4, CU52-3/5, CU52-3/6, CU52-3/11, and CU5-4/4) were identified as *R. vitis*, each harboring genes in different plasmid regions (*pehA*, *virA*, *virF*, *octopine*). According to the MALDI-TOF MS analysis of pathogenic isolates, 10 strains (CU39-13/2, CU39-11/2, CU52-3/2, CU14-3/7, CU39-11/5, CU39-8/1, CU52-3/11, CU52-2/2, CU52-3/1, and CU52-3/5) showed high similarity to *R. vitis*, with an index value of 2.00 or above.

In conclusion, it has been determined that the dominant species causing grapevine galls in vineyards is *R. vitis*, and the pathogen is widespread in the region's vineyards. Furthermore, classical and molecular tests support each other in the identification of *R. vitis*. This study reveals the impact of pathogenic isolates with different virulence on the same grapevine variety. However, studies on different grape varieties can be conducted to develop *R. Vitis-resistant* or tolerant varieties, addressing this problematic pathogen in Turkish viticulture.

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

Conflict of Interest Statement

The authors of the article declare that there is no conflict of interest between them.

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