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RESEARCH PAPERS - 9TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

Fungal trunk pathogens of Sultana Seedless vineyards in Aegean region of Turkey

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Summary. In recent years, grapevine trunk diseases have become a problem in Sultana Seedless vineyards of Manisa and Izmir provinces (Aegean Region, Turkey). A field survey was conducted in 2013 in these provinces (in 8 cities and 80 vineyards) to determine disease incidence, fungal species associated with grapevine trunk diseases and pathogenicity. Symptomatic vines were grouped by two different grapevine trunk disease symptoms: (1) typical tiger-striped leaves, (2) dead arm, shoot decline or apoplexy. Over 80% of vineyards in these areas were positive for at least one characteristic trunk disease symptom. Incidence of tiger-stripe symptom ranged from 2.9-15% and incidence of apoplexy ranged from 0–4.2%. Eight fungal species in five fungal families were identified from declining grapevines based on morphological and molecular (ITS, β-tubulin and EF1-α) studies including, *Botryosphaeria dothidea, Diplodia seriata, Lasiodiplodia theobromae, Neofusicoccum parvum, Diaporthe ampelina, Phaeomoniella chlamydospora, Togninia minima* and *Fomitiporia mediterranea*. Overall, *D. ampelina* was the most frequently recovered fungus from symptomatic grapevine tissues followed by botryosphaeriaceous fungi, *P. chlamydospora, F. mediterranea* and *T. minima*. Pathogenicity tests confirmed all eight fungi as pathogens of grapevine in these regions with *N. parvum* being the most virulent among the fungi tested.

Key words: Botryosphaeriaceae, esca, *Diaporthe ampelina, Togninia minima, Vitis vinifera*

Introduction

Grapevine (*Vitis vinifera* L.) is one of the major fruit crops in Turkey with over 4.2 million metric tones of grapes produced in 2012, which accounted for 6.3% of the total world production (FAO, 2013). Turkey is one of the leading countries on raisin exports in the world with 85% of raisins exported to European Union countries. Approximately 49.2% of Turkey's total grape production is from the Aegean Region (Western Turkey) with Sultana Seedless as the most prevalent cultivar, which is primarily planted in the Manisa Province.

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Grapevine trunk diseases (GTD) have become an important problem of grape-growing areas all over the world. These diseases may affect vineyard productivity and longevity by causing cost increases and yield losses. When disease occurs in a vineyard, a variety of characteristic symptoms may appear on leaves, roots, trunks, inner wood tissues and vascular bundles. Chlorotic rounded irregular spots or tiger-striped leaves, dead arm, wedge-shaped discoloration and deterioration of wood, delayed bud burst of vines, reduced vigor, cane bleach and streaking in xylem vessels are some of the well-known symptoms of GTD. GTD are caused by various fungal species from different families including Botryosphaeriaceae, Diatrypaceae (dead arm, wood canker, and dieback), *Phaeomoniella (Pa.) chlamydospora, Phaeo-*

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acremonium spp., *Fomitiporia* spp., (Esca syndrome), *Campylocarpon* spp., *Dactylonectria* spp., *Ilyonectria* spp., and *Neonectria* spp., (black foot and dieback) in young and older vines (Mugnai *et al*., 1999; Halleen *et al*., 2006; Slippers and Wingfield, 2007; Trouillas *et al*., 2010; Lombard *et al*., 2014). When favorable conditions are present, these fungi can cause disease individually or together, hence some of the characteristic symptoms may appear in a single vine. In Turkey, the first study on GTD, aimed at determining the main fungal pathogens, was conducted 17 years ago. Erkan and Larignon (1998) first detected *Pa. chlamydospora* and *Togninia minima* in the Aegean Region's Sultana Seedless, Kozak Beyazi, Kozak Siyahi and Alphonse Lavalleé cultivars. In addition to these fungi, *Stereum hirsutum* (Willd.) Pers. and *Phellinus igniarius* (L.) Quel. were isolated and identified in that study. Köklü (2000) conducted a survey study in the Thrace Region (northwest of Turkey) to rate esca disease occurrence in 14 table-and wine-grape cultivars in 26 vineyards. The rate of vines showing typical esca symptoms was found to be 1.6%, from those the tiger striped leaf necrosis rate was 1.4% (133) and apoplexy rate was 0.2% (17) in total inspected vines (9291). Özben *et al*. (2012a and 2012b) screened 67 vineyards in the Ankara Region (Midwest Turkey) to determine fungal trunk pathogens associated with declining grapevine in this region and reported *Phaeoacremonium scolytii* and *Dactylonectria macrodidyma* associated with grapevine for the first time in this location and Turkey. Akgül *et al*. (2013 and 2014a) isolated four species of Botryosphaeriaceae fungi from vineyards having wood canker and decline symptoms in 15 different locations (Ankara, Çorum, İzmir and Manisa cities) of Turkey. These species were identified and reported as *Botryosphaeria dothidea*, *Diplodia seriata*, *Neofusicoccum parvum* and *Lasiodiplodia theobromae*. In addition, one isolate of *Diaporthe neoviticola* and *Campylocarpon fasciculare* were isolated for the first time from 13 different vineyards and 15 grapevine nurseries in Manisa city (Akgül *et al*., 2014b and 2014c).

During the last 10 years, GTD have dramatically increased in Sultana Seedless vineyards in Manisa and Izmir provinces. Most of the grape growers requested local government agencies for more information about the etiology of vine decline, dead arm, or apoplexy diseases in their vineyards creating a need to determine the current status of diseases and their fungal pathogens in this region. Most of the propagation materials (such as scions, rootstocks and buds) or grafted young plants are sold and transported from these locations to the other grape growing regions of Turkey. Doing accurate identification of the pathogens and determination of diseases an imperative preliminary step to prevent GTD spreading in Turkey vineyards.

The purpose of this study was to (i) index disease symptoms in Sultana Seedless vineyards in Manisa and Izmir provinces (ii) determine the occurrence and prevalence of GTD in these vineyards (iii) identify fungi associated with declining grapevine using morphological characteristics and (iv) assess the pathogenicity of fungi associated with declining grapevine in Manisa and Izmir Sultana Seedless vineyards in Turkey.

Materials and methods

Field survey, disease symptoms and isolation of fungi

Field surveys were conducted throughout 2013 in 97 vineyards cv. Sultana Seedless in Manisa (Ahmetli, Alasehir, Merkez, Salihli, Sarigol, Saruhanli, Turgutlu cities) and Izmir (Menemen cities) provinces in the Aegean Region (Figure 1). Eighty vineyards (100 vines from each) were inspected for symptoms incidence used here in the total survey area.

Approximately one-ha-area vineyards (10 to 25 years old) were selected to determine the incidence of GTD symptoms in mid-September. Ten rows from each vineyard (three rows from left-right sides and four rows from center) were examined and 10 vines were counted from the center of the each row. The symptomatic vines were recorded into two groups; 1) having typical tiger-striped leaves, 2) dead arm, shoot decline and apoplexy. Occurrence of the symptoms was expressed as a percentage in a vineyard and mean percentages were calculated for each city. Three to five symptomatic wood samples (showing 1: wedge-shaped brown canker lesions, 2: dark brown or black spots in xylem vessels and 3: yellowish spongy rot in cross sections) were taken from each vineyard in 8 cities totaling 232,500 ha area (Table 1). These samples were taken from the vines including in the visual inspection.

Samples were transported in a cooler to the laboratory at Manisa Viticulture Research Station for examination and isolation. Trunk and branch parts were washed with tap water to remove rough debris and dried with a paper towel. Woody parts were surface

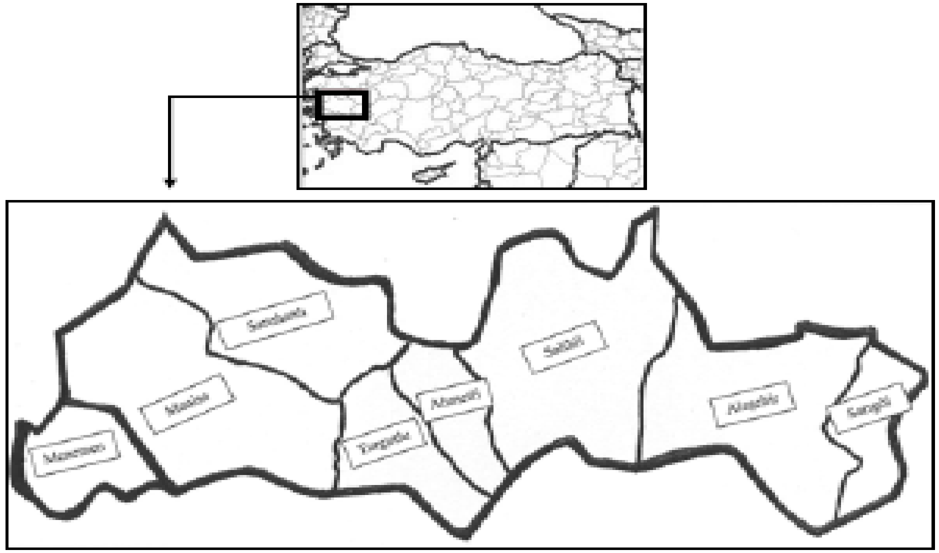

Figure 1. Map of Turkey showing the Sultana Seedless raisin-grape production region of the Aegean where the vineyards were surveyed.

disinfested with 96% ethanol and flame sterilized to burn off ethanol. The outer bark was removed and 5–6 mm^2 sized pieces at the margin of necrotic tissues were cut with a sterile scalpel, then six to seven pieces were placed onto potato dextrose agar (PDA; Merck) amended with 0.015% streptomycin sulphate (Sigma-Aldrich) (PDA-str). Petri plates were incubated in the dark for 5–6 days at 24°C. Colonies of the fungal isolates were sub-cultured onto fresh PDA-str by hyphal tipping and after colony development pure fungal cultures were stored as fungal plugs in 30% glycerol and water at 4°C. Isolation frequency was calculated by counting fungal colonies growing from wood chips placed on petri plates and proportions of fungi for each vineyard expressed as total colony number of each fungus to total wood chips (plated onto PDA).

Morphological identification

Morphological identification was done on the basis of colony morphology, pycnidiospore formation, conidia or conidiophore shapes on PDA. Botryosphaeriaceae isolates were inoculated on 25-cm-long Sultana Seedless dormant cuttings to induce pycnidial formation. Mycelial agar plugs (six-day-old) were placed into the wounds done on the cuttings and the inoculation sites were covered with parafilm. The bottom of the cuttings were placed into beakers containing tap water and maintained in a growth chamber with the following conditions (25°C temp., 85% RH, 12-h photoperiod) for 25–30 days. After pycnidia formation, pycnidia were collected with a sterile surgical blade and crushed on a slide before microscopic examination. For the remaining fungal isolates, sterilized wood chips were placed onto PDA-str and fungi allowed to colonize at 24°C, 12-h photoperiod, for 15–20 days. Conidial dimensions (length and width of 25 conidia per isolate) were measured using a compound microscope (Olympus BX-51 attached with Olympus Camedia-4501X) with ocular and objective micrometer. Average dimensions were recorded and compared with previous studies (Table 2).

Table 1. Information regarding vineyards sampled, average incidence of disease symptoms and fungal isolation frequency by grape growing province and cities in Turkey.

Survey locations		Number of vineyards sampled	Total vineyard area (ha)	Average incidence of GTD symptoms in sampled vineyards (%)		Isolation frequency (%)				
Province	City			Group 1[a]	Group 2	Botryosphaeriaceae[b]	*D. ampelina*	*F. mediterranea*	*P. chlamydospora*	*T. minima*
Manisa	Ahmetli	8	5042.5	4.1	2.7	19.7	17.2	-	11.1	2.0
	Alasehir	18	18250	4.6	2.4	4.3	20.2	-	4.0	-
	Merkez	5	8560	7.5	3.3	2.0	10.0	6.8	-	-
	Salihli	15	9621.5	4.5	2.4	5.8	22.6	1.5	-	-
	Sarigol	7	7845	3.0	-	1.3	19.7	-	-	-
	Saruhanli	7	8252.5	15.0	2.0	14.5	3.8	0.2	5.7	-
	Turgutlu	15	7680	14.0	2.8	13.8	11.2	0.6	2.7	1.5
Izmir	Menemen	5	2732	2.9	4.2	3.6	18.2	1.5	1.9	1.6
	TOTAL	80	67983.5	-	-	-	-	-	-	-

[a] Group numbers, (1) typical tiger-striped leaves; (2) local dead arm, shoot decline or apoplexy
[b] Includes *B. dothidea*, *D. seriata*, *L. theobromae* and *N. parvum*

DNA extraction and PCR amplification

Fungal DNA was extracted using a slight modification of the protocol of Cenis (1992). Mycelial mats (approximately 50 mg) were taken from fresh cultures of the isolates with a sterile surgical blade and crushed with a plastic pestle in micro-centrifuge tubes containing 550 µL DNA extraction buffer (200 mM Tris-HCl (pH:8.5), 250 mM NaCl, 25 mM EDTA and 2% Sodium Dodecyl Sulphate). After homogenization, 150 µL of 3M Sodium Acetate (NaOAc) was added into tubes and tubes were placed at -20°C for 15 min. The homogenates were centrifuged for 10 min at 14,000 rpm and the supernatants (200 µL) were transferred to the new tubes. An equal volume of isopropanol (2-propanol) was added and mixed gently about five times, and the tubes were placed at 0°C for 10 min. Precipitated DNA was pelleted by centrifugation at 14,000 rpm for 10 min and supernatant was discarded. The DNA pellet was washed with 1 mL of 70% ethanol and the pellet was air-dried for 10 min. DNA was re-suspended in 75 µL of TE (1M Tris-HCl, pH:8 and 0.5M EDTA) buffer and stored at -20°C.

Oligonucleotide primers ITS4 and ITS5 were used to amplify the ITS1, 5.8S, and ITS2 region of the rDNA (White *et al.*, 1990). A partial sequence of the β-tubulin nuclear gene and translation elongation factor (EF) 1-α were amplified using the Bt2a and Bt2b (Glass and Donaldson, 1995) and EF1-728F and EF1-986R (Carbone and Kohn, 1999) primer pairs respectively. PCR reactions were conducted in a real-time thermal cycler (Roche Light-Cycler Nano). Each of the 30-µL PCR reaction tubes contained 15 µL of FastStart Essential DNA Green Master mix (Roche), 11.1 µL nuclease free PCR-grade water, 0.45 µL of 20 mM primer, and 3 µL template DNA. The reaction protocols for ITS, β-tubulin and EF1-α were as follows; 95°C for 10 min (initial denaturation), followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 10 s (ITS4-ITS5 and EF1-728F & EF1-986R), 55°C for 10 s (Bt2a-Bt2b), extension at 72°C for 20 s, and a final extension at 72°C for 10 min. After amplification, PCR products were separated by gel electrophoresis in 2.0% agarose (Sigma) gels in 1x Tris-Acetic acid-EDTA (TAE) buffer to check DNA quality visually. PCR products were

Table 2. Morphological characteristics of the GTD fungi used in this study

	Colony morphology (on PDA)	Conidium morphology	Growth rate at 24°C in dark	Conidial size (μm)		
				In this study	In previous studies	Reference
Botryosphaeria dothidea	Aerial mycelium initially colorless, turning dark olive from center	Hyaline, ellipsiod to fusoid, smooth and aseptate	65–70 mm for 5 days	28.1 × 7.1	28.8 × 7.4	Smith and Stanosz (2001)
Diplodia seriata	Greyish-black color with dense fluffy aerial mycelium	Hyaline, ellipsoid, becoming dark brown, moderately thick-walled, generally aseptate but rarely one-septate	70–75 mm for 5 days	28.3 × 10.9	29.1 × 11.8	Adesemoye *et al.*, (2014)
Diaporthe ampelina	Mycelium superficial, slightly raised with white undulating growth. Colonies produce pycnidia which exuded light-cream cirrhi containing both alpha and beta conidia.	Alpha conidia hyaline, ellipsoidal and unicellular. Beta conidia hyaline, filiform and slightly curved	50–55 mm for 20 days	10.0 × 2.4 (α) 22.5 × 1.0 (β)	10.0 × 2.5 (α) 23.0 × 1.0 (β)	Gomes *et al.*, (2013)
Fomitiporia mediterranea	Abundant, yellowish-brown aerial mycelium, hyphae septate and branched	-	40–40 mm for 7 days	-	-	Fischer (2002)
Lasiodiplodia theobromae	Greyish-brown to black with dense, fluffy aerial mycelium	Producing abundant conidia on PDA, sub-ovoid to ellipsoid, thick-walled, with longitudinal striaitons and one-septate. Conidia color initially hyaline, turning dark-brown with age	65–70 mm for 5 days	21.9 × 10.4	22.6 × 10.0	Costa *et al.*, (2010)
Neofusicoccum parvum	Color white with fluffy aerial mycelium, turning pale olivaceous gray but turning black with age	Ellipsoidal with round apices and aseptate	65–70 mm for 5 days	18.1 × 5.0	19.0 × 5.2	Costa *et al.*, (2010)
Phaeomoniella chlamydospora	Olive-green to white (at margin) and yeast like growing	Abundant, hyaline, aseptate and generally aggregated	18–20 mm for 14 days	3.2 × 1.4	3.5 × 1.5	Crous and Gams (2000)
Togninia minima	Mycelium greyish white at first, slightly raised and reverse greyish-brown to dark brown	Simple, hyaline, aggregated and ellipsoidal	17–18 mm for 20 days	3.1 × 1.2	2-5 × 1-1.5	Pascoe *et al.*, (2004)

sequenced by Macrogen Co. (South Korea) and the sequences were compared with those deposited in the NCBI GenBank database using the BLAST program (version 2.0; National Center for Biotechnology Information, United States National Institutes of Health). The sequences of the three gene locations (ITS, β-tubulin and EF1-α) were also submitted to the NCBI GenBank and accession numbers were obtained (Table 3).

Pathogenicity tests

Pathogenicity tests were conducted under greenhouse conditions (25°C temp., 80% RH) on 1-year-old rooted grapevine (*Vitis vinifera* L.) cv. Sultana Seedless plants using four isolates of each species. The dormant cuttings (containing five to six buds) were planted in 2:1:1, soil: peat moss: vermiculite mixture in 1 L plastic bags and they were maintained in the greenhouse for 30–40 days to encourage rooting. Stems of the grapevine plants were wounded by removing bark with a 5-mm-diameter cork-borer and mycelial agar plugs were placed into the holes (Van Niekerk *et al*., 2004). Control plants were inoculated with sterile agar plugs. Inoculation points were covered with parafilm and plants were maintained for 15 to 16 weeks to evaluate pathogenicity after which plants were uprooted and inspected for lesion development. The extent of discolored wood (lesions) was measured acropetally and basipetally from the inoculation point. To assess differences in the extent of lesions, analysis of variance (ANOVA) was performed and means were compared using Fisher's least significant difference (LSD) test at the 5% significance level (Gomez and Gomez, 1984). In order to fulfill Koch's postulates, small pieces of discolored tissues were cut from the inoculated plants and placed onto PDA-str and incubated in 24°C. Developing colonies were morphologically compared with previously inoculated colonies and isolation frequency was calculated. The pathogenicity tests were arranged in a completely randomized design with four replications and were conducted twice.

Results

Field survey, disease symptoms and isolation of fungi

Of the vineyards surveyed, 82.5% were observed to have most of the GTD symptoms in the survey region. The incidence of vines showing typical tiger stripe symptom ranged between 2.9–15.0% in all surveyed area. The lowest (2.9%, average of five vineyards) and highest (15.0%, average of the seven vineyards) mean was obtained from Menemen and Saruhanli cities, respectively. The average incidence of this symptom from the other cities were; 14.0% (Turgutlu), 7.5% (Merkez), 4.6% (Alaşehir), 4.5% (Salihli), 4.1% (Ahmetli) and 3.0% (Sarigol). Dead arm and/or apoplexy symptoms were seen in all the Sultana Seedless vineyards (except Sarigol) in surveyed areas, but the incidence of these symptoms were lower than tiger striped symptom. The highest rate (4.2%) was from Menemen, while the lowest rate (0%) was from Sarigol (Table 1). When main branches and woody shoots of vines were inspected, wedge-shaped brown discolored tissues or black necrotic spots were found in the inner parts.

Approximately 350 wood samples were collected from 80 vineyards and eight different fungal species associated with GTD were isolated (Table 1). According to morphological characteristics and molecular analyses, the fungi associated with GTD were found to be the members of the Botryosphaeriaceae; *Botryosphaeria dothidea*, *Diplodia seriata*, *Lasiodiplodia theobromae* and *Neofusicoccum parvum*, Diaporthaceae; *Diaporthe ampelina*, Calosphaeriaceae; *Phaeomoniella chlamydospora*, *Togninia minima* and Hymenochaetaceae; *Fomitiporia mediterranea*. Though GTD symptoms were seen throughout the vineyards surveyed, the fungal species associated with esca syndrome could not be isolated from all vineyards.

D. ampelina and Botryosphaeriaceae members were the most commonly isolated fungi from all survey areas. Generally, the frequency of *D. ampelina* was higher than that of Botryosphaeriaceae fungi. The maximum percentage of *D. ampelina* was obtained from Salihli (22.6%) and most of the cities, except Sarigol, had higher isolation frequencies (more than 10%). Botryosphaeriaceae members were the second most frequently isolated fungi in all survey areas. While minimum percentage (1.3%) was recorded from Sarigol, maximum percentage (19.7%) was obtained from Ahmetli city (Table 1). The maximum isolation frequency of *P. chlamydospora*, *T. minima* and *F. mediterranea* was 11.1% (in Ahmetli), 2.0% (in Ahmetli) and 6.8% (in Merkez, Manisa) respectively. Considering all survey areas, Turgutlu and Menemen were two cities in which all GTD pathogens mentioned above were isolated.

Table 3. GTD fungi from Sultana Seedless vineyards of the Aegean Region that were used in this study.

Isolate	Identity	Host (*V. vinifera* cv.)	Origin	GenBank Accession Number		
				ITS	β-tubulin	EF1-α
MBAi13AG	*Phaeomoniella chlamydospora*	Sultana Seedless	Horozkoy	KP083211	KP721669	KP721637
MBAi20AG	*P. chlamydospora*	S. Seedless	Yunidagi	KP083212	KP721670	KP721638
MBAi156AG	*P. chlamydospora*	S. Seedless	Turgutlu	KP083213	KP721671	KP721639
MBAi157AG	*P. chlamydospora*	S. Seedless	Ahmetli	KP083214	KP721672	-
MBAi169AG	*P. chlamydospora*	S. Seedless	Saruhanli	KP083215	KP721673	KP721640
MBAi18AG	*Togninia minima*	S. Seedless	Horozkoy	KP083216	KP721674	KP721641
MBAi40CL	*T. minima*	110 Richter	Horozkoy	KF460428	KP721675	KP721642
MBAi133AG	*T. minima*	S. Seedless	Menemen	KP083230	KP721676	-
MBAi150AG	*T. minima*	S. Seedless	Ahmetli	KP083218	KP721677	KP721643
MBAi151AG	*T. minima*	S. Seedless	Turgutlu	KP083217	KP721678	KP721644
MBAi152AG	*T. minima*	S. Seedless	Turgutlu	KP083219	KP721679	KP721645
MBAi153AG	*T. minima*	S. Seedless	Menemen	KP083231	KP721680	-
MBAi155AG	*T. minima*	S. Seedless	Saruhanli	KP083220	KP721681	KP721646
MBAi170AG	*T. minima*	S. Seedless	Ahmetli	KP083232	KP721682	-
MBAi35AG	*Diaporthe ampelina*	S. Seedless	Horozkoy	KP083221	KP721683	KP721647
MBAi93AG	*D. ampelina*	S. Seedless	Saruhanli	KP083222	KP721684	KP721648
MBAi95AG	*D. ampelina*	S. Seedless	Saruhanli	KP083223	KP721685	-
MBAi190AG	*D. ampelina*	S. Seedless	Salihli	KP083224	KP721686	-
MBAi191AG	*D. ampelina*	S. Seedless	Alasehir	KP083225	KP721687	KP721649
MBAi72AG	*Fomitiporia mediterranea*	S. Seedless	Muradiye	KP083226	-	-
MBAi83AG	*F. mediterranea*	S. Seedless	Menemen	KP083227	-	-
MBAi99AG	*F. mediterranea*	S. Seedless	Cobanisa	KP083228	-	-
MBAi132AG	*F. mediterranea*	S. Seedless	Muradiye	KP083229	-	-
MBAi25AG	*Botryosphaeria dothidea*	Red Globe	Horozkoy	KF182329	KP721688	KP721650
MBAi48AG	*B. dothidea*	S. Seedless	Muradiye	KJ596525	KP721689	KP721651
MBAi98AG	*B. dothidea*	S. Seedless	Cobanisa	KJ921846	KP721690	KP721652
MBAi126AG	*B. dothidea*	S. Seedless	Turgutlu	KJ921848	KP721691	KP721653
MBAi135AG	*B. dothidea*	S. Seedless	Cobanisa	KJ596531	KP721692	KP721654
MBAi130AG	*Diplodia seriata*	S. Seedless	Ahmetli	KJ921851	KP721693	KP721655
MBAi145AG	*D. seriata*	S. Seedless	Salihli	KJ921852	KP721694	KP721656
MBAi164AG	*D. seriata*	S. Seedless	Salihli	KJ921854	KP721695	KP721657
MBAi183AG	*D. seriata*	S. Seedless	Salihli	KJ594528	KP721696	KP721658
MBAi185AG	*D. seriata*	S. Seedless	Salihli	KJ596530	KP721697	KP721659

(continued)

Table 3. Continued.

Isolate	Identity	Host (*V. vinifera* cv.)	Origin	GenBank Accession Number		
				ITS	β-tubulin	EF1-α
MBAi28AG	*Lasiodiplodia theobromae*	110 Richter	Horozkoy	KF182331	KP721698	KP721660
MBAi39AG	*L. theobromae*	S. Seedless	Horozkoy	KJ596523	KP721699	KP721661
MBAi128AG	*L. theobromae*	S. Seedless	Turgutlu	KJ921850	KP721700	KP721662
MBAi184AG	*L. theobromae*	S. Seedless	Alasehir	KJ596529	KP721701	KP721663
MBAi51AG	*Neofusicoccum parvum*	S. Seedless	Salihli	KJ921840	KP721702	KP721664
MBAi53AG	*N. parvum*	S. Seedless	Saruhanli	KJ921842	KP721703	KP721665
MBAi54AG	*N. parvum*	S. Seedless	Muradiye	KJ921843	KP721704	KP721666
MBAi84AG	*N. parvum*	S. Seedless	Saruhanli	KP083233	KP721705	KP721667
MBAi131AG	*N. parvum*	S. Seedless	Turgutlu	KJ596527	KP721706	KP721668

Morphological identification

The morphological characteristics of eight GTD species, used in this study, were detailed according to colony morphology on PDA, conidium morphology, growth rate at 24°C in dark and their conidial sizes (Table 2).

Pathogenicity tests

Average lesion lengths of wood discoloration caused by the 32 isolates and the non-inoculated control are shown in Table 4. Approximately four months after inoculation, blackish-brown lesions were observed both acropetally and basipetally from inoculation points in potted one-year-old grapevines. No lesions were observed in control plants, however a wound response of approximately 6 mm was visible. Lesion lengths varied among fungal species and within isolates of the same species. On average, isolates of *N. parvum* produced the largest lesions (79.1 mm) among all fungi tested followed by *L. theobromae* (59.8 mm), *D. ampelina* (34.6 mm), *P. chlamydospora* (27.5 mm), *T. minima* (25.5 mm), *B. dothidea* (24.8 mm) and *D. seriata* (21.4 mm). *F. mediterranea* produced the smallest lesions (8.5 mm) of all fungi tested (Figure 2). Fungal recovery from inoculated plants ranged from 43.9 to 92.3% with average recovery frequencies for botryosphaeriaceous fungi above 70% for *D. ampelina* and average recovery frequencies below 70% for *T. minima*, *P. chlamydospora* and *F. mediterranea*. No GTD fungi were recovered from control plants.

Discussion

Sultana Seedless is an economically important grape cultivar for Turkish raisin export and viticulture. Due to its ecological suitability and high profit, raisin production is an important economic source for most grape growers in this region. Symptomatic grouping, isolation frequency values and fungal species, obtained from the survey study, revealed that grapevine trunk diseases are a serious problem for Sultana Seedless vineyards. So far some studies were done to identify causal agents of some symptomatic vines suffering from GTD in Turkey (Erkan and Larignon, 1998). Köklü (2000) did a symptomatic grouping of esca in the Thrace Region to demonstrate the status of disease, but no further work was done to determinate the fungi associated with declining grapevine. Poyraz and Onogur (2013) surveyed 15 grapevine nurseries and 42 Sultana Seedless vineyards of the Aegean Region to assess Petri Disease and Esca pathogens. According to morphological characteristics (just compared with the reference isolates), *P. chlamydospora*, *Phaeoacremonium aleophilum* and *F. mediterranea* species were found to cause the disease in this region. However no detailed survey results, molecular and phylogenetic analyses were carried out in their work. Sofia *et al.* (2013) conducted

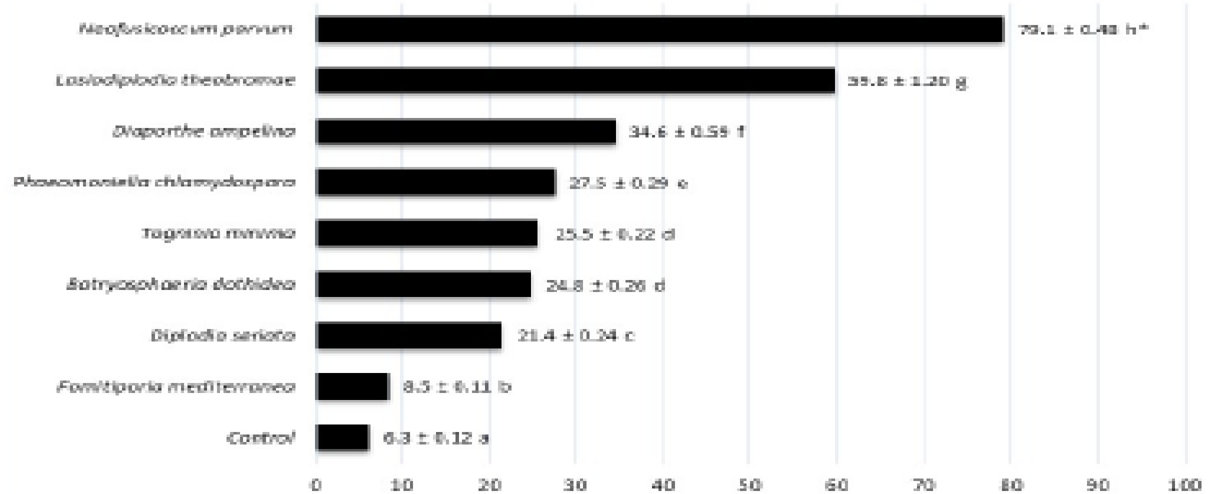

Figure 2. Mean lesion lengths (mm) of the species in pathogenicity test.
* Mean values within a bar are significantly different at the 0.05 level based on LSD test. Mean values (from four isolates in each species) correspond to the extent of wood discoloration measured in pathogenicity tests. LSD (5%): 1.3.

a 62-questionnaire-study to investigate awareness of wine grape growers for GTD in Dao Wine Region of Portugal. A leaflet describing esca, Phomopsis cane and leaf spot, black dead arm (BDA) and young grapevine decline symptoms was given to growers to estimate the frequency of these disorders in their vineyards. More than 88% of the growers declared their vineyards were positive with esca symptoms. Their results demonstrated that esca was the most well-known GTD and Phomopsis cane / leaf spot, BDA and young vine decline followed it with 82, 58 and 30% frequency respectively. Martin and Cobos (2007) collected 84 wood samples from 22 vineyards to investigate GTD fungi in Castilla y Leon Region of Spain. The isolated fungi were identified with morphological characteristics on growth media and verified with DNA-based molecular (PCR-RFLP) techniques. *Phaeomoniella chlamydospra*, *Phaeoacremonium aleophilum*, *Diplodia seriata*, *D. mutila*, *Dothiorella iberica*, *Do. sarmentorum*, *Botryosphaeria dothidea* and *Neofusicoccum parvum* were the most commonly isolated fungi but *Phomopsis viticola*, *Fomitiporia mediterranea*, *Stereum hirsutum* and *Eutypa lata* were rarely obtained from the symptomatic vines in survey region. Among the most common species, isolation frequencies of *P. chlamydospora*, *Pm. alephilum* and Botryosphaeria-like fungi were 18, 16 and 40% respectively. In our study, *Diaporthe ampelina*, Botryosphaeria-like fungi and *Pa. chlamydospora* were among the most commonly isolated ones with 22.6, 19.7 and 11.1% isolation frequencies respectively. Likewise *P. viticola* was rarely isolated from the symptomatic wood samples. These findings were parallel with the findings of Martin and Cobos (2007).

Phomopsis viticola and *Diaporthe* spp. are two important species that cause wood cankers on grapevines (Van Niekerk *et al*., 2005; Udayanga *et al*., 2011). Baumgartner *et al.* (2013) identified two species of *Phomopsis* (*P. viticola* and *P. fukushii*) and *Diaporthe eres* from wood cankers of Concord and Chardonnay grapes in the Northeastern United States. They suggested that wood infecting *Diaporthe* spp. frequently co-occurred with the foliar symptoms of Phomopsis cane, leaf spot and wood cankers, but the latter was not always due to *P. viticola*. Among the pathogens from the current study, *Diaporthe ampelina* was the most frequently isolated species from wedge-shaped cankers of vines. Úrbez-Torres *et al.* (2013) reported that several *Diaporthe* species (*D. ambigua*, *D. eres* and *D. neotheicola*) had been isolated from wood cankers of grapes along with *Phomopsis* spp. and these species have been proved to be associated with GTD symptoms. In our study, *Phomopsis viticola* was rarely obtained from cankers (data not shown) but *D. ampelina* was almost isolated from all. This finding reveals that *Diaporthe* is the most common genus among the GTD fungi in Sultana Seedless vineyard of the survey region. It also corroborates the study of Baumgartner *et al.* (2013)

In the present study, botryosphaeriaceous fungi were the second most predominate fungi associated with GTD and four species were identified including *B. dothidea*, *D. seriata*, *L. theobromae* and *N. parvum*. Members of the Botryosphaeriaceae are well known pathogens of woody hosts worldwide, especially of grapevine where at least 21 species are known as pathogens causing various disorders in grapevine (Úrbez-Torres, 2011). The four botryosphaeriaceous fungi reported herein have been found associated with grapevine in other growing regions including Australia, Spain, South Africa and the United States of America (Crous *et al.*, 2000; Van Niekerk *et al.*, 2004; Úrbez-Torres *et al.*, 2006; Aroca *et al.*, 2008; Luque *et al.*, 2009; Úrbez-Torres *et al.*, 2009; Pitt *et al.*, 2010). Pathogenicity tests revealed *N. parvum* to be the most virulent Botryosphaeriaceae spp. in the current study followed by *L. theobromae*, *B. dothidea* and *D. seriata*, which is supported by other studies showing *N. parvum* and *L. theobromae* to be more virulent fungal species than *B. dothidea* and *D. seriata* (Luque *et al.*, 2009; Úrbez-Torres *et al.*, 2009). As the two most virulent pathogens determined in this study, *N. parvum* and *L. theobromae* should be considered important pathogens of Seedless Sultana grapevine in the Aegean region and efforts should be made to limit pathogen spread and disease progression of these fungi through proper management programs.

Current research suggests that *Fomitiporia* spp. are the most important basidiomycetes as they relate to esca disease (Fischer *et al.*, 2005). Several *Fomitiporia* spp. have been reported from grapevine in association with esca throughout various continents, however the distribution of these species in grape growing regions appears to be defined by geographic region. In Europe, *F. mediterranea* has been reported from grapevines in Germany and Italy, however *F. australiensis* and *F. capensis* have been reported from Australia and South Africa respectively (Fischer, 2002; Fischer and Kassemeyer, 2003; Fisher *et al.*, 2005; Cloete *et al.*, 2014). The confirmation of *F. mediterranea* associated with GTD in the Aegean region of Turkey supports the idea that this fungus is likely restricted to Europe and is likely to be the most important if not the only *Fomitiporia* sp. associated with grapevine in these regions. More remains to be understood regarding the overall contribution of *F. mediterranea* in GTD in this region when considering the larger fungal complex that exists in these trunk disorders of grapevine.

P. chlamydospora, and *Togninia minima* are two pathogenic fungi causing esca disease on grapevines (Mugnai *et al.*, 1999; Eskalen and Gubler, 2001; Mostert *et al.*, 2005). These species are commonly associated with decline, dieback and apoplexy symptoms (Gramaje *et al.*, 2012). *Phaeoacremonium* spp. infecting grapevines have been studied extensively and 29 species in this genus were described to date (Mostert *et al.*, 2006; Essakhi *et al.*, 2008). White *et al.* (2011) conducted a field survey to characterize fungal trunk pathogens associated with esca disease in grape-growing regions of South Africa from 2001 to 2008 years. The isolates were identified by cultural growth patterns, morphology and phylogenetic analysis. Three Botryosphaeriaceae species (*Diplodia seriata*, *Neofusicoccum australe* and *N. parvum*), six *Phaeoacremonium* species (*Pm. aleophilum*, *Pm. alvesii*, *Pm. parasiticum*, *Pm. iranianum*, *Pm. mortoniae*, and *Pm. sicilianum*), *Phaeomoniella chlamydospora*, *Eutypa lata*, *Phomopsis viticola*, *Pho. theicola*, *Diaporthe ambigua* and *F. mediterranea* were identified in that study and they were found to be associated with esca disease. *Pm. iranianum*, *Pm. mortoniae* and *Pm. sicilianum* were reported for the first time in South Africa.. In our study, we isolated four Botryosphaeria-like fungi (*B. dothidea*, *D. seriata*, *L. theobromae* and *N. parvum*), *F. mediterranea*, *D. ampelina*, *Pa. chlamydospora* and *T. minima* but did not isolate *E. lata*, and other *Phaeoacremonium* and *Diaporthe* species presently. The obtained pathogens were considered to be the predominant GTD fungi in Sultana Seedless vineyards of the Aegan Region. From the black-streaking wood lesions, *P. aleophilum*-like isolates were isolated and they were found to be *T. minima* with phylogenetic analysis (not shown). Actually, we did not examine perithecia occurrence on inoculated plants in pathogenicity tests but these results revealed that com-

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Derleme
(Review)

Derleme (Review)

- Deneyimli yazarlar tarafından yazılan,
- Bir konu hakkında şimdiye kadar yapılmış makalelerden bir özet çıkaran,
- Bu araştırmalara yorum getiren ve araştırmaların hangi yönde ilerlediğini anlatan,
- Güncel bilgileri bulunduran,
- Okuyucuya geniş bir bakış açısı sunan yazılardır

Derlemelerin Önemi

- Bir konuda araştırmaya başlayacak kişiler için ideal bir kaynaktır.
- Derlemelerin sonundaki literatür listesinde bir çok kaynağı bir arada görmek mümkündür.

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Crown Gall of Grape

Biology of *Agrobacterium vitis* and the Development of Disease Control Strategies

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Several early descriptions of crown gall on grape from different European countries and the United States were thoroughly reviewed by Hedgcock (29). In 1897, Cavara (20) first demonstrated the infectious nature of a bacterium causing crown gall of grape (*Bacillus ampelopsorae*) in Italy. The crown gall bacterium was first isolated in the United States from Paris daisy by Smith and Townsend in 1907 (54). By inoculating several plants, they demonstrated the tumorigenic nature of the bacterium. Hedgcock then isolated strains of a bacterium from grape crown gall and demonstrated their tumorigenicity on peach and apricot (29). Today, *Agrobacterium vitis* is recognized as the predominant species causing grape crown gall. Within the past 25 years, grape crown gall has been reported from China, Japan, and South Africa and from several other countries in Europe, the Middle East, and North and South America. In a recent investigation carried out in 1996 by the O.I.V. (Office International de la Vigne et du Vin, France), the sporadic occurrence of grape crown gall was highlighted in France, Spain, Germany, Italy, Chile, and Israel; it is endemic in South Africa, with recurrent and severe attacks. We will discuss how the pathogen survives and spreads, how it infects plants, and various strategies that are being used commercially or are being researched for disease management.

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The Disease Cycle

Survival in vines. A key point in the disease cycle of grape crown gall (Fig. 1) is the systemic survival of *A. vitis* in grape. Lehoczky presented the first direct evidence that *A. vitis* survives systemically in grape and induces crown gall at injuries caused by twisting and other wounding of canes (38). This information has been particularly important for explaining the induction of crown gall following vine injuries as well as the systemic nature of the pathogen and how it is disseminated in propagation material. Further studies by Lehoczky and others demonstrated that *A. vitis* could be isolated from sap of bleeding vines (39,40,42). Samples of sap sometimes yielded nearly pure cultures of *A. vitis*, whereas other samples from the same or other infected vines yielded none, demonstrating an uneven distribution of the bacterium. Lehoczky hypothesized that the pathogen overwinters primarily in the root system and suggested that under moist conditions in the spring, root pressure causes xylem fluids to "sweep" bacterial cells from the roots upward, where they may be attracted to wounds. *A. vitis* has been isolated directly from 1-year-old canes, but the percentage of cuttings from which *A. vitis* is detected is often highly variable. This may reflect uneven distribution of the bacterium in vines or the sensitivity of the methods used for detection of the bacterium. Stover et al. (58) demonstrated that by prefreezing dormant cuttings, the number of *A. vitis* cells detected using a vacuum flushing method (discussed below) was greatly increased. In this case, it appears that the physical effects of freezing facilitated the increased numbers of cells flushed from the cuttings. Related research done by Bauer et al. showed that populations of *A. vitis* were greatest at inoculation sites on shoots of vines and that population curves were similar for Riesling and Müller-Thurgau cultivars, with the highest numbers of bacterial cells being detected in the spring and fall (1). The greatest populations were detected in Riesling, and it required at least 15 weeks for *A. vitis* to move internally from inoculation sites to the root system.

Translocation of *A. vitis* in green shoots was demonstrated by Tarbah and Goodman (64). They inoculated shoots by submersing their basal ends in a bacterial suspension and then monitoring the movement of the bacterium. Bacteria translocated up to 30 cm from the point of entry within 24 h. Lehoczky indirectly demonstrated the presence of *A. vitis* in green shoots by grafting plants with shoots from infected vines and finding that 6.75% of the grafted vines became infected (41). The stage at which current-season shoots are invaded by *A. vitis* (moving from woody cane tissue) may be related to the development of vasculature between canes and shoots. Burr et al. (13) did not detect the bacterium in green shoots on grapevines growing in a crown gall–infested vineyard until late in the growing season (when shoots were becoming lignified) and never detected *A. vitis* in shoot tips. More recently, Szegedi

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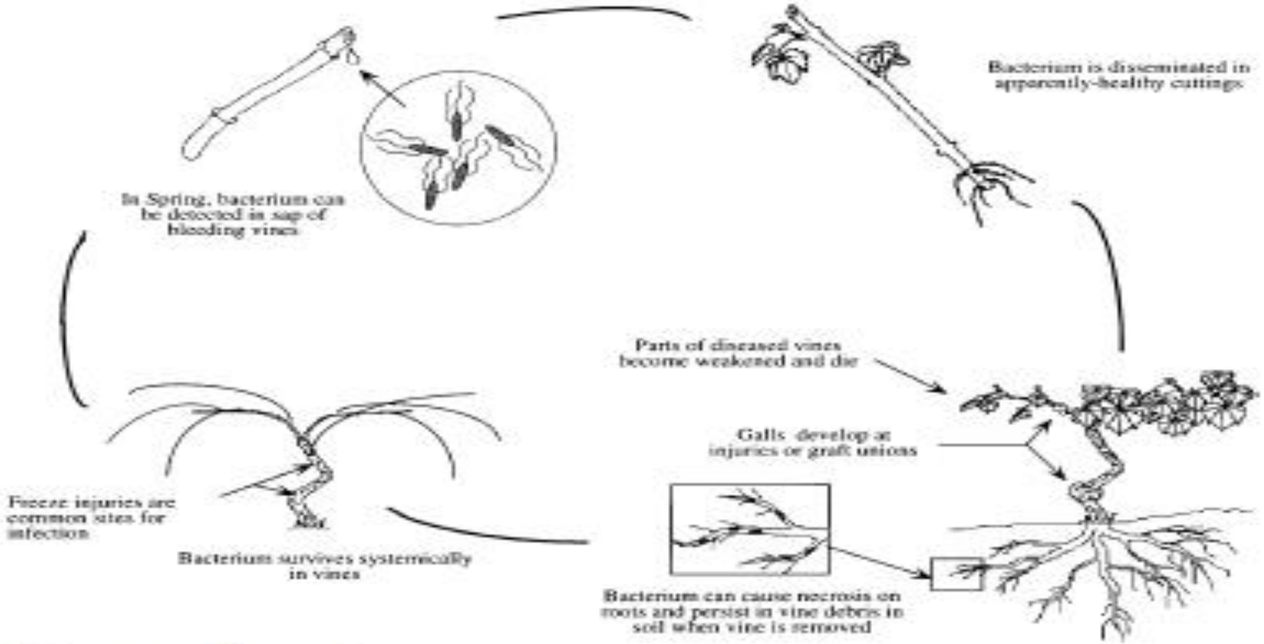

Fig. 1. Grape crown gall disease cycle.

and Nemeth (63) also did not detect *A. vitis* in green shoots of crown gall–infected grapevines.

Although most research has focused on survival and translocation of *A. vitis* in xylem tissue, Jager et al. provided convincing evidence that the bacterium persists in the rind (layer of tissue directly beneath bark) of canes (32). Therefore, the specific survival sites of *A. vitis* within vines are not clearly defined and may vary with cultivar and time of year.

Soil survival and root necrosis. The genus *Agrobacterium* is a common soil inhabitant, but the vast majority of strains that are detected in soil are nontumorigenic. Where soils have been analyzed specifically for *Agrobacterium* spp., *A. vitis* was not detected (8,9). The potential for *A. vitis* to persist in grape tissue debris in soil was investigated (18). Strains of *A. vitis* remained tumorigenic in vine debris and were detected over the 2-year course of the study. In this case, tumorigenic *A. vitis* persisted in a saprophytic phase, since no galls were formed on any of the grape tissues. These findings indicate that once a vineyard becomes contaminated with *A. vitis*, the bacterium will survive in grape debris for years after vines are removed. Therefore, the effectiveness of eradicating the pathogen from vineyard sites by removing infected vines and leaving soil fallow or planting to nonhosts will vary depending on the amount of grape debris in soil and its rate of decomposition.

A. vitis does not typically cause crown gall on roots of grape but instead induces a localized necrosis (Fig. 2) (11). A mutant strain that no longer produces polygalacturonase was found to cause a reduced level of necrosis and tumorigenicity on grape (50). In this case, mutant CG50 did not induce necrosis when inoculated to Concord grape seedling crowns at a concentration of 10^6 CFU/ml, whereas the wild type strain CG49 did. At higher concentrations (10^8 CFU/ml), CG50 induced necrosis, indicating that polygalacturonase is only one factor associated with necrosis.

Wild grape species as hosts for *A. vitis*. The ability of *A. vitis* to survive in *Vitis* spp. growing in the wild is being investigated in Italy and in the United States. Root samples of *V. riparia* were collected at several locations near and far-removed (more than 150 km [100 miles]) from commercial vineyards in New York. *A. vitis* strains were commonly isolated (49); they were identified based on colony morphology on the medium of Roy and Sasser (RS) (12), the testing scheme of Kerr and

Fig. 2. Necrosis of grape roots caused by *Agrobacterium vitis*.

Panagopoulos (35), reaction to a species-specific monoclonal antibody (6), production of polygalacturonase (50), and ability to induce necrosis of grape seedling roots (11). All strains from wild grape were found to be nontumorigenic.

The Italian study involved numerous samples of *V. vinifera silvestris* cuttings and roots from viticultural and nonviticultural areas (C. Bazzi, *unpublished*). Fifty-five *A. vitis* strains were isolated and identified by enzyme-linked immunosorbent assay (ELISA). All were nontumorigenic on indicator plants and did not generate polymerase chain reaction (PCR) products from tumor-inducing (Ti) plasmid–derived primer sets, as described by Schulz et al.

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(53). It is interesting that, thus far, all strains from wild grape species are nontumorigenic and crown gall has not been observed on these feral vines. The potential role of these strains in the spread and development of crown gall disease is being investigated. Can they acquire Ti plasmids, thus becoming tumorigenic? At least some nontumorigenic *A. vitis* are able to inhibit gall formation by tumorigenic strains on grape, as discussed below.

How *A. vitis* infects. Vineyards often remain crown gall–free for several years until conditions conducive to infection (generally freeze injuries) occur. This was the case during the winter of 1984-85, which was followed by severe outbreaks on several susceptible cultivars in Italy. In the United States, the frequency of occurrence and severity of the disease have increased dramatically as wine industries expand into regions where vines are exposed to injuries caused by freezing temperatures.

Crown gall infections are initiated at plant injuries for reasons that will be discussed below. Whereas in the majority of grape-growing regions, freeze injuries provide sites for initiating crown gall, in South Africa and Israel, high temperatures and humidity are considered equally important. Crown galls commonly form on the trunks of vines (Fig. 3); however, they even develop on 1-year-old canes (Fig. 4). Crown gall can also be significant in grape nurseries. Galls develop at wounds made by disbudding, at the base of rooted cuttings, and at grafts (Fig. 5). However, a more significant consideration for nurserymen is that propagation material may be systemically infested with *A. vitis*. Infected vines often produce inferior growth, and in some cases, the disease may cause partial or complete vine death. The importance of crown gall in reducing the vigor and yield of the cultivar Zinfandel was quantitatively shown by Schroth et al. (52). Another less common expression of the disease may develop when established vines are top-grafted or budded with new scion cultivars. In this case, severe crown gall may form at grafting and budding sites (Fig. 6), resulting in poor growth or death of scion shoots.

Infection of plants by *Agrobacterium* is a multistage process (71,72). It represents the only known example of DNA transfer from a bacterium to a plant in nature. It has been known for several years that a part of the bacterial Ti plasmid (the T-DNA) is transferred to the plant cell and expressed in the plant genome. Not all steps of the infection process for *A. vitis* have been identified, but many are known to be very similar to those of *A. tumefaciens*. The first steps are chemotactic attraction toward wounded plant cells and attachment of the bacterium to them. In the case of grapes, freeze injuries often provide the wounded cells that are considered to be highly susceptible to infection by *A. vitis*. Certain phenolic and other compounds released by wounded cells induce the virulence genes of *Agrobacterium*. Acetosyringone is a common inducer that is studied in the *A. tumefaciens* infection system, and methyl syringate was identified as a signal compound from grape (55). A protein encoded by the bacterial Ti plasmid, VirA, detects the chemical signals from the plant. When a phenolic compound of the right structure binds to VirA, the complex acquires the capacity to phosphorylate another protein, VirG, which is inside the bacterium, and this in turn leads to the activation of various other genes that make up the virulence (or vir) region of the Ti plasmid.

Although much is already known about the functions of the virulence genes, certain important points remain to be elucidated. Basically, the *virD2* gene codes for the enzyme VirD2, which liberates a linear fragment of the T-DNA (the T-strand), or T-DNAs in some strains. VirD2 becomes firmly attached to the T-strand and pilots it out of the bacterium and into the plant cell nucleus, where it gets incorporated into one of the plant chromosomes. The *virB* genes (which make at least 11 different proteins) probably enable the bacterium to construct a porelike structure through which the T-strand passes. It is remarkable that similar proteins are used in several bacterial conjugation systems where plasmid DNA passes from one bacterium into another. It has therefore been proposed that in the course of evolution, the *Agrobacterium* plant infection process originated from a bacterial conjugation system (47). Other vir genes (*virE* and *virF*) play a role in protecting the T-strand on its way to the plant cell chromosome. Once inserted in a plant chromosome, the T-strand will be transcribed. This leads to the synthesis of various proteins, which can be divided into two functional groups: those involved in opine synthesis and those that stimulate cell growth, resulting in crown gall disease.

Plant gall–inducing genes (oncogenes) on the T-DNA turn on the rapid multiplication of plant cells. The oncogenes encode auxin synthesis (a two-step process requiring the *iaaM* and *iaaH* genes) and cytokinin synthesis (*ipt* gene), and include various genes that induce or modify the growth of crown galls. The infected cells also produce opines, thereby further benefiting the survival of the pathogen.

Opines are small molecules that are secreted from the plant crown gall cells and then are utilized by *Agrobacterium* living in the vicinity of galls. Many types of opines have been described; all result from the combination of two small, abundant

Fig. 3. Severe crown gall on multiple trunks of a *Vitis vinifera* vine.

Fig. 4. Crown gall on a 1-year-old cane that is wrapped around the trellis wire.

plant cell compounds like keto acids, amino acids, or sugars. The combination into larger opine compounds makes it more difficult for most microorganisms to use them; *Agrobacterium* carries special enzymes that are able to degrade opines. In this way, *Agrobacterium* selectively exploits opines synthesized following crown gall infections. Within the genus *Agrobacterium*, and even within a given species, there is fierce competition for the opines around plant galls, as is evident from the existence of different types of opine synthesis and degradation genes within these groups. We will discuss the Ti plasmids and T-DNAs found so far in *A. vitis* strains.

Diversity of *Agrobacterium vitis*. It was assumed for many years that grape crown gall was caused by strains of *A. tumefaciens*, the cause of crown gall on numerous dicotyledenous plants. However, in 1977, Kerr and Panagopoulos classified tumorigenic *Agrobacterium* strains from grape as biovar 3 of *A. tumefaciens* (35). In subsequent years, researchers in several parts of the world identified strains from grape as biovar 3. Further taxonomic studies by Ophel and Kerr resulted in the distinction of biovar 3 as a separate species, *A. vitis* (43). *A. vitis* is now recognized as the predominant species causing grape crown gall; however, tumorigenic strains of biovars of *A. tumefaciens* are also infrequently isolated from grape.

The first Ti plasmid and T-DNA of an *A. vitis* strain were described in 1984 (10). Since then, many strains have been investigated, and several Ti and T-DNA types have been defined. The *A. vitis* group is the only *Agrobacterium* species that has been systematically investigated with regard to T-DNA, Ti plasmid, and chromosome structures. These studies were made possible because the natural host range of *A. vitis* is restricted to grape and because *A. vitis* strain collections exist all over the world.

Three basic T-DNA structures have been defined in *A. vitis* (Fig. 7). Each differs in the number of T-DNAs and in the makeup of oncogenes. The most abundant structure (found in about 60% of the strains) consists of two independent T-DNAs (TA-DNA and TB-DNA) on the Ti plasmid. The TA region contains oncogenes *5*, TA-*iaaM*, TA-*iaaH*, *ipt*, *6b*, and opine genes *acs* and *ocs* (the latter two coding for synthesis of agrocinopine and octopine). The TB region contains oncogenes TB-*iaaM*, TB-*iaaH*, and opine genes *acs* and *cus* (for cucumopine synthesis). This TA/TB T-DNA makeup has been called the octopine/cucumopine type. Detailed studies of cloned and sequenced TA and TB regions have revealed the existence of numerous variants. Remarkably, these variants differ not so much in nucleotide sequence as by insertion and deletion of so-called insertion (IS) elements (mobile DNA fragments that can insert randomly in the bacterial genome, thereby leading to mutations). One such mutant (having a small TA region) no longer carries TA-*iaaM*, TA-*iaaH*, or *ipt* but still induces tumors through the activity of genes *6b* and TB-*iaaM* and TB-*iaaH*. This is exemplified by strain AB3 in Figure 7. Functional studies of the different oncogenes (31) have shown that the TA/TB oncogene combination is to a certain extent redundant (some of the genes in TA have the same function as genes in TB). This probably explains the existence of the variants. These variants are recent on the scale of evolution and represent either random mutations that provide different selective advantages to *A. vitis* or mutants that have already passed through the sieve of natural selection, allowing them to occupy special niches in nature. Only competition experiments that compare mixtures of variants under controlled conditions can answer these questions.

The second T-DNA structure (about 30% of strains) is the nopaline type, having a single T-DNA with oncogenes *5*, *iaaM*, *iaaH*, *6b*, and *3'*, and opine genes *acs* and *nos* (nopaline synthase). No variants have yet been detected, indicating that this group either is very recent, contains less-active IS elements, or does not easily generate viable mutants.

The third group (about 10% of the strains) is the vitopine type, which carries three independent T-DNAs, a situation so far only found in *A. vitis*. T1 carries *6b* and *vis* (vitopine synthesis), T2 has *iaaH* and *iaaM*, and T3 has *ipt* and a mutated, inactive *vis* gene (19). Why these genes, which normally occur together, are distributed over three different T-DNAs is unknown. It may represent a primitive condition occurring before T-DNA genes were combined into a single structure.

Interestingly, we have recently detected a new T-DNA form in strain CG474. This form is related to the TA region of o/c strains but is clearly different from it. This exceptional strain may have acquired its Ti plasmid and T-DNA from an *A. tumefaciens* strain. It is not known whether the different strain types are specialized for specific grape cultivars or they have adapted to particular environments. However, the exhaustive molecular knowledge of *A. vitis* now allows us to carry out such ecological studies and to follow the fate of the different types over time.

Although T-DNA structures are eminently important to the infection process, the remainder of the bacterial DNA should

Fig. 5. Crown gall that developed at graft unions on vines growing in a nursery.

Fig. 6. Crown gall at a site where a mature trunk was budded.

not be forgotten. Little is known about the Ti plasmid virulence genes of the different *A. vitis* subgroups. Whereas the *vir* region of the o/c strains seem to be similar to that found in classical *A. tumefaciens* strains, the vitopine strains have a different *vir* region organization and possibly additional *vir* genes (26). Ti plasmids have been cloned and physically mapped to identify major types, but they have not been fully analyzed. Other genes that influence grape infection, such as the opine utilization genes and plasmid replication genes, remain to be studied. A further complication is the difference in chromosome structure; although most crown gall induction genes are on the Ti plasmid, the chromosome also contains virulence genes that may be important for survival in the soil and in the grape plant. It is therefore important to know the overall extent of chromosomal variation in *A. vitis*. This problem has been addressed recently using PCR to amplify specific chromosomal regions of *A. vitis*. The intergenic space between the 16S and 23S rRNA genes was digested with different endonucleases, and DNA fingerprints were generated (46). Several chromosomal groups have been defined and their phylogenetic relationships established; however, more work is needed to relate these forms to pathological properties. The chromosomal data show that most Ti plasmids, with their characteristic T-DNAs, are associated with a particular chromosomal background, suggesting that Ti plasmid exchange or transfer is rare under natural conditions. In practice, this means that chromosomal markers can be used to predict the Ti plasmid type.

Among the potential *A. vitis* host range factors, two have been studied in considerable detail; the polygalacturonase gene (*pehA*), which codes for an enzyme associated with grape tissue necrosis, and the tartrate degradation (*tar*) genes. *PehA*-minus mutants are less pathogenic on grapevine but not on other hosts (50).

The TAR region codes for tartrate utilization and is found in most *A. vitis* strains (45). Three different types have been isolated and sequenced; their basic structures and functions are the same, but they are found on different plasmids. Once again, the vitopine strains are exceptional in that their TAR region seems to be quite different. Competition experiments between a TAR-plus strain and a TAR-minus mutant showed that the TAR system confers a clear advantage to the bacterium on grapevine but not on other hosts and is therefore a major host range determinant (L. Otten, *unpublished data*).

Management of Crown Gall

Varietal susceptibility. Evaluations of grape cultivars for crown gall susceptibility were done as early as 1910 (29). In more recent years, many *Vitis* species, rootstocks, and scion cultivars have been evaluated in different ways for crown gall susceptibility (25,27,58,61). *V. vinifera* cultivars, including Chardonnay, Riesling, Merlot, Cabernet Sauvignon, and many others, are highly susceptible, as determined by field observation and controlled inoculations. In contrast, *V. riparia* clones (such as Riparia Gloire) are generally resistant to *A. vitis*, although some are susceptible. *V. labrusca* and hybrid cultivars are generally more resistant than *V. vinifera*, although some (such as Chancellor and Niagara) can become severely infected with crown gall. Ferreira and van Zyl evaluated 40 rootstocks for crown gall susceptibility using five *A. vitis* strains (25). Although some discrepancies exist between their results and those of Stover et al. (58), it is evident that certain rootstocks used worldwide, such as Couderc 3309, 101-14 Mgt, and Riparia Gloire, are resistant; whereas Teleki 5C and 110 Richter are susceptible. A summary of crown gall susceptibility ratings for various scion and rootstock cultivars from different countries is given in Table 1. Complicating the resistance screening process is the fact that *A. vitis* strains are genetically variable (as previously described), affecting their tumorigenicity on different *Vitis* germ plasm. For example, in the papers cited, various rootstocks and *Vitis* spp. were screened against five strains of *A. vitis*. Not all strains caused the same numbers and sizes of galls on the same genotypes. Also, some genotypes were resistant to certain strains but developed large galls when inoculated with other strains. Therefore, crown gall susceptibility of grape appears to be determined by genetic determinants of the plant and pathogen.

The strategy of planting relatively resistant cultivars and rootstocks as a means of disease management has been suggested. This is not practical for situations where susceptible cultivars are preferred by the producer, who is growing them to make premium wine. Recently, however, it was demonstrated that under field conditions, crown gall was significantly reduced

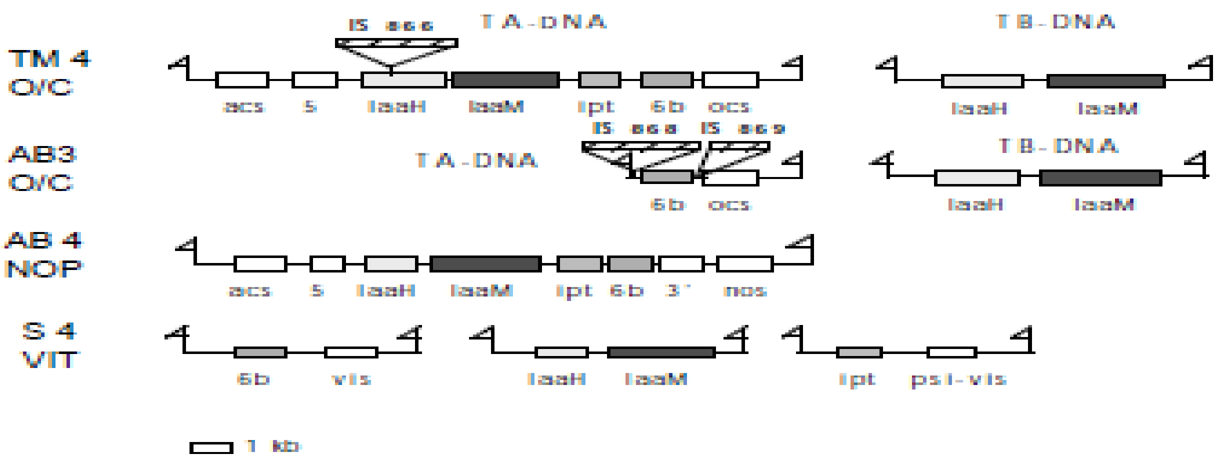

Fig. 7. T-DNA gene arrangements in *Agrobacterium vitis* strains. TM4 and AB3 are O/C (octopine/cucumopine) strains with large and small TA regions, respectively. Strain AB4 has a NOP (nopaline) T-DNA arrangement, and S4 has a VIT (vitopine) T-DNA. Flag symbols denote border sequences of T-DNAs. Genes are described in text. Regions where IS elements IS866, IS868, and IS869 have inserted are denoted.

trigging. Models predicting the evolution of T-DNAs have provided great insight into *A. vitis* tumorigenesis; however, little is known about the rest of that bacterial genome. It appears from DNA fingerprinting and sequencing of a few chromosomal genes that the *A. vitis* chromosome may be quite divergent from that of other *Agrobacterium* spp. Presently, it is speculated that the ability to produce polygalacturonase, to induce a grape-specific necrosis, and to utilize tartrate may all be related to the specificity of *A. vitis* on grape. It will now be possible to identify genes that are associated with *A. vitis*–grape interactions and to specifically determine their roles. Such information will not only be of academic interest but is also likely to provide information useful for developing novel disease control strategies.

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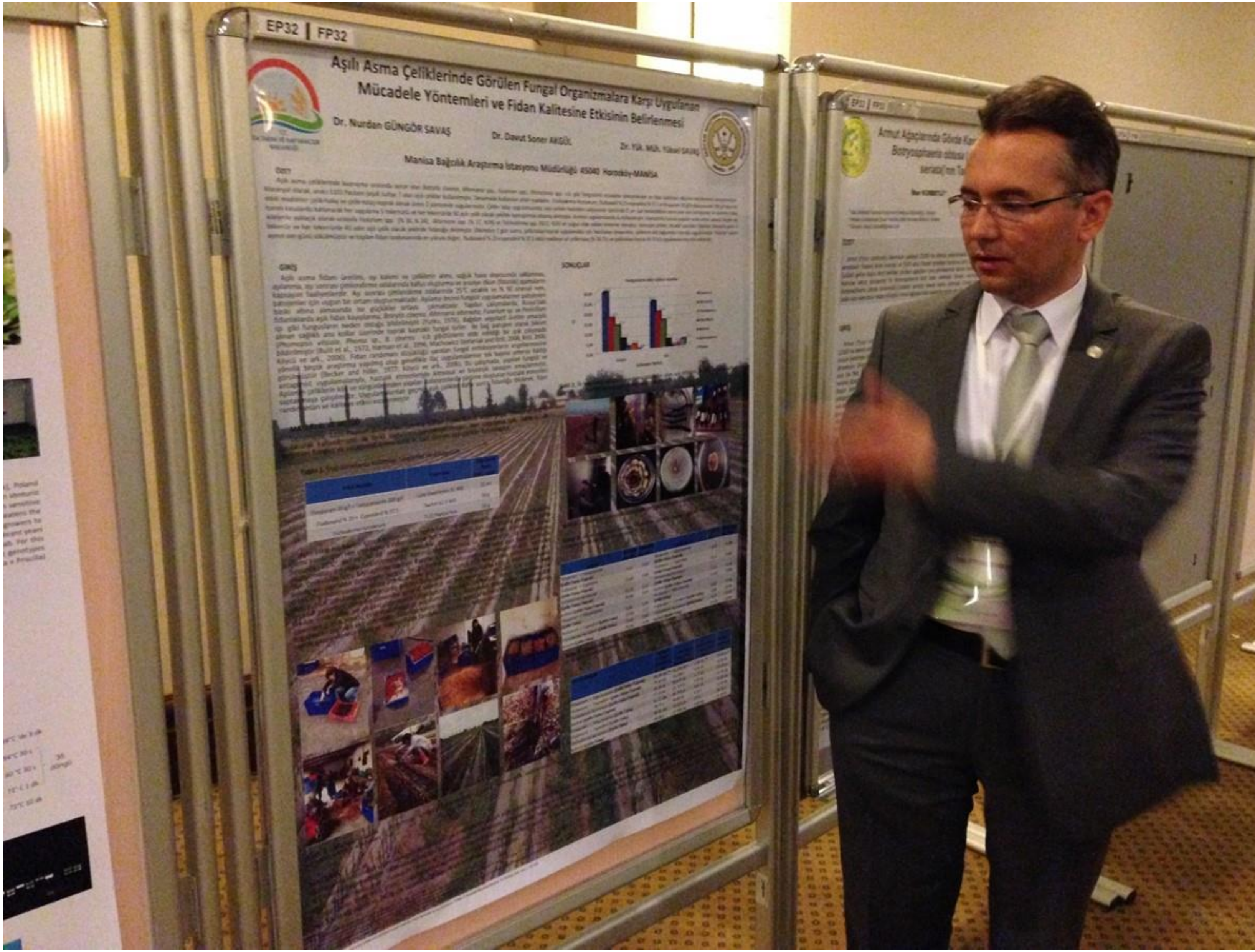
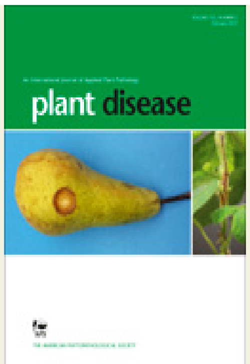


Teknik Rapor ya da Vaka Takdimi

Teknik Rapor ya da Vaka Takdimi

- **Ülkemizde şimdiye kadar rastlanmamış bir patojenin ya da zararlının varlığını rapor etmek için yazılan, kısa ve tanıtıcı bir rapordur.**



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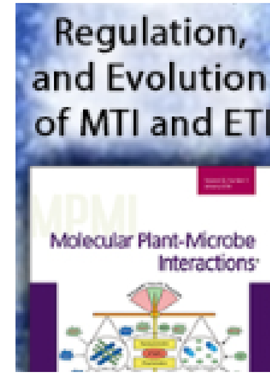
Nurdan Gungor Savas

Mumine Ozarslandan

Grapevine trunk diseases associated with Botryosphaeriaceae fungi are common and important diseases in Turkey vineyards (Akgül et al. 2015). In June 2017, entire 20 year-old symptomatic vines (*Vitis vinifera* cv. Cabernet Sauvignon and cv. Horoz Karasi) were received in the laboratory from vineyards in Manisa (three vines) and Gaziantep (one vine) provinces. Wedge-shaped dark brown cankers, xylem necrosis, and lack of spring growth, symptoms resembling Botryosphaeria dieback, were observed in cordons and arms from all samples. Surface sterilization in 2.5% NaOCl solution for 3 min and rinsing in sterile distilled water twice of symptomatic wood was followed and wood chips were plated onto potato dextrose agar (PDA) amended with 150 mg·L-1 streptomycin-sulphate in an attempt to isolate the fungal pathogens. Plates were incubated at 25°C in dark for 21 days and growing colonies were examined for colony morphology and conidia shapes under light microscope. Three *Lasiodiplodia*-like and one *Neoscytalidium*-like isolates were obtained from Manisa and Gaziantep respectively. *Lasiodiplodia*-like conidia (having dark brown color, one-septate, thick-walled with longitudinal striations, produced on inoculated grapevine canes) and *Neoscytalidium*-like conidia (light brown, 0-2 septate arthroconidia with rounded apices, some of them have triangular appearance) were observed on PDA and their sizes were measured (n=50 *Lasiodiplodia* isolates: 20.9 x 13.2 µm and for *Neoscytalidium* isolate 26.9 x 3.2 µm). For molecular identification, DNA was extracted from mycelia and ITS and translation elongation factor 1-alpha gene regions were from Manisa and Gaziantep respectively. *Lasiodiplodia*-like conidia (having dark brown color, one-septate, thick-walled with longitudinal striations, produced on inoculated grapevine canes) and *Neoscytalidium*-like conidia (light brown, 0-2 septate arthroconidia with rounded apices, some of them have triangular appearance) were observed on PDA and their sizes were measured (n=50 *Lasiodiplodia* isolates: 20.9 x 13.2 µm and for *Neoscytalidium* isolate 26.9 x 3.2 µm). For molecular identification, DNA was extracted from mycelia and ITS and translation elongation factor 1-alpha gene regions were amplified with PCR using primers ITS4/ITS5 and ITS4/V9G (for *Neoscytalidium*) and ITS4/ITS5 and EF1-688F/EF1-1251R (for *Lasiodiplodia*) respectively (White et al. 1990; Alves et al. 2008; Polizzi et al. 2011) and subsequently sequenced. NCBI-BLAST analysis was performed with the sequences and their similarities were minimum 99-100% with the sequences of MH863173 and KF766207 (for *N. novaehollandiae*) and KU886816 and KU886939 (for L. exigua) in NCBI database. After phylogenetic analysis, they were found to be *N. novaehollandiae* (CUZFnovo1) and *L. exigua* (CUZFLexi1, CUZFLexi2, CUZFLexi3) and morphological features were also in agreement with the studies of Ray et al. (2010) and Linaldeddu et al. (2015). The sequences were deposited in GenBank under accession numbers MK064153, MK056265 (ITS) for *N. novaehollandiae* and MK045639, MK04640, MK045641 (ITS) and MK050968, MK050969, MK050970 (EF1-α) for *L. exigua* respectively. Pathogenicity tests were conducted under greenhouse conditions (24°C, 16/8h day/night, 75% RH) and one-year old potted grapevines (cv. Cardinal) were used. Stem internodes were laterally drilled with a sterile 2 mm-diameter needle and 2 mm2 mycelial agar pieces were inserted into the holes with toothpicks (12 vines per isolate and for 4 isolates, 48 vines totally). The inoculation points were wrapped with a parafilm and plants were grown for 4 months. Sterile agar pieces were inoculated in the control plants. After that, plant stems were divided with a knife longitudinally, brown discoloration and streaks (69.6, 70.3 and 71.2 mm for *L. exigua*; 51.7 mm for *N. novaehollandiae* isolates) were observed in wood tissues of inoculated plants but not in un-inoculated (7.8 mm) ones. Koch's postulates were confirmed by re-isolation of the isolates from the inoculated canes. This report is important for the new studies aiming at determination of Botryosphaeriaceae diversity in Turkey viticulture. References Akgul, D. S. et al. 2015. Phytopathologia Mediterranea. 54:380. Alves, A. et al. 2008. Fungal Divers. 28:1. Linaldeddu, B. T. et al. 2015. Fungal Divers. 71:201. Ray, J. D. et al. 2010. Australasian Plant Dis. Notes. 5:48. Polizzi, G. et al. 2011. Acta Hort. 892:237. White, T. J. et al. 1990. Page 315 in: PCR Protocols



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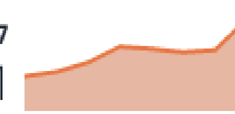

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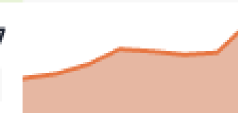

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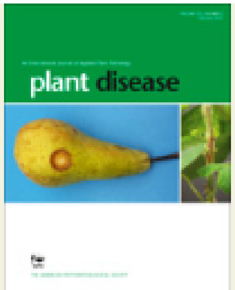

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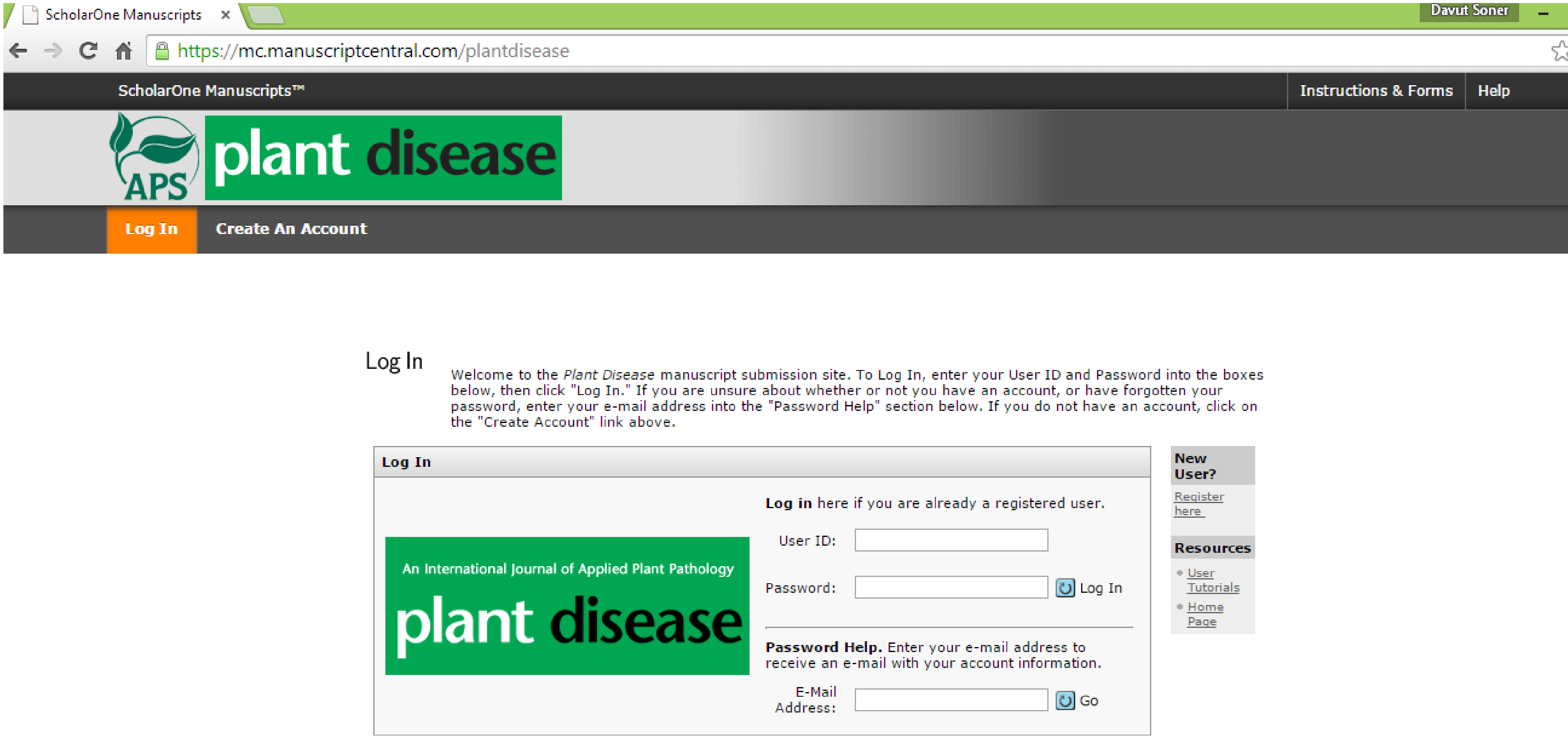
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