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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

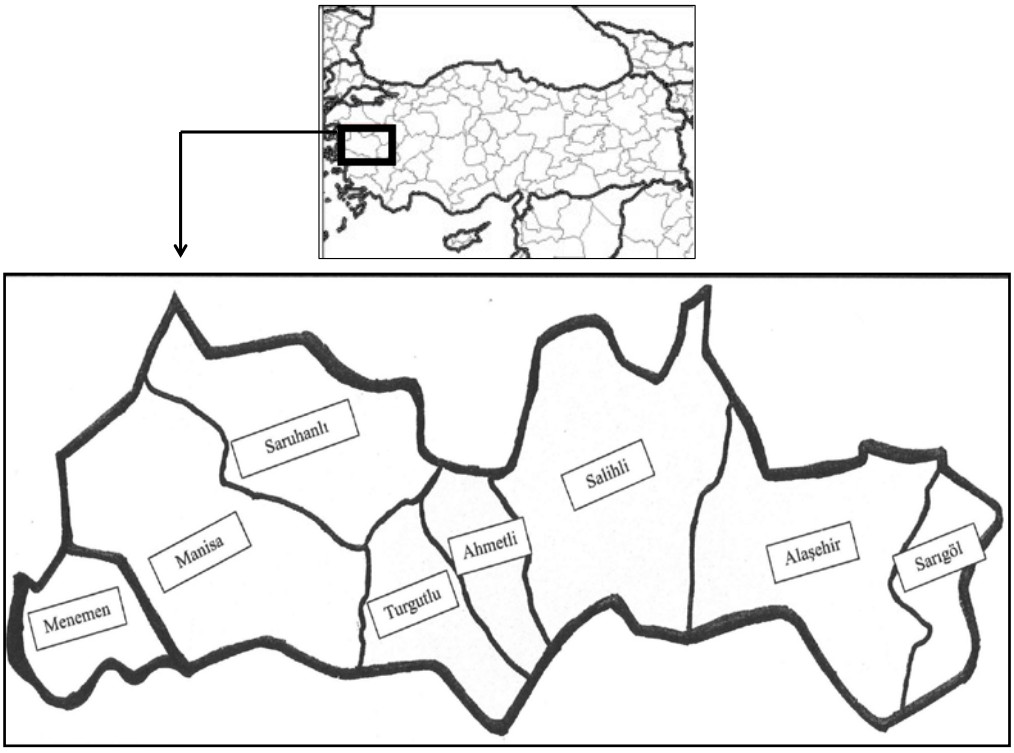

Figure 1. Map of Turkey showing the Sultana Seedless raisin-grape production region of the Aegean where the vineyards were surveyed.

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	Yuntdagi	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

Table 4. Mean lesion length and re-isolation frequency for different fungal species on rooted grapevine.

Isolate	Species	Mean lesion length (mm) ± SE	Re-Isolation Frequency (%)
Control		6.1 ± 0.06 a*	-
MBAi132AG	*Fomitiporia mediterranea*	8.2 ± 0.27 b	66.5
MBAi99AG	*F. mediterranea*	8.5 ± 0.26 b	63.8
MBAi72AG	*F. mediterranea*	8.6 ± 0.51 b	69.4
MBAi83AG	*F. mediterranea*	8.7 ± 0.19 b	75.2
MBAi183AG	*Diplodia seriata*	20.8 ± 0.39 c	74.3
MBAi145AG	*D. seriata*	21.3 ± 0.17 c	76.3
MBAi130AG	*D. seriata*	21.5 ± 0.49 c	71.9
MBAi126AG	*B. dothidea*	21.5 ± 0.30 c	70.5
MBAi48AG	*Botryosphaeria dothidea*	24.2 ± 1.13 d	72.7
MBAi164AG	*D. seriata*	24.5 ± 0.47 d	66.4
MBAi98AG	*B. dothidea*	24.9 ± 0.87 d	67.1
MBAi151AG	*Togninia minima*	25.0 ± 0.58 d	51.8
MBAi152AG	*T. minima*	25.2 ± 0.28 de	50.7
MBAi135AG	*B. dothidea*	25.4 ± 0.26 de	76.9
MBAi133AG	*T. minima*	25.8 ± 0.29 def	49.7
MBAi150AG	*T. minima*	25.9 ± 0.19 def	43.9
MBAi157AG	*Phaeomoniella chlamydospora*	26.9 ± 0.54 efg	56.4
MBAi20AG	*P. chlamydospora*	27.4 ± 0.40 fg	58.7
MBAi156AG	*P. chlamydospora*	27.5 ± 0.68 fg	54.0
MBAi190AG	*Diaporthe ampelina*	28.3 ± 0.90 g	79.8
MBAi169AG	*P. chlamydospora*	33.0 ± 0.62 h	56.3
MBAi95AG	*D. ampelina*	34.4 ± 0.70 hi	80.9
MBAi93AG	*D. ampelina*	35.0 ± 0.75 i	81.4
MBAi35AG	*D. ampelina*	35.8 ± 0.44 i	75.4
MBAi39AG	*Lasiodiplodia theobromae*	58.0 ± 0.63 j	90.8
MBAi128AG	*L. theobromae*	58.7 ± 1.00 j	91.3
MBAi184AG	*L. theobromae*	59.0 ± 1.50 j	79.6
MBAi28AG	*L. theobromae*	63.3 ± 0.58 k	78.6
MBAi53AG	*Neofusicoccum parvum*	77.7 ± 1.43 l	91.6
MBAi51AG	*N. parvum*	79.3 ± 0.64 l	90.8
MBAi54AG	*N. parvum*	79.7 ± 0.34 m	89.5
MBAi84AG	*N. parvum*	79.8 ± 0.34 m	92.3

* Mean values within a column are significantly different at the 0.05 level based on LSD test. Mean values correspond to the extent of wood discoloration measured upward and downward from the point of inoculation. LSD: 1.8 (5%).

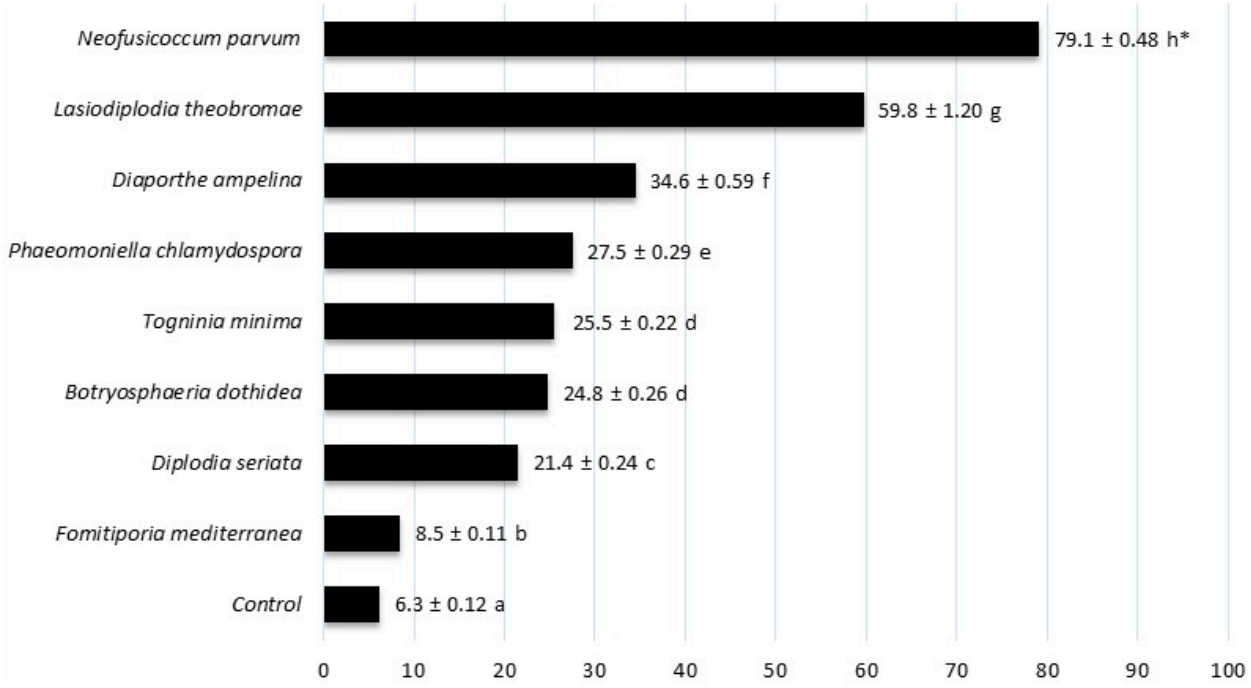

Figure 2. Mean lesion lengths (mm) of the species in pathogenicity test.
* Mean values within a bar are significantly different at the 0.05 level based on LSD test. Mean values (from four isolates in each species) correspond to the extent of wood discoloration measured in pathogenicity tests. LSD (5%):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

patible mating types of this species were present in Aegean Region, therefore high genetic diversity may be expected. In the study of Mostert *et al.* (2003), *T. minima* was proposed to have a biallelic heterothallic mating system and high genetic diversity might be observed among the isolates of this species. It is estimated that this case may be associated with our results for our *T. minima* isolates.

Pathogenicity tests conducted with 32 isolates of eight species confirmed that they were pathogenic on Sultana Seedless vines. While the most virulent isolate was *N. parvum* MBAi84AG (with 79.8 mm lesion length), the least virulent one was *F. mediterranea* MBAi132AG (with 8.2 mm lesion length). Luque *et al*. (2009) conducted a survey study in 79 vineyards (Macabeo or Tempranillo) to determine GTD pathogens in Northeast Spain. *B. dothidea, D. seriata, E. lata, F. mediterranea, N. luteum, N. parvum, Pa. chlamydospora* and *T. minima* were among the most commonly isolated fungi in symptomatic wood samples. In their pathogenicity tests, *N. parvum* was determined to be the most virulent species, while *F. mediterranea* was the least virulent on Macabeo and Tempranillo cultivars. *N. parvum* (isolate 396) produced 13.8 and 10.8 mm lesion lengths on Macabeo and Tempranillo but *F. mediterranea* just produced 0.7 and 1.5 mm lesion lengths nine months later. The second most virulent species was *N. luteum* with 8.6 and 8.2 mm and following one was *Pa. chlamydospora* with 2.5 and 4.5 mm respectively. Other lesion lengths produced by the other fungi ranged by 0.8–8.6 mm. Their findings corroborate our pathogenicity test results since *N. parvum* was the most virulent and *F. mediterranea* was the least virulent species in our study. However, despite longer incubation period (nine months), the lesion lengths of their isolates were shorter than ours. Laveau *et al*. (2009) tested aggressiveness of six fungal species (59 isolates) including *Pa. chlamydospora, T. minima, F. mediterranea, E. lata, D. seriata* and *N. parvum* on rooted cuttings of Cabernet Sauvignon plants. Fifteen months after inoculation, *Pa. chlamydospora* and *N. parvum* produced the largest cankers and the longest internal lesions and they were grouped as the most aggressive species. However, *E. lata, D. seriata* and *T. minima* were included in the second and *F. mediterranea* was included in the third group. According to our results, virulency of *Pa. chlamydospora* isolates was not so severe as in the case of *N. parvum* isolates.

Fungal phytotoxins are known to be effective on symptom expression of infected plants during host-pathogen interactions. As in other fungal pathogens, *F. mediterranea, Pa. chlamydospora, T. minima, E. lata* and Botryosphaerious fungi have been shown to produce some phytotoxic metabolites, such as scytalone, isosclerone, naphtalenone pentaketides, eutypine, methyl eutypinol, mellein, 4-hydroxymellein, tyrosol etc. in their culture filtrates (Evidente *et al*., 2000; Tabacchi *et al*., 2000, Martos *et al*., 2008). Luque *et al*. (2009) reported some pathogenicity test results demonstrating *N. parvum* and *N. luteum* as highly virulent species, *B. dothidea* as moderately virulent and *D. seriata* as the least virulent species. Our pathogenicity tests also demonstrated that the most virulent species was *N. parvum* following *L. theobromae* and *B. dothidea* but the least virulent one was *D. seriata* among the Botryosphaerious fungi. However, Úrbez-Torres and Gubler (2009) found that *L. theobromae* was the most virulent species followed by *N. luteum, N. parvum* and *N. australe* categorized as highly virulent. *B. dothidea* was intermediately virulent and *D. seriata, D. mutila, Dothiorella iberica* and *D. viticola* were weakly virulent pathogens collected from California vineyards. Our results were parallel with these findings in some extent but not at all. Based on these results, it may be suggested that cultivar susceptibility and fungal isolate diversity might have played an important role on different lesion lengths in our study.

The Aegean Region is the center of Turkish viticulture with most of the grafted young vines used throughout the country produced and sold from the Aegean region. This study points out that GTD is an important problem in Sultana Seedless vineyards of the Aegean Region and disease control measures should be taken to prevent dissemination of pathogenic fungi and trunk infections. Further studies are under way to assess hot water treatments and their combinations with systemic fungicides to produce healthy grapevine plants.

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