Fungal pathogens associated with young grapevine decline in the Southern Turkey vineyards

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Abstract. Young grapevine decline is a common and important disease caused by fungal plant pathogens in Turkey vineyards. Every year many grape growers face this problem in their vineyards and seek solutions to cope with it. The aims of the study were to examine fungal pathogens of young grapevine decline in Southern Turkey and to determine pathogenicity of fungi involved in the disease. Twenty vineyards (2–3 years-old, located in Adana, Mersin and Gaziantep cities) were surveyed in March 2018 and declining whole plants were sampled and processed for mycological procedures. Sub-cultured fungal colonies were examined for colony morphology and conidia-conidiophore shapes under light microscope. For molecular identification, ITS, beta-tubulin, histone and TEF1-alpha gene regions were amplified with PCR using appropriate primers and PCR products were subsequently sequenced. The sequences were compared with those deposited in the NCBI GenBank database using the BLASTn program and fungal identifications were confirmed by getting accession numbers. Pathogenicity tests were fulfilled under greenhouse conditions for two months. The results indicated that Botryosphaeria Dieback and Black Foot fungi were two most common pathogen groups, while Petri Disease and Diaporthe Dieback pathogens had minor incidence. Although a variety of *Fusarium* species were isolated from declined vines, only *F. brachygibbosum* and *F. solani* were found to have considerable role in disease occurrence.

1. Introduction

Turkey is an important country among the grape producing countries in the world and it ranks sixth after China in the world grape production. Approximately, 4.1 million tons of grapes are produced in 435.000 ha area in Turkey vineyards [1]. With the expansion of vineyard areas, some phytopathological problems have emerged during the last 50 years in Turkey. Young grapevine decline has been an important disease which encountered in majority of newly established vineyards in Turkey. Failure of grafted materials and dying of newly planted vines are common symptoms in planting year. Stunted and retarded growth, lack of sprouting, chlorotic leaves and dieback are the other symptoms, encountered in subsequent five years [2]. A variety of fungal pathogens including Botryosphaerieaceae fungi, black-foot; *Campylocarpon fasciculare, Cylindrodendrum* spp., *Dactylonectria* spp., *Neonectria* spp. and *Ilyonectria* spp., Petri Disease; *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., Diaporthe fungi: *Diaporthe* spp. and *Cadophora luteo-olivacea* and *Pleurostomophora richardsiae* have been reported to be associated with young grapevine decline [3–5]. These fungi survive in soil, plant debris or grapevine propagation materials and are mainly transport with infected saplings to many grape growing areas [6]. In case of severe infections, young vines die within 2–5 years and growers face economic losses due to re-planting costs and losing of time.

When young grapevine decline emerged in any vineyard, it is necessary to identify the causal pathogens and to track where disease was originated. A survey study was conducted in commercial grapevine nurseries of Portugal and *Ilyonectria liriodendri, Botryosphaeriaceae fungi, Phomopsis viticola* and *Phaeomoniella chlamydospora* were found to be the common species causing young grapevine decline [7]. In another study conducted in California (U.S.), *Armillaria mellea, Phytophthora* spp., *Phaeomoniella chlamydospora, Phaeoacremonium* spp. *Ilyonectria liriodendri* and *Verticillium dahliae* species were reported as causal agents associated with root rots and young grapevine decline diseases [8].

Up to date, some studies were conducted to manifest fungal pathogens in young grapevines and certain pathogens were recorded in the Aegean and Central Anatolia Region of Turkey. Poyraz and Onogur (2013) surveyed 21 vineyards, younger than 10 years, and they detected *Phaeomoniella chlmydospora* and *Phaeoacremonium aleophilum* species leading to Petri Disease in the Aegean Region of Turkey [9]. Akgul et al. (2014) and Gungor-Savas et al. (2015) reported that *Campylocarpon fasciculare* and *Ilyonectria liriodendri* were the fungal pathogens responsible for black foot disease of grapevine nurseries in the Aegean Region of Turkey [10,11]. In the Central Anatolia (Turkey), *Ilyonectria macrodidyma, Phaeoacremonium scolyti* and *Pleurostoma richardsiae* species were found to be common pathogens associated with young grapevine decline disease [12–14].

The Southern Turkey has the third largest vineyard region and in this region, climate is warmer than that of the Aegean and Central Anatolia Regions. Moreover, there are very few grapevine nurseries in there, so that most of the saplings come from the Aegean Region. In recent years, grape growers have reported that young

Figure 1. Locations where samples were collected in the Southern Turkey.

grapevine decline has become a common disease in the Southern Turkey vineyards. Up to now, no detailed study was conducted to reveal the fungal pathogens in this region, although some vineyards have been visited and a couple of symptomatic vines were analysed in laboratory. Therefore, a mycological study was arranged to reveal fungal pathogens in the vineyards of this region.

The aims of the study were to determine fungal pathogens associated with young grapevine decline and to understand common species. It was also aimed to raise awareness of the grape growers against the disease.

2. Material and methods

2.1. Sample collection and laboratory studies

To obtain fungal isolates, 20 vineyards (2–3 years old, located in Adana, Mersin and Gaziantep cities, Fig. 1), having young grapevine decline symptoms, were examined and whole vine samples were uprooted to do laboratory analysis. The samples were taken from local and common table grape varieties (*Vitis vinifera* cv. Cardinal, Eraly Sweet, Hatun Parmagi, Italia, Prima, Tarsus Beyazi, Yalova Incisi, Victoria) and root, rootstock, graft-union and trunk were separately packed in paper bags.

The samples were washed under running water, briefly dried with paper towels and superficially disinfected with NaOCl solution (4%) for 3 minutes, after that they rinsed twice in sterile distilled water and dried with sterile papers under laminar air flow. Tissues (3–5 mm) were placed on PDA (Potato Dextrose Agar; Merck) amended with streptomycin-sulphate ($150\,\mathrm{mg}\cdot\mathrm{L}^{-1}$), plates were incubated at 24 °C for 3 days. After colony growth, mycelia were sub-cultured on PDA, the representative isolates, having different colony morphology, were archived and stored in sterile centrifuge tubes (containing 30% glycerol-water) at −20 °C for further identification studies. Isolation frequency was calculated by counting colonies and proportions of fungal genera were found by dividing colony numbers of each genus to total wood chips in each vineyard. From these data, average isolation rates of the genera (for all regions) were calculated for each different parts of vines (for example: root, rootstock, graft-union and trunk).

2.2. Morphological and molecular identifications

In morphological discrimination, colony colour, mycelial growth pattern, conidia and conidiophore shapes, conidiomata and chlamydospore occurrence and picnidial paraphyses were examined under light microscope (Olympus BX51) and the species were estimated by following the descriptions in literature [15–18].

For molecular identification, DNA was extracted using CTAB extraction protocol, modified by Porebski et al. (1997), their concentration was measured with nanodrop device and final DNA concentration ($50\,\mathrm{ng}\cdot\mu\mathrm{l}^{-1}$) was adjusted by dilution with PCR grade water [19]. Universal primer pair ITS4-ITS5 were used to amplify ribosomal DNA regions (rDNA) for all fungal isolates [20]. Additionally, one of the following genes; beta-tubulin, elongation factor (EF) $1-\alpha$ and histone were amplified with Bt2a-Bt2b, EF728F-EF986R and CYLH3F-CYLH3R primers [21–23] using a thermocycler (Applied Biosystems) for identification of some isolates. Each of the PCR reaction tubes contained 10X Thermo Scientific Green PCR buffer® (2.5 µl), dNTP mix. (1 µl), forward and reverse primers (0.5 µl, $10\,\mathrm{pmol}\cdot\mu\mathrm{l}^{-1}$), Thermo Taq polymerase® (5 iu, 0.125 µl), PCR grade water (19.4 µl) and target DNA (1 µl). The thermocycler was adjusted as follows; 95 °C for 3 min (initial denaturation) followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min (ITS4-ITS5 and EF728F-EF986R), 62 °C for 1 min (Bt2a-Bt2b), 54 °C for 1 min (CYLH3F-CYLH3R), extension at 72 °C for 1 min and a final extension 72 °C for 7 min. After PCR amplification, the products were separated by agarose gel electrophoresis (1.5%) in 1X TAE buffer to check DNA quality. After that, PCR products sequenced (sanger sequencing) and obtained nucleotides were compared with those deposited in the NCBI database using the BLASTn software. For each fungal isolate two different gene sequences were submitted to the GenBank and accession numbers were obtained (Table 1).

2.3. Pathogenicity tests

Pathogenicity tests were performed in growth-room conditions (27 °C stable temp., 12h dark/light, 85% RH) on Cardinal grape saplings. Dormant grapevine cuttings (cv. Cardinal, 30 cm long, three buds) were taken from vineyard of Plant Protection Dept., University of Cukurova in December 2018. To prevent dehydration, they were immersed into clean tap water at ambient temperature for overnight. After that, cuttings were superficially disinfected with NaOCl (2%) for 2 min, rinsed with sterile distilled water twice and dried in laboratory. Cane internodes were laterally drilled with 2 mm-diameter needle and 2 mm-mycelial plugs (for hardly sporulating fungi) were inserted into cores of the cuttings, or 25 µl conidial suspensions (10^7 conidia $\cdot$ ml^{-1} water) were injected to the holes with a micropipetor, then the holes were sealed with parafilm® . The inoculated canes were planted to plastic bags containing growing mixture (fine sawdust, perlite, sand, soil and peat in equal volumes), watered and maintained at growth room conditions (at 28 °C temp., 90% relative humidity, 12 h dark/light) for two months. After this time, cuttings were longitudinally divided with a knife and lesion lengths were measured around inoculation points. To reveal differences between means of the lesion lengths, variance analysis was performed on data and the statistical groups were determined by Fisher's Least Significant Difference (LSD) test at 5% significance level in all studies [24].

Table 1. Lesion lengths in wood tissues of inoculated grapevine plants (*Vitis vinifera* cv. Cardinal) after two-month incubation.

Isolate Numbers and Species Names		Lesion Lengths (mm)
Adana Isolates		
MH136Cyh	*Diplodia seriata*	11.9 ± 0.8 e*
MH151Cyh	*Fusarium oxysporum*	2.5 ± 0.2 a
MH126Cyh	*Fusarium solani*	4.9 ± 0.5 b
MH103Cyh	*Neofusicoccum parvum*	39.2 ± 0.1 m
Gaziantep Isolates		
MH146IsL	*Fusarium oxysporum*	2.2 ± 0.1 a
MH91IsL	*Neofusicoccum parvum*	39.4 ± 0.4 m
Mersin Isolates		
MH25Trs	*Botryosphaeria dothidea*	25.9 ± 0.4 j
MH145Trs	*Diporthe ampelina*	25.6 ± 0.1 j
MH143Trs	*Diaporthe ampelina*	27.2 ± 0.6 k
MH84Srv	*Diplodia seriata*	11.4 ± 0.1 de
MH19Trs	*D. seriata*	11.4 ± 0.5 de
MH21Trs	*D. seriata*	10.8 ± 0.4 d
MH137Trs	*D. seriata*	11.9 ± 0.7 e
MH261Srv	*D. seriata*	12.0 ± 0.2 e
MH40Trs	*Dactylonectria macrodidyma*	19.3 ± 0.3 gh
MH116Trs	*Dactylonectria torresensis*	17.8 ± 0.4 f
MH153Trs	*D. torresensis*	18.6 ± 0.1 fg
MH154Trs	*D. macrodidyma*	20.6 ± 0.5 i
MH127Trs	*Fusarium brachygibbosum*	8.7 ± 0.2 c
MH111Trs	*Fusarium proliferatum*	2.1 ± 0.5 a
MH113Trs	*F. proliferatum*	2.3 ± 0.1 a
MH118Trs	*F. proliferatum*	2.3 ± 0.2 a
MH94Trs	*F. proliferatum*	2.0 ± 0.6 a
MH139Trs	*Fusarium oxysporum*	2.2 ± 0.7 a
MH100Trs	*Fusarium solani*	4.9 ± 0.4 b
MH119Trs	*F. solani*	4.8 ± 0.6 b
MH134Trs	*F. solani*	5.2 ± 0.1 b
MH12Trs	*Lasiodiplodia theobromae*	33.1 ± 0.2 l
MH31Trs	*L. theobromae*	33.8 ± 0.1 l
MH45Trs	*L. theobromae*	33.1 ± 0.5 l
MH89Trs	*Neofusicoccum parvum*	39.2 ± 0.3 m
MH90Trs	*N. parvum*	39.0 ± 0.4 m
MH121Trs	*N. parvum*	39.0 ± 0.2 m
MH108Trs	*Phaeoacremonium minimum*	19.6 ± 0.4 h
MH115Trs	*Phaeoacremonium minimum*	18.9 ± 0.4 gh
MH114Trs	*Pleurostomophora richardsiae*	11.2 ± 0.4 de
Un-inoculated Control		1.7 ± 0.2 a

* 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.

3. Results and discussion

3.1. Isolation and identification results

Totally 36 fungal isolates were obtained from the 20 vineyards in the Southern Turkey Region. 15 isolates were belonged in Botryosphaeriaceae fungi, 12 isolates were from *Fusarium* genus, four of them from black-foot, two were from Petri Disease, and Diaporthe Dieback, and one isolate was *Pleurostomophora richardsiae*.

Botryosphaeriaceae were the most frequently isolated fungi, so that they could be isolated from all parts of the symptomatic, declined vines. The isolation rates were 36.8%, 30.8%, 20% and 4.9% from the trunk, graft-union, rootstock and roots respectively. Following that *Fusarium* was the second commonly isolated genus from the roots and the other parts of the vines with 26.7%, 14.7%, 7.4% and 11.4% rates (from bottom to top). Besides that, black-foot fungi were also found from all parts of the vines but maximum incidence (35.2%) was recorded from the root tissues. The incidence of this fungi decreased below 10% as going up from the roots to trunk. On the contrary, Petri Disease pathogens could not be isolated from all examined sections of the vines and it was found just in rootstock tissues with the minimum incidence (0.8%). *Diaporthe* was another genus which was found in most of the parts (except roots) in vines but its incidence was lower (minimum: 2.2% and maximum: 6.3%) than that of Botryosphaeriaceae and *Fusarium* fungi (Fig. 2). According to morphological-microscopic examinations and molecular methods, four different species of Botryosphaeriaceae fungi (*Botryosphaeria dothidea, Diplodia seriata, Lasiodiplodia theobromae* and *Neofusicoccum parvum*), four species of *Fusarium* (*F. barchygibbosum, F. oxysporum, F. proliferatum* and *F. solani*), two species of *Dactylonectria* (*D. macrodidyma, D. torresensis*), *Diaporthe ampelina*, *Phaeoacremonium minimum* and *Pleurostomophora richardsiae* were identified and their species names were confirmed by recording sequences to the NCBI GenBank with accession numbers (Table 2). On the other hand, a variety of endophytic species (*Alternaria, Aspergillus*, *Penicillium Rhizopus* sp. etc.) and *Clonostachys rosea* (potential biocontrol agent) were detected from the young vines but no GenBank numbers were obtained for these fungi.

3.2. Pathogenicity tests results

The fungal isolates included in pathogenicity tests produced lesions (generally blackish-brown streaks), which their lengths ranging from 2.0 to 39.4 mm, in wood tissues of the inoculated grapevine plants within two months. In control plants treated with sterile water, average 1.7 mm brown lesions were occurred but most of the isolates lead to significantly longer streaks than that of control. Two members of Botryosphaeriaceae fungi (*Neofusicoccum parvum* and *Lasiodiplodia theobromae*) lead to longest lesions in inoculated plants, so that the lesion lengths of these species ranged between 33.1 and 39.4 mm. *N. parvum* was more virulent than *L. theobromae*. In this family, *Diplodia seriata* and *Botryosphaeria dothidea* could not show remarkable virulence as observed in the former two species. Considering the other lesion lengths, *Diaporthe ampelina, Dactylonectria torresensis, Dactylonectria macrodidyma, Phaeoacremonium minimum* and *Pleurostomophora richardsiae* were classified the other species following *N. parvum and L. theobromae*.

On the other hand, *Fusarium* isolates were not so successful in producing symptomatic wood streaks, when compared the other pathogen groups. The average lesion lengths produced by *Fusarium oxysporum* and

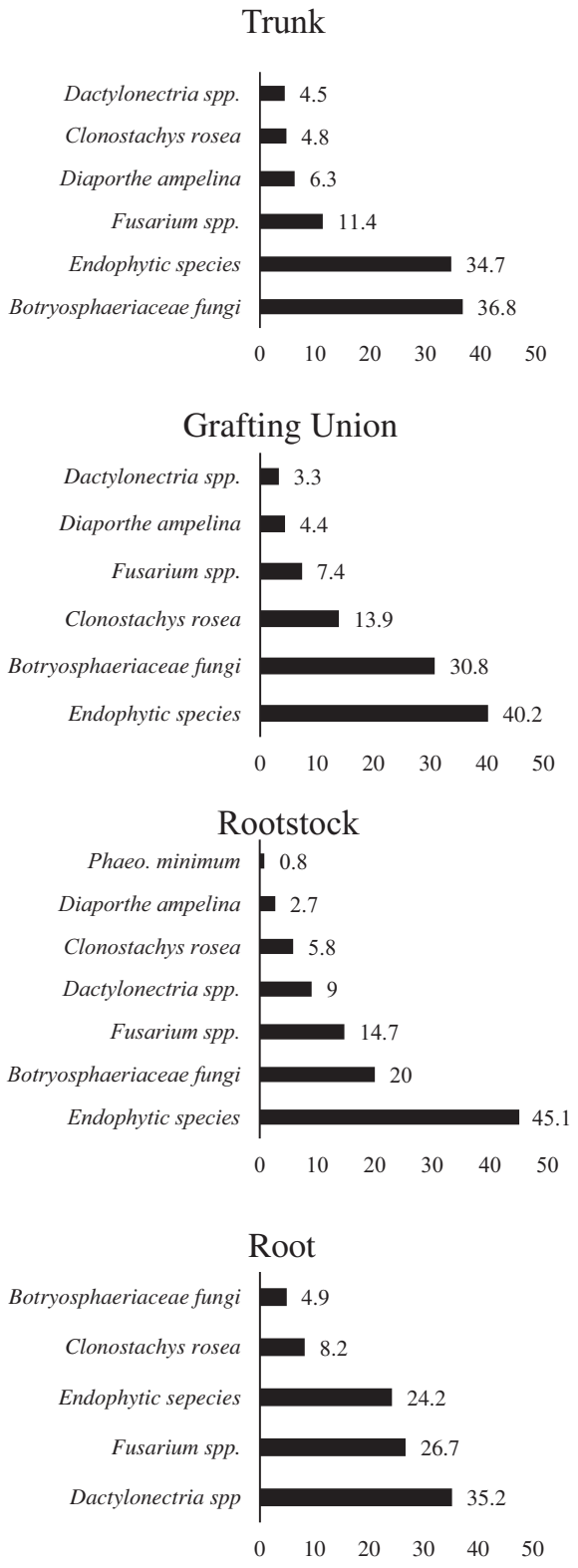

Figure 2. Isolation rates (%) of fungi from different parts of symptomatic vines in the vineyards sampled.

F. proliferatum were not different from those produced by sterile water statistically. However, longer lesions were recorded when grapevine cuttings were inoculated with *F. brachygibbosum* and *F. solani*. According to these results, it can be concluded that *F. oxysporum* and *F. proliferatum* isolates were not to be pathogenic but *F. brachygibbosum* and *F. solani* were pathogenic on grapevines (Table 1).

Aroca et al. (2006) surveyed 13 young vineyards (one-year old) in Spain and conducted a study to reveal main fungal pathogens causing decline symptoms. 208 plants were collected in 2002–2004 years, of which 94 plants (45.2%) were found to be infected with *Phaeomoniella chlamydospora, Phaeoacremonium, Botryosphaeria, Ilyonectria* and *Phomopsis* species [25]. In another study conducted in Italy (2013–2015 years), totally 45 young grapevine plants (aged from 12–18 months) were examined and Petri Disease, Botryosphaeria Dieback and Black Foot pathogens were isolated. Some of the species (*Phaeoacremonium minimum, Pleurostoma richardsiae, Diplodia seriata, Lasiodiplodia theobromae, Neofusicoccum parvum* and *Dactylonectria torresensis*) isolated in this research were the same with that we isolated in our study. So our findings corroborate the results of Carlucci et al. (2017) [26].

Pintos et al. (2018) determined the phytosanitary status of grapevine nurseries (2 in Spain, 1 in France) by analysing 150 young grapevine plants. From 449 fungal isolates, five species of Botryosphaeria Dieback, six from Black Foot Disease, six from Diaporthe Dieback and three species from Petri Disease have been characterized [27].

Botryosphaeriaceae fungi have been frequently obtained from symptomatic young grapevines and they have been considered to have an important role in disease occurrence [28]. These fungi can be found in any asymptomatic propagation material as an endophyte or latent pathogen and can easily be disseminated to large areas [29]. In the current study, high incidence percentages of Botryosphaeriaceae indicate that these fungi were highly prevalent in all parts of the symptomatic vines and most of the propagation materials were thought to be contaminated as a latent infection in the Southern Turkey vineyards. Climate is a critical factor influencing geographical distribution of this fungi [30]. While the climate is mild and rainy (average precipitation; 750 mm · year^{-1}) in winters, it is very hot (34–42 °C) during summer in this region. Hot weather and water stress have been shown to contribute disease development by Botryosphaeriaceae pathogens [31]. These factors may be one of the reasons for the high level of disease incidence in Southern Turkey.

Black foot fungi were the second group having high percentage incidence in that region. Interestingly, they have been isolated from the roots to trunks of sampled plants. It has been demonstrated that inoculum of black foot pathogens was found at different stages of vine nursery propagation process and suggested that these pathogens could infect plant tissues during vine production process [32]. The reasons why these fungi were found in all processed tissues during laboratory studies could be related with these events. There are a few grapevine nurseries in the Southern Turkey and most of the grafted plants are brought from the Aegean Region where many commercial nurseries are found. Therefore, most of the black foot pathogens was thought to contaminate the Southern vineyard areas from the Aegean Region, Turkey. *Dactylonectria* species can survive in soil as chlamydospore and infect newly planted vines from their roots [33]. Root infections are important and may be responsible for disease incidence. However, we could not see decline symptoms in the vineyards established on their own roots that we visited.

Table 2. Fungal species obtained from the isolate collection study in the Southern Turkey vineyards.

Isolate Numbers and Species Names		Host (*Vitis vinifera* cv.)	GenBank Accession Numbers			
			ITS	Beta-tubulin	TEF1-α	Histone H3
Adana Isolates						
MH136Cyh	*Diplodia seriata*	5BB-Italia	MK817029	–	MK838563	–
MH151Cyh	*Fusarium oxysporum*	1103P-Victoria	MK817030	–	MK838564	–
MH126Cyh	*Fusarium solani*	1103P-Cardinal	MK817031	–	MK838565	–
MH103Cyh	*Neofusicoccum parvum*	1103P-Cardinal	MK817032	–	MK838566	–
Gaziantep Isolates						
MH146IsL	*Fusarium oxysporum*	5BB-Hatun Parmagi	MK817033	–	MK838567	–
MH91IsL	*Neofusicoccum parvum*	5BB-Hatun Parmagi	MK817034	–	MK838568	–
Mersin Isolates						
MH25Trs	*Botryosphaeria dothidea*	1103P-Victoria	MK817035	–	MK838569	–
MH145Trs	*Diporthe ampelina*	5BB-Yalova Incisi	MK817037	MK838571	–	
MH143Trs	*Diaporthe ampelina*	SO4-Victoria	MK817036	MK838570	–	–
MH84Srv	*Diplodia seriata*	1103P-Prima	MK817040	–	MK838574	–
MH19Trs	*D. seriata*	110R-Yalova Incisi	MK817041	–	MK838575	–
MH21Trs	*D. seriata*	110R-Victoria	MK817042	–	MK875258	–
MH137Trs	*D. seriata*	Tarsus Beyazi	MK817038	–	MK838572	–
MH261Srv	*D. seriata*	1103P-Victoria	MK817039	–	MK838573	–
MH40Trs	*Dactylonectria macrodidyma*	1103P-Prima	MK817043	MK875273	–	MK955244
MH116Trs	*Dactylonectria torresensis*	1103P-Victoria	MK817044	MK875274	–	MK955245
MH153Trs	*D. torresensis*	1103P-Prima	MK942432	MK875276	–	MK955246
MH154Trs	*D. macrodidyma*	1103P-Victoria	MK942433	MK875277	–	MK955247
MH127Trs	*Fusarium brachygibbosum*	1103P-Victoria	MK817053	–	MK875267	–
MH111Trs	*Fusarium proliferatum*	1103P-Early Sweet	MK817046	–	MK875260	–
MH113Trs	*F. proliferatum*	1103P-Victoria	MK817047	–	MK875261	–
MH118Trs	*F. proliferatum*	1103P-Prima	MK817048	–	MK875262	–
MH94Trs	*F. proliferatum*	Tarsus Beyazi	MK817049	–	MK875263	–
MH139Trs	*Fusarium oxysporum*	1103P-Victoria	MK817045	–	MK875259	–
MH100Trs	*Fusarium solani*	1103P-Early Sweet	MK817050	–	MK875264	–
MH119Trs	*F. solani*	5BB-Yalova Incisi	MK817051	–	MK875265	–
MH134Trs	*F. solani*	1103P-Victoria	MK817052	–	MK875266	–
MH12Trs	*Lasiodiplodia theobromae*	Tarsus Beyazi	MK817054	–	MK875268	–
MH31Trs	*L. theobromae*	1103P-Early Sweet	MK817055	–	MK875269	–
MH45Trs	*L. theobromae*	1103P-Victoria	MK817056	–	MK875259	–
MH89Trs	*Neofusicoccum parvum*	1103P-Prima	MK817058	–	MK875271	–
MH90Trs	*N. parvum*	1103P-Early Sweet	MK817059	–	MK875272	–
MH121Trs	*N. parvum*	5BB-Yalova Incisi	MK817057	–	MK875270	–
MH108Trs	*Phaeoacremonium minimum*	1103P-Victoria	MK817061	MK875275	–	–
MH115Trs	*Phaeoacremonium minimum*	5BB-Yalova Incisi	MK817062	–	MK955243	–
MH114Trs	*Pleurostoma richardsiae*	1103P-Prima	MK817060	–	MK955242	–

The pathogenicity of some *Fusarium* isolates that we isolated from the symptomatic vines demonstrates that these fungi could contribute to disease occurrence. In a study conducted in Canada, five *Fusarium* species (*Fusarium* sp. *F. oxysporum, F. proliferatum, F. solani* and *F. ramigenum*) were isolated from declined vines and their role on disease incidence were investigated with pathogenicity tests. Results indicated that these species caused root necrosis similar to those observed by *Dactylonectria pauciceptata, D. macrodidyma* and *Ilyonectria liriodendri* and have an important role on young grapevine health [34].

This study showed that some of the fungal pathogens grouped in grapevine trunk diseases (GTD) were responsible for young grapevine decline occurrence in the Southern Turkey vineyards. Isolation of GTD pathogens (more than one) from the same plant indicates that grapevine saplings have been infected with these fungi

during propagation process in the nurseries. Therefore, grapevine sapling producers should follow cultural, physical, biological and chemical management practices to reduce young grapevine decline incidence and to produce healthier plants.

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