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Determination of grapevine stem fungal diseases in Malatya province

Malatya ilinde asma gövde fungal hastalıklarının belirlenmesi

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ABSTRACT

Grapevine (*Vitis vinifera* L.) is one of the most prevalent and long-standing cultivated crops in the world due to its non-selective climate and soil demands, types of usage and having a wide range of varieties. Turkey has a rich grapevine gene pool because of its favorable climate zone. Viticulture, which has an important role in agriculture, faces many problems in the process of production to marketing. Fungal diseases take an important place by limiting the production wherever grapevine is cultivated. It is aimed to determine the stem fungal diseases that cause drying in viticulture areas of Malatya province with this research. Arapgir, Yesilyurt, Battalgazi and Darende districts of Malatya province, where grapevine is intensely cultivated, were surveyed in two different vegetation periods, and samples were taken from symptomatic plants. Identification of these isolates were performed based on morphological and molecular examinations. As a result of this study, *Botryosphaeria* spp., *Phaeomoniella chlamydospora*, *Fomitiporia mediterranea*, *Cytospora viticola*, *Neoscytalidium dimidiatum*, *Dothiorella* spp., *Lasiodiplodia* spp. were identified as causal agents for the diseases observed in Malatya province.

INTRODUCTION

Turkey is a country located on the most ideal climate zone for vineyard cultivation in the world. It has a viticulture culture dating back to the past and is gene source of the vine (*Vitis vinifera* L.). Anatolia is a very rich gene center not only with its vineyards and grape production but also with its culture vine and wild vine species.

When the evaluation possibilities of the grape, which is also called the "Fruit of Heaven" among the people, are examined, it is one of the rare plants with many alternatives in the world and in our country. The main evaluation methods of grape can be listed as follows; besides its fresh consumption, it is used in various ways such as vinegar, wine, molasses and dried nuts. Pickled vine leaves are also a product whose economic value has increased in recent years (Cabaroğlu 2015).

The most important grape-producing countries are Spain, France, Italy, USA, Turkey and China. According to 2019 data from FAOStat, Turkey ranks 5th (400.997 ha) in terms of vineyard area and 6th (4.208.908 tons) in terms of production in the world (FAOStat 2019). Of the 4.208.908 tons of grapes produced, approximately 38.3% are table grapes, 11.6% seedless table grapes, 26.1% seedless raisins,

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14% raisin with seed and 10% wine grape. Turkey exports 40-45% of seedless raisins, nearly half of the world production, hereby been a significant income for the country's economy.

After the Aegean, Mediterranean, Central Anatolia and Western Anatolia regions, Eastern Anatolia ranks fifth in terms of grape production and vineyard area as it has 4.4% of the vineyards. When evaluated provinces in the region, Malatya is the second after Elazig in terms of both cultivation area and production (Table 1) (Anonymous 2020).

Table 1. The production areas (da) and production amounts (tons) of the provinces located in Eastern Anatolia

Province	Production area (da)	Production amount (tons)
Elazığ	108.568	94.463
Malatya	38.920	22.812
Bingöl	1.600	640
Tunceli	2.781	2.869
Van	410	232
Muş	3.848	2.114
Bitlis	5.315	3.139
Hakkari	6.485	5.131
Eastern Anatolian	167.927	131.400
Turkey	4.009.970	4.208.908

Grape is an important agricultural product whose production faces many problems from growing to storage and from processing to marketing. The most important of these problems are fungal diseases, which are increasing in importance day by day in all countries where vineyards are grown, reaching economic dimensions and limiting grape production due to the damage, they cause (Göktaş 2008).

Among the fungal diseases that cause significant losses, powdery mildew (*Uncinula necator* Burr.), grape downy mildew (*Plasmopara viticola* Berl. & De Toni), anthracnose (*Elsinoe ampelina* Shear.) and gray mold (*Botrytis cinerea* Pers.) are of great importance due to direct damage to the product. Apart from these, the dead arm (*Phomopsis viticola* Sacc.), which causes significant damage to the shoots, the esca (kav) disease (Anonymous 2008) determined to be caused by a group of factors (*Stereum hirsutum*, *Phellinus igniarius*, *Phaeoacremonium* spp., *Phaemoniella chlamydospora*), petri disease caused by *Phaeoacremonium oleophilum*, *Phaemoniella chlamydospora* (Crous and Gams 2000, Crous et al. 1996), retrograde death disease by *Eutypa lata* which causes branch and trunk drying (Rappaz 1984), cancer in the ligaments and black dead arm disease (*Botryosphaeria* spp.) are the diseases frequently encountered in the cultivation areas (Urbez-Torres 2011). *Fusarium* spp., *Pythium* spp., *Phytophthora* spp., *Macrophomina phaseolina*, *Verticillium dahliae*, *Armillaria mellea*, *Rhizoctonia solani* and *Rosellinia necatrix* cause root rot and plant death (Gubler et al. 2004, Petit and Gubler 2005, van Coller et al. 2005).

Although the vineyard area is spread over a large area in Malatya province, the average yield per unit area is low. Diseases and pests are among the main reasons for the low yield. Fungal diseases observed in different stages of production cause significant product losses. Considering the share of agriculture in the increasing population and total income, it is important for our country to ensure high efficiency and quality in agriculture and to fight fungal diseases in this respect (Kiracı et al. 2015).

There are many wood tissue diseases that cause economic losses in vineyard cultivation. The observation of intense drying in the vineyards of Malatya province in recent years and the fact that research conducted upon the complaints from the producers did not reveal the cause of the fungal stem diseases in the vineyard areas. Thus, this study aimed to determine the fungal agents in the wood tissue in the vineyards of Malatya province.

MATERIALS AND METHODS

Survey

The villages of Arapgir, Yesilyurt, Battalgazi and Darende districts of Malatya province where viniculture is concentrated were determined as survey areas to determine fungal stem diseases (Figure 1). Sampling was carried out in two different periods considering the vegetation period in the vineyard areas selected to represent the region. The first sampling was performed in June-July when fungal ligament

Figure 1. The surveyed area in the province of Malatya

diseases were widespread, and the second sampling was performed in September and October just before harvesting in the region. The guided sampling method reported by Bora and Karaca (1970) was used.

Isolation and morphologic identification

Transverse sections were taken from the part/tissue of plants including trunk and 2-year-old shoots of vine stocks. About 3-4 mm pieces containing both diseased and healthy tissue were cut out with the help of sterile scalpel from parts where symptoms observed, kept in 1% sodium hypochlorite for 2-3 minutes, and dried between sterile blotting papers (Whatman Filter Papers 110 mm, Germany) for 15 minutes. The excised sections were placed in Petri dishes (90 mm diam.) containing potato dextrose agar (PDA; Merck, Germany) and Malt Extract Agar (MEA; Merck, Germany) supplemented with streptomycin (Sigma Aldrich, USA) (100 mg/l) and incubated at 22 ± 2 °C and 12 hours light photoperiod in an incubator (ME-352 PE, Panasonic, Japan). After about 7-10 days of incubation, hyphal tips of each fungal colonies were transferred into another plate containing fresh PDA to obtain pure cultures.

Morphological features of fungal agents (colony color, micelle growth, pycnidia formation, and conidial shape) was evaluated in PDA medium. The conidia of the fungal isolates were measured and photographed in a trinocular research microscope (Nikon Eclipse E200, Japan). Diagnostics were done according to Fischer (2006), Fischer et al. (2016), Larignon and Dubos (1997), Lawrence et al. (2017), Urbez-Torres et al. (2010).

Pathogenicity studies

Pathogenicity was conducted with three inoculated plants per selected isolates on a 2-years-old potting Köhnü grape variety in a completely randomized experimental design. Plants were left in the glasshouse (23–26 °C) for 3 weeks. Dense suspensions of conidia (~1.107 conidia/ml) were prepared by flooding the surface of two-week-old PDA cultures of selected isolates with sterile water and gently rubbing the surface with a sterile rod. Grapevine stems were injured with a sterile scalpel, 200 µl of the spore suspension were poured directly on a wound. Control plants were inoculated with sterile distilled water. Three weeks after inoculation, vascular lesions were recorded, by removing the bark from the stem and measuring the necrotic lesions downwards and upwards at the inoculation site. The presence of necrosis was used as an indicator of pathogenicity.

Molecular studies

Pathogenicity tests of morphologically pre-diagnosed isolates were conducted and isolation of total genomic DNA from mycelia (50 mg) taken from single spore cultures grown on PDA medium of the isolate isolated from disease symptoms was carried out using DNeasy Blood and Tissue kit (Qiagen, Germany) and the protocols suggested by the manufacturer.

PCR studies were performed using ITS4 and ITS5 primer pair for the internal transcribed spacers (ITS) rDNA site (White et al. 1990), Bt2a and Bt2b primer pair for the β-tubulin (TUB2) gene region (Glass and Donaldson 1995), LROR and

Table 2. Primers and PCR conditions used in molecular studies

Locus	Primers	PCR condition	
ITS	ITS4 (5'-TCC TCC GCT TAT GC-3') ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3')	95 °C 3 min 35 cycle 95 °C 1 min 54 °C 45 sec 72 °C 45 sec 72 °C 10 min	White et al. (1990)
β-Tubulin	BT2a (5'GGT AAC CAA ATC GGT GCT GCT TTC-3') BT2b (5'-ACC CTC AGT GTA GTG ACC CTT GGC-3')	95 °C 3 min 35 cycle 95 °C 1 min 58 °C 45 sec 72 °C 45 sec 72 °C 10 min	Glass and Donaldson (1995)
LSU	LROR (5'-ACC CGC TGA ACT TAA GC-3') LR5 (5'-TCC TGA GGG AAA CTT CG-3')	95 °C 3 min 35 cycle 95 °C 1 min 58 °C 45 sec 72 °C 45 sec 72 °C 10 min	Woudenberg et al. (2009)
TEF1- α	EF1-986R 5'-TA CTT GAA GGA ACC CTT ACC-3' EF1-728F 5'-CA TCG AGA AGT TCG AGA AGG-3'	95 °C 3 min 35 cycle 95 °C 1 min 58 °C 45 sec 72 °C 45 sec 72 °C 10 min	Carbone and Kohn (1999)

LR5 primer pair for large subunit (LSU) (Woudenberg et al. 2009), EF1-728F and EF1-986R primer pair for Translation elongation factor 1-alpha (TEF1-α) (Carbone and Kohn 1999) (Table 2). In DNA amplification for each reaction, 5 µl of 10 × enzyme buffer, 0.2 mM of dNTPs, 2 mM MgCl2, 0.5 µl of each primer, 1-unit Taq DNA polymerase and 2 µl of DNA were added to a total of 50 µl of PCR mixture. PCR amplification conditions were 3 min initial denaturation at 95 °C, 1 min at 95 °C, 54 °C for ITS and 58 °C for TUB2, LSU and TEF 1-α, 72 °C for 45 seconds (35 cycles) and 10 min at 72 °C as a final step. Depending on the quality of the DNA bands obtained, PCR products were selected for sequencing. DNA nucleotide sequences of PCR products were matched with existing fungal species listed in the NCBI (National Center for Biotechnology Information) library using the BLASTn algorithm (Boratyn et al. 2013). Access numbers of some isolates diagnosed according to these results were deposited in the library of NCBI GenBank. The dendrogram showing the genetic kinship between the isolates was created using the maximum likelihood method and the obtained phylogenetic tree was confirmed with 1,000 repetitions (Bootstrap, p-distance, pairwise deletion) (Kumar et al. 2016).

RESULTS

Survey studies were carried out in the districts of Arapgir, Yesilyurt, Darende and Battalgazi (Table 3) and their villages, which are the most important vineyard areas of Malatya, in June-July and September-October, and the disease symptoms (Figure 2) were detected in 2018, considering the phenology of the vine.

A total of 133 vineyard samples were collected; 47 from Arapgir, 30 from Yesilyurt, 27 from Battalgazi, and 29 from Darende. The collected samples were transferred to the laboratory and the fungi were isolated and purified. Morphological diagnoses of the purified isolates were made by considering criteria such as colony shape, culture color, micelle structure, and asexual reproduction structures (Figure 3). The number of isolates obtained from each district as a result of morphological diagnosis are presented in Table 4.

Figure 2. Symptoms of apoplexy in grapevine (a), mottling on grains (b), white rot in vascular tissue (c), color change in the form of the letter 'V' (d) and bending and discoloration in the plant stem (e)

Pathogenicity test of isolates diagnosed at genus or species level as a result of diagnoses made by considering morphological criteria were tested against 2-years-old Köhnü grape variety. Brown-black discoloration of the peeled wood in the cut part and the bark tissue of the plant were evaluated as pathogen at the end of the 21 days (Figure 4). At the same time, the plant was cut horizontally from the part where the cut was, and a brown-black color change was observed. As a result of pathogenicity, *Botryosphaeria* spp., *Fomitiporia mediterranea*, *Phaeomoniella chlamidospora*, *Cytospora viticola*, *Neoscytalidium dimidiatum*, *Dothiorella*

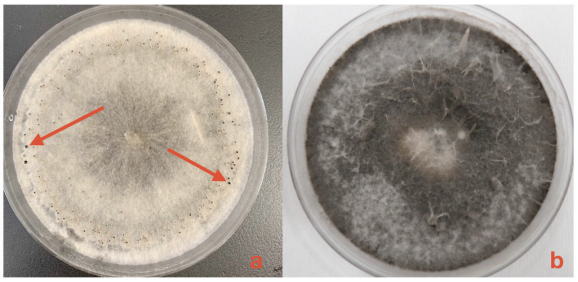

Figure 3. *Cytospora viticola* colony growth and pycnidia on PDA (a) and colonies of *Neoscytalidium dimidiatum* on PDA (b)

Table 3. The number of samples taken in from each district in Malatya province

Province	District	Number of samples		Total
		1st Period	2nd Period	
Malatya	Arapgir	25	22	47
	Yesilyurt	18	12	30
	Battalgazi	15	12	27
	Darende	14	15	29
Total	72	61	133	

Table 4. Number of isolates obtained from each district

Species	Number of isolates				
	Arapgir	Yeşilyurt	Battalgazi	Darende	Total
Botryosphaeria spp.	33	10	13	7	63
Fomitiporia mediterranea	18	7	5	5	35
Phaeomoniella chlamidospora	12	3	3	3	21
Cytospora viticola	4	2	1	1	8
Dothiorella spp.	3	2	2	7	14
Neoscytalidium dimidiatum	6	4	3	3	16
Phaeoacremonium spp.	7	4	3	2	16
Lasiodiplodia spp.	2	1	-	-	3
Alternaria spp.	12	4	2	2	20
Fusarium spp.	8	4	3	2	17
Epicoccum nigrum	5	3	1	1	10
Cladosporium spp.	7	5	3	2	17
Aspergillus spp.	5	7	5	4	21
Torula herbarum	3	3	1	2	9
Curvularia spp.	8	4	5	2	19
Beauveria bassiana	6	4	2	2	1
Total	139	67	52	45	290

spp., *Phaeoacremonium* spp. and *Lasiodiplodia* spp. were noted to cause necrosis in the stem of Köhnü grape variety; however, *Alternaria* spp., *Fusarium* spp., *Epicoccum nigrum*, *Cladosporium* spp., *Aspergillus* spp., *Torula herbarum*, *Curvularia* spp. and *Beauveria bassiana* did not cause any symptoms. Pathogens were re-isolated from symptomatic tissues of inoculated plants to fulfill Koch postulates and stored in oblique agar and sterile filter papers.

The sequences of randomly selected fungal isolates (*Phaeomoniella chlamydospora*, *Dothiorella viticola*, *Botryosphaeria dothidea*, *Fomitiporia mediterranea*, *Lasiodiplodia viticola* and *Phaeoacremonium viticola*) amplified with ITS primers were deposited in GenBank with the accession nos. MW692120, MW692121, MW692122, MW692123, MW692124 and MW692125, respectively.

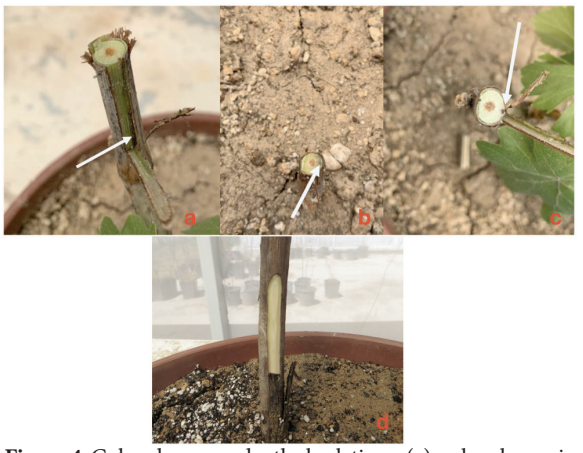

Figure 4. Color change under the bark tissue (a), color change in vascular tissue (b, c) and control (d)

Among the isolates identified as causing stem diseases in vineyards using morphological diagnosis and pathogenicity test, a detailed molecular diagnosis of *Neoscytalidium dimidiatum* and *Cytospora viticola* isolates which were detected for the first time in Turkey, was done using ITS, BTU, LSU and Tef 1- α primers for PCR analysis (Figure

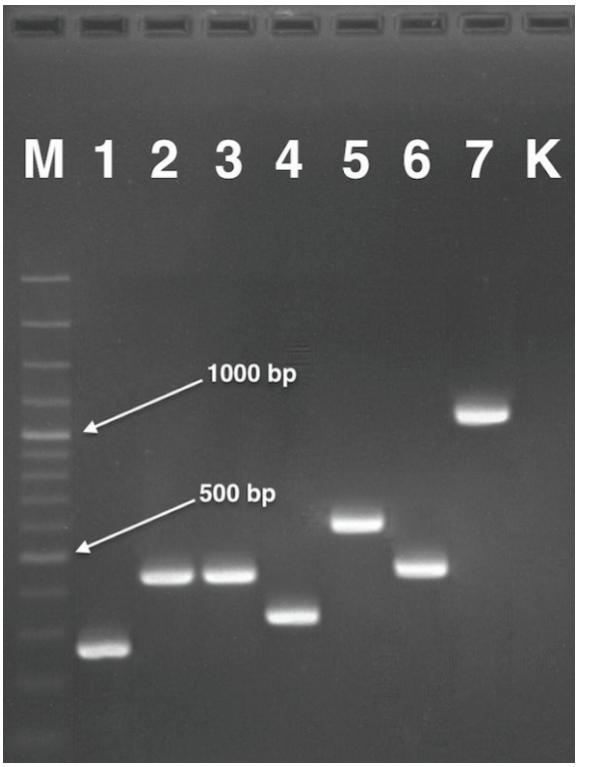

Figure 5. Agarose gel with DNA marker (M, Termoscientific, USA), TEF 1-α (1), ITS (2), β-Tubulin (3) of *Cytospora viticola* isolate and TEF 1-α (4), ITS (5), b-Tubulin (6) LSU primers (7) of *Neoscytalidium dimidiatum* and water control (K)

5) and similarity ratio was assessed by molecular analysis of sequences obtained with species existing in the GenBank database by using the BLAST analysis program and Mega 7 software on NCBI's web page.

Cytospora viticola isolate formed bands of different sizes depending on the primer used. In PCR processes using TEF 1-α primers 200-300 bp bands, in PCR processes using ITS primers a 400-450 bp and in PCR processes using β-Tubulin primers bands between 400-450 bp were obtained. Accession numbers (MK706295, MK715441 and MK715442) of the *C. viticola* isolate were obtained from NCBI, and the species verification was made by comparing the ITS, BTU, TEF1-alpha sequences of the isolate with the sequences of the same species.

Neoscytalidium dimidiatum isolate formed bands of different sizes depending on the primer used. In PCR processes using TEF 1-α primers, band formation between 250-350 bp occurred, band formation between 550-600 bp in PCR processes using ITS primers, band formation between 450-500 bp in PCR processes using b-Tubulin primers band formation between 1100-1200 bp occurred in PCR processes using LSU primers. Accession numbers (MK816354, MK816355, MK813853, and MK813852) of *N. dimidiatum* isolate were obtained from NCBI, and species verification was made by comparing the ITS, LSU, BTU, TEF1-alpha sequences of the isolate with the sequences of the same species.

DISCUSSION AND CONCLUSION

Within the scope of the study, surveys were carried out taking into account vine phenology in the villages of Arapgir, Yesilyurt, Darende and Battalgazi districts of Malatya in June-July and September-October when fungal disease symptoms began to be observed in 2018. A total of 133 plant samples and a total of 2.400 decares were surveyed and samples were taken from the main stem and shoots of plants showing signs of diseases. Of the diseased plant samples collected, 290 fungus isolates were isolated and 176 of these were determined as pathogens.

Moreno-Sanz et al. (2013), carried out a study in Northern Spain after considering that the low yield in the vineyards in the last 10 years was related to fungal stem diseases. Non-pathogenic fungi detected (55%) were *Cladosporium* spp., *Penicillium* spp., *Fusarium* spp., *Alternaria* spp., *Gliocladium* spp., *Epicoccum* spp., *Aspergillus* spp., *Ulocladium* spp., *Phialophora* spp., *Sporothrix* spp., *Nigrosporra* spp. and pathogenic fungi detected (31%) were *Cylindrocarpon* spp., *Botryosphaeria* spp., *Phaeoacremonium* spp. In our study, sampling was done on plants showing similar symptoms such as reverse death, wilt, dead arms and color change in the leaves in the vineyards. Among the samples collected 60.6% were identified as pathogenic and 39.4% were non-pathogenic. When other studies were examined in terms of pathogen and saprophyte factors, it was seen that the distribution of pathogen and saprophyte fungal agents that cause drying in the vineyards was almost the same.

Results of surveys and diagnostic studies we conducted in the study showed that the most common pathogen detected at a rate 21.7% in Malatya vineyards was *Botryosphaeria* spp. Siebert (2001) stated that *Botryosphaeria* spp. fungus has become the most common in vineyard areas in the world in recent years, has a wide host range and is widespread in worldwide. It has been reported that this disease causes high amounts of economic losses in viticulture.

Lawrence et al. (2017) reported that because *Cytospora* canker has some similar general symptoms as esca, it is considered a part of connective body diseases. In our study, eight *Cytospora viticola* isolates were detected. The symptoms seen in diseased plants are not caused by a single pathogen but by the pathogen complex. For this reason, it is not possible to diagnose the agent by just observing the symptoms.

Similar to our work, as a result of isolations made from plants showing symptoms such as wilting and necrosis in leaves, drying and shrinkage in fruits, and in some cases collapse (apoplexy) of the whole vine in the middle of the growing season in vineyards areas in California in 2012, *Rolshausen* et al. (2013) detected the presence of *Neoscytalidium dimidiatum* based on morphological characters. For the first time Akgül (2019) reported two different isolates, *Lasiodiplodia exigua* and *Neoscytalidium novaehollandiae*, after fungal isolation from symptoms of xylem necrosis and wedge-shaped dark brown spots in the wood tissue in the study in the bond areas. In our study, 16 isolates of *N. dimidiatum* were determined to cause trunk diseases in Malatya vineyards. *Neoscytalidium* agent has been reported to cause disease in different hosts in our country in recent years (Dervis et al. 2019, Dervis et al. 2020, Kurt et al. 2019, Oksal and Özer 2021, Oksal et al. 2020a, 2020b, Ören et al. 2020)

Fungal diseases of the stem are thought to enter from the wound formed in the plant during pruning and training (Eskalen and Gubler 2001). It has been stated that the greatest factor in the spread of stem fungal pathogens in vineyards is pruning tools (Mugnai 1999).

The fight against fungal diseases that cause yield and quality losses in the vineyards in the world and in our country should be done on time and following the conditions. One of the ways to increase yield and quality during production

of cultivated plants is to protect the plant from diseases and pests. The agents that we obtained from vineyards in Malatya, many of which are wound and vulnerability pathogens, enter the plants during cultural processes or through natural openings and continue their lives in the plant body for a period of 2-3 years without any symptoms. When the plants reach the harvest period, symptoms occur depending on climatic conditions. It has been determined that none of the fungal agents we have identified in the vineyard areas of Malatya province are found in a single plant, but generally in a complex. This situation makes control difficult and in some cases impossible. The reason for this is that the periods and forms of transmission of each pathogen are different and that a single cultural measure or a single chemical is not sufficient to combat these diseases.

Producers beginning to abandon cultural measures, the lack of quarantine and hygiene measures, and climate change have caused major problems in the vineyards, especially in recent years. In control of fungal stem diseases, many of which are wound pathogens, it is of great importance to remove or destroy pruning residues from the field, avoid pruning on rainy and cool days, and apply a protective fungicide within 24 hours after pruning.

This study aimed to determine the fungal pathogens in the vineyard areas that have an important place in plant production and to perform morphological and molecular diagnoses. *Botryosphaeria* spp., *Phaeomoniella chlamydospora*, *Fomitiporia mediterranea*, *Cytospora viticola*, *Neoscytalidium dimidiatum*, *Dothiorella* spp. and *Lasiodiplodia* spp. were found to be the most common fungal agents in the vineyards of Malatya province at the end of the study.

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ÖZET

İklim ve toprak istekleri yönünden çok seçici olmayışı, değerlendirme şekilleri ve çeşit açısından zengin olması nedeniyle asma (*Vitis vinifera* L.) dünyada en yaygın ve en eski kültür bitkilerinden biridir. Bağcılık için dünyanın en elverişli üretim alanlarından biri olan ülkemiz zengin asma gen potansiyeline sahiptir. Bitkisel üretimde önemli bir yere sahip olan bağcılık, günümüzde üretimden pazarlamaya kadar geçen süreç içerisinde bir çok sorunla karşı karşıyadır. Bu sorunlar içerisinde üretimi sınırlandıran fungal hastalıklar önemli yer tutmaktadır. Çalışmada Malatya ili bağ alanlarında kurumalara sebep olan gövde fungal etmenlerinin saptanması amaçlanmıştır. Bu amaçla Malatya ilinde bağcılığın yoğun olduğu Arapgir, Yeşilyurt, Battalgazi ve Darende ilçelerinde vejatasyon peryodu dikkate alınarak iki farklı dönemde örnekleme yapılmış ve hastalık belirtisi görülen bitkilerden numuneler alınmıştır. Hastalıklı bitki numunelerinden 290 adet fungal izolat elde edilmiştir. Fungal izolatların tanısı morfolojik özelliklerine ve moleküler yöntemlere göre yapılmıştır. Tanılama çalışmaları sonucunda *Botryosphaeria* spp., *Phaeomoniella chlamydospora*, *Fomitiporia mediterranea*, *Cytospora viticola*, *Neoscytalidium dimidiatum*, *Dothiorella* spp., *Lasiodiplodia* spp. hastalık etmenleri saptanmıştır.

Anahtar kelimeler: *Vitis vinifera*, fungal hastalıklar, Botryosphaeriaceae, Malatya

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