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# Characterization of *Diaporthe ampelina* isolates and their Sensitivity to Hot-Water Treatments and Fungicides in *in vitro*

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## ABSTRACT
*Diaporthe ampelina* (=*Phomopsis viticola*) is one of the most important pathogens causing both cane/leaf spot and wood canker diseases in grape growing countries in the world. In this research, morphological, molecular and pathogenic characterization of 23 *D. ampelina* isolates were studied and their sensitivity was tested against hot-water treatments and some of the fungicides used in vineyards. Morphologically, the isolates were grouped according to "W type" and "G type" colony appearance and microscopic features. In molecular characterization, beta-tubulin, calmodulin and translation elongation factor (tef1-α) gene regions were amplified with PCR. The nucleotide sequences were analyzed using NCBI-BLAST search and recorded in GenBank, through which species identity was also confirmed. Mycelial viability was tested against hot-water treatments (46 – 50°C for 30 and 45 min) in centrifuge tubes and thermal inactivation point was determined. It was also tested against some of the fungicides (azoxystrobin, boscalid, cyprodinil, tebuconazole, azoxystrobin + cyproconazole + tebuconazole, cyprodinil + fludioxonil, azoxystrobin + tebuconazole and fludioxonil) *in vitro* and $EC_{50}$ values were calculated. The morphological and molecular study results showed that all the isolates were *D. ampelina* and they were pathogenic on wood tissues of vines. Thermal inactivation of "W type" isolates was ensured at 48°C-30 min hot-water treatments. Although this treatment also reduced colony growth of "G type" isolates, it did not inhibit it completely and 48°C-45 min treatment was needed to reach full eradication. Considering fungicide sensitivity, fludioxonil or tebuconazole containing fungicides were the most effective in suppressing the mycelial growth of the fungus. However, azoxystrobin, boscalid, cyprodinil could not perform a strong inhibition when compared to fludioxonil and tebuconazole.

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# *Diaporthe ampelina* İzolatlarının Karakterizasyonu ve *in vitro*'da Bazı Fungisitlere ve Sıcak Su Uygulamalarına Duyarlılıkları

## ÖZET
*Diaporthe ampelina* (=*Phomopsis viticola*) dünyadaki üzüm yetiştirilen ülkelerde hem sürgün/yaprak lekesi hem de odun kangrenine neden olan en önemli patojenlerden biridir. Bu araştırmada, 23 *D. ampelina* izolatının morfolojik, moleküler ve patojenik karakterizasyonu çalışılmış ve bunların bağcılıkta kullanılan bazı fungisitlere ve sıcak su uygulamalarına karşı duyarlılığı test edilmiştir. Morfolojik olarak izolatlar, "W" tipi ya da "G" tipi koloni görünümleri ve mikroskobik özelliklerine göre gruplandırılmıştır. Moleküler karakterizasyonda beta-tubulin, calmodulin ve translation elongation factor (tef1-α) gen bölgeleri PCR ile çoğaltılmıştır. Nükleotid dizileri NCBI-BLAST yazılımı kullanarak analiz edilmiş, gen bankasına kaydedilmiş ve tür tanısı doğrulanmıştır. Miseliyal canlılık sıcak su uygulamalarına karşı

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(46–50°C'de 30-45 dk.) santrifüj tüplerinde test edilmiş ve termal inaktivasyon noktası saptanmıştır. Bu aynı zamanda *in vitro*'da bazı fungisitlere karşı (azoxystrobin, boscalid, cyprodinil, tebuconazole, azoxystrobin + cyproconazole + tebuconazole, cyprodinil + fludioxonil, azoxystrobin + tebuconazole and fludioxonil) test edilmiş ve $EC_{50}$ değerleri hesaplanmıştır. Morfolojik ve moleküler çalışma sonuçları tüm izolatların *D. ampelina* olduğunu ve bunların asma odunsu dokularında patojen olduklarını göstermiştir. "W" tipi izolatların termal inaktivasyonu 48°C-30 dakikalık sıcak su uygulamasıyla sağlanmıştır. Bu uygulama "G" tipi izolatların koloni büyümesini azaltmasına rağmen tamamen inhibe etmemiş, tam eradikasyona ulaşmak için 48°C-45 dakikalık uygulamaya gerek duyulmuştur. Fungisit duyarlılığı ele alındığında, fludioxonil ve tebuconazole içeren fungisitler fungusun miseliyal büyümesini baskılamada en etkili olmuşlardır. Ancak azoxystrobin, boscalid ve cyprodinil, fludioxonil ve tebuconazole ile kıyaslandığında güçlü bir inhibisyon sergileyememiştir.

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## INTRODUCTION

*Diaporthe ampelina* (Berk. & M.A. Curtis) R.R. Gomes, C. Glienke & Crous, *comb. nov.* is an important fungal pathogen causing cane and leaf spot of *Vitis* species (*Vitaceae*) in the world (Mostert et al. 2001). It can infect all green parts, canes and pruning wounds of vines and causes yield losses up to-30%, when favorable conditions are available for pathogen progress (Erincik et al. 2001). The pathogen also causes wedge-shaped cankers, discolorations and sectoral necrosis in perennial wood resembling those caused by Eutypa and Botryosphaeria Dieback Disease fungi. Until the 2000s, it was a controversial issue that *D. ampelina* was involved in these perennial wood symptoms in vines and most of the researchers were estimating they were associated with diseases caused by *Eutypa lata* and Botryosphaeriaceae species. But Urbez-Torres et al. (2013) proved the pathogenicity of *Diaporthe* (=*Phomopsis*) species on wood tissues of grapevines in field conditions and stated this species was overlooked for many years. Baumgartner et al. (2013) also demonstrated that *Phomopsis viticola*, *P. fukushii* and *Diaporthe eres* (isolated from wood cankers) were pathogenic on pruning wound tissues of *Vitis labruscana* (cv. Concord) and *V. vinifera* (cv. Chardonnay) grapes. Some of the studies performed with *D. ampelina* have revealed that there may be significant differences in virulence between the pathogen's isolates. Schilder et al. (2005) characterized seventy-five *Phomopsis* isolates obtained from typical *Phomopsis* cane and leaf spot symptoms in vineyards of the Great Lakes Region in the US. They tested 13 representative isolates for pathogenicity on leaves, internodes and fruit clusters of a *Vitis* interspecific hybrid 'Seyval and observed a significant difference in terms of virulence between isolates. Kanematsu et al. (1999) discriminated *Phomopsis* isolates according to colony color and sporulation types as white (W) or gray (G) and they suggested that "G" type isolates were more virulent than "W" type ones. They emphasized that pathogenicity tests and biological characterization were fundamental and important stages in a study focusing on the management of any plant pathogen.

Although *Diaporthe ampelina* mainly spreads with airborne spores, it is also able to spread within grapevine propagation materials latently. This case may lead to the production of unhealthy vine plants in nurseries. Rego et al. (2009) detected mother-plants of rootstocks and scions to be infected with *Diaporthe ampelina* and Botryosphaeriaceous fungi before grafting in some nurseries of Portugal. We detected *D. ampelina* from the necrotic rootstock tissues of the young vines showing decline symptoms in the Mediterranean Region of Turkey (Akgül and Ahioğlu 2019). In another study, we found that *D. ampelina* was the most encountered species (with high isolation frequency ranging 3.8 - 22.6%) from the wood cankers of Sultana Seedless vines in the Aegean Region. The isolates showed considerable virulence on wood tissues of vines in pathogenicity tests performed at greenhouse conditions (Akgül et al. 2015).

Hot-water treatment (HWT) is a reasonable method for eradication of latent pathogens in vine propagation materials. Pathogen eradication or inoculum reduction could be achieved if this treatment is properly applied. Commonly followed

hot-water treatment (at 50ºC for 30 min) is stated to reduce or eradicate some pests and pathogens in some studies (Caudwell et al. 1997; Crous et al. 2001) but there are also opposite results indicating it is not sufficient for most of the pathogens associated with grapevine trunk diseases in the other studies (Rooney and Gubler 2001; Waite et al. 2018). The growth responses and thermal inactivation points of fungal pathogens exposed to hot-water treatments are different from each other. While some of the pathogens could be eliminated with a certain regime of hot-water treatment, the others may not be affected by this treatment. Gramaje et al. (2008) determined that *Phaeomoniella chlamydospora* had tolerated 53°C temperature but *Phaeoacremonium aleophilum* and *Pm. parasiticum* had tolerated 54°C even in *in vitro* conditions. They suggested that further studies should focus on above 51°C to reduce fungal infections in vegetative propagation materials in grapevine nurseries. Elena et al. (2015) indicated that the mycelial viability of *Neofusicoccum parvum* was about 15% from HWT at 50°C for 30 min in test vials and higher temperatures were needed to reduce viability in *in vitro*. Combining of HWTs with fungicides may increase the chance of fungal eradication in vine propagation materials during the soaking stage. Görür and Akgül (2019) found that HWTs (at 40°C for 2h and 50°C for 30 min) combined with tebuconazole were more effective (34% reduction) than standard HWT alone (50°C for 30 min) in dormant grapevine canes infected with *Neofusicoccum parvum*. The eradicative effects of the other fungicides (thiophanate-methyl and cyprodinil + fludioxonil) at the same HWT combinations were inferior (11.4% and 2.9%) when compared to tebuconazole. Their study revealed the importance of fungicide selection and HWT regime for curing endogenous fungal infections in dormant propagation materials.

So far, there is little information about the effects of fungicides and HWTs (different temperatures and durations) on the mycelial viability of *Diaporthe ampelina in vitro*. Therefore, it is necessary to determine which treatment and fungicide could eliminate *D. ampelina* firstly in *in vitro* conditions. The objectives of the study were to evaluate the sensitivity of *D. ampelina* isolates to HWTs and different fungicides in *in vitro* and to test their pathogenicity in field conditions. This study may be useful to develop an effective HWT strategy to reduce *Diaporthe* infections in grapevine propagation materials.

## MATERIAL AND METHODS

### Materials

In the present study, 23 *Diaporthe ampelina*-like isolates were obtained (in 2014) from the symptomatic wood cankers of grapevines (*Vitis vinifera* cv. Sultana Seedless) located in different districts of the Aegean Region, Turkey and they were included in identification, pathogenicity, fungicide and hot-water sensitivity tests. Eight fungicides (Table 1) with different modes of action were selected for *in vitro* studies and all of them are commercially registered for grape and other fruit crops in Turkey.

Table 1. Fungicides used in this study
*Çizelge 1. Çalışmada kullanılan fungisitler*

| Active Ingredients | Fungicide Group | Trade Name and Formulation | Manufacturer |
|---|---|---|---|
| Azoxystrobin (250 g $\cdot L^{-1}$) | Strobilurin | Nazatem® 250 SC | Koruma |
| Azoxystrobin (100 g $\cdot L^{-1}$) + Cyproconazole (30 g $\cdot L^{-1}$) + Tebuconazole (125 g $\cdot L^{-1}$) | Strobilurin + Triazole | Amistar Trio® 255 EC | Syngenta |
| Azoxystrobin (120 g $\cdot L^{-1}$)+ Tebuconazole (200 g $\cdot L^{-1}$) | Strobilurin + Triazole | Azimut® 320 SC | Adama |
| Boscalid (50 %) | Succinate dehydrogenase inhibitor | Cantus® WG | BASF |
| Cyprodinil (300 g $\cdot L^{-1}$) | Anilinopyrimidine | Qualy® 300 EC | Adama |
| Cyprodinil (37.5 %) + Fludioxonil (25 %) | Anilinopyrimidine + Phenylpyrrole | Switch® 62.5 WG | Syngenta |
| Fludioxonil (50 %) | Phenylpyrrole | Scholar® 50 SC | Syngenta |
| Tebuconazole (250 g $\cdot L^{-1}$) | Triazole | Orius® 25 EW | Adama |

### Methods

#### Morphological and Molecular Characterization

For morphological characterization, colony growth pattern, colony color and conidia shapes (consisting of just alpha conidia or both alpha and beta conidia, n=30) were examined on PDA (Potato Dextrose Agar, Difco®) at 25°C in dark for 25-28 days of incubation (Kanematsu et al. 1999; Mostert et al. 2001). Thirty conidia were measured, minimum and maximum spore dimensions were recorded using a light microscope (Olympus® BX51 attached with ToupTek

Xcam1080 Camera®) and the average dimensions were calculated. After that, fungal DNA was extracted using CTAB protocol suggested by O'Donnell et al. (1998) and stored at -20°C until use. PCR amplification of the TEF1-α (translation elongation factor), calmodulin and β-tubulin genes were performed using EF728F/EF986R, CAL228F/CAL737R (Carbone and Kohn 1995) and Bt2a/Bt2b (Glass and Donaldson 1995) primer pairs. The PCR mixture of each sample (totally 50 µl) contained 5 µl of 10X Green Buffer, 2 µl of dNTP mix., 0.25 µl of Taq polymerase (Thermo Scientific® EP0702), 1 µl of primers (10 pmol) and 38.75 µl of PCR grade water. The thermocycler (Applied Biosystems; Simpliamp A24811®) conditions were adjusted as follows; 95°C for 3 minutes (initial denaturation), followed by 35 cycles each of denaturation at 95°C for 1 min, annealing at 52°C for 1 min TEF1-α, 55°C for 1 min (calmodulin) and 62°C for 1 min (β-tubulin) and extension at 72°C for 1 min, and a final extension at 72°C for 10 minutes. After amplification, PCR products (stained with SYBR Green I® dye, Lonza) were visualized by agarose gel electrophoresis (1.5%), purified and sequenced by MedSanTek Co. (Istanbul, Turkey). Nucleotide sequences of the isolates were contiged using Chromas Lite free software (Technelysium®) and the consensus sequences were compared with the other ones in the NCBI Gen Bank database using the BLASTn program. Molecular identification of the isolates was decided according to maximum score results (with 99% and 100% similarity), the sequences were submitted to NCBI GenBank and accession numbers were obtained.

### Pathogenicity Test

The ability of *Diaporthe ampelina* isolates to cause disease and to which extent these isolates can cause necrosis (virulence) were evaluated in this test. The pathogenicity of 23 isolates was evaluated on green shoots of ten-years-old vines (*V. vinifera* cv. Tarsus Beyazı, located in the implementation area of Cukurova University, Agriculture Faculty) in field conditions. The isolates were grown on PDA at 25°C in dark for 20 days, after conidiomata formation, 4-mm mycelial agar plugs were used as inoculum. The green shoots (not so young, approximately 8 to 10 mm in diameter) were cleaned with 70% ethanol saturated cotton and slightly incised with a sterile scalpel. After that, conidial agar plugs were inoculated into the wounds and these points (between two nodes) were sealed with parafilm to induce fungal penetration (Rawnsley et al. 2004). Sterile agar plugs were inoculated into the wounds as control, five shoots were used for an isolate and each inoculation point was considered as a replication (arranged in a completely randomized design). The vines used in these tests were not sprayed with any pesticide. 35-40 days after inoculation, green shoot lesion lengths were recorded and variance analysis was performed on data using one-way ANOVA test to reveal the statistical differences between means. The statistically similar groups were determined by Fischer's LSD test ($P \leq 0.05$) (Gomez and Gomez 1984). Pathogenicity of the isolates was confirmed by re-isolating of fungi from the inoculation points and emerging colonies were morphologically compared with previously inoculated ones.

### Effect of Hot-Water Treatments on Mycelium Viability of *D. ampelina*

Six isolates of *D. ampelina* were selected for hot-water treatment and fungicide sensitivity tests. Half of them were "G" type (CUZF70, CUZF78, CUZF97) the others were "W" type (CUZF62, CUZF101, CUZF144) colonies. Hot-water treatments at 46, 47 and 48°C for 30 and 45 min were applied to 25-days old mycelia using a dry block thermostat (Bio TDB-100, Biosan®, Riga, Latvia). One milliliter of sterile distilled water (SDW) was added to sterile plastic centrifuge tubes (1.5 ml) and 5-mm mycelial agar plugs were plunged into the water. After that, the tubes were hold in a hot-block thermostat according to specified temperature and time combinations and then they were floated to cool on tap water at ambient temperature (19°C) for 30 min. The mycelial agar discs were briefly dried on sterile filter papers, then transferred onto PDA and Petri plates were incubated at 25°C for 10 days. Control agar discs were not treated in a dry block thermostat but placed in tubes containing SDW (19°C). The experiment used a completely randomized design, each of the plates was considered as a replicate and four plates were used for each isolate and untreated control. Colony growth was assessed on each Petri plate after 10 days by measuring the perpendicular diameters with a caliper. Percent inhibition was calculated using the Abbott formula, which is ((mean diameter of control – mean diameter of treatment)/control) (Gomez and Gomez 1984). After incubation, the temperature and time combination at which no fungal growth was determined as the thermal death point.

### *In vitro* Fungicide Screening on Mycelial Growth of *Diaporthe ampelina* Isolates

In this part of the study, the mycelial growth of *Diaporthe ampelina* isolates (formerly used in *in vitro* hot water treatment tests) was tested on PDA against eight different fungicides. The glass tubes containing 15 ml of PDA were autoclaved and then cooled to 50°C in a water-bath (Memmert WB 10, Germany). The stock solutions/suspensions of fungicides were prepared in sterile distilled water, they were added into tubes with micropipettes to give desired the final

concentrations (1, 5, 10, 25 and 50 µg·ml$^{-1}$). After pouring fungicide-PDA mixtures into plates, fresh mycelial agar plugs were placed onto the media and plates were incubated at 25°C for 11 days in the dark. Control PDA plates had just sterile distilled water instead of fungicide. The experiment was arranged in a completely randomized design, there were four replicates of each fungicide concentration. Colony growth of each isolate was measured after 11 days of inoculation, mean colony diameters were recorded and $EC_{50}$ values were calculated using LeOra POLO Plus® software. Variance analysis was performed on data using one-way ANOVA test to reveal statistical differences between means. The statistically similar groups were determined by Fischer's LSD test ($P \leq 0.05$) (Gomez and Gomez 1984).

## RESULTS and DISCUSSION

### Morphological and Molecular Identification

According to colony growing type on PDA, all the isolates (23) used in this study exhibited a typical *Diaporthe* colony morphology at 25°C for 28-days incubation in dark. Average colony diameters reached 76 mm at that time, aerial hyphal rings, white and gray pigmentation was observed. Of the 23 isolates, while CUZF70, CUZF78, CUZF97 and CUZF140 showed gray and the others showed white pigmentation on PDA, so they were characterized as "G" type and "W" type isolates respectively. All the isolates produced creamy conidiomata on scattered black pycnidia. The alpha conidia were hyaline, biguttulate, fusoid to ellipsoid and aseptate, their dimensions were 9.4 - 10.1 x 2.3 – 2.5 µm (av.= 9.9 x 2.4 µm, n=30). The beta conidia were straight, curved, aseptate and hyaline, their dimensions were 21 – 25 x 0.5 – 1.0 µm (av.= 22 x 1 µm, n=30). While "G" type isolates were observed to produce only alpha conidia, the other ones to produce both alpha and beta conidia. After these observations and examining descriptive publications (Gomes et al. 2013; Baumgartner et al 2013) the isolates were identified as *Diaporthe ampelina*.

Using triple locus gene sequencing and the nucleotide BLAST search, molecular identification was completed. The BLAST search results also confirmed morphological identification results, so that their nucleotide sequences matched with the sequences of *Diaporthe ampelina* isolates previously deposited in GenBank at least 99.0%. The accession numbers provided by NCBI GenBank and isolate location info were shown in Table 2.

Table 2. *Diaporthe ampelina* isolates, used in this study, recovered from grapevine (cv. Sultana Seedless) wood cankers and their accession numbers provided by NCBI GenBank

*Çizelge 2. Çalışmada kullanılan asma (çeş. Tarsus Beyazı) odun kanserlerinden izole edilmiş Diaporthe ampelina izolatları ve NCBI GenBank tarafından verilen kayıt numaraları*

| *Diaporthe ampelina* isolates | Location | GenBank accession numbers | | |
|---|---|---|---|---|
| | | BT | CAL | EF-1α |
| CUZF01 | İzmir, TR | KY887663 | MZ634304 | KY950392 |
| CUZF16 | İzmir, TR | MZ634295 | KY887662 | KY950394 |
| CUZF61 | Turgutlu, TR | KY923779 | MZ634305 | KY950393 |
| CUZF63 | Menemen, TR | KY923780 | MZ634306 | KY950395 |
| CUZF68 | Salihli, TR | MZ634296 | KY930662 | KY950396 |
| CUZF70 | Manisa, TR | KY923781 | MZ634307 | KY950397 |
| CUZF78 | Menemen, TR | MZ634297 | KY930663 | KY950398 |
| CUZF81 | Manisa, TR | KY923782 | KY930664 | KY950399 |
| CUZF86 | Alaşehir, TR | KY923783 | KY930665 | KY950400 |
| CUZF92 | Sarıgöl, TR | KY923784 | KY930666 | KY950401 |
| CUZF97 | Alaşehir, TR | KY923785 | KY930667 | KY950402 |
| CUZF101 | Alaşehir, TR | KY923786 | KY930668 | KY950403 |
| CUZF109 | Menemen, TR | MZ634298 | MZ634308 | KY950404 |
| CUZF111 | Sarıgöl, TR | KY923787 | KY930669 | KY950405 |
| CUZF114 | Ahmetli, TR | MZ634299 | KY930670 | KY950406 |
| CUZF119 | Turgutlu, TR | KY923788 | MZ634309 | KY950407 |
| CUZF120 | Turgutlu, TR | MZ634300 | MZ634310 | KY950408 |
| CUZF123 | Salihli, TR | MZ634301 | KY930671 | KY950409 |
| CUZF136 | Alaşehir, TR | KY923789 | KY930672 | KY950410 |
| CUZF140 | Salihli, TR | MZ634302 | MZ634311 | KY950411 |
| CUZF142 | Ahmetli, TR | MZ634303 | MZ634312 | KY950412 |
| CUZF144 | Manisa, TR | KY923790 | KY930673 | KY950413 |
| CUZF146 | Manisa, TR | KY923791 | KY930674 | KY950414 |

## Pathogenicity Tests

Pathogenicity of the *D. ampelina* isolates was tested on slightly woody shoots in vineyard conditions. All the isolates were found to be pathogenic on these shoots so that the lesion lengths were longer than that of control (sterile agar inoculated ones). While restricted discoloration (1.5 mm) occurred around the inoculation point of the control, obviously larger and blackish-brown discoloration was observed on *D. ampelina* inoculated shoots. The average lesion lengths produced by the isolates ranged from 6.5±0.9 to 20.3±1.4 for 35-40 days of incubation in vineyard conditions. While the largest lesion lengths were obtained from the isolates; CUZF97 (20.3 mm), CUZF70 (16.0 mm) and CUZF63 (14.8 mm) and smallest ones obtained from the isolates; CUZF136 (6.5 mm), CUZF140 (7.3 mm) and CUZF101 (8.0 mm) respectively (Figure 1). When the lesion lengths produced by “W” and “G” type isolates were compared with each other, no significant correlation was observed between the type and virulence of the isolate. After measurement, the isolates were successfully recovered from the inoculation points of shoots except for control.

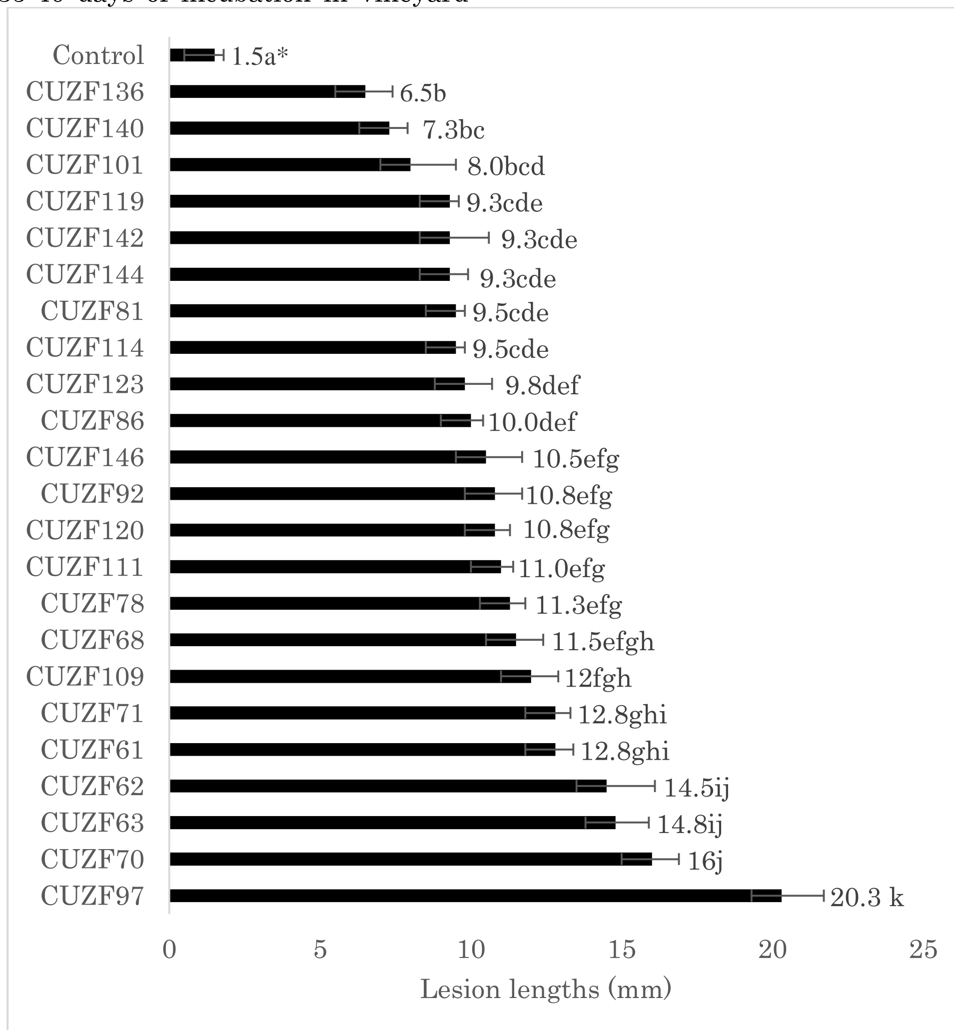


Figure 1. Mean lesion lengths (mm) caused by *D. ampelina* isolates after 35-40 days of inoculation on *Vitis vinifera* (cv. Tarsus Beyazı)

*Mean values within a bar are significantly different at the 0.05 level based on LSD test. LSD value:2.44

*Şekil 1. D. ampelina izolatlarının inokulasyondan 35-40 gün sonra Vitis vinifera (Tarsus Beyazı)'da oluşan ortalama lezyon uzunlukları (mm)*

**Bir bardaki ortalama değerler LSD testine göre (0.05 seviyesinde) önemli derecede farklıdırlar. LSD değeri:2.44*

## *In vitro* Hot-Water Treatments

Mycelial growth of *D. ampelina* isolates was affected with hot-water treatments in *in vitro* tests and was inhibited as the water temperature and duration increased. Depending on the isolates, mycelial growth was reduced (at 46°C – 30 min) ranging from 11.3% to 27.1%. When the temperature was increased by 47°C, mycelial growth was inhibited by 23.6 – 47.1% rates in all isolates at 30-min duration. From all HWT combinations, the thermal death point was found to

be 48°C-30 min for "W" type isolates (CUZF62, CUZF101, CUZF144), at which these isolates were completely inhibited (Figure 2). However, "G" type isolates (CUZF70, CUZF78, CUZF97) were significantly inhibited (max. 87%) at 48°C – 30 min but their thermal death point was 48°C – 45 min. So "G" type isolates were found to be more resistant to HWTs than "W" types.

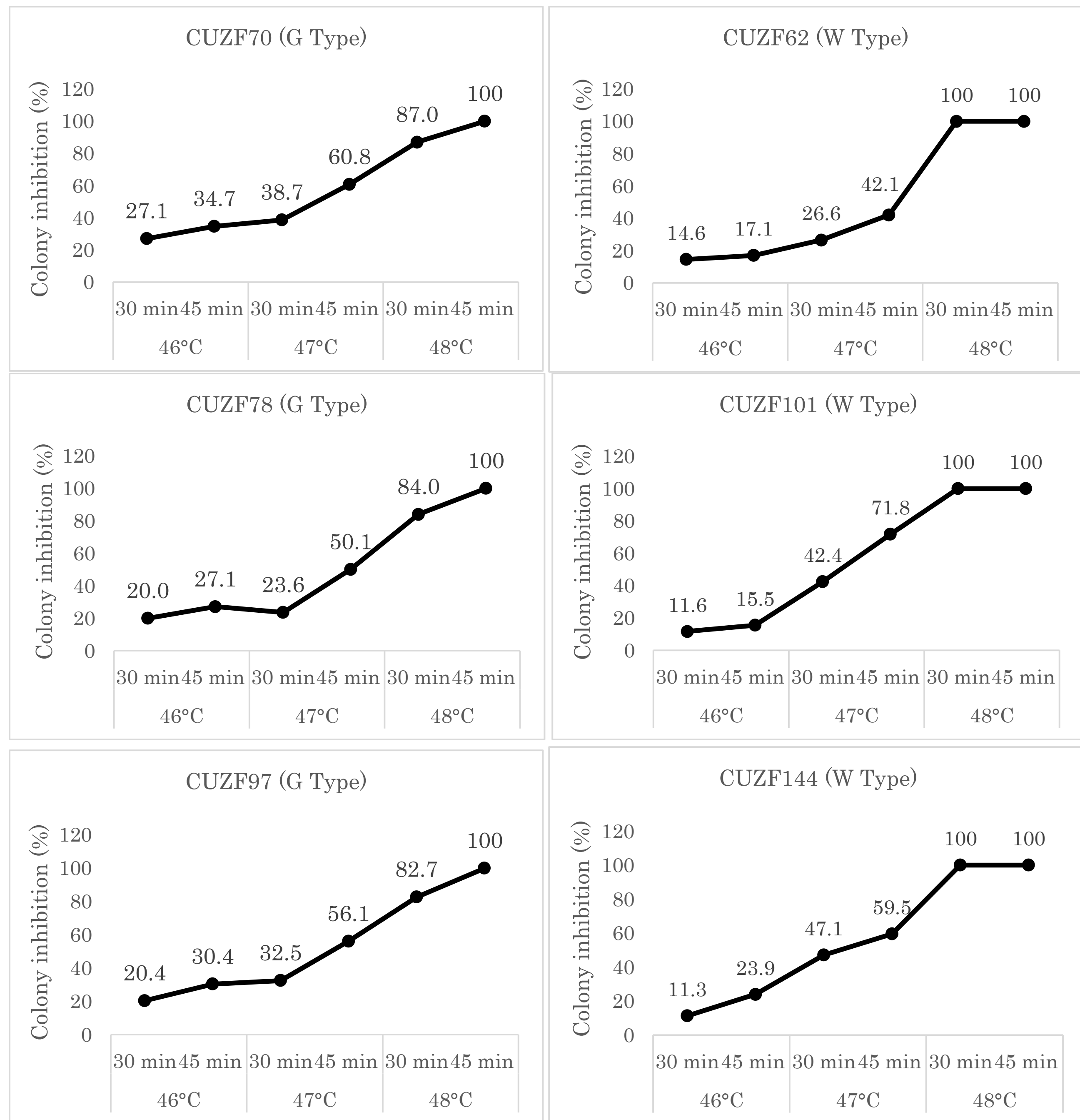


Figure 2. Percentage colony growth inhibition of "G" and "W" types of *D. ampelina* isolates treated with HWTs in *in vitro* at different temperatures and durations.

*Şekil 2. In vitro'da farklı sıcaklık ve uygulama sürelerinde "G" ve "W" tipi D. ampelina izolatlarının koloni büyüme inhibisyon yüzdesi*

### *In vitro Fungicide Screening*

Effect of different fungicides on mycelial the growth of *D. ampelina* isolates was investigated in *in vitro* conditions. Among the fungicides, fludioxonil and cyprodinil + fludioxonil inhibited colony growth at 1.0 µg·ml$^{-1}$ concentration with 76.3 and 89.5% rates, so they were found to be the most effective fungicides. While the average colony diameters of the six isolates were recorded as 70.5 – 72.8 mm in control (no fungicide), they were 10.1 – 17.0 mm on the colonies subjected to fludioxonil alone and 7.5 – 15.9 mm in

cyprodinil + fludioxonil at 1.0 µg·ml$^{-1}$. On the other hand, tebuconazole and azoxystrobin + tebuconazole caused significant inhibition at lower concentration when compared to azoxystrobin, boscalid and cyprodinil alone (Table 3). Propiconazole + azoxystrobin + cyproconazole mixture caused more than 50% inhibition at 5 µg·ml$^{-1}$ concentration and colony growth was completely inhibited at 25 µg·ml$^{-1}$ and 50 µg·ml$^{-1}$ concentrations. When effective concentration ($EC_{50}$) values were examined, the inhibitory effect of tested fungicides on mycelial growth was clearly demonstrated. $EC_{50}$ values ranged from <0.001 to >100. Cyprodinil + fludioxonil, tebuconazole, fludioxonil had consistently lower $EC_{50}$ when tested with the other fungal isolates. However, azoxystrobin, boscalid and cyprodinil had consistently higher $EC_{50}$ values. Depending on isolates, the highly effective fungicides were fludioxonil and tebuconazole containing ones (Table 4).

In this study, 23 *Diaporthe* isolates were identified and screened for pathogenicity, susceptibility against some fungicides and hot-water treatments. Morphological and molecular studies showed all isolates were *Diaporthe ampelina*. The isolates were obtained from wood cankers of vines along with vines exhibiting cane and leaf spot symptoms. *Diaporthe ampelina* is a predominant pathogen in the Aegean Region (climatically different form the Mediterranean Region) of Turkey. We found this species the most frequently isolated fungus from wood cankers (just five isolates were morphologically and molecularly identified) when compared to the other fungal trunk pathogens in this region, so that its isolation frequency was ranged between 3.8% and 22.6% (Akgül et al. 2015). Mostert et al. (2001) discriminated 61 *Diaporthe* isolates from 58 different vineyards in South Africa by morphological, molecular and pathogenic features. *Diaporthe amygdali, D. perjuncta* and some *Phomopsis* isolates are not virulent on shoots of vines and they were described as lesser pathogenic or endophytic species. However, *Diaporthe ampelina* was found to be a highly virulent, most common and widely distributed species in vineyards of Western Cape province. Guarnaccia et al. (2018) conducted a broad survey in Croatia, Czech-Republic, France, Hungary, Israel, Italy, Spain and England to determine species diversity of *Diaporthe* species and their pathogenicity. *Diaporthe eres* and *D. ampelina* were the most commonly isolated ones and further *D. bohemiae, D. celeris, D. hispaniae* and *D. hungariae* were described for the first time in Europe. Our identification results corroborate these studies, because of *D. ampelina* was a predominant pathogen in the Aegean Region of Turkey, up to now, no other species was found on grapevines. Morphologically, the isolates that we used in pathogenicity tests were included in two groups according to description of Kanematsu et al. (1999).

Most of the isolates had “W type” colonies producing both alpha and beta conidia and four isolates had “G type” colonies producing just alpha conidia on PDA. All “G type” colonies did not produce severe and longest lesions on green shoots as expected, the lesion lengths of some isolates were moderate or shorter for 35-40 days of incubation in field conditions. These findings were not entirely parallel with the suggestions of Kanematsu et al (1999) who described “W type” colonies were less virulent on hosts. Virulence of a pathogen may vary depending on the genetic characteristics of the isolate, environmental conditions and host susceptibility and significant differences may occur between the isolates used in a study. Urbez-Torres et al. (2013) tested virulence of *D. ampelina, D. ambigua* and *D. neotheicola* species on six different *V. vinifera* cultivars. Among the inoculated fungi, *D. ampelina* always produced the longer vascular discolorations on vine cordons and its re-isolation rates were higher than 60%. The lesion lengths varied according to isolate and vine cultivars used in the study. Our pathogenicity results confirm this virulence measured in this study. In a vineyard survey conducted in China, *Diaporthe eres, D. hongkongensis, D. phaseolorum* and *D. sojae* were isolated from symptomatic wood tissues of vines but *D. ampelina* not. The pathogenicity tests revealed *D. eres* and *D. hongkongensis* were the most virulent species as compared to *D. phaseolorum* and *D. sojae* (Dissanayake et al. 2015). In a similar study, Baumgartner et al. (2013) compared the virulence of *P. viticola* isolates (isolated from wood cankers and leaf spots) with those of virulence of *P. fukushii* and *D. eres* on the stems of Concord and Chardonnay grapes. At the end of one-year incubation, longer lesions were observed with inoculation of *P. viticola* when compared to *P. fukushii*, *D. eres* and non-inoculated control. Thermal death point of pathogens to hot-water treatment may differ to species and their isolates. Gramaje et al. (2008) screened sensitivity some of the Petri disease pathogens to hot-water treatments in *in vitro* and found that *Phaeomoniella chlamydospora* were more sensitive than *Phaeoacremonium* species. While *Pa. chlamydospora* tolerated 53°C-HWT, *Pm. parasiticum* and *Pm. aleophilum* could tolerate even 54°C-HWT. Elena et al. (2015) tested mycelial viability of *Diplodia seriata, Neofusicoccum luteum, N. parvum, N. vitifusiforme, Lasiodiplodia theobromae* and *Spencermartinsia viticola* in tubes subjected to 50-54°C HWT regime. They found that *L. theobromae* and *N. vitifusiforme* were very tolerant but the others were susceptible to HWTs. In our study, 48°C-30 min HWT completely inhibited mycelial vitality of “W type” isolates of *D. ampelina* but not the others, so 48°C-45 min-treatment was needed to reach complete eradication

Table 3. Mean colony diameters of six *D. ampelina* isolates at different concentrations of fungicides on PDA
*Çizelge 3. PDA'daki farklı fungisit konsantrasyonlarında altı D. ampelina izolatının ortalama koloni çapları*

| Fungicide Doses (µg·ml-1) | *Diaporthe ampelina* isolates | | | | | |
|---|---|---|---|---|---|---|
| | CUZF62 | CUZF70 | CUZF78 | CUZF97 | CUZF101 | CUZF144 |
| | Colony diameter (mm) | | | | | |
| | **Azoxystrobin** | | | | | |
| 0 | 71.1±0.5 f* | 71.4±0.5 f | 70.5±0.5 f | 72.8±0.6 e | 72.4±0.3 e | 71.0±0.4 d |
| 1 | 48.9±0.4 e | 52.1±0.8 e | 59.9±0.6 e | 58.9±0.6 d | 59.4±0.5 d | 57.5±0.5 c |
| 5 | 38.5±0.5 d | 48.9±0.6 d | 55.3±0.5 d | 51.1±0.7 c | 59.3±0.5 d | 56.4±0.4 c |
| 10 | 29.5±0.5 c | 42.5±0.7 c | 50.6±0.4 c | 50.1±0.4 c | 55.8±0.5 c | 54.0±0.4 b |
| 25 | 25.8±0.5 b | 35.9±0.6 b | 45.9±0.7 b | 47.9±0.6 b | 50.3±0.3 b | 50.0±0.6 a |
| 50 | 23.5±0.7 a | 30.8±0.4 a | 38.6±0.3 a | 34.0±0.5 a | 42.3±0.4 a | 38.8±0.8 a |
| | **Boscalid** | | | | | |
| 0 | 71.3±0.3 f | 71.9±0.3 f | 71.0±0.5 f | 71.4±0.4 f | 71.1±0.6 d | 72.1±0.2 f |
| 1 | 62.9±0.6 e | 63.6±0.5 e | 58.4±0.4 e | 64.5±0.5 e | 58.4±0.6 c | 54.6±0.3 e |
| 5 | 55.3±0.3 d | 55.8±0.3 d | 52.8±0.3 d | 55.5±0.4 d | 55.4±0.2 b | 51.1±0.5 d |
| 10 | 52.5±0.5 c | 47.4±0.7 c | 42.1±0.5 c | 52.0±0.5 c | 54.6±0.4 b | 47.3±0.5 c |
| 25 | 45.4±0.7 b | 43.1±0.4 b | 36.0±0.5 b | 46.3±0.4 b | 54.3±0.6 ab | 45.6±0.4 b |
| 50 | 40.8±0.6 a | 35.4±0.5 a | 27.0±0.2 a | 41.6±0.4 a | 49.3±0.5 a | 42.5±0.2 a |
| | **Cyprodinil** | | | | | |
| 0 | 71.4±0.2 f | 70.4±0.2 f | 70.9±0.2 f | 71.1±0.2 f | 71.8±0.3 f | 71.6±0.2 f |
| 1 | 59.9±0.4 e | 59.8±0.7 e | 55.8±0.6 e | 59.3±0.8 e | 61.9±0.2 e | 61.9±0.2 e |
| 5 | 46.0±0.5 d | 49.1±0.8 d | 52.8±0.3 d | 54.4±0.4 d | 56.4±0.5 d | 50.1±0.6 d |
| 10 | 37.8±0.5 c | 45.3±0.4 c | 51.3±0.8 c | 50.9±0.4 c | 51.3±0.9 c | 30.0±0.8 c |
| 25 | 34.3±0.6 b | 21.3±0.4 b | 41.5±0.5 b | 39.9±0.5 b | 30.4±0.2 b | 19.6±0.4 b |
| 50 | 23.5±0.5 a | 10.3±0.3 a | 12.9±0.1 a | 12.3±0.5 a | 9.0±0.5 a | 7.9±0.2 a |
| | **Fludioxonil** | | | | | |
| 0 | 72.4±0.1 e | 70.5±0.4 a | 72.6±0.3 f | 72.0±0.4 e | 71.6±0.1 e | 72.1±0.2 e |
| 1 | 10.1±0.6 d | 12.3±0.5 d | 11.1±0.5 e | 15.8±0.5 d | 17.0±0.5 d | 11.3±0.6 d |
| 5 | 7.0±0.2 c | 4.8±0.1 c | 6.5±0.2 d | 8.0±0.2 c | 9.8±0.3 c | 6.0±0.4 c |
| 10 | 5.0±0.2 b | 4.3±0.1 c | 3.5±0.3 c | 6.1±0.1 b | 9.3±0.3 bc | 6.4±0.1 b |
| 25 | 0.0±0.0 a | 3.0±0.0 b | 2.3±0.3 b | 5.3±0.3 b | 8.4±0.4 b | 4.8±0.1 b |
| 50 | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 1.6±0.4 a | 4.8±0.5 a | 3.2±0.1 a |
| | **Cyprodinil + Fludioxonil** | | | | | |
| 0 | 71.6±0.4 e | 71.5±0.5 c | 71.5±0.2 c | 72.8±0.3 f | 71.1±0.4 d | 71.9±0.4 d |
| 1 | 7.5±0.2 d | 8.6±0.2 b | 15.9±0.4 b | 9.1±0.2 e | 14.1±0.6 c | 9.3±0.3 c |
| 5 | 3.4±0.4 c | 0.0±0.0 a | 0.0±0.0 a | 5.5±0.2 d | 3.4±0.4 b | 1.8±0.3 b |
| 10 | 2.0±0.2 b | 0.0±0.0 a | 0.0±0.0 a | 3.8±0.1 c | 0.0±0.0 a | 0.0±0.0 a |
| 25 | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.6±0.1 b | 0.0±0.0 a | 0.0±0.0 a |
| 50 | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a |
| | **Tebuconazole** | | | | | |
| 0 | 71.4±0.4 d | 72.8±0. d | 73.3±0.1 c | 71.3±0.3 d | 72.4±0.3 c | 73.3±0.3 c |
| 1 | 33.8±0.6 c | 55.3±0.6 c | 51.6±0.5 bc | 48.0±0.4 c | 26.1±0.3 b | 37.0±0.5 b |
| 5 | 8.4±0.6 b | 32.4±0.6 b | 0.0±0.0 a | 3.0±0.0 b | 0.0±0.0 a | 0.0±0.0 a |
| 10 | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a |
| 25 | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a |
| 50 | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a |
| | **Azoxystrobin + Tebuconazole** | | | | | |
| 0 | 71.8±0.5 e | 71.1±0.2 d | 71.9±0.3 c | 71.6±0.5 d | 72.4±0.6 d | 71.6±0.6 d |
| 1 | 34.3±0.7 d | 46.4±0.4 c | 40.6±0.6 b | 50.5±0.7 c | 57.5±0.6 c | 48.0±0.6 c |
| 5 | 9.6±0.4 c | 32.0±0.5 b | 0.0±0.0 a | 5.8±0.5 b | 9.4±0.2 b | 6.1±0.3 b |
| 10 | 3.4±0.7 b | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a |
| 25 | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a |
| 50 | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a |
| | **azoxystrobin + cyproconazole + propiconazole** | | | | | |
| 0 | 70.5±0.5 e* | 71.5±0.5 e | 71.4±0.6 e | 72.0±0.4 e | 71.4±0.4 e | 71.3±0.5 e |
| 1 | 45.8±2.2 d | 47.5±0.6 d | 57.1±0.7 d | 54.0±0.8 d | 57.3±0.3 d | 47.3±0.4 d |
| 5 | 31.8±0.5 c | 31.3±0.5 c | 37.1±0.4 c | 33.4±0.6 c | 33.9±0.6 c | 31.6±0.7 c |
| 10 | 27.8±0.7 b | 10.8±0.5 b | 12.3±0.4 b | 15.0±0.4 b | 16.3±0.6 b | 18.5±0.5 b |
| 25 | 6.9 ±0.2a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a |
| 50 | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a |

*Means within a column followed by the same letter is not significantly different ($P \leq 0.05$) based on LSD test.
** Sütun içerisinde aynı harfle takip eden ortalamalar LSD ($P \leq 0.05$) testine göre farklı değildir.*

Table 4. Means of effective concentrations ($EC_{50}$) for 50% reduction of mycelial growth of *Diaporthe ampelina* isolates during the *in vitro* fungicide screening experiment

*Çizelge 4. In vitro fungisit tarama denemeleri sırasında Diaporthe ampelina izolatlarının miseliyal gelişimini %50 azaltan etkili konsantrasyon ($EC_{50}$) ortalamaları*

| Fungicides | EC50 values (µg·ml -1) | | | | | |
|---|---|---|---|---|---|---|
| | CUZF62 | CUZF70 | CUZF78 | CUZF97 | CUZF101 | CUZF144 |
| Azoxystrobin | 93.4 d* | 87.7 f | >100 e | >100 e | >100 e | >100 e |
| Azoxystrobin + Cyproconazole + Tebuconazole | 3.69 b | 2.8 c | 3.9 c | 3.6 b | 4.0 b | 2.9 b |
| Azoxystrobin + Tebuconazole | 1.2 a | 2.2 c | <0.001 a | 1.7 b | 2.0 b | 1.7 b |
| Boscalid | >100 e | 66.6 e | 33.9 d | 99.5 d | 86.5 d | 79.7 d |
| Cyprodinil | 21.7 c | 13.9 d | 28.5 d | 24.7 c | 17.5 c | 10.7 c |
| Cyprodinil + Fludioxonil | 0.11 a | <0.001 a | <0.001 a | 0.05 a | <0.001 a | <0.003 a |
| Fludioxonil | 0.17 a | 0.08 b | 0.01 b | 0.01 a | <0.001 a | <0.001 a |
| Tebuconazole | 1.18 a | <0.001 a | <0.001 a | 1.7 b | <0.001 a | <0.001 a |
| LSD (0.05) | 1.15 | 1.06 | 1.92 | 2.01 | 1.48 | 1.08 |

*Means within a column followed by the same letter is not significantly different ($P \leq 0.05$) based on LSD test.

** Sütun içerisinde aynı harfle takip eden ortalamalar LSD ($P \leq 0.05$) testine göre farklı değildir.*

for all types of isolates. It is suggested that the differentiation between two types of isolates may have related to melanization in gray type colonies. Many researchers state that melanin contributes not only to pathogenicity, but also to the adaptation of fungi to environmental factors (Kim et al. 2003; Gessler et al. 2014). Rehnstrom and Free (1996) compared conidial viability in melanized (wild-type) and non-melanized (mutant) *Monilinia fructicola* isolates to hot-water treatment (40°C – 30 min) in *in vitro*. While this treatment killed all conidia of mutant isolate, 50% of conidia have been detected to survive in wild-type isolate. So melanin has been suggested to play an important role in resistance of conidia to a variety of environmental stresses. In another study, virulence of *Magnaporthe grisea* (rice blast disease fungus) was found to be affected by melanin synthesis ability of fungus. While virulent isolate being successful to reveal typical disease symptoms, the other plants inoculated with albino mutant isolate stayed asymptomatic (Howard and Valent 1996). In the current study, the fungicides containing fludioxonil and tebuconazole showed highly inhibitory effect even at 1.0 µg·ml$^{-1}$ concentration. However, azoxystrobin, cyprodinil and boscalid performed inferior effect on mycelial growth of *D. ampelina* isolates, when compared to fludioxonil and tebuconazole. Mostert et al. (2000) determined efficacy of different fungicides (azoxystrobin, flusilazole, folpet, fosetyl-al + mancozeb, kresoxym-methyl, penconazole, propineb, mancozeb, spiroxamine and trifloxystrobin) on mycelial growth and spore germination of *Phomopsis viticola* in *in vitro*. Azoxystrobin, kresoxym-methyl and trifloxystrobin have been found to be the most effective fungicides with 0.35, 1.67 and 0.05 µg·ml$^{-1}$ $EC_{50}$ values in this study. However, azoxystrobin could not perform a strong inhibition in our study (minimum $EC_{50}$ value was 87.7 µg·ml$^{-1}$), so our results were not parallel with the findings of Mostert et al. (2000). This difference may have been due to the ratio of the active ingredients in fungicide (azoxystrobin) used. Gramaje et al. (2009) tested 14 fungicides on mycelial growth and conidial germination of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* in *in vitro*. Azoxystrobin, carbendazim and tebuconazole were found to be the most effective ones against *P. chlamydospora* and *Pa. aleophilum*. Especially carbendazim exhibited a satisfactory performance in hydration tanks by eliminating conidia of these fungi. In a similar study of Rego et al. (2009), cyprodinil + fludioxonil and pyraclostrobin + metiram were detected to decrease the percentage of infected plants in grapevine nurseries when they used in hydration tanks before grafting stage. Sosnowski et al. (2013) examined the efficacy of 24 fungicides and naturally obtained active substances for protection of pruning wounds against ascospore infections of *Eutypa lata* both in laboratory and vineyard conditions. They found tebuconazole and carbendazim to be the most effective fungicides on ascospore germination and mycelial growth of the fungus in both conditions. Pyrimethanil, fluazinam, cyprodinil + fludioxonil were also found to be effective but their performance was inferior when compared to tebuconezole and carbendazim. The results of our fungicide experiment somewhat overlap with the findings of the studies mentioned above. However, there are few studies examining suppressive effects of modern fungicides on growth of *D. ampelina*.

## CONCLUSION

In the current study, *D. ampelina* has been revealed to be an important pathogen associated with grapevine trunk disease in Turkey vineyards. We have also detected that tebuconazole and fludioxonil containing fungicides were very effective and 48°C-45 min. hot-water treatment completely inhibited mycelial growth of this fungus in laboratory conditions. These results would contribute to further

studies aiming at reduction of latent infections of the pathogen during soaking and pre-grafting stages of dormant cuttings in grapevine nurseries.

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### Contribution statement of researchers

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

### Conflict of interest

The authors state that there are no conflict of interests.

## REFERENCES

Akgül DS, Savaş NG, Teker T, Keykubat B, Mayorquin JS, Eskalen A 2015. Fungal Trunk Pathogens of Sultana Seedless Vineyards in Aegean Region of Turkey. Phytopathol Mediterr 54 (2):380-393.

Akgül DS, Ahioğlu M 2019. Fungal pathogens associated with young grapevine decline in the Southern Turkey vineyards. 42. World Congress of Vine & Wine, 15-19 July 2019, Geneva, Switzerland, 15 (01027):1-6.

Baumgartner K, Fujiyoshi PT, Travadon R, Castlebury LA, Wilcox WF, Rolshausen PE 2013. Characterization of Species of *Diaporthe* from Wood Cankers of Grape in Eastern North American vineyards. Plant Dis 97: 912-920.

Carbone L, Kohn LM 1995. A method for Designing Primer Sets for Speciation Studies in Filamentous Ascomycetes. Mycologia 91: 553–556.

Caudwell A, Larrue J, Boudon-Padieu E, McLean GD 1997. Flavescence Dorèe Elimination from Dormant Wood of Grapevines by Hot-Water Treatment. Aust J Grape Wine R 3: 21–25.

Crous PW, Swart L, Coertze S 2001. The Effect of Hot-Water Treatment on Fungi Occurring in Apparently Healthy Grapevine Cuttings. Phytopathol Mediterr 40: 464–466.

Dissanayake AJ, Liu M, Zhang W 2015. Morphological and Molecular Characterization of *Diaporthe* Species Associated with Grapevine Trunk Disease in China. Fungal Biol 119: 283–294.

Elena G, Di Bella V, Armengol J, Luque J 2015. Viability of Botryosphaeriaceae Species Pathogenic to Grapevine After Hot Water Treatment. Phytopathol Mediterr 54: 325–334.

Erincik O, Madden LV, Ferree DC, Ellis MA 2001. Effect of Growth Stage on Susceptibility of Grape Berry and Rachis Tissues to Infection by *Phomopsis viticola*. Plant Dis 85:517-520.

Gessler NN, Egorova AS, Belozerskaia TA 2014. Melanin Pigments of Fungi Under Extreme Environmental Conditions (review). Prikl Biokhim Mikrobiol 50: 125–134.

Glass NL, Donaldson GC 1995. Development of Primer Sets Designed for Use with the PCR to Amplify Conserved Genes from the Filamentous Ascomycetes. Appl Environ Microbiol 61: 1323–1330.

Gomes RR, Glienke C, Videira SIR, Lombard R, Groenewald JZ, Crous PW 2013. *Diaporthe*: A Genus of Endophytic, Saprobic and Plant Pathogenic Fungi. Persoonia 31: 1–41.

Gomez KA, Gomez AA 1984. Statistical Procedures for Agricultural Research. 2nd ed. Wiley, New York, USA.

Görür V, Akgül DS 2019. Fungicide Suspensions Combined with Hot-Water Treatments Affect Endogenous *Neofusicoccum parvum* Infections and Endophytic Fungi in Dormant Grapevine Canes. Phytopathol Mediterr 58(3): 559-571.

Gramaje D, Garcia-Jimenez J, Armengol J 2008. Sensitivity of Petri disease Pathogens to Hot-Water Treatments *in vitro*. Ann Appl Biol 153: 95-103.

Gramaje D, Aroca A, Raposo R, Garcia-Jimenez J, Armengol J 2009. Evaluation of fungicides to control Petri disease pathogens in the grapevine propagation process. Crop Prot 28: 1091-1097.

Guarnaccia V, Groenewald JZ, Woodhall J, Armengol J, Cinelli T 2018. *Diaporthe* Diversity and Pathogenicity Revealed from A Broad Survey of Grapevine Diseases in Europe. Persoonia 40: 135–153.

Howard RJ, Valent B 1996. Breaking and Entering: Host Penetration by the Fungal Rice Blast Pathogen *Magnoporthe Grisea*. Annu Rev. Microbiol 50: 491-512.

Kanematsu S, Kobayashi T, Kudo A, Ohtsu Y 1999. Conidial Morphology, Pathogenicity and Culture Characteristics of *Phomopsis* Isolates from Peach, Japanese Pear and Apple in Japan. Ann Phytopathol Soc Jpn 65: 264–273.

Kim DS, Park SH, Kwon SB, Joo YH, Youn SW, Sohn UD, Park KC 2003. Temperature Regulates Melanin Synthesis in Melanocytes. Arch Pharm Res 26:840–845.

Mostert L., Denman S, Crous PW (2000) *In vitro* Screening of Fungicides Against *Phomopsis viticola* and *Diaporthe perjuncta*. S. Afr. J. Enol Vitic. 21 (2): 62-65.

Mostert L, Crous PW, Kang JC, Phillips AJL 2001. Species of *Phomopsis* and a *Libertella* sp. Occurring on Grapevines with Specific Reference to South Africa: Morphological, Cultural,

Molecular and Pathological Characterization. Mycologia 93: 146-167.

O'Donnell K, Cigelnik E, Nirenberg HI 1998. Molecular Systematics and Phylogeography of the *Gibberella Fujikuroi* Species Complex. Mycologia 90(3): 465-493.

Rawnsley B, Wicks TJ, Scott ES, Stummer BE 2004. *Diaporthe perjuncta* Does Not Cause Phomopsis Cane and Leaf Spot Disease of Grapevine in Australia. Plant Dis 88: 1005–1010.

Rehnstrom AL, Free SJ 1996. The Isolation and Characterization of Melanin Deficient Mutants of *Monilinia fructicola*. Physiol Mol Plant P 49: 321–330.

Rego C, Nascimento T, Cabral A, Silva MJ, Oliviera H 2009. Control of Grapevine Wood Fungi in Commercial Nurseries. Phytopathol. Mediterr 48: 128-135.

Rooney SN, Gubler WD 2001. Effect of Hot Water Treatments on Eradication of *Phaeomoniella chlamydospora* and *Phaeoacremonium inflatipes* from Dormant Grapevine Wood. Phytopathol Mediterr 40: 467–472.

Schilder AMC, Erincik O, Castlebury L, Rossman A, Ellis MA 2005. Characterization of *Phomopsis* spp. Infecting Grapevines in the Great Lakes Region of North America. Plant Dis 89: 755–762.

Sosnowski MR, Loschiavo AP, Wicks TJ, Scott ES 2013. Evaluating Treatments and Spray Application for the Protection of Grapevine Pruning Wounds from Infection by *Eutypa lata*. Plant Dis. 97: 1599-1604.

Úrbez-Torres JR, Peduto F, Smith RJ, Gubler WD 2013. *Phomopsis* dieback: A Grapevine Trunk Disease Caused by *Phomopsis viticola* in California. Plant Dis. 97: 1571-1579.

Waite H, Armengol J, Billones-Baaijens R, Gramaje D, Halleen F 2018. A Protocol for the Management of Grapevine Rootstock Mother Vines to Reduce Latent Infections by Grapevine Trunk Pathogens in Cuttings. Phytopathol Mediterr 57: 384−398.