http://dergipark.org.tr/trkjnat
Trakya University Journal of Natural Sciences, 22(1): 93-100, 2021
ISSN 2147-0294, e-ISSN 2528-9691
DOI: 10.23902/trkjnat.863506

Research Article

MOLECULAR IDENTIFICATION OF *Diplodia seriata* De Not. CAUSING DIEBACK EFFECT ON GRAPEVINES AND EVALUATION OF *in vitro* EFFICACY OF FIVE DIFFERENT SYNTHETIC FUNGICIDES AGAINST THIS DISEASE

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Cite this article as:

Güngör Savaş N. & Yıldız M. 2021. Molecular identification of *Diplodia seriata* De Not. causing dieback effect on grapevines and evaluation of *in vitro* efficacy of five different synthetic fungicides against this disease. *Trakya Univ J Nat Sci*, 22(1): 93-100, DOI: 10.23902/trkjnat.863506

Received: 18 January 2021, Accepted: 13 April 2021, Published: 15 April 2021

Abstract: The aim of this study was to realize the molecular identification of *Diplodia seriata* De Not., a member of the Botryosphaericea family, isolated from 2-10 years old vines in vineyards showing symptoms of dieback disease. The susceptibility of the pathogen against the fungicides with the fosetyl-Al+triadimenol, azoxystorbin+difecanozole, fludioxanil+cyprodinil, metrafenone, fluopyram+tebuconazole combinations were evaluated. The isolates obtained from the root and crown parts of the vine samples were identified as *D. seriata* according to the morphological and molecular methods. In molecular identification, the ITS (Internal Transcribed Spacer) and TUB2 (β-tubulin) gene regions of the isolates were amplified by Real-Time PCR and the nucleotide sequences were obtained in these gene regions. After using the MEGA 7 software, ITS and TUB2 sequences were aligned and a combined phylogenetic tree was made. It has been molecularly confirmed that the *D. seriata* isolate has a 100% similarity index with *Diplodia* species according to the phylogenetic analyses. The mean effective concentration (EC_{50}) values of fungicides used with different concentrations (0, 1, 3, 10, 30, 50, 100 µL mL-1) were determined by Probit analysis. Cyprodinil + fludioxonil showed the highest efficacy (100%) at a concentration of 1 µL mL^{-1}. According to EC_{50} values, cyprodinil + fludioxonil (0.001 µL mL^{-1}) was recorded as the most effective fungicide followed by fluopyram + tebuconazole (0.520 µL mL^{-1}) and, azoxystrobin + difenoconazole (2.958 µL mL^{-1}), respectively.

Özet: Bu çalışmanın amacı, geriye doğru ölüm hastalığı belirtileri gösteren bağlardaki 2-10 yaşındaki asmalardan izole edilen Botryosphaericea ailesinin bir üyesi olan *Diplodia seriata* De Not.'nın, moleküler tanılamasını gerçekleştirmektir. Patojenin duyarlılığı, fosetil-Al+triadimenol, azoxystorbin+difecanozole, fludioxanil+cyprodinil, metrafenon, fluopyram+tebuconazole dâhil olmak üzere çeşitli fungisitlere karşı değerlendirilmiştir. Üreticiler tarafından getirilen asma örneklerinin kök ve kök boğazı kısımlarından elde edilen izolatlar, morfolojik ve moleküler yöntemlere göre *D. seriata* olarak tanımlanmıştır. Moleküler tanımlamada izolatların ITS (Internal Transcribed Spacer) ve TUB2 (β-tubulin) gen bölgeleri Real-Time PCR ile çoğaltılmış ve bu gen bölgelerinden nükleotid dizileri elde edilmiştir. Daha sonra MEGA 7 yazılımı kullanılarak ITS ve TUB2 dizileri hizalanmış ve kombine bir filogenetik ağaç çizilmiştir. *Diplodia seriata* izolatının filogenetik analizlere göre *Diplodia* türleri ile % 100 benzerlik indeksine sahip olduğu moleküler olarak doğrulanmıştır. Farklı konsantrasyonlarda (0, 1, 3, 10, 30, 50, 100 µL mL^{-1}) kullanılan fungisitlerin ortalama etkili konsantrasyon (EC_{50}) değerleri Probit analiziyle belirlenmiştir. Cyprodinil+fludioxonil 1 µL mL^{-1} konsantrasyonunda en yüksek etkinliği (%100) göstermiştir. EC_{50} değerlerine göre cyprodinil+fludioxonil (0.001 µL mL^{-1}) en etkili fungisit olarak kaydedilmiş, ardından fluopyram+tebuconazole (0.520 µL mL^{-1}) ve azoxystrobin+difenokonazol (2.958 µL mL^{-1}) izlemiştir.

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Key words:
Botryosphaeriaceae
Dieback
Diplodia seriata
Vitis vinifera L.

Introduction

Turkey is one of the countries including the most geographically favorable areas for viticulture. According to FAO 2019 statistics, Turkey has the 6th largest land area devoted to vineyards with 470,000 hectares and is the 5th largest grape producer in the world with 4.1 million tons per year (FAO 2019). In the country, the Aegean region ranks first in terms of both area (1,392,082 da) and production (1,952,356 tons per year) and the Manisa

province ranks first in Aegean region with 809,123 da and 1,372,571 tons per year (Anonymous 2019). More than 30% of the country's production is provided by this region. Manisa province supplies 90% of Turkey's dried grape production and is the leader in Sultani seedless grape production used for export (Anonymous 2019).

Botryosphaeria dieback, caused by members of the fungi family Botryosphaeriaceae, is an important disease in vines seen all over the world and in Turkey. Members of Botryosphaeriaceae in the Dothideomycetes class are found largely as endophytes, parasites, and saprophytes in both annual and perennial plants under different ecological conditions in many regions of the world (Slippers & Wingfield 2007). The importance of these disease agents in vines was understood after it was first reported as a pathogen in the 2000s (Phillips 2002). The Eutypa dieback disease caused by *Eutypa lata* (Pers.) Tul. & C. Tul. has been thought to be responsible for cancers and deaths seen on vines in Australia for many years (Highet & Wicks 1998, Pascoe & Cottral 2000, Castillo-Pando *et al*. 2001, Siebert 2001). In Turkey, dieback, symptoms on vine leaves, and the development of brown color in wood tissue, which the shape of a “V”, have been associated with Esca or Eutypa dieback disease as in Australia.

Identification of *Botryosphaeria* Ces. & De. Not. diseases is problematic because symptoms occurring on vines in the field are very similar to other diseases such as Phomopsis dead arm disease caused by *Phomopsis viticola* (Sacc.) and Eutypa dieback caused by *Eutypa lata* (Castillo-Pando *et al*. 2001). Species of *Botryosphaeria* Ces. & De. Not., *Diplodia* Fr., *Lasiodiplodia* Ellis & Everh., *Neofusicoccum* Slippers & A.J.L. Phillips in the family Botryosphaeriaceae have been isolated and described for the first time from vines showing dieback symptoms (Akgül *et al*. 2014). *Diplodia seriata* De Not. and *Neofusicoccum luteum* (Pennycook & Samuels 1985) Crous, Slippers & A.J.L. Phillips 2006 were often isolated from stems, branches and shoots that show symptoms during surveys conducted in vineyards in the sub-tropical region of eastern Australia (Savocchia *et al*. 2007).

Members of the genera *Diplodia* and *Botryosphaeria* have often been isolated from the root and crown parts of vine samples delivered to Manisa viticulture research institute plant health laboratory by producers in Aegean region. It has been reported that varieties of *Vitis vinifera* L. are generally more susceptible to disease (Larignon *et al*. 2001). *Diplodia* and *Botryosphaeria* species shorten the life of the vineyards, allow late awakening of the vines, lead to the formation of yellow dots on the leaves followed by necrotic spots with zebra pattern, killing of the shoots and the stem and eventually drying the vine backward, which are defined as important signs of the disease (Gramaje *et al*. 2018, Kühn *et al*. 2017). In addition to the *Botryosphaeria* species, fungi from the fungal families *Botryosphaeria* species, as well as fungi from *Phaeoacremonium* W. Gams, Crous, M. J. Wingf. & Mugnai, *Phaeomoniella* Crous & W. Gams, and *Phomopsis* Sacc. & Roum were generally isolated the necrotic wood tissues showing symptoms of the disease. *Botryosphaeria* colonies are similar to *Alternaria* Nees colonies and are not noticed during the diagnostic phase based on colony development (Pitt *et al*. 2010, Úrbez-Torres 2011). The asexual spores of *Diplodia* spp. with spores of anamorphic of *Botryosphaeria* species are very similar, and a classification based only on the sexual situation is not suitable, especially since it is known that some species have only the asexual structures, and in some species, sexual development is extremely rare. Given these conditions, it has been determined that there are too many features in Botryosphaeriaceae that make it difficult to classify species (Slippers *et al*. 2013). For this reason, molecular diagnostics and even phylogenetic analysis are recommended. Ozben (2011) pointed to the presence of *Botryosphaeria obtusa* (Schwein.) Shoemaker and *B. rhodina* (Pat.) Griffon & Maubl. in the vineyard areas of Ankara, but the presence of these species has not been approved molecularly and phylogenetically. In a study conducted in the Aegean region, Akgül *et al*. (2015) morphologically and molecularly identified the presence of *B. dothidea* and *D. seriata* in Sultani seedless vineyards, but phylogenetic analyses were not performed. Between 2015 and 2018, 22 Botryosphaeriaceae cultures were isolated from the vineyard areas in the Mediterranean and Southern Anatolia regions and they were phylogenetically separated (Akgül *et al*. 2020). It is very difficult to combat wood trunk diseases contained in wood tissue, and the applications for accurate diagnostics and combating are extremely important. Therefore, the present study was performed in order to evaluate the molecular characterization of *Diplodia seriata* isolated from the vineyards in Aegean Region and its sensitivity against five different fungicides.

Materials and Methods

Isolation of the Disease Pathogen

Sultani seedless vines (N=23 samples) showing dieback disease symptoms were obtained from growers in 2019 and 2020 from the vineyard areas in Manisa and Denizli province in Aegean Region. In addition to the symptoms the samples (including root regions) were those which dried in the vineyards. The information about the rootstock of the vines and the area where the vineyards were located were noted for those where *D. seriate* could have been isolated (Table 1). Preliminary examinations of the root, crown, and trunks revealed thick-fine lines in the wood tissue or brown necrosis spread over a wider area in the form of "V". 23 symptomatic samples from roots and cordons were cut, surface disinfection was performed by dipping into 1% (v/v) sodium hypochlorite for 2 min, and small pieces from the edge of necrotic and healthy tissues were removed and plated on potato dextrose agar (PDA) (Darmstadt, Germany). Petri dishes containing PDA were incubated for 4-5 days in the dark at 25°C, and micelle structures taken from fungal colonies that developed around tissues were purified by transferring them to new Petri dishes containing PDA according to colony properties and morphological structures (Phillips *et al*. 2007).

Table 1. Provincial, county-village, and rootstock information of Sultani seedless vine samples showing signs of dieback.

Sample No	Isolate code	Province	County-village	Vine rootstock	Age of the vines in years
1	*MBAE234N	Manisa	Yunusemre-Horozköy	5BB	9
2	MBAE244N	Manisa	Yunusemre-Muradiye	1103Paulsen	6
3	MBAE275N	Manisa	Yunusemre-Evrenos	110R	3
4	MBAE288N	Manisa	Yunusemre-Horozköy	1103Paulsen	5
5	MBAE312N	Manisa	Yunusemre-Horozköy	1103Paulsen	4
6	MBAE313N	Manisa	Akhisar-Sazoba	SO4	10
7	MBAE336N	Manisa	Şehzadeler-Hacıhaliller	1103Paulsen	4
8	MBAE344N	Manisa	Gölmarmara-Kayaaltı	1103Paulsen	2
9	MBAE358N	Manisa	Saruhanlı-Nuriye	5BB	7
10	MBAE359MN	Denizli	Çal	5BB	3
11	MBAE368MN	Denizli	Buldan-Yenicekent	110R	6

* Naming MBAE is an abbreviation for the research institute where isolates were isolated. Number is the sequence number in the cultural collection. N/NM next to the number is the name abbreviation of the person/persons isolating it.

The mycelial discs from pure cultures were then transferred to Eppendorf tubes containing sterile 30% glycerol and stored at -80°C.

Molecular diagnosis of Diplodia seriata and phylogenetic analysis

Molecular diagnosis and phylogenetic analyses were carried out by selecting two isolates (MBAE359NM, MBAE368NM) from 11 *D. seriata* isolates obtained purely and very similar to each other from morphological-microscopic aspects. A total of 50 mg fresh mycelial mass was taken from the colonies of these two isolates, and DNA extraction was carried out. The micelle were put into sterile 1.5 mL Eppendorf tubes and crushed with Evolution Homogenizer (Precellys® Evolution, Paris, France), and 550 µL DNA extraction buffer (200 mM Tris-HCl) was added. DNA was obtained according to the proposed method of Cenis (1992) using 250 mM NaCl, 25 mM EDTA and 2% Sodium Dodecyl Sulphate. The concentration and purity of the isolated DNA were determined with a Multiscan GO µ-drop plate (Thermo Scientific, Vantaa, Finland). In PCR studies, the internal transcribed spacers (ITS) rDNA region was amplified with the ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) primer pair (White *et al.* 1990), and the β-tubulin (TUB2) gene region was amplified with βt-2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and βt-2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) primer pair (Glass & Donaldson 1995). In real-time PCR reactions, 0.3 µL 20 µM forward primer, 0.3 µL 20 µM reverse primer, 2 µL DNA, and 10 µL 2x FastStart Essential DNA Green Master Mix were added into sterile PCR tubes, and the final volume was completed with DNase/RNase pure water to 20 µL. Real-time PCR (Roche Light Cycler® Nano) amplification conditions included the initial denaturation for 10 min at 95°C, denaturation for 30 s at 95°C, 54°C for annealing temperature of ITS, 58°C for TUB2, and 35 cycles for 1 min. at 72°C. After PCR amplification, melting analysis eliminated non-specific amplifications such as primer dimers and determined whether the replicated area was the target region. Sequence data of the PCR products were obtained by a two-way genome sequencing service from a laboratory that provides Sanger sequence service (by TrioGene firm). Chromatogram files of sequence data were analyzed with ChromasPro 1.7.6 chromatogram analysis software, and consensus sequences were obtained by combining sequence data with forward and reverse sequences. BLASTn analyzes fungal species were performed using the consensus sequences obtained for each gene region in the National Center for Biotechnology Information (NCBI) GenBank database. According to these results, access numbers were obtained from the NCBI GenBank library of diagnosed isolates. For phylogenetic analyses, sequences belonging to an isolate were primarily aligned with Bioedit 7.2.5 sequence alignment software, then similarity ratios of nucleotide sequences were determined by the Clustal W software. The dendrogram of data of nucleotide sequences was created using the Mega 7 software and the Maximum likelihood model (Tamura *et al.* 2011) and confirmation of the obtained phylogenetic tree was made with 1000 repetitions (Bootstrap, p-distance, pairwise deletion). The access numbers of the isolates obtained in this study and the references from the GenBank database in the phylogenetic tree are listed in Table 2.

Evaluation of susceptibility of Diplodia seriata to fungicides in in-vitro conditions

To test the effect of fungicides on the development of mycelia in *in vitro* conditions, the fungicides given in Table 3 were used. The MBAE368MN isolate was used for this experimental step. The fungicidal activity of the fungicides was evaluated in the growth culture containing the same concentrations (0, 1.0, 3.0, 10.0, 30.0, 50.0, 100.0 PDA containing µg/mL). Stock solutions of each fungicide were prepared and concentrations from stock solutions were mixed into a sterile PDA medium cooled to 50°C and poured into Petri dishes (Isolab, 90x100 mm). As a control, Petri dishes with a PDA medium free from fungicides were used. Six mm diameter mycelial discs cut from the edges of the fungal colony of the 5-day-old *D. seriata* isolate (MBAE368NM) were placed to the center

Table 2. Isolates obtained from the GenBank database and references in the phylogenetic tree.

Species	Isolate	Host	Origin	GenBank Accession Nos.	
				ITS	TUB2
Diplodia seriata	MBAE359MN*	*V. vinifera*	Turkey	MT880771	MT914171
Diplodia seriata	MBAE368MN*	*V. vinifera*	Turkey	MT880774	MT914174
Diplodia seriata	CBS:114791	*V. vinifera*	South Africa	KX464107	KX464833
Botryosphaeria obtusa	CBS 112555	*V. vinifera*	Portugal	AY259094	DQ458856
Diplodia seriata	CBS 113527	*V. vinifera*	South Africa	KX464106	KX464832
Diplodia mutila	CBS 136014	*Populus alba* L.	Portugal	KJ361837	MG015815
Diplodia mutila	CBS 431.82	*V. vinifera*	France	KU198424	KU198426
Lasiodiplodia theobromae (Pat.) Griffon & Maubl.	CBS 111530	Proteaceae	Netherlands	FJ150695	KU887531
Lasiodiplodia theobromae	CBS 164.96	Fruit along coral reef coast	Papua New Guinea	NR_111174.1	EU673110.1
Neofusicoccum parvum (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips	CBS 123652	*Syzygium cordatum* (Hochst.)	South Africa	KX464184	KX464996
Neofusicoccum parvum	CBS 257.77	*Cocos nucifera* L.	India	KX464197	KX465012
Neofusicoccum luteum	CBS 562.92	*Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson	New Zeland	KX464170	KX464968
Neofusicoccum luteum	CBS 612.83	*Persea Americana* Mill	USA	KX464171	KX464969
Tiarosporella tritici B. Sutton & Marasas	CBS 118719	*Triticum* sp.	South Africa	KF531830	KF531810

* Isolates registered in GenBank and their accession numbers.

Table 3. The active ingredient, formulation, and company information of the fungicides used in determining fungicidal activity in *in vitro* conditions.

Active ingredient %	Commercial Name	Firm	Formulation Type*
Cyprodinil+Fludioxonil, %37.5+%25	Switch 62.5	Syngenta	WG
Fluopyram+Tebuconazole, 200 g/L +200 g/L	Luna Experience	Bayer	SC
Azoxystrobin+Difenoconazole, 200 g+125 g	Quadris Maxx	Syngenta	SC
Folpet+Triadimenol, 700 g/kg+20 g/kg	Shavit F 72	Adama	WDG
Metrafenone, 500 gr/L	Vivando	Basf	SC

*WG, WDG: Water dispersible granule of formulation type, SC: Water soluble concentrate of formulation type.

of the growth culture containing fungicide concentrations. All inoculated application and control Petri dishes were incubated in darkness at 20°C for 7 days. During this period, the rates of blocking micelle by fungicides (%) were examined by measuring the radial development of fungal micelles (Uysal & Kurt 2019).

In the trials, randomized controlled trials were established so that each Petri dish represented one replication and each concentration has 3 replications. The experiment was repeated at 2 different times.

Calculations and Statistical Evaluation

In vitro trials were set up to have 3 replications for each application, according to the randomized controlled trial. For establishing antifungal efficacy in petri dishes containing fungicide concentrations, variation analysis was carried out with one-way ANOVA using SPSS Statistics (Version 17.0, SPSS Inc., Chicago, IL, USA) without converting the blocking rates of micelle development in petri dishes ($p \leq 0.05$). Effective concentrations of fungicides that inhibit the development of micelles at 50% level (EC_{50}) were determined by Probit analysis with the help of the SPSS statistics using the values obtained in different concentrations for each chemical.

Results

Isolation and morphological characteristics of the disease agent

Eleven *Diplodia seriata* isolates were obtained during the isolation from the root and crown regions of the samples taken from Sultani seedless vine varieties showing signs of dieback in the vineyards in Manisa and Denizli provinces. Wedge-shaped brown lines and necrotic tissues ranging from brown to black color were observed in the root areas of the isolated samples. On the upper part of the vine, yellow spots in the form of dots on the leaves, necrotic spots with zebra pattern, and backward drying on the shoots were found to be remarkable.

Colonies that develop in a PDA medium were initially colorless or light olive green-gray in color but darkened and blacken in time (Fig. 1A and B). The conidia were dark brown in colour and oval, broad at the apex and truncated or rounded at the base, and its wall was rough. Immature spores were without compartment and formed darker brown single-compartment spores as they matured. Forty conidia per isolate were measured by light microscopy (Olympus BX-51 connected with Olympus Camedia-4501X, Hamburg, Germany). The

size of the conidia was measured as 27.4 ± 0.31 x 10.8 ± 0.15 µm, with no significant differences between the isolates, and it was determined to be the same as the characteristics of *D. seriata* (teleomorph: *Botryosphaeria obtusa*) specified in morphological diagnostics made by Phillips *et al*. (2007).

Molecular diagnosis and phylogenetic analyses

By using primers specific to ITS (ITS-1 and ITS-4), TUB2 (βt-2a and βt-2b) gene regions, PCR, and DNA sequencing of MBAE359MN and MBAE368MN isolates selected among the fungal isolates resulted in the 580 bp and 434 bp bands, respectively. By comparing the nucleotide sequences with sequences in the NCBI GenBank (BLASTn), the isolates were found to be *D. seriata*. As a result of Blast comparison with species registered in GenBank, they were found to have a 97-100% resemblance to MBAE359MN and MBAE368MN *D. seriata* isolates (for ITS and TUB gene regions, the access numbers are KX464107, KX464833, and KX464106, KX464832, respectively). The nucleotide sequences of *D. seriata* MBAE359MN and MBAE368MN isolates, which were morphologically and molecularly determined, were uploaded to the GenBank database and obtained the access number MT880771 and MT880773 were obtained for the ITS gene and MT914171 and MT914174 for the TUB2 gene. A combined dendrogram was obtained for the ITS and TUB 2 genes with MEGA 7 software using the maximum likelihood method (Fig. 2). According to the dendrogram, MBAE359MN and MBAE368MN isolates were in the same group as *D. seriata* CBS: 114791, CBS 112555, and CBS:113527 isolates. *Tiarosporella tiritici* CBS 118719 was determined as an external group (Fig. 2).

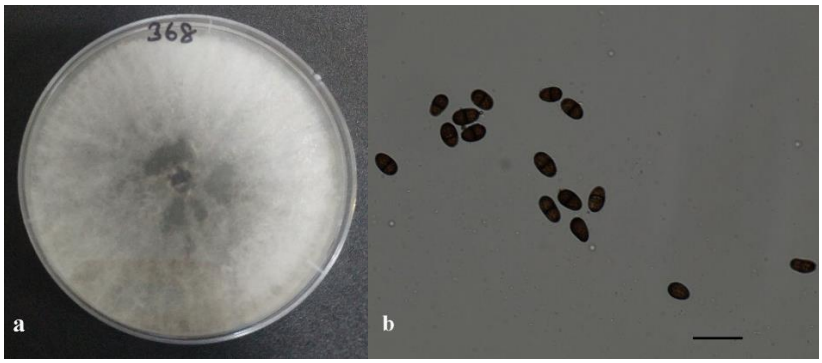

Fig. 1. *Diplodia seriata;* **a.** 7-day views of young colonies on PDA, **b.** Conidia, scale 10 µm.

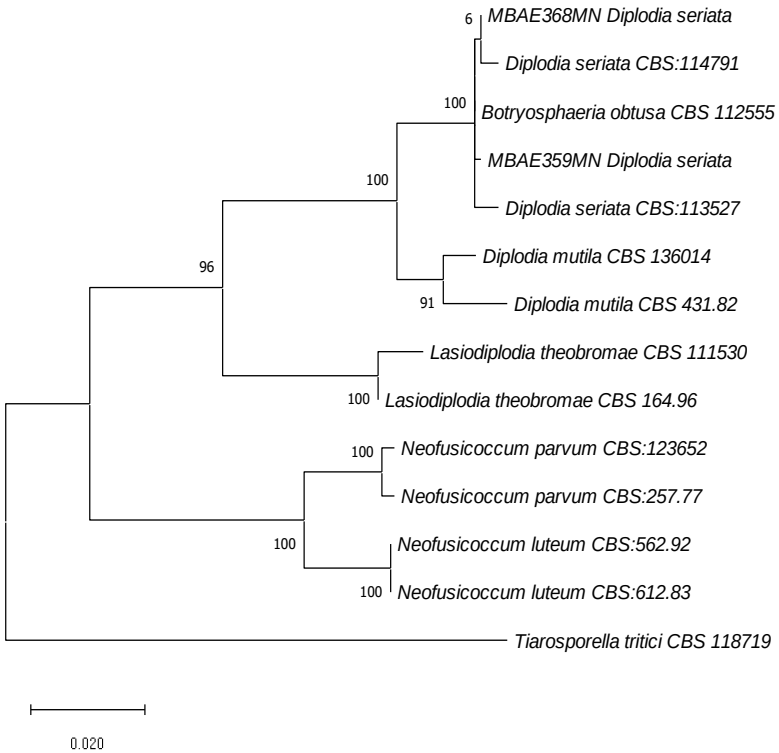

Fig. 2. Phylogenetic relationships of some Botryosphaeriaceae species according to the maximum similarity model obtained by the combination of ITS and TUB2 gene regions (Maximum Likelihood). The reliability of the phylogenetic tree was calculated with 1000 replications by the Bootstrap method. Bootstrap values below 50% were not shown. *Tiarosporella tritici* (CBS 118719) is the outside group. The scale bar shows the number of nucleotide changes.

Table 4. Antifungal effects of the tested fungicides on *Diplodia seriata* MBAEM368MN isolate as revealed by *in vitro*-micelle growth prevention.

Active substance	Replication 1 EC_{50} value ** (µg mL^{-1})	Replication 2 EC_{50} value (µg mL^{-1})	Replication 3 EC_{50} value (µg mL^{-1})	Average EC_{50} value (µg mL^{-1})*
Cyprodinil+Fludioxonil,	0.001	0.001	0.001	0.001±0.00 a
Fluopyram+Tebuconazole	0.453	0.434	0.673	0.520±0.08 a
Azoxystrobin+Difenoconazole	2.847	2.006	4.021	2.958±0.58 a
Folpet+Triadimenol	4.962	5.721	7.546	6.076±0.76 ab
Metrafenone	4.996	21.090	7.585	11.223±4.99 b

***** Different letters next to the mean EC_{50} (µg/mL^{-1} ± S.E) in the row indicate that the difference between the applications is statistically significant according to the Duncan Multiple Comparison Test ($p<0.05$).
******Effective concentrations (EC_{50}) of fungicides that inhibit micelle growth by 50% were estimated by using the values obtained at different concentrations for each fungicide by Probit analysis with the help of SPSS statistical software (Version 17.0, SPSS Inc., Chicago, IL, USA).

Determination of susceptibility of the disease agent to fungicides in in-vitro conditions

The effective concentration (EC_{50}) values required for fungicides used in the study to prevent micelle development of disease factor *D. seriata* by 50% were determined by the SPSS statistical software Probit analysis. When the antifungal effects of fungicides with different active substances preventing micelle growth of *D. seriata* MBAEM368MN isolate in *in vitro* conditions were examined, it was observed that the disease agent was quite sensitive to other fungicides except for metrafenone. Among the fungicides, cyprodinil+fludioxonil showed the highest efficacy (100%) at a concentration of 1µg mL^{-1}. The highest activity was recorded as cyprodinil+fludioxonil with the lowest EC_{50} value as 0.001 µg mL^{-1}, followed by fluopyram+tebuconazole with 0.520 µg mL^{-1}, azoxystrobin+difenoconazole with 2.958 µg mL^{-1}, and folpet+triadimenol with 6.076 µg mL^{-1} respectively. The lowest efficacy was obtained from metrafenone active substance with the highest EC_{50} value of 11.223 µg mL^{-1} (Table 4). Cyprodinil+fludioxonil, fluopyram+tebuconazole, azoxystrobin+difenoconazole fungicides were in the same statistical group with low EC_{50} values and were determined as the most effective fungicides against *D. seriata* MBAEM368MN isolate.

Discussion

Diplodia seriata was isolated from the root and crown parts of the Sultani seedless vine varieties between of 2-10 years old, grafted on different rootstocks in Denizli and Manisa in Aegean Region. In a study examining cross infections of *Ilyonectria* spp. and Botryosphaeriaceae family that cause dieback in young vines in Australia; *D. seriata*, *D. mutila* (Fries) Montagne, and *Lasiodiplodia theobromae* were isolated from 79.8% and 8% of Chardonny and Ramsey rootstocks, respectively. *Diplodia seriata* and *D. mutila* were detected from the roots of 10% and 5% of the plants showing the symptoms, respectively (Pitt *et al.* 2010). In a study conducted in 2015 in the Sultani seedless vineyard areas in Aegean Region, it was reported that at least one grapevine stem disease was seen in more than 80% of the vineyards who were at least 10 years old, and *Botryosphaeriaceae* species were the most isolated pathogens with 1.3% (Akgul *et al*. 2015). However, Billones-Baaijens *et al.* (2013) found that *D. seriata* and *D. mutila* were less virulent in green shoots compared to the three *Neofusicoccum* species. These *Diplodia* species were equally virulent when grafted into rooted strawberries, and these species may be tissue-specific pathogens also may be explain the reason for the endophytic behavior of these pathogens as their high availability from asymptomatic breeding materials and nurseries.

It is difficult to differentiate *Diplodia* species based on their conidial morphology because they are very similar to each other. The time of onset of conidia pigmentation in *Diplodia* species, and very small differences in color and compartments aid in distinction (Phillips *et al*. 2008, 2012). It is noted that the conidia in most *Diplodia* species remains hyaline for a long time and may never actually be brown. However, species within the group are characterized by brown, aseptate conidia (such as *D. seriata* and *D. sapinea* (Fries) Fuckel), pigmented conidia (Phillips *et al*. 2013). In our study, the isolates obtained based on morphological characters of fungal structures of diseased plants were described as *D. seriata* De Not (teleomorph "*Botryosphaeria*" obtusa) (Phillips *et al*. 2007, Chebil *et al*. 2017). By phylogenetic analysis, the two *Diplodia* species were clustered on two different branches in the same group. It has been molecularly confirmed that the *Diplodia seriata* isolate has a 100% similarity index with *Diplodia* species. In a study on species in the Botryosphaeriaceae family associated with dieback in Vine in China, *Lasiodiplodia theobromae*, *Neofusicoccum parvum, Botryosphaeria dothidea* and, *D. seriata* were determined as pathogens by their molecular characterization of the ITS, TUB, and EF1-α gene regions (Yan *et al*. 2013).

A number of studies have been carried out on members of the Botryosphaeriaceae family, which leads to dieback in vines (Luque *et al.* 2009, Urbez-Torres & Gubler 2009). In a study that evaluated fungicides for the control of Botryosphaeria dieback disease in vines in New Zealand, 8 of the 16 tested fungicides were been found to be effective in preventing conidial germination and mycelial growth in against *Neofusicoccum australe* (Slippers, Crous & Wingfield) Crous, Slippers & Phillips,

N. luteum, and *D. mutila*. Flusilazole, carbendazim, tebuconazole, prochlorase, procymidone, iprodione, fenarimol, thiophanate methyl, chlorothalonil, and mancozeb 1 µg mL^{-1} were found to be the most effective against these three *Botryosphaeria* species in *in vitro* with lower average EC_{50} values (Amponsha *et al*. 2012). The effectiveness of 20 different fungicides used against *Botryosphaeria* cancer in the vineyards in Australia was revealed by *in vitro* trials using *B. dothidea*, *N. parvum*, *L. theobromae*, and *D. seriata*. Fludioxonil, carbendazim, fluazinam, tebuconazole, flusilazole, penconazole, procymidone, iprodione, myclobutanil, and pyraclostrobin, with a value of <1.0 mg L^{-1}, were identified as the most effective fungicides (Pitt *et al*. 2012).

Diplodia seriata, isolated in the present study was determined as the dieback disease in vines in Manisa and Denizli provinces in Aegean region. It is thought that this agent can be found in the root and crown region and accelerates dieback according to the region in which it is located.

The effects of fungicides with different chemical structures against the disease agent isolated from the vineyard were tested for the first time in *in vitro* conditions. Cyprodinil + fludioxonil and fluopyram + tebuconazole were found to be the most effective fungicides against *D. seriata* MBAEM368MN isolate with low EC_{50} values. For complete control of dieback disease, an integrated control system that encompasses the entire life cycle of grapevines is necessary. In future studies, integration of well-planned cultural methods and chemical control practices that will provide optimum protection in field conditions can be realized. It may also be recommended to add the Switch 62.5 and Luna Experience fungicides with good results according to EC_{50} values in chemical control applications.

Acknowledgement

This study was carried out in the laboratory of The Plant Health Clinic of the Directorate of Manisa Viticulture Research Institute. A part of this study was presented as a short summary at II. International Agricultural, Biological & Life Science Conference (E-Agbiol), held in Edirne, Turkey, from September 1 to 3, 2020.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Author Contributions: Concept: N.G.S., M.Y., Desing: N.G.S., M.Y., Execution: N.G.S., M.Y., Material supplying: N.G.S., M.Y., Data acquisition: N.G.S., M.Y., Data analysis/interpretation: N.G.S., M.Y., Writing: N.G.S., M.Y.

Conflict of Interest: The authors have no conflicts of interest to declare.

Funding: The authors declared that this study has received no financial support.

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