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Sensitivity of *Alternaria* spp. Isolates to Some Fungicides Used on Sultani Çekirdeksiz Grapes in Aegean Region Vineyards

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Abstract

This study aimed to identify *Alternaria* species morphologically and molecularly, that is one of the important pathogens causing cluster rot, and determine their reactions to some fungicides in *in vitro*. The fungal isolates obtained from leaves, flowers, stems, and shoots in almost every development period of the Sultani Çekirdeksiz vineyards in 2009 and 2010 from Manisa province in the Aegean region were identified as *Alternaria alternata* (Fr.) Keissler according to morphological and molecular methods. In molecular studies, the ITS (Internal Transcribed Spacer) gene region was amplified by real-time PCR and the nucleotide sequences of 570 bp long isolates were stored in the NCBI GenBank database. ITS sequences were aligned using the Mega X software, and a phylogenetic tree was constructed. Sensitivity levels of isolates to cyprodinil+fludioxanil, mancozeb, pyrimethanil, kresoxim-methyl+boscalid, azoxystrobin, and basic copper sulfate were tested under laboratory conditions. In vitro antifungal effect of different concentrations (0.01-100 µg ml^{-1} and 3-3000 µg ml^{-1}) of six fungicides against 24 *A. alternata* isolates were evaluated. Cyprodinil+fludioxanil was recorded as the most effective fungicide that, according to ED_{50} value, 23 isolates were in the range of 0.01-0.1 µg ml^{-1}. BAC 1/2 and GMK 2/1 isolates were found to have the highest virulence with values of 100% and 86.67%, respectively.

Keywords: *Alternaria alternata*, Fungicides, Grapevine, Real-time PCR, Sensitivity**,** Sultani Çekirdeksiz

Ege Bölgesi Bağlarında Sultani Çekirdeksiz Üzüm Çeşidinde Kullanılan Bazı Fungisitlere *Alternaria* spp. İzolatlarının Duyarlılığı

Öz

Bu çalışmada, salkım çürüklüğüne neden olan patojenlerden biri olan *Alternaria* türlerinin morfolojik ve moleküler olarak tanımlanması ve *in vitro* olarak bazı fungisitlere karşı etkilerinin belirlenmesi amaçlanmıştır. Ege bölgesinde Manisa ilindeki, Sultani Çekirdeksiz bağlarının 2009 ve 2010 yıllarında hemen her gelişme döneminde yaprak, çiçek, gövde ve sürgünlerinden elde edilen izolatlar morfolojik ve moleküler yöntemlere göre *Alternaria alternata* (Fr.) Keissler olarak tanımlanmıştır. Moleküler tanıda ITS (Internal Transcribed Spacer) gen bölgesi real-time PCR ile amplifiye edilmiş ve 570 bp uzunluğunda nükleotid dizisi elde edilerek, NCBI Gen bankasında kayıt altına alınmıştır. ITS dizileri Mega X yazılımı kullanılarak hizalanmış ve bir filogenetik ağaç çizilmiştir. İzolatların cyprodinil+fludioxanil, mancozeb, pyrimethanil, kresoxim-methyl+boscalid, azoxystrobin ve temel bakır sülfata karşı duyarlılık düzeyleri laboratuvar koşullarında test edilmiştir. Altı fungisitin farklı konsantrasyonlarda (0.01-100 µg ml^{-1} ve 3-

3000 µg ml^{-1}) 24 *A. alternata* izolatına karşı *in vitro* antifungal etkisi değerlendirilmiştir. EC_{50} değerine göre 23 izolatın 0.01-0.1 µg ml^{-1} aralığında olması ile cyprodinil+fludioxanil en etkili fungisit olarak kaydedilmiştir. BAC 1/2 ve GMK 2/1 izolatları sırasıyla %100 ve %86,67 değerleri ile en yüksek virülans değerine sahip bulunmuştur.

Anahtar Kelimeler: *Alternaria alternata*, Fungisitler, Asma, Real-time PCR, Duyarlılık, Sultani Çekirdeksiz

1. Introduction

In Turkey, a total of 4 million 200 thousand tons of grapes were produced, of which 2 million 218 thousand tons are given in the table, 1 million 535 thousand tons for raisins (346 thousand tons seeded-1 million 188 thousand tons seedless), and 456 thousand tons for wine. Aegean is a region in the west of Turkey where table grapes and especially raisins, are cultivated in about 809 thousand decares (FAO, 2020). Due to the fairly convenient humidity and temperature levels for fungal growth in this region, bunch rots have been causing severe yield losses in recent times.

Several soil borne pathogens have been reported infecting different plants (Erdoğan et al., 2014; Erdoğan et al., 2016; Koç et al., 2018). The genus *Alternaria*, one of the largest one, includes pathogenic species in many crops of economic importance, such as cereals, oil plants, vegetables, and fruits (Pitt and Hocking, 1997). The pathogen, which can be isolated in different phenological stages of the vineyard, causes cluster rot and damages fruits, peduncle, and pedicels (Swart et al., 1995; Mostert et al., 2000). Species such as *A. alternata*, *A. arborescens*, and *A. tenuissima* have often been isolated from grapes in the vineyard or post-harvest storage (Serra et al., 2006; Polizzotto et al., 2012). *A. alternata* infected both the vineyard and cold-stored grapes through wounds, stomata, and lenticels (Swart et al. 1995). There is limited information about the existence of different *Alternaria* spp. as a bunch rot pathogen in vineyards. There are main strains diversity between the isolates *Alternaria* isolated from different plant hosts, including grapes (Polizzotto et al., 2012; Rotondo et al., 2012). Moreover, the pathogenicity of *Alternaria* as a cause of post-harvest rot in grapes is not fully known. As a post-harvest rot factor, the rate of rotten grape production during drying or cold storage conditions is not clear.

Alternaria is generally easy to identify but difficult to diagnose different species based on morphological and cultural characteristics (Simmons, 2007). Different molecular diagnostic methods such as RAPD, AFLP, RFLP, and DNA sequences have been used to correctly identify and classify the species and diagnose according to the morphological features of *Alternaria* spp. (Peever et al., 2002). To the best of our knowledge no report has been identified related to the grape bunch rot agent, *Alternaria* spp.

Alternaria spp. is a secondary microorganism, which causes bunch rot, can become the primary pathogen by infection on grapes caused by the rain, hail, and insect damage (Rotem, 1994). Although *Alternaria* spp. is often isolated from the vineyards, its damage is rarely encountered on bunches because of the effectiveness of almost all fungicides used against powdery mildew on vineyards. *Alternaria* spp. among powdery mildew fungicides, boscalid and tebuconazole, are effective against *B. cinerea* and *Alternaria* spp. (Lu et al., 2004; Delen, 2001). Fungicides used against powdery mildew and gray mould diseases *Alternaria* spp. was effective (Delen, 2001). Azoxystrobin prevents the lesions caused by powdery mildew, *Phomopsis viticola*, and downy mildew. It also controls the causes of the disease considerably by preventing *B. cinerea* and *Alternaria* spp. from colonizing these places (Koplay, 2004). In recent years azoxystrobin and pyraclostrobin have been reported to be very effective in controlling *Alternaria* diseases (Farra et al., 2004). In addition, fungicide resistance has been detected in some *Alternaria* spp. (Farra et al., 2004).

This study aimed to morphologically and molecularly identify *Alternaria* species, one of the pathogens causing cluster rot, and determine their reactions to some fungicides with different chemical structures in vitro.

2. Materials and Methods

2.1. Isolates and Morphological Characterization

A total of 141 sampling and isolations were made from the Aegean region in 2009 and 2010 to determine the vineyard prevalence of the fungal-borne cluster rot pathogen *Alternaria* spp. Sample collecting started in the pre-blooming period and continued in the inflorescence initiation, blooming, fruit set phase, green berry phase,

veraison, and finally, the full ripening phase. Plant sections of 3-4 mm in size, including rot and healthy tissues resulting from infection, were disinfected from the surface for 4 minutes in 2% NaOCl, rinsed in sterile distilled water 2 times, and left to dry on sterile blotting papers. The disinfected plant tissues were transferred to potato dextrose agar (PDA) medium containing 50 µg ml^{-1} streptomycin sulfate. Petri dishes containing PDA were incubated at 25°C for 5 days in the dark. After incubation, hyphal tips of fungi emerging from diseased tissues were re-cultured, conidia were collected from naive colonies, and single conidia culture was prepared following the techniques outlined by Choi et al. (1999) on PDA plates. The morphological features of colonies growing from a single conidium were examined under a 40x resolution optical microscope (Olympus BX-51 attached with Olympus Camedia-4501X, USA). In this study, the morphological identification of *A. alternata* was made according to Barnett and Hunter (1998) and Simmons (2007).

2.2. Molecular and Phylogenetic Analysis

One year after the completion of the study, molecular diagnostic analyzes were performed on 24 isolates used in the project. Fungal DNA was extracted by following the extraction protocol of Cenis (1992). Mycelia mats (approximately 50 mg) were taken from the fresh cultures of the isolates with a sterile surgical blade. They were crushed in micro-centrifuge tubes (1.5 ml) with a sterile plastic pestle by adding 550 µl DNA extraction buffer (a 2% Sodium Dodecyl Sulphate 200 mM Tris-HCl (pH: 8.5), 250 mM NaCl, and 25 mM EDTA). After homogenization, 150 µl 3M Sodium Acetate (NaOAc) was added into tubes and placed at -20°C for 15 minutes. The homogenates were centrifuged for 10 minutes at 14.000 rpm, and the supernatants (200 µl) were transferred to the new tubes. An equal volume of isopropanol (2-propanol) was added and mixed gently about five times, and the tubes were placed at 0°C for 10 minutes. After that, the DNA pellet was precipitated by centrifugation at 14.000 rpm for 10 minutes, and the supernatant was discarded. DNA was washed with 1 ml of 70% ethanol for 1-2 seconds, and the pellet was dried for 10 minutes under the hood. Finally, DNA was suspended with 75 µl of TE (1M Tris-HCl, pH: 8, and 0.5M EDTA) buffer and stored at -20°C.

In study, Real-time PCR mixtures, 25 µl each, consisted of 10 µl of 2x FastStart Universal SYBR Green Master Mix (Roche Biotechnology, Switzerland), 0.3 µl of each primer (Triogen, Germany) at 20 µM, and 2 µl of DNA. Real-time PCR was performed in a LightCycler® Nano Instrument (Roche, Basel, Switzerland). *Alternaria* isolates were identified molecular the species level using ITS4 and ITS5 primers as described by White et al. (1990). Ribosomal DNA fragments (ITS4, 5.8S ITS5 rDNA loci) were amplified with ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') and ITS5 (5' GGA AGT AAA AGT CGT AAC AAG G 3') universal primers in real-time PCR (The Roche LightCycler® Nano Instrument) according to the amplification protocol (initial denaturation 95°C for 10 minutes and followed by 35 cycles at 95°C 10 s., 54°C 10 s., 72°C 15 s., and 72°C 10 minutes final extension) (White et al., 1990). Real-time PCR has been preferred because it provides great convenience and eliminates the need for post-amplification processing required in conventional PCR.

After that, PCR products were sequenced (to Macrogen Co, South Korea), and the sequences were compared with those deposited in the NCBI Gene Bank database. The ITS sequences with an average size of 570 bp were also submitted to the National Center for Biotechnology Information (NCBI) GenBank. Accession numbers of these isolates were obtained. Chromatogram files of sequence data were analyzed with the ChromasPro 1.9.9 chromatogram analysis software, and consensus sequences were obtained by combining sequence data with forward and reverse sequences. BLASTn fungal species were tested using the consensus sequences obtained for each gene region in the NCBI GenBank database. Based on these results, accession numbers of the identified isolates were obtained from the NCBI GenBank library. For phylogenetic analysis, the sequences of an isolate were first aligned with the Bioedit 7.2.5 sequence alignment software. Then the similarity ratios of the nucleotide sequences were determined with the Clustal W software. The dendrogram of nucleotide sequences data was generated using the Mega 7 software and the Jukes-Cantor distance model, neighbour-joining (NJ) method (Saitou and Nei, 1987). Validation of the obtained phylogenetic, the tree was built with 1000 repetitions (Bootstrap, p-distance, double deletion).

2.3. Pathogenicity Studies

During the growth period of vines in 2010-2011, pathogenicity of *Alternaria* spp. isolates were determined by testing the berries under fungicide-free conditions. 18 isolates of *Alternaria* spp., which had sporulation ability, were used in the virulence tests. Healthy grape berries were wounded once with the help of sterile injectors to avoid wounds on the grapes. Styrofoam was then placed in plastic boxes, and pure, sterile water was added to provide moisture. Five berries were then inoculated with 10 µl micropipette of *Alternaria* spp. spore suspension was at $1.0x10^{-6}$ spor ml (Swart and Holz, 1991). The grapes were kept at 25±1 °C in the climate room and were evaluated after seven days of incubation. The research was established with three replications, and 5 of those berries were used in each repetition. Berries were regularly monitored for disease development. The lesion lengths on the berries were measured to better demonstrate the virulence difference between isolates.

2.4. Determination of the Susceptibility of the Disease Agents to Fungicides in In vitro Conditions

In experiments used fungicides and this fungicides including commercial products are: basic copper sulfate (Rooster SC 139 g/L, Hektaş Crop Science AG), mancozeb (Dithane M 45 Special, %80, Dow Agro Science AG), cyprodinil+fludioxonil (Switch 62.5 WG, %37.5+%25, Syngenta Crop Protection AG), azoxystrobin (Quadris SC, 250 g/L, Syngenta Crop Protection AG), pyrimethanil (Mythos 30 SC, 300 g/L, Bayer Crop Science AG), and kresoxim-methyl + boscalid (Collis SC, 100+200 g/L, Basf Chemical Company). The determined concentrations of each fungicide were mixed into a sterile PDA medium cooled to 50°C and poured into Petri dishes (90x100 mm). Petri dishes containing PDA medium without any fungicide were used as control.

The sensitivity of 24 isolates to different concentrations of fungicides was tested with 0.01-100 µg ml^{-1}, 0 (control), and PDA. For basic copper sulfate fungicide, 3-3000 µg ml^{-1} e.m dosages were tested on WA. The four-day-old cultures of isolates developed in the dark at 23°C were used for inoculation. Plugs, 4 mm in diameter, obtained from the periphery of 4-day-old cultures of *Alternaria* spp. cultures with a cork-borer were inoculated in fungicide-containing and fungicide-free (control) Petri dishes. Three replicates were used for each treatment. After inoculation, the Petri dishes were kept in the dark incubators at 23°C (Delen et al., 1984). After 4 days of incubation, radial growth of colonies of *Alternaria* spp. isolates were measured in 2 orthogonal directions. From these values, the effective dose (ED_{50} µg ml^{-1}) rates that prevented mycelial growth of 50% were determined.

2.5. Determination of the Effectiveness of Fungicides by Berry Tests under Controlled Conditions

Two isolates were selected for each fungicide. The selection of isolates was defined according to their ED_{50} (µg ml^{-1}) values. The isolate with the highest ED_{50} (µg ml^{-1}) value was characterized with the symbol fungicide-R; the isolate with the least EC_{50} value was characterized with the symbol fungicide-S. R/S isolates of *A. alternate* fungicides were tested on Sultani Çekirdeksiz grape berries, according to Koplay (2004). Half and quarter dosages were used in the tests considering each fungicide's recommended dosage as full. Grape clusters were cut off with their stalks to prevent any injury and were exposed to surface disinfection in 1% NaOCl for 1-2 minutes, and then the berries were wounded at one site with a dissecting needle (Zahavi et al., 2000). Berries were then fixed on sterilized Styrofoam. After that, 1×10^{6} spor ml^{-1} *A. alternata* conidia suspensions that had been adjusted beforehand were applied to the wound sites on each berry with a 10 µl micropipette. The fruits were placed in plastic boxes, and distilled water was added. The boxes were closed to provide 90-95% relative humidity. They were incubated for 6 days at 25°C. The diameters of the lesions on the fruits were measured, and fungicide efficacy was calculated according to Abbot's formula (Abbott, 1925).

Abbot's formula = 100*[Untreated control grapes (A)-treated grapes/A] (1**)**

2.6. Statistical Evaluation

Each isolate's ED_{50} value (µg ml^{-1}) was calculated from data subjected to probit-analysis. Mean values of the efficacy of each fungicide concentration against resistant/sensitive isolates were analyzed by Duncan's multiple range test ($P \leq 0.01$). In the suitability experiments of selected isolates, differences were calculated by Student's

t-test. The effective dose of fungicides that inhibit the development of micelles at the 50% level (ED_{50}) was determined by Probit analysis with the help of the SPSS statistics using the values obtained in different concentrations for each chemical.

3. Results and Discussion

3.1. Morphological Characterization of Isolates

For the preliminary identification of the fungi, the morphological characters of the pathogen are thought to be isolated. Approximately 100 isolates from all visited vineyards (all isolates not presented in this study) were obtained from all structures of the cluster, including its leaves. Morphological studies were performed on 24 randomly selected isolates. Colony growth of fungi on PDA was observed as a white-grayish airy mycelium with light to dark green interiors emerging from a common center during the initial stage of fungal growth. The mycelium color in the Petri dish is dark brown, and then green, surrounded by gray mycelium on edge. Conidiophores, flat-walled approximate conidia, sometimes also had a beak that was one-third as long. It was 24.7 x 11.5 µm long and 3-6 µm thick, with 3-8 transverse and 1-3 longitudinal or oblique septa. *Alternaria* species classification is mostly based on conidia and conidiophore morphology and development, and to a lesser extent on colony morphology and plant host interactions (Ellis, 1993; Barnett and Hunter, 1998) as similar in all isolates.

Rotem (1994) reported that the diagnosis of *A. alternata* within the genus based on morphological and physiological characters should not be used as a criterion for distinguishing species and that there is high variability in terms of characters. However, most *Alternaria* spp. show significant and variable morphological features dependent on various factors such as temperature, wetness, and light intensity (Simmons, 1992). Due to the problems mentioned above, molecular tests of the individual isolates one year after the project's completion were carried out to support their morphological diagnosis.

3.2. Molecular and Phylogenetic Analysis

24 isolates randomly selected for pathogenicity and fungicide susceptibility tests within the project's scope were included in molecular diagnostic tests. However, the DNA of 7 isolates could not be extracted, and ITS4/ITS5 gene regions on DNA in 6 isolates could not be amplified in real-time PCR (RT-PCR) tests. For this reason, molecular diagnostic tests were performed according to ITS4/ITS5 gene regions of eleven isolates, and the phylogenetic tree was drawn.

Real time PCR was performed using SYBR Green flourescence dye and ITS4/ITS5 universal primer. *A. alternata* isolates produced peaks that passed the threshold (Ct) level and produced values between 9.53 and 18.35 on average. The Negative control reaction value, which we diluted with TE buffer, was 0.0 (Figure 1b). The SYBR melting curve was conducted in 54-95°C temperature profiles at a ramp rate of 0.02 C/s to determine the single amplification product of target *A. alteranata* obtained by real-time PCR. Each of the isolates produced a single melting peak in the mean range of 85.17-85.98 (Figure 1a).

Sequence and NCBI-BLASTn analysis of the PCR products (amplified by universal primer pairs, ITS4/ITS5) revealed that eleven *Alternaria* species were obtained from identifying *Alternaria* cultures. The species names, their accession number records, maximum identity percentages are presented in Table 1.

The rDNA-ITS regions of the present isolates were amplified by real time-PCR with ITS4 and ITS5 primer pairs and sequenced to confirm the molecular identity of *A. alternata*. The sequenced RT-PCR products were aligned, and BLAST was carried out using the BLASTn program. The rDNA sequence was deposited in the GenBank database with the accession numbers in Table 2. The sizes of the PCR products were average 570 bp BLAST analysis of the amplicon showed 100% similarity with *A. alternata* sequences reported from other hosts from another country. To assess the relationships with other *Alternaria* spp. the corresponding genomic regions of 11 *Alternaria* spp. showing 100% - 99% sequence identity with present isolate were obtained from GenBank, and a phylogenetic tree was constructed. Phylogenetic analysis revealed that the present *Alternaria alternate* infecting grapevines grouped separately from other *Alternaria* spp. reported from other host species (Figure 2).

The isolates obtained in the study were in the same group as the strains accessions available of *A. alternata* with similar sequences has a 100% similarity index. In the study of *Alternaria* blight caused by *A. alternata* in pigeon pea in India, *A. alternata* sequences were included in the same

group with a 100% similarity rate in the phylogenetic tree based on rDNA-ITS analysis (Sharma et al., 2013). Basım et al. (2017) carried out on identifying and characterizing *A. alternata*, which causes leaf spot disease in olive trees. Phylogenetic analysis with general primers of *A. alternata* isolates ITS and Beta Tubulin (BT) and species-specific AaltFor/AaltRev primer fragments yielded a high rate of evolutionary ancestors between 95-100%. The study obtained 100% high homology between the ITS gene region sequences of *A. alternata* obtained from the vine and GenBank species.

Table 1. GenBank nucleotide accessions of *A. alternata* isolated on grapevines with other fungal isolates based on ITS region used for phylogenetic analysis

Çizelge 1. Filogenetik analiz için kullanılan ITS bölgesi bazında asmalardan izole edilen A. alternata'nın diğer fungal izolatları ile GenBank nükleotid erişimleri

Strain/Isolates *Tür/Izolatlar*	**Location** *Lokasyon*	**GenBank Accession No** *GenBank Kayıt No*
***Alternaria alternata* (in this study)**	Manisa -Turkey	KJ739870
***Alternaria alternata* (in this study)**	Salihli-Turkey	KJ739871
***Alternaria alternata* (in this study)**	Güzelköy-Turkey	KJ739872
***Alternaria alternata* (in this study)**	Salihli-Turkey	KJ739873
***Alternaria alternata* (in this study)**	Alaşehir-Turkey	KJ739874
***Alternaria alternata* (in this study)**	Manisa Merkez-Turkey	KJ739875
***Alternaria alternata* (in this study)**	Turgutlu-Turkey	KJ739876
***Alternaria alternata* (in this study)**	Manisa Merkez-Turkey	KJ739877
***Alternaria alternata* (in this study)**	Manisa Merkez-Turkey	KJ739878
***Alternaria alternata* (in this study)**	Harmandalı-Turkey	KJ739879
***Alternaria alternata* (in this study)**	Harmandalı-Turkey	KJ739880
***Alternaria alternata* CBS 130265**	India	KP124391
***Alternaria alternata* CBS 127672**	United State of America (USA)	KP124382
***Alternaria alternata* CBS 127334**	USA	KP124380
***Alternaria alternata* CBS 126908**	USA	KP124378
***Alternaria cerealis* CBS 119544**	Spain	KP125031
***Alternaria iridiaustralis* CBS 118487**	Australia	KP125060
***Alternaria iridiaustralis* CBS 118404**	New Zealand	KP125058
***Alternaria tomato* CBS 114.35**	Unknown	KP125070
***Alternaria gossypina* CBS 102601**	Colombia	KP125057
***Alternaria gossypina* CBS 100.23**	Unknown	KP125053
***Alternaria alternantherae* CBS 124392**	China	KC584506
***Colletotrichum boninense* CBS:128547**	New Zealand	JQ005159

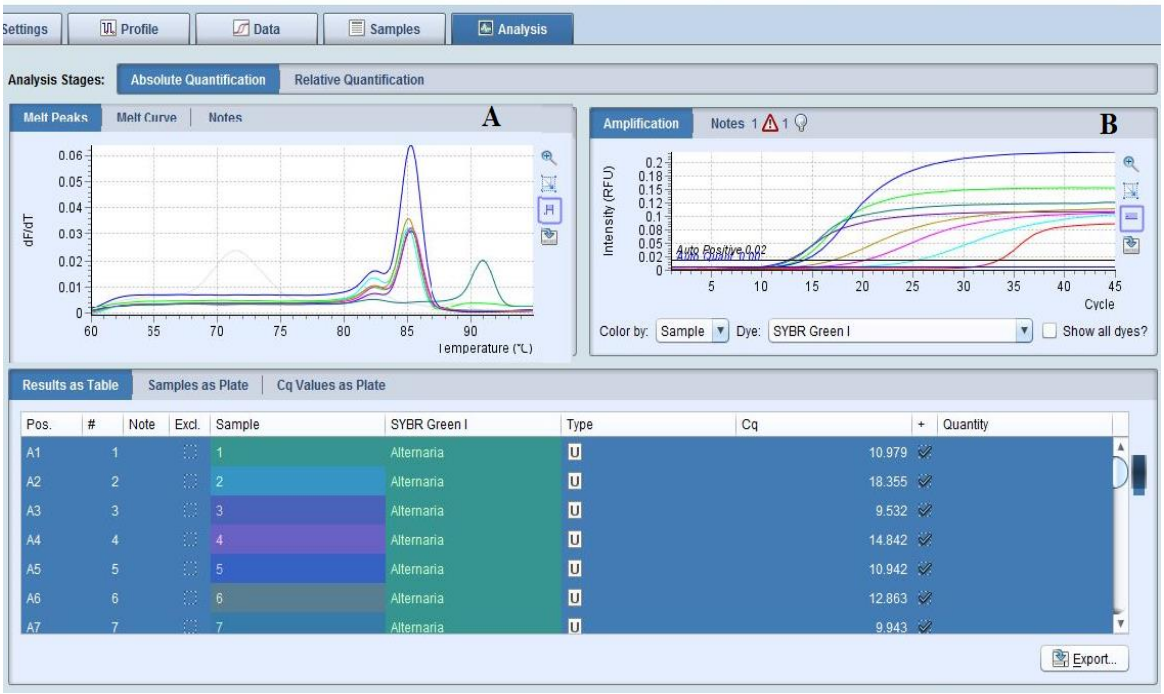

Figure 1. a) Melting curve temperature analysis of *A. alternata* by real-time PCR, b) Detection of *A. alternata* by real-time PCR with SYBR Green stain and ITS 4-5 primers.

Şekil 1. a) Real-Time PCR tarafından A alternata'nın erime eğrisi sıcaklığı analizi, b) SYBR Green boyası ve ITS 4-5 primerleri ile Real-Time PCR ile A alternata'nın tespiti.

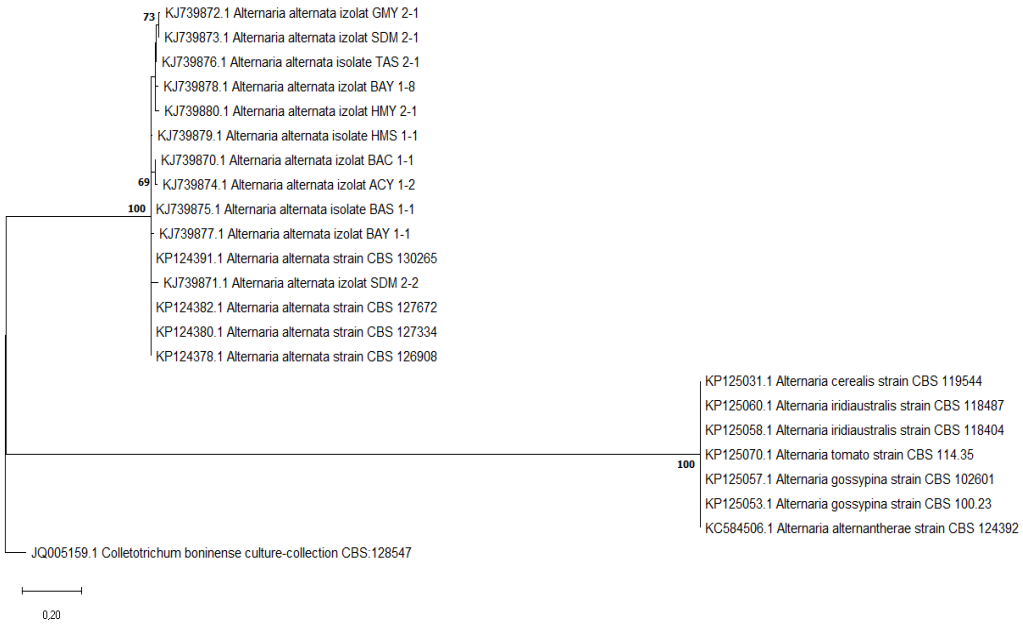

Figure 2. Maximum likelihood tree was plotted to show the phylogenetic relationship between the isolates in the study and other isolates in GenBank using the ITS4/ITS5 primer of A. alternata based on the ITS region sequence. Bootstrap values (expressed as a percentage of 1000 replicas) are shown in the nodes. The bar indicates the estimated 20/100 nucleotide substitutions per site.
Şekil 2. A. alternata'nın, ITS bölge sekansına dayalı olarak ITS4/ITS5 primeri kullanılarak çalışmadaki izolatlar ile GenBank'taki diğer izolatlar arasındaki filogenetik ilişkiyi göstermek için Maksimum benzerlik ağacı çizilmiştir. Bootstrap değerleri (1000 çoğaltma yüzdesi olarak ifade edilir) düğümlerde gösterilir. Ölçek çubuğu, her bölgede hesaplanan tahmini nükleotit değişiminin (20/100) olarak gösterir.

The ITS sequencing was a rapid and reliable method to identify different species of date palm fungal contamination; many published reports have proved the effectiveness and reliability of ITS products as a tool of fungal discrimination (Koç et al. 2020), including the species of *Aspergillus*, *Alternaria*, and *Penicillium* (Henry et al., 2000; Izumi et al., 2012; Pashley et al., 2012).

3.3. Pathogenicity of Alternaria alternata isolates

Alternaria isolates inoculum with a density of 1 x 10^6 spor ml^{-1} were given on the grapes with a micropipette of 10 µl. In the measurements made 6 days after inoculation, the tissues of the affected fruits shrank and turned brown. Grape berries flesh structure slightly changed but remained intact. Grape berries skins were dull and easily separated from the fruit flesh. Gray, white or black mycelial structures developed on the grape berries and covered the berries. It was determined that all isolates on the berries were pathogenic, and the lesion lengths were compared to the control (Table 2). BAÇ 1/2 isolate appeared to be R to azoxystrobin fungicide (EC_{50} value 70.67 µg/ml), and its lesion growth was 7.6 mm. BAÇ 1/2, SDM 2/1, GMK 2/1, TAS 2/1, AÇYS 1/4, AÇY 1/2, and BAÇ 1/1 isolates were observed to have high virulence levels (Table 2). It was observed that BAY 1/1 and BAS 1/2 showed lower virulence with lesion lengths of 0.13 and 0.40 mm, respectively. In this study, *A. alternata* was detected as a bunch rot pathogen in grapes for the first time. It was first reported in 2009 as an *A. alternata* bunch rot pathogen of the vine in Slovakia. It has been identified as a bunch rot pathogen in grapevines (Kakalíková et al., 2009). 6 isolates among the 24 tested for their sensitivity to fungicides were not taken to the virulence test. While some isolates achieved very slow mycelial development, 2 isolates achieved considerably well mycelia development; however, they had a low sporulation efficiency.

Table 2. ED_{50} values, lesion growth and virulence of fungicides resistant and fungicides sensitive isolates of *A. alternata*

Çizelge 2. A. alternata'nın fungisitlere dirençli ve fungisitlere duyarlı izolatlarının ED_{50} değerleri, lezyon gelişimi ve virülansı

Isolate names *İzolat İsmi*	**Fungicides** *Fungisitler*	**ED_{50} values (µg/ml)** *ED_{50} Değerleri (µg/ml)*	**Lesion growth on berry (mm) **** *Lezyon gelişimi (mm)***	**Percent Virulence (%)** *Virülanslık Yüzdesi (%)*
BAY 1/1	Mancozeb Cyprodinil+fludioxonil	91.33 0.04	0.134 c	6.66
BAY 1/6	Azoxystrobin Cyprodinil+fludioxonil	91.33 0.08	1.07 c	33.33
BAY 1/8	Azoxystrobin Cyprodinil+fludioxonil	98.67 0.05	1 c	33.33
BAY 1/9	Mancozeb Cyprodinil+fludioxonil	100 0.05	0.87 c	33.33
BAÇ ½	Azoxystrobin Cyprodinil+fludioxonil	70.67 0.2	7.6 a	100
GMY 2/1	Azoxystrobin Kresoxim-methyl + boscalid	100 0.07	1 c	46.66
AÇÇ 1/3	Mancozeb Cyprodinil+fludioxonil	70 2.81	0.93 c	40
KMÇ 1/5	Pyrimethanil Cyprodinil+fludioxonil	17.4 0.05	1.07 c	53.33
AÇY ½	Mancozeb Kresoxim-methyl + boscalid	70.933 0.02	2.60 bc	53.33
BAS ½	Azoxystrobin Cyprodinil+fludioxonil	86.67 0.19	0.4 c	6.66
BAS 1/1	Azoxystrobin Cyprodinil+fludioxonil	100 0.34	1.53 bc	46.66
AÇYS 1/1	Azoxystrobin Cyprodinil+fludioxonil	84 0.11	5.6 ab	46.66
AÇYS ¼	Azoxystrobin Cyprodinil+fludioxonil	100 0.09	3 abc	53.33
BAÇ 1/1	Azoxystrobin Cyprodinil+fludioxonil	100 0.08	2 bc	53.33
SDM 2/1	Azoxystrobin Cyprodinil+fludioxonil	90.67 0.02	7.53 a	100
HMY 2/1	Azoxystrobin Cyprodinil+fludioxonil	100 0.13	1.33 bc	33.33
TAS 2/1	Azoxystrobin Cyprodinil+fludioxonil	95 0.01	2.867 abc	80
GMK 2/1	Azoxystrobin Cyprodinil+fludioxonil	100 0.03	4 abc	86.67

* Each value is the average of three replications.

** Values within columns followed by different letters are significantly different according to Duncan's multiple range test (P = 0.01).

** Her değer, üç tekrarın ortalamasıdır.*

*** Farklı harflerle takip edilen sütunlardaki değerler Duncan'ın çoklu aralık testine göre önemli ölçüde farklıdır (P = 0.01)*

3.4. In vitro Determination of Fungicide Sensitivity on Mycelial Growth of Alternaria Alternata Isolates

As summarized in Figure 1, the ranges of ED_{50} values for the isolates varied between the different fungicides. The sensitivity tests showed that *A. alternata* isolates appeared more sensitive to cyprodinil+fludioxanil than pyrimethanil, kresoxim-methyl+boscalid mancozeb, azoxystrobin, iprodione, and basic copper sulfate (Figure 3). The ED_{50} value of three isolates was determined to be 0.01-0.03 µg ml^{-1} for cyprodinil+fludioxanil and between 0.03 and 0.1 µg ml^{-1} for 4 isolates. Cyprodinil+fludioxonil fungicide was the most effective fungicide preventing mycelial growth in *A. alternata* isolates. The highest value of ED_{50} for this fungicide was ≤ 1µg ml^{-1}. The ED_{50} value of six isolates for kresoxim-methyl + boscalid appeared to be 0.1-1 µg ml^{-1}, while it ranged from 1 to 3 µg ml^{-1} for the seven other isolates. The ED_{50} values of 10 isolates for the fungicide azoxystrobin ranged between 30 and 100 µg ml^{-1}.

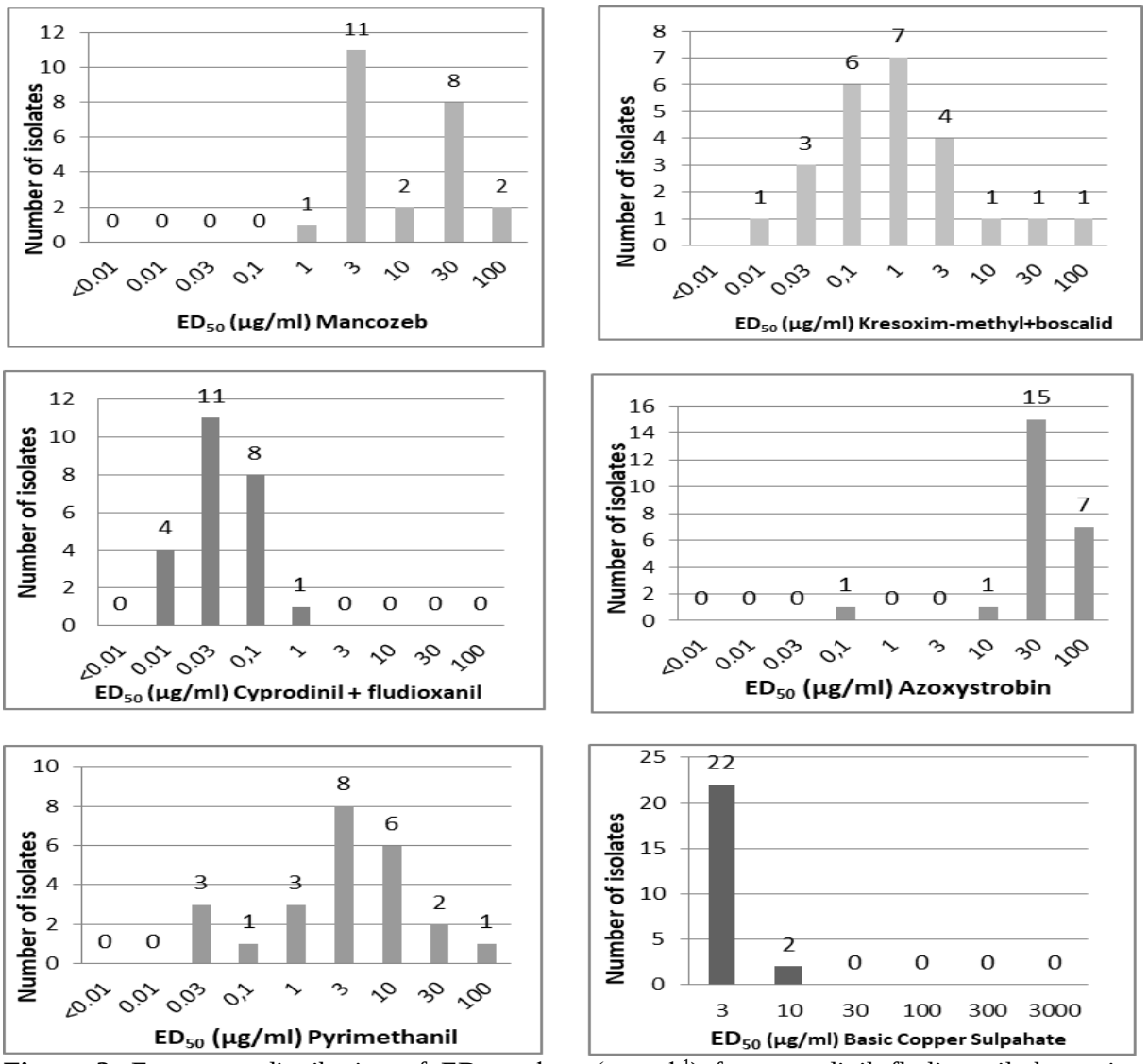

Figure 3. Frequency distribution of ED_{50} values (µg ml^{-1}) for cyprodinil+fludioxonil, kresoxim-methyl+boscalid, mancozeb, pyrimethanil, azoxystrobin and basic copper sulphate determined with *A. alternata* isolates.

Şekil 3. A. alternata izolatları ile belirlenen cyprodinil+fludioxonil, kresoxim-methyl+boscalid, mancozeb, pyrimethanil, azoxystrobin ve bazik bakır sülfat için ED_{50} değerlerinin (µg ml^{-1}) frekans dağılımı.

Azoxystrobin can prevent lesions caused by powdery mildew and *Phomopsis viticola* and prevent *B. cinerea* colonizing in these parts. Iprodione, registered for *B. cinerea*, is a very important fungicide in controlling bunch rots in grapevines. It is categorized in the dicarboximide group. Efficacy tests of iprodione on the mycelial growth of *A. alternata* showed that ten isolates were sensitive with an ED_{50} value of more than 0.1< µg ml^{-1}. Six isolates, however, were observed to develop resistance with an ED_{50} value ranging between 1≤ and 10≥ µg ml^{-1}. The ED_{50} values of seven isolates for mancozeb and eleven isolates for basic copper sulfate were between 3 and 10 µg ml^{-1} (Figure 3). Copper compounds are the most widely used fungicides in Turkey. The ED_{50} values of basic copper sulfate for *A. alternata* isolates were determined to be between 3 and 10.73 µg ml^{-1}.

In the current study, the fungicides containing fludioxonil+cyprodinil and kresoxim-methyl + boscalid showed a highly inhibitory effect at 0.01-3.0 µg ml^{-1} concentration (Figure 3). However, azoxystrobin, basic copper sulfate, mancozeb, iprodione, and pyrimethanil had an inferior effect on the mycelial growth of *A. alternata* isolates compared to fludioxonil and tebuconazole. According to other studies carried out in vineyards and greenhouses, some *B. cinerea* isolates (EC_{50} value 2.90-4.84 µg ml^{-1}) collected at harvest were resistant against cyprodinil after 4-5 years of usage of cyprodinil and fludioxonil. There was no

change in the sensitivity of isolates against fludioxonil. Therefore it was reported that these two fungicides would be more effective if used as a mixture of cyprodinil+fludioxanil (Forster and Staub, 1996; Leroux et al., 2002). This supports collected without any mutagen (Lorenz and Eichhorn, 1978; Delen, 2008). Resistance to dicarboximide group fungicides has been observed to appear very slowly in vineyards and greenhouses contrary to laboratory conditions (Prusky et al., 1983). Although adaptation of *B. cinerea* to copper sulphate in culture conditions has been reported (Pary and Wood, 1958), no resistance problems have been reported, which could cause a decrease in the efficacy of copper-containing fungicides on fungal organisms. In the study, the ED_{50} values of *A. alternata* isolates obtained from the vineyard were determined to be very high against copper sulfate in vitro. It was thought that adaptation might have occurred.

3.5. Effectiveness of Fungicides on Selected Isolates

Effectiveness studies show the ability of *A. alternata* R and S isolates to cause infection on fruits, as indicated in Table 3. Efficacy tests showed that the active ingredients in cyprodinil+fludioxanil were the most effective fungicides against R (80%) and S (57.14%) isolates (Table 3). Only cyprodinil is not licensed for use against *B. cinerea* in Turkey. In a study carried out in 1994, a decline was observed in the sensitivity of *B. cinerea* isolates collected from vineyards where cyprodinil was applied four times (Delen, 2008). Baroffio et al. (2003) observed a decline in the sensitivity against cyprodinil of isolates while reporting no change in isolates' sensitivity against fludioxonil in seven years of observations. Pyrimethanil was the most effective fungicide against R (63.64%) and S (66.67%) isolates in berry tests (Table 3). As a result, it was determined that anilinopyrimidine fungicides should be used carefully in controlling bunch rot pathogens, and they should also be used following anti-resistance strategies.

The average efficacy level in an application dosage of 59.99 % to R-isolates and 25.00% to S-isolates of kresoxim-methyl+boscalid against *Alternaria* spp. in grape tests reinforce the idea the conclusion that cyprodinil+fludioxanil was effective against *A. alternata* and therefore should be used together. It was determined under laboratory conditions that isolate resistant to dicarboximide group fungicides can be easily
that it should be used in early powdery mildew spraying periods when latent infections are also observed.

It was determined in this study that the application dosage of iprodione was more effective (66.67% to R-isolate and 50% to S-isolate) in grape tests after cyprodinil+fludioxanil.

The other efficient fungicide which belongs to the dithiocarbamate group and has a very wide range of efficiency is mancozeb. It is considered to have no resistance problems because it has no specified area of influence. However, recent studies have suggested an opposite idea because it has been used for many years (Lorbeer and Vincelli, 1990). An increase in the sensitivity of *B. cinerea* isolates to thiram and mancozeb over time has been found. It has been understood experimentally that these fungicides fail to prevent growth in the isolates, with decreased sensitivity through recommended dosages (Delen et al., 1984, 2000). The effectiveness of all concentrations of mancozeb, azoxystrobin, and basic copper sulfate on fungicides-S and fungicides-R was determined to be less than 50%. As indicated in Table 3, it was determined that azoxystrobin fungicide-R (0.0%) and S (0.0%) isolates were not sufficiently effective in their suggested concentrations. Adaptation of *Alternaria* spp. isolates to nature were detected through leaf and grape tests. Ranganath et al. (2003) reported that conidia of *Alternaria* spp., which had a very dense concentration in the atmosphere, achieved a very weak and low growth on leaves.

4. Conclusions

In the current study, *A. alternata* has been identified as a significant pathogen associated with bunch rot disease in Turkey vineyards for the first time. We have also detected that cyprodinil+fludioxonil containing fungicide was very effective, and berries treatment inhibited the growth of this fungus on the grape in laboratory conditions.

Table 3. Effectiveness of fungicides against sensitive and resistant isolates of *A. alternata* on berries.
Çizelge 3. Tane üzerinde A. alternata'nın duyarlı ve dirençli izolatlarına karşı fungisitlerin etkinliği.

Fungicides ***Fungisitler***	**Isolate and Phenotype*** ***İzolat ve Fenotipi****	**ED_{50} value (µg ml^{-1})** ***ED_{50} değeri (µg ml^{-1})***	**Concentration (µg a.i ml^{-1})**** ***Konsantrasyon (µg a.i ml^{-1})****	**LesionGrowth (mm)***** ***LezyonGelişmi (mm)*****	**Percent Disease (%)***** ***Hastalık Yüzdesi (%)*****	**Percent Inhibition (%)** ***Engelleme Yüzdesi (%)***
Cyprodinil+ fludioxonil	AÇÇ 1/3 (R)	2.81	125	0.52 a	20.00 a	80
			62.5	2.25 a-e	53.33 a-d	46.67
			32.15	3.23 a-h	66.67 a-d	33.33
			0	7.72 ı-k	100.00 d	0
	GMK 2/1 (S)	0.01	125	1.50 a-d	40.00 a-d	57.14
			62.5	2.38 a-e	60.00 a-d	35.71
			32.15	2.78 a-g	66.67 a-d	28.57
			0	6.78 h-k	93.33 cd	0
Pyrimethanil	KGM 1/1 (R)	100	250	1.40 a-d	26.67 cd	63.64
			125	2.54 a-f	33.33 abc	54.55
			62.5	1.67 a-d	46.67 a-d	36.36
			0	3.53 a-h	73.33 a-d	0
	AÇY 1/2 (S)	0.05	250	1.00 ab	26.67 cd	66.67
			125	1.20 abc	40.00 a-d	50
			62.5	1.87 a-d	53.33 a-d	33.33
			0	3.67 a-ı	80.00 a-d	0
Kresoxim-methyl+ boscalid	KGM 1/1 (R)	100	75	0.67 a	26.67 cd	59.99
			37.5	1.40 a-d	33.33 abc	50
			18.75	3.87 a-i	40.00 a-d	40
			0	4.47 a-i	66.67 a-d	0
	AÇY 1/2 (S)	0,02	75	1.80 h-l	60.00 a-d	25
			37.5	2.00 a-d	60.00 a-d	25
			18.75	2.33 a-f	66.67 a-d	16.67
			0	3.73 a-i	80.00 a-d	0
Mancozeb	KGM 1/1 (R)	100	500	7.00 h-k	66.67 a-d	9.09
			250	3.93 a-i	46.67 a-d	36.36
			125	8.40 ijk	66.67 a-d	9.09
			0	7.66 ijk	73.33 a-d	0
	TAS 2/1 (S)	2,16	500	2.53 a-f	73.33 a-d	26.67
			250	3.27 a-h	86.67 bcd	13.33
			125	4.67 b-j	93.33 cd	6.67
			0	4.93 b-j	100.00 d	0
Azoxystrobin	BAS 1/1 (R)	100	187.5	6.87 h-k	93.33 cd	0
			93.75	6.40 f-k	80.00 a-d	14.29
			46.87	5.87 e-k	66.67 a-d	28.57
			0	6.93 h-k	86.67 abc	0
	AÇY 1/2 (S)	0,68	187.5	4.87 b-j	93.33 cd	0
			93.75	5.13 c-j	80.00 a-d	14.29
			46.87	4.27 a-i	86.67 bcd	7.14
			0	5.33 d-j	93.33 cd	0
Basic copper sulfate	AÇYS 1/1 (R)	11,23	1000	8.40 jkl	100.00 d	0
			500	7.00 h-k	93.33 cd	6.67
			250	9.66 kl	93.33 cd	6.67
			0	11.46 l	100.00 d	0
	AÇÇ 1/3 (S)	0,03	1000	4.00 a-i	66.67 a-d	33.33
			500	5.07 c-j	86.67 bcd	13.33
			250	4.93 a-j	86.67 bcd	13.33
			0	6.60 g-k	100.00 d	0

*R, isolate with highest ED_{50} value; S. isolate with lowest ED_{50} value. **Quarter, half and recommended commercial dose of fungicides, respectively. ***Values within columns followed by different letters are significantly different according to Duncan's multiple range test (P = 0.05).

****Efficacy data calculated according to Abbott's formula with respect to percent inhibit.

**R, en yüksek ED_{50} değerine sahip izolat; S, en düşük ED_{50} değerine sahip izolat. ** Fungisitlerin sırasıyla çeyrek, yarım ve önerilen ticari dozu.*

****Farklı harflerle takip edilen sütunlardaki değerler, Duncan'ın çoklu karşılaştırma testine göre önemli ölçüde farklıdır (P = 0.05).*

*****Yüzde engellemeye göre Abbott formülüne göre hesaplanan etkinlik verileri*

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6. Conflict of Interest

Under personal responsibility, the authors declare the absence of a conflict of interest.

7. Declaration of Author Contribution

N.G. Savas: Developed the theory and performed computations, writing the manuscript; **F. Yıldız:** Provided in theory, research and consultancy; and **N. Delen:** Provided consultancy.

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