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Research Article

MOLECULAR CHARACTERIZATION OF BLACK FOOT DISEASE PATHOGENS IN GRAPEVINE NURSERIES AND EVALUATION OF SOME FUNGICIDES FOR CONTROL OF THE MOST VIRULENT ISOLATES

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Abstract: Black foot disease, which is mainly caused by *Ilyonectria* Chaverri & Salgado and *Dactylonectria* L. Lombard & Crous species, causes serious destruction in most of the wine and grape producing regions of the world, particularly in nurseries and young vineyards. The aim of this research was to evaluate the present situation of black foot disease in grapevine nurseries (*Vitis Vinifera* L.), to identify its potential agents at morphological and molecular levels and to develop a control strategy against isolates found as the major virulent ones in pathogenicity tests. This study also represents the first attempt to control black foot disease in grapevine saplings in Turkey. Preliminary surveys were carried out in 21 grapevine nurseries in the Aegean Region during 2019 growing season. Black foot species obtained from vine saplings were identified as *Ilyonectria liriodendri* (Halleen, Rego & Crous), *Dactylonectria macrodidyma* (Halleen, Schroers & Crous) and *D. torresensis* (A. Cabral, Rego & Crous), using morphological and molecular methods. The pathogenicity studies revealed that the most virulent isolates belonged to *I. liriodendri*. Twelve chemical and one biological fungicides were screened *in vitro* for mycelial inhibition of *I. liriodendri* isolates. The experiments were performed in three replicates for each of the chemical fungicides. Sterile distilled water, instead of fungicide solution, was used for control trials. Mixtures of cyprodinil + fludioxonil, thiabendazole + fludioxonil + metalaxyl–m + azoxystrobin and azoxystrobin + metalaxyl-m + fludioxonil were the most effective chemical fungicides tested in mycelial inhibition of *I. liriodendri* isolates. Chemical fungicides mixtures of boscalid + kresoxim-methyl, promocarb hydrochloride (HCL) + fosetyl-al, azoxystrobin + difenoconazole, fludioxonil + metalaxyl–m and individual fungicides; cyprodinil, fluoxapyroxad, hymexazole, fenhexamid and 8-hydroxyquinoline sulphate were not effective on mycelial inhibition. Biocontrol activity of *Trichoderma harzianum* Rifai KRL-AG2 strain against *I. liriodendri* was evaluated. The experiment was performed in four replicates for each isolate. Petri dishes with only *I. liriodendri* mycelial discs used as controls. Treatment with *Trichoderma harzianum* Rifai KRL-AG2 strain provided 60.1% to 80.6% inhibition against *I. liriodendri* isolates. The most effective fungicides tested *in vitro* were evaluated in 2020 and 2021 under controlled conditions against possible infections of *I. liriodendri* during the rooting process of 1103 Paulsen grapevine rootstocks. Mixtures of thiabendazol + fludioxonil + metalaxyl–m + azoxystrobin and cyprodinil + fludioxonil were the most effective fungicides in the experiments conducted in 2020 and 2021, followed by azoxystrobin + metalaxyl-m + fludioxonil and *Trichoderma harzianum* Rifai KRL-AG2 strain.

Özet: Esas olarak *Ilyonectria* Chaverri & Salgado ve *Dactylonectria* L. Lombard & Crous türlerinin neden olduğu karabacak hastalığı, dünyanın şarap ve üzüm üreten bölgelerinin çoğunda, özellikle fidanlıklarda ve genç bağlarda ciddi yıkımlara sebep olmaktadır. Bu çalışma ile karabacak hastalığının, asma fidanlıklarındaki (*Vitis Vinifera* L.) durumunun saptanması, etmenlerinin morfolojik ve moleküler düzeyde tanılanması ve patojenisite testlerinde en virülent olarak belirlenen türlerin mücadelesi amaçlanmıştır. Bu çalışma aynı zamanda Türkiye'de asma fidanlarında karabacak hastalığının mücadelesine yönelik olarak ilk girişimi temsil etmektedir. İlk olarak, 2019-2020 yıllarında Ege Bölgesi'ndeki 21 asma fidanlığında sürveyler gerçekleştirilmiştir. Asma fidanlarından elde edilen karabacak türleri, morfolojik ve moleküler

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yöntemler kullanılarak *Ilyonectria liriodendri* (Halleen, Rego & Crous), *Dactylonectria macrodidyma* (Halleen, Schroers & Crous) ve *D. torresensis* (A. Cabral, Rego & Crous) olarak tanımlanmıştır. Patojenisite çalışmaları sonucunda en virülent izolatların *I. liriodendri* türüne ait olduğu belirlenmiştir. Şu ana kadar Türkiye'de karabacak hastalığına karşı ruhsatlı bir fungisit bulunmamaktadır. On iki kimyasal ve bir biyolojik fungisit, *I. liriodendri* izolatlarının miselyal inhibisyonu için *in vitro* olarak taranmıştır. Deney, her bir kimyasal fungisit için üç tekerrür halinde gerçekleştirilmiştir. Kontroller için fungisit solüsyonu yerine steril distile su kullanılmıştır. Cyprodinil + fludioxonil, thiabendazole + fludioxonil + metalaxyl–m + azoxystrobin ve azoxystrobin + metalaxyl-m + fludioxonil karışımları, *I. liriodendri* izolatlarının miselyal inhibisyonunda test edilen en etkili kimyasal fungisitler olmuştur. Kimyasal fungisit karışımları boscalid + kresoxim-methyl, promocarb hidroklorür (HCL) + fosetyl-al, azoxystrobin + difenoconazole, fludioxonil + metalaxyl–m ve tekli fungisitler cyprodinil, fluoxapyroxad, hymexazole, fenhexamid ve 8-hidroksikinolin sülfat, *I. liriodendri*'nin miselyal inhibisyonu üzerinde etkili bulunmamıştır. *Trichoderma harzianum* Rifai KRL-AG2 ırkının, *I. liriodendri*'ye karşı *in vitro* biyokontrol aktiviteside değerlendirilmiştir. Deney, her izolat için dört tekrarlı olarak gerçekleştirilmiştir. Sadece *I. liriodendri* miselyal diskleri olan petri kapları kontrol olarak kullanılmıştır. Denemede, *Trichoderma harzianum* Rifai KRL-AG2 ırkı ile muamele *I. liriodendri* izolatlarına karşı %60.1 ve %80.6'lık miselyal inhibisyon sağlamıştır. Ayrıca *in vitro*'da test edilen en etkili fungisitler, 1103 Paulsen asma anaçlarının köklenme işlemi sırasında *I. liriodendri*'nin olası enfeksiyonlarına karşı kontrollü koşullarda 2020 ve 2021 yıllarında test edilmiştir. 2020 ve 2021 yıllarında yapılan deneylerde thiabendazol + fludioxonil + metalaxyl–m + azoxystrobin ve cyprodinil + fludioxonil en etkili fungisitler olmuş, bunu azoxystrobin + metalaxyl-m + fludioxonil ve *Trichoderma harzianum* Rifai KRL-AG2 ırkı izlemiştir.

Introduction

Turkey is one of the key countries with an active role in world grape production. Turkey is the world's largest raisin producer and exporter with a production of 1 million 188 thousand tons of seedless raisins. Raisins are mostly produced in the Aegean Region, particularly in Manisa (87%), İzmir (9%) and Denizli (4%) provinces (TÜİK 2021). The Aegean Region, which is the most important viticulture region of Turkey, also holds approximately 80% of the country's grafted grapevine saplings producing facilities and most of these nurseries are located in Manisa and Denizli provinces (Dardeniz *et al.* 2015).

A number of diseases are known to cause economical losses by negatively effecting the production and quality of the grape berries (Ari & Tosun 2004, Atak *et al.* 2017, İçli & Tahmas 2020). Black foot disease directly causes the death of vines in nurseries and young vineyards around the world, causing serious economic losses, one of which is replanting costs (Cabral *et al.* 2012c, Úrbez-Torres *et al.* 2014).

The fungal genera *Ilyonectria* Chaverri & Salgado and *Dactylonectria* L. Lombard & Crous include the major species of black foot disease affecting grapevines (*Vitis* spp.) worldwide (Lombard *et al.* 2014). Recently, the prevalence of this disease has increased dramatically in main viticulture areas, including South Africa, New Zealand, Italy, Spain, Portugal, Australia, North America and Brazil (Halleen *et al.* 2004, Petit & Gubler 2005, Alániz *et al.* 2009, Cabral *et al.* 2012c, Úrbez-Torres *et al.* 2014, Dos Santos *et al.* 2014).

Black foot pathogens survive in soil, plant debris or vine propagation material and are transported to other vineyards mostly by infected cuttings (Waite *et al.* 2004). Generally, new vineyard plantations seem normal during planting, but variances in vigour could be noticed including declined caliper size of the trunk, shortened internodes, declined foliage, and declined leaf size. Beginning of foliar symptoms may be seen as severe leaf blight, followed by necrosis and early defoliation following 3 to 5 years after planting. Wilting and dieback may also occur. Irregular wood maturity, often linked with quick dehydration, is an additional prevalent indicator. The cross-section investigation of trunks of the weakening grapevines, reveals dark brown to black zipping apparent in the vascular parts because of plugging of single or aggregates of xylem vessels with amber to black gum (gummosis) and establishment of tyloses. Necrosis spreading from the bark to the pith is typical of black foot disease (Gramaje *et al.* 2018).

Campylocarpon fasciculare (Schroers, Halleen & Crous), *Ilyonectria liriodendri* (Halleen, Rego & Crous), *Dactylonectria alcacerensis* (A. Cabral, Oliveira & Crous), *D. torresensis* (A. Cabral, Rego & Crous), *Cylindrocarpon macrodidymum* (Halleen, Schroers & Crous), *Cylindrodendrum alicantinum* (C. Agust-Brisach, J. Armengol & A. Cabral) and *Pleiocarpon strelitziae* (L. Lombard & D. Aiello) have been reported sof far as species associated with grapevine black foot disease in Turkey (Özben *et al.* 2012, Akgül *et al.* 2014, Güngör-Savaş *et al.* 2015, 2020, Özben 2020).

In nurseries where there are many possibilities for infection by black foot pathogens, there has recently been a particular focus on the development of certain procedures and products in many countries to prevent or reduce the infection of woody tissues with these pathogens. There exists no rootstocks known to be fully resistant against black foot pathogens. Therefore, preventive measures are applied in sapling production process to ensure the use of healthy plants and to reduce the spread of the disease. More recently, researches have specifically focused on fungicides and biological control agents to prevent or reduce black foot disease infection of the woody tissues of vine (Halleen & Fourie 2016, Gramaje *et al.* 2018). However, there are no reports covering the countermeasures against black foot disease

in Turkey, and there are no registered fungicide that can be used to control, and prevent the disease. Therefore, the prevalence of the disease is increasing rapidly.

Rego *et al.* (2006) found that treating the roots of vines with mixtures of benomyl, tebuconazole, and carbendazim with flusilazole and cyprodinil with fludioxonil prior to transferring them to disease-inoculated media significantly reduced black foot disease severity. Recently, the worlds' attention has turned to finding sustainable, safe and environmentally friendly options in the control of the disease. Biological control agents refer to the use of certain microorganisms to overpower the development of plant pathogens (Pal & McSpadden Gardener 2006). The most commonly used and recommended biological control agents in the management of black foot disease were reported as *Trichoderma* Pers. species (Santos *et al.* 2015). It has been found that *Trichoderma* members promote root growth of the plants which were predisposed to black foot disease and make them less vulnarable (Fourie & Halleen 2001, Agustí-Brisach & Armengol 2013).

In the present study, we aimed to determine the species that cause black foot disease in grapevine nurseries (*Vitis vinifera* L.) in the Aegean Region by morphological and molecular methods. There is no registered fungicide used to control black foot pathogens in grapevine nurseries or young vineyards in Turkey. Therefore, we also evaluated, the *in vitro* efficacy of 12 chemicals and one commercial biological fungicide against *I. liriodendri*, which was obtained from grapevine nurseries in Aegean Region and was found to be the most virulent in pathogenicity tests, was evaluated. Fungicides found to be effective *in vitro*, were also tested *in vivo* to establish their potential to prevent infections caused by *I. liriodendri* isolates during the rooting stage of the vine.

Materials and Methods

Materials

The black foot isolates were obtained from grapevine saplings with different rootstock types from 21 different nurseries in Manisa and Denizli provinces (Table 1).

Table 1. Samples and rootstock types of Sultani Seedless grapevine saplings collected from Manisa and Denizli provinces and used in disease isolation studies.

District	Province	Number of Nurseries Sampled	Rootstock Type
Şehzadeler	Manisa	3	1613 (1)*, 5 BB (1), 1103P (1)
Yunusemre	Manisa	6	1103P (3), 5 BB (3)
Akhisar	Manisa	1	Sultani
Turgutlu	Manisa	1	1103P
Salihli	Manisa	3	41 B (1), Sultani (2)
Alaşehir	Manisa	4	Sultani (4)
Sarıgöl	Manisa	2	Sultani (2)
Çal	Denizli	1	41 B

* Distribution of the number of producers by rootstock type

The isolates were stored as pure cultures in 1.5 mL Eppendorf tubes (20% glycerol-water) at -80 °C before use. Potato Dextrose Agar (PDA), (Oxoid), Malt Extract Agar (MEA) and Synthetic Nutrient Agar (SNA) were used for isolation and identification of fungi. In pathogenicity tests, 1103 Paulsen, which is the most planted rootstock of the Aegean Region, was used. The properties of fungicides listed in Table 2 were evaluated *in vitro* and those in Table 3 *in vivo* against *Ilyonectria liriodendri*.

Table 2. Fungicides used *in vitro* against *I. liriodendri* isolates.

Active ingredient (a.i)	Formulation*	Commercial Name/Firm
Cyprodinil %37.5 + Fludioxonil %25	WG	Switch 62.5 WG, Syngenta
Cyprodinil %50	WG	Vektör 50 WG, Agri Sciences
Boscalid 200 g/L + Kresoxim-methyl 100 g/L	SC	Collis SC-BASF
Thiabendazole 300 g/L + Fludioxonil 37.5 g/L + Metalaxyl -M 30 g/L + Azoxystrobin 15 g/L	FS	Maxim Quattro FS, Syngenta
Fludioxonil 25 g/L + Metalaxyl -M 10 g/L	FS	Maxim XL 035 FS, Sygenta
Azoxystrobin 75 g/L + Metalaxyl -M 37.5 g/L + Fludioxonil 12.5 g/L	FS	Dynasty CST 125 FS, Syngenta
Trichoderma harzianum Rifai KRL-AG2	WP	T-22 Planter Box, Bioglobal
Fluoxapyroxad 300 g/L	SC	Sercadis-BASF
Promocarb Hydrochloride (HCL) 530 g/L + Fosetyl-Al 310 g/L	SL	Previcur Energy SL 840, Bayer
Hymexazole 360 g/L	SL	Tachigaren 30 L, Sumi agro
8-hydroxyquinoline sulphate 500 g/L	SL	Beltanol L, Probelte S.A
Fenhexamid 500 g/L	SC	Teldor SC 500, Bayer
Azoxystrobin 200 g + Difenoconazole 125 g	SC	Quadris Maxx, Syngenta

*WG: Water-dispersible granule, WP: Wettable powder, SC: Water-soluble concentrate, SL: Soluble concentrate, FS: Fluid concentrate for seed treatment.

Table 3. Fungicides used *in vivo* trials.

Active ingredient (a.i)	Trade name	Dose of application	Field rate
Cyprodinil 37.5 %+ Fludioxonil 25%	Switch 62.5 WG	50 g/100 L water	1.00 g/L
Thiabendazole 300 g/L + Fludioxonil 37.5 g/L + Metalaxyl -M 30 g/L + Azoxystrobin 15 g/L	Maxim Quattro FS	8.5 mL/unit 50.000 seeds	0.40 mL/L
Azoxystrobin 75 g/L + Metalaxyl -M 37.5 g/L + Fludioxonil 12.5 g/L	Dynasty CST 125 FS	250 mL/da	0.50 mL/L
Trichoderma harzianum Rifai KRL-AG2	T22 Planter Box	7.5 g/ 1 kg seed	2.00 g/L

Nursery survey and fungal isolation

Surveys were carried out in 21 grapevine nurseries producing Sultani Seedless in Manisa and Denizli provinces between April and June 2019. 10 vine samples were collected from each nursery for mycological analysis. Since it is known that the disease is also transmitted through healthy saplings, sampling was distributed randomly and performed in the direction of diagonals in the nurseries (Bora & Karaca 1970).

Firstly, the sapling samples were washed under running water and dried with sterile paper towels. For the purpose of isolation of black foot pathogens, sections (1 cm^2) were taken from the root, rootstock, grafting union and inner tissues of the trunk region of the grape saplings, which have typical colour changes and show brown spots, partially diseased and healthy tissue. Removed tissue pieces were sterilized by soaking in 0.5% sodium hypochlorite (NaOCl) solution for three minutes, rinsed twice in distilled water and dried under laminar airflow. Tissues were placed on PDA modified with streptomycin-sulfate (150 mg/L) and the petri dishes were incubated at 24°C for three days. Growing colonies were purified and representative isolates with different colony morphology were archived and stored at -80°C in 1.5 mL sterile eppendorf tubes (20% glycerol-water) for further diagnostic studies.

Determination of disease prevalence and incidence

According to the diagnosis of black foot disease isolates obtained from nurseries, the disease prevalence and incidence at provincial and district level were calculated according to the formulas [1 and 2] below (Bora & Karaca 1970).

$$\text{Prevalence (\%)} = \frac{\text{Number of Diseased Nurseries}}{\text{Total Number of Nurseries Sampled}} \times 100 \quad [1]$$

$$\text{Incidence (\%)} = \frac{\text{Number of Diseased Grapevine Saplings}}{\text{Total Number of Grapevine Saplings}} \times 100 \quad [2]$$

Morphological identification and characterization

Pure cultures stored at -80 ˚C were planted in PDA medium for morphological and microscopic diagnosis and the cultures were kept for seven days in the incubation chamber at 22 ± 2 ˚C under 12 hours daylight and darkness regime. Conidial dimensions (length and width of 40 conidia per isolate) were measured with a microscope camera software (Olympus BX-51 and Olympus Camedia-4501X). The isolates associated with black foot disease were identified based on colony colour, colony morphology, macroconidia, microconidia, conidiophore and chlamydospore structures and pigmentation as described by Halleen *et al.* (2004) and Alániz *et al.* (2007).

Molecular identification and phylogenetic analyses

Total genomic DNA was obtained with the extraction protocol described by Cenis (1992) for molecular identification. Universal primers that amplify the histone H3 (Crous *et al.* 2004), Internal Transcribed Spacer (ITS) (White *et al.* 1990) and β-tubulin (Glass & Donaldson 1995) regions of fungi were targeted to identify the isolates in PCR tests. 15 µl Roche FastStart Essential DNA Green Master Mix, 11.1 µl nuclease-free water, 0.45 µl forward and reverse primer (20 mM concentration), and 3 µl DNA were added to the PCR tubes. DNA samples were replicated with the amplification protocol presented in Table 4 and the Real-Time PCR system (Roche Light Cycler® Nano) for the 3 primers. PCR products were sequenced by Macrogen Inc., Sequencing Center (The Netherlands, Europe). Reliability of the sequences was guaranteed by sequencing the amplicons in both directions by applying the same primer pairs used for amplification. The sequencing data chromatogram files were analyzed with ChromasPro 2.1.9 chromatogram analysis software, and consensus sequences were developed by combining the sequencing data for forward and reverse sequences. Consensus sequences were compared with reference sequences in the National Center for Biotechnology Information (NCBI) Genbank database, using the Basic Local Alignment Search Tool (BLAST).

Analyses were conducted according to Maximum Likelihood (ML) and were performed with a combined alignment of the three loci. GenBank sequences from different species were selected in consonance with their high similarity compared to our query sequences (Table 5). Isolate sequences selected for phylogenetic analysis were first aligned with the Clustal W routine in MEGAX software (Thompson *et al.* 1994). The sequences of each loci aligned individually were combined into a single file. The Maximum Likelihood analysis (ML) determining the

Table 4. Primers and PCR cycles used in Real Time PCR studies.

Primer	Primer Sequence (5'-3')	Product size (bp)	Real-time PCR cycles
ITS1: **ITS4:**	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	700	1 X (95°C 10 dk) 35 X (95°C 20 sn / 53 °C 20 sn / 72°C 20 sn)
Bt2a: **Bt2b:**	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	650	1 X (95°C 10 dk) 35 X (95°C 20 sn / 60 °C 20 sn / 72°C 20 sn)
CYLH3-F: **CYLH3-R:**	AGGTCCACTGGTGGCAAG AGCTGGATGTCCTTGGACTG	500	1 X (95°C 10 dk) 35 X (95°C 20 sn / 53°C 20 sn / 72°C 20 sn)

Table 5. Reference species used for phylogenetic analysis.

Isolate number and the species		Host	Origin	GenBank Accession Numbers*		
				His3	ITS	BT
CBS 117640	*Ilyonectria liriodendri*	*Vitis vinifera*	Torres Vedras, Portugal	JF735510	DQ178166	DQ178173
CBS 112596		*V. vinifera*	Western Cape, S. Africa	JF735511	AY677264	AY677239
CBS 112607		*V. vinifera*	Western Cape, S. Africa	JF735512	AY677269	AY677241
CBS 112603	*Dactylonectria macrodidyma*	*V. vinifera*	Western Cape, S. Africa	JF735645	AY677285	AY677232
CBS 112605		*V. vinifera*	Malmesbury, S. Africa	JF735646	AY677287	AY677230
CBS 112615		*V. vinifera*	Malmesbury, S. Africa	JF735647	AY677284	AY677229
CBS 129086	*D. torresensis*	*V. vinifera*	Torres Vedras, Portugal	JF735681	JF735362	JF735492
CBS 112598		*V. vinifera*	Western Cape, S. Africa	JF735662	JF735351	JF735479
Cy133	*D. alcacerensis*	*V. vinifera*	Valencia, Spain	JF735628	JF735331	JF735459
CBS 100819	*D. pauciseptata*	*Erica melanthera*	New Zealand	JF735582	EF607090	EF607067
CBS 120171		*Vitis sp.*	Krsko, Slovenia	JF735587	EF607089	EF607066
CBS 112613	*Campylocarpon fasciculare*	*V. vinifera*	Western Cape, S. Africa	JF735502	AY677301	AY677221
CBS 112679	*Ca. pseudofasciculare*	*V. vinifera*	S. Africa	JF735503	AY677306	AY677214

* His3: Histone H3, ITS: Internal Transcribed Spacer, BT: β-tubulin.

best nucleotide substitution model settings for each locus was performed with MEGAX 10.1.7 (Kumar *et al.* 2018). The statistical reliability of the phylogenetic tree and certain sequences in the tree were determined with the Bootstrap method in 1000 repetitions. *Campylocarpon fasciculare* (CBS 112613) and *Ca. pseudofasciculare* (CBS 112679) were used as outgroups in phylogenetic analyses

Pathogenicity tests

Cuttings of 1103 Paulsen grapevine rootstocks were used to determine the virulence of pathogens causing black foot disease. 14-day-old fungal cultures with three different colony growth cultured at 20 ± 2°C in PDA medium were used for inoculation. Each isolate was inoculated onto five test plants and the trials were conducted with a randomized plot design in five replicates. Each cutting was accepted as a replicate. Initially, cutting surfaces were disinfected with 95% ethanol. 4 mm mycelial agar discs were placed in the 4 mm deep wounds incised under the peel with a cork-bore, and these sections were closed with Vaseline and covered with paraffin. Only PDA agar discs were inserted into the wounds in control plants. The inoculated cuttings were planted in pots including 1:1 sterile perlite and peat. The pots were incubated for 4 months in climate chambers (25°C temperature, 70% humidity, 16 hours daylight and eight hours darkness) and the development was inspected every morning. At the end of this period, the changes in wood colour and vascular system lesions were examined. After the incubation, each test plant was cut longitudinally at the point of inoculation with a knife and the lesion size was measured from the bottom to the top of the point of inoculation. The isolates that caused the longest lesion length (mm) were determined and assigned as the most virulent isolates (Úrbez-Torres *et al.* 2014). Seven small pieces (each approximately 1 cm^2) of the necrotic tissue from the edge of each lesion were cut and placed on PDA medium with streptomycin-sulphate in an attempt to recover the inoculated fungus and complete Koch's postulates. Fungal identifications were confirmed according to morphological and microscopic examinations.

Determination of the effect of fungicides on mycelial growth

PDA medium was used to determine the fungicidal activity of the fungicides (see Table 2) against *I. liriodendri* isolates. PDA plates were prepared with each fungicide at concentrations of 0, 0.01, 0.05, 0.1, 0.5, 1.0,

5.0, 10.0, 50.0 and 100.0 ppm. Stock solutions (10,000, 1,000 and 100 ppm) of each fungicide were prepared and concentrations from the stock solutions were mixed into a sterile PDA medium cooled to 50 °C and poured into Petri dishes (Isolab, 90 x 100 mm). Control PDA plates were prepared similarly, but sterile distilled water was added instead of the fungicide solution. Three mycelial discs of 4 mm diameter cut from the edges of the fungal colony of 10-day-old *I. liriodendri* isolates (MBAE160MY and MBAE205MY) were placed in the center of the growth culture containing the fungicides. The experiment was performed in three replicates for each fungicide. All inoculated treatment and control Petri dishes were incubated in dark at 23°C for seven days. Mycelial growth of the isolates of black foot disease at seven days after incubation was determined by measuring the diameter. The efficacy of the fungicides was demonstrated according to the EC_{50} (50% inhibiting dose of mycelial growth) and the lowest concentration (MIC) values that inhibit mycelial growth, which were determined based on the diameter measurement values of the fungicides.

Determination of in vitro efficacy of Trichoderma harzianum Rifai KRL-AG2 strain

In order to examine the antagonistic effect of *Trichoderma harzianum* Rifai KRL-AG2 strain against *I. liriodendri* isolates (MBAE160MY and MBAE205MY), a disc (4 mm diameter) was taken from the edges of seven-day-old *Trichoderma* and pathogen colonies and placed 4 cm apart on the surface of 9 cm PDA-containing petri dishes. They were simultaneously placed 1 cm away from the edge of the petri dishes in both directions. Petri dishes with only *I. liriodendri* mycelial discs served as controls. Petri dishes were incubated at 27°C. The experiments were performed in four replicates for each isolate. The development of *Trichoderma* against *I. liriodendri* isolates was followed daily for seven days. On the fifth day, assessment of antagonism was performed according to Bell's recommended classification from grade 1 to grade 5 as below (Bell *et al.* 1982).

Grade 1-*Trichoderma* completely overgrow the pathogen and cover the entire medium surface; grade 2–*Trichoderma* occupies 75% surface of the medium surface; grade 3-*Trichoderma* occupies 50% of the medium surface; grade 4-*Trichoderma* occupies 25% of the medium surface; grade 5-the pathogen completely overgrow the *Trichoderma*.

After seven-days of incubation period, the percent inhibition rate of *I. liriodendri* was calculated using the following formula (Royse & Riese 1978):

$$\text{Inhibition (\%)} = \frac{(R1 - R2)}{R1} \times 100$$

R1 = Mycelia growth of the pathogen without *Trichoderma* (control),

R2 = Mycelia growth of the pathogen in the presence of *Trichoderma*

Effect of selected fungicides on the rooting phase

In order to determine their potential to prevent infections caused by *I. liriodendri* at the rooting stage of 1103 Paulsen vine rootstocks, four fungicides found to be most effective in *in vitro* experiments were selected (see Table 3).

Care was taken to ensure that the vine rootstocks used in the experiments were approximately 35 cm tall and had four buds. Firstly, rooted plants were carefully removed from plastic bags containing peat/perlite and soaked in tap water for 24 hours at 20 °C. Then, the root parts of the rootstocks were cut lightly, wounds were created, and the root parts of the plants (approximately 15 cm) were dipped separately for 30 minutes in a 2×10^6 conidia suspension of each 10-day old isolates (MBAE160MY and MBAE205MY) grown on PDA at 25°C. Rootstocks were planted in plastic bags containing sterile peat/perlite mixture (media: 80% peat, 20% perlite) immediately after conidial suspension treatment. In addition, the experiment was established by pouring 100 mL of fungicide suspension into each plastic bag right after the plants were planted. In controls, distilled water was used instead of fungicide application. In addition to the application of the biological control agent, plastic bags containing sterile peat-perlite mixture were wetted with *T. harzianum* biopreparation 14 days prior to the experiment, allowing the biological control agent to colonize there, and then the pathogen was inoculated (Santos *et al.* 2014). The second fungicide application was repeated 14 days later. The experiment was established in a randomized plot design with four replicates and three rootstocks in each replication. The controls were consisted of 2 groups which are; pathogen only and no treatment. The development of the plants was observed for four months in the climate chamber with a temperature of 25ºC and a humidity of 70%. The experiment was repeated for a second time.

After four months of incubation, disease assessment was performed according to 0-5 scale, in which 0 = healthy with no lesions, 1 = slight discoloration with 0-25% of root mass reduction, 2 = slight to moderate discoloration with 26-50% of root mass reduction, 3 = moderate discoloration with 51-75% of root mass reduction, 4 = severe discoloration with >75% of root mass reduction and 5 = dead plant (Alániz *et al.* 2010). Disease severity was calculated using the scale values according to the Townsend–Hauberger (1943) formula, as follows: [Σ (number of plants in disease scale category × disease scale category) / (total number of plants × maximum disease scale category] × 100). The efficacy of fungicides was calculated with the Abbott's formula: [(control– treatment)/control) × 100)] (Abbott 1925). In addition to the root dry weight, plant height, root length and shoot dry weight were also measured.

Statistical Analyses

SPSS Statistics 17 (Ver.17.0, SPSS Inc., Chicago, IL, USA) was used in statistical analyses. One-way ANOVA was used to determine differences between applications

and data were compared with Duncan multiple comparison test based on 5% error margin ($P \leq 0.05$). In addition, for the mycelial growth, analysis of variance (ANOVA) was performed without converting the EC_{50} values to % ratios, and the differences between the means were evaluated with a 5% margin of error according to the Duncan multiple comparison test (Kurt *et al.* 2020).

Results and Discussion

Black foot disease in nurseries

A total of 210 Sultani Seedless grapevine saplings were collected from each nursery in Manisa (20 nurseries) and Denizli (one nursery) provinces. During the isolation of vascular tissues, the presence of necrosis occluded xylem vessels was detected in the cross-sections of the basal end of the symptomatic trunks (Fig. 1a). The necrotic area completely affected the root base of some saplings (Fig. 1b). It has been determined that some of these symptomatic vine saplings have a root structure called "J-rooting" or secondary root development (Figs 1c, d).

The isolation from vascular tissues revealed that, fungal species associated with black foot disease occurred in 8 of 21 (38.1%) surveyed grapevine nurseries and were isolated from 56 of 210 (26.6%) samples collected (Table 6).

Black foot fungi were identified as common pathogens, especially in grapevine nurseries and young vineyards. In a study conducted in Spain, black foot disease was found in 23.8% of the grapevine samples examined (Armengol *et al.* 2001). Pathogens of black foot disease were isolated from the roots to trunks of the sampled vines. Agustí-Brisach *et al.* (2013), validated that inoculum of *Ilyonectria* spp. is also existing at the different phases of the grapevine nursery propagation routine and proposed that infections instigated by these pathogens can also come about during this routine. These results confirm those obtained by Cardoso *et al.* (2012), who discovered inoculum causes of black-foot pathogens in a commercial grapevine nursery in Portugal.

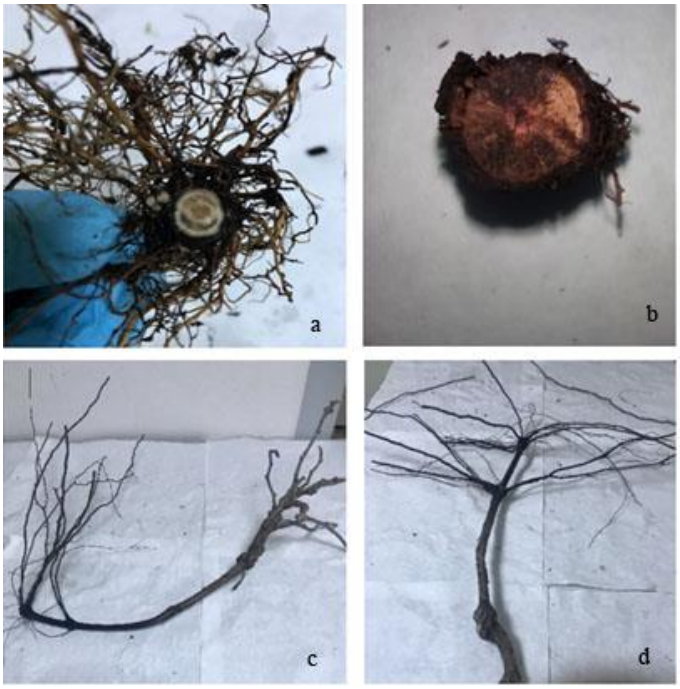

Fig. 1. Symptoms of black foot disease observed in vine saplings and different root growths. a. Vascular necrosis in wood tissue, b. necrotic area at the root base, c. "J rooting" observed in symptomatic vine saplings, d. secondary root development observed in symptomatic vine saplings.

Table 6. Prevalence and incidence of black foot pathogens in grapevine nurseries in Aegean Region (%).

Districts	Number of diseased nurseries / Total number of nurseries sampled	Disease prevalence (%)	Number of diseased grapevine saplings / Total number of grapevine saplings	Disease incidence (%)
Yunusemre	2/6	33.3	14/60	23.3
Şehzadeler	1/3	33.3	10/30	33.3
Turgutlu	1/1	100	6/10	60
Akhisar	-/1	-	-/10	-
Salihli	2/3	66.6	12/30	40
Alaşehir	1/4	25	8/40	20
Sarıgöl	-/2	-	-/20	-
Çal	1/1	100	6/10	60

Obtained results suggest that new plants are infected during the propagation process in nurseries and that even the planting material used in the propagation process might be infected with these pathogens (Rego *et al.* 2000, Cardoso *et al.* 2012, Agustí-Brisach *et al.* 2013). The reasons for the presence of these fungi in all tissues of the plants during the isolation studies may be related to these events.

Morphological identification and characterization

20 isolates associated with black foot disease were divided into three different groups as a result of morphological and microscopic examinations according to Halleen *et al.* (2004) and Alániz *et al.* (2007):

The first group consisted of 17 isolates. The isolates were characterized by a grayish-white colony margin and a mixture of brown-white smooth mycelium (Fig. 2a). This group were found to be compatible with the previously published colony morphology of *I. liriodendri* (Agustí-Brisach & Armengol 2013). Conidiospores are usually simple, unbranched or sparsely branched. Macroconidia usually showed a structure with 1-3 or rarely more than three septa. 1-septal conidia (10-) 17.5 (-21) × (3-) 4.5 (-5) µm; 2-septal conidia (20-) 26.5 (-35) × (4.5) 5.5 (-6.5) µm; The 3-septal conidia (20-) measured 32.5 (-35) × (5-) 5.5 (-7) µm. The microconidia were observed as oval shaped ellipsoidal, although abundant and without septa. 0-1 septa; It was measured as (6-) 10 (-15) × (2.5-) 4 (-5) µm. Chlamydospores generally showed a chain arrangement and measured (10-) 16.5 (-20.5) × (7.5) 11.5 (16) µm (Fig. 2d). The second group consisted of one isolate. It is characterized by brown colony margin and white light yellow mycelium (Fig. 2b). Colony morphology was found to be compatible with previous descriptions of *D. macrodidyma* (Alániz *et al.* 2007, Petit *et al.* 2005, Úrbez-Torres *et al.* 2014). Macroconidia predominantly has 3 and rarely 4 or 5 septa (Fig. 2e). 1-septal conidia (6.5–) 11 (–13.5) × (2.5–) 3 (–3.5) µm; 2-septal conidia (27.5-) 33.5 (-35) × (5-) 5.5 (-6.5) µm; The 3-septal conidia measured (33-) 34.5 (-37.5) × (5.5) 6 (-7.5) µm. The microconidia were elliptical, abundant and measured as (5–) 6.5 (–8.5) × (2.5–) 3 (–4.5) µm. The third group consisted of two isolates and the colony colour was characterized as amber yellow in the center and cream colour on the colony margins (Fig. 2c). The macroconidia was observed as cylindrical and (1–) 3 septa. 1-septal conidia (12–) 23.5 (–29) × (4.5–) 5 (–5.5) µm; 2-septal conidia (22–) 29.5 (–33.5) × (3.5–) 5 (–6.5) µm; and 3-septal conidia (30.5–) measured 32.5 (–34.5) × (3–) 5.5 (–6) µm. Microconidia were elliptical, without septa, and measured (5–) 8.5 (–12) × (2–) 2.5 (–3) µm (Fig. 2f).

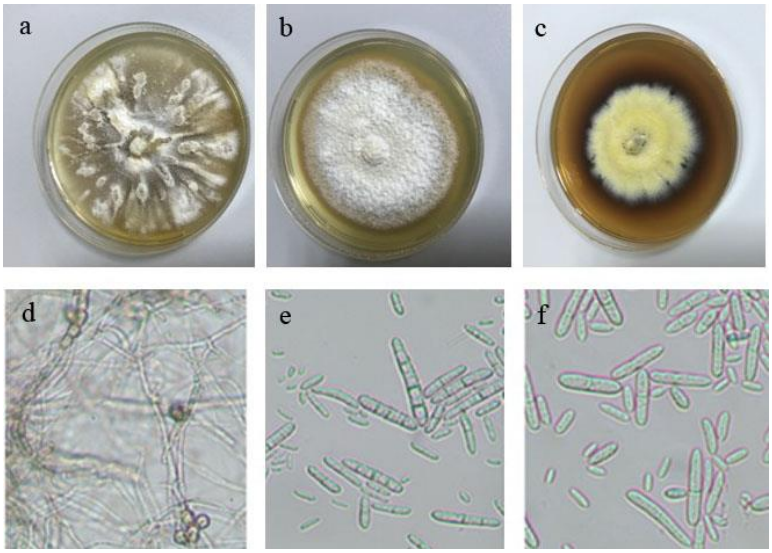

Fig. 2. Colony and conidial morphology of *Ilyonectria* and *Dactylonectria* species isolated from symptomatic grapevine saplings in Aegean Region. a. *Ilyonectria liriodendri* colony, b. *Dactylonectria macrodidyma* colony, c. *D. torresensis* colony, d. *I. liriodendri* conidia and chlamidospore morphology, e. *D. macrodidyma* conidial morphology, f. *D. torresensis* conidial morphology, Scale (DF)=20 µm.

Molecular identification and phylogenetic analyses

As a result of molecular analyzes, three species associated with black foot disease were identified; *Ilyonectria liriodendri* (17 isolates), *Dactylonectria macrodidyma* (two isolates) and *D. torrsensis* (one isolate) (Table 7). DNA sequence data for these species showed high similarity (>99%) with reference sequences in the NCBI Genbank database. Some isolates (MBAE65MY, MBAE73MY, MBAE159MY, MBAE160MY, MBAE205MY) showed 99% similarity when compared with reference sequences of his3 due to the nucleotide differences. Therefore, combined phylogenetic analysis was performed with the his3, tub2 and ITS sequences of the isolates. Amplification products of approximately 411-682 bases for ITS, 349-539 bases for tub2, and 445-500 bases for his3 were obtained from the isolates listed in Table 7.

Phylogenetic analysis was performed using the Maximum Likelihood method and the Tamura-Nei model (Tamura & Nei 1993). The combined phylogenetic analysis divided 20 local isolates into three distinct groups. Sequences from all native *I. liriodendri* isolates were included in the first group (99% bootstrap support)

along with the reference sequences of *I. liriodendri* from GenBank (CBS 117640, CBS 112596 and CBS 112607). The MBAE68MY isolate was in the second group (97% boostrap support) with the reference sequences of *D. macrodidyma* from GenBank. MBAE15MY and MBAE135MY isolates were included in the third group with a high support value (90% boostrap support) without forming any subclasses as separate branches with the reference sequences of *D. torresensis* (Fig. 3). Since morphological features alone are insufficient to distinguish black foot species, DNA sequencing was performed to identify these species most accurately (Lombard *et al.* 2014). The proposed his3, ITS and tub2 gene regions were used to identify black foot species (Cabral *et al.* 2012a, b, Lawrence *et al.* 2019). Because these three gene regions contain unique polymorphisms in each species, they have also been used in previous studies to identify species associated with black foot disease. The results are in parallel with previous studies performed to characterize *Cylindrocarpon*-like fungi using these gene regions (Cabral *et al.* 2012a, b, Lombard *et al.* 2014, Lawrence *et al.* 2019).

It is not surprising that *I. liriodendri*, which constitutes 85% (17 isolates) of the black foot isolates was detected as such a common species because this species found in France, Portugal, South Africa (Rego *et al.* 2000, Halleen *et al.* 2006), Spain (Alániz *et al.* 2009), Iran (Mohammadi *et al.* 2009) and New Zealand (Pathrose *et al.* 2014) has been reported as the main pathogen of black foot disease in many grape growing regions. Some previous studies showed that *I. liriodendri* is a dominant species (Agustí-Brisach & Armengol 2013, Úrbez-Torres *et al.* 2014, Mundy 2015).

Pathogenicity tests

1103 Paulsen, which is the most planted rootstock variety in Aegean Region, was used in the pathogenicity test performed with the fungal disc method. At the end of the four-month trial, all black foot isolates (20 isolates) caused dark brown necrosis in both sub-bark wood tissue (Aigoun-Mouhous *et al.* 2019) and vascular tissues (Úrbez-Torres *et al.* 2014) in grapevine cuttings (Fig. 4).

The length of the necrotic area measured cross-sectionally at the base of the trunk was successfully used to evaluate the experiments. While an average of 6 mm lesion length was observed in controls inoculated with sterile agar due to oxidation, the same length ranged from 22.8 to 45.4 mm in cuttings inoculated with pathogens. MBAE160MY (45.4 ± 1.2) and MBAE205MY (43.6 ± 1.3) isolates of *I. liriodendri* collected from Yunusemre and Alaşehir districts of Manisa province were determined to be more virulent than other isolates and were statistically included in the same group (Table 8). These findings confirming previous studies that reported cross-sectional necrotic areas at the base of the trunk can be used successfully to quantify disease symptoms (Úrbez-Torres *et al.* 2014). In pathogenicity tests, these pathogens were successfully re-isolated from necrotic areas in 45.7% to 85.7%, and the ability of these pathogens to cause disease was confirmed.

Table 7. Information on DNA sequencing and registered data in NCBI GenBank of local isolates.

Isolate number and species		Host	Origin	GenBank Accession Numbers		
				His3	ITS	BT
MBAE5MY	*Ilyonectria liriodendri*	41 B/Sultani	Denizli/Çal	MT708551	MT711168	MT748009
MBAE7MY		41 B/Sultani	Denizli/Çal	MT732971	MT711169	MT748010
MBAE10MY		41 B/Sultani	Denizli/Çal	MT732972	MT711170	MT748011
MBAE15MY	*Dactylonectria torresensis*	1103P/Sultani	Manisa/Yunusemre	MW995961	MW979404	MW995964
MBAE65MY	*I.liriodendri*	Sultani	Manisa/Salihli	MT732973	MT711171	MT748012
MBAE67MY		Sultani	Manisa/Salihli	MT732974	MT711172	MT748013
MBAE68MY	*D.macrodidyma*	Sultani	Manisa/Salihli	MW995962	MW979405	MW995965
MBAE73MY	*I.liriodendri*	Sultani	Manisa/Salihli	MT732975	MT711173	MT748014
MBAE103MY		41 B/Sultani	Manisa/Salihli	MT732976	MT711174	MT748015
MBAE128MY		1103P/Sultani	Manisa/Yunusemre	MT740297	MT711175	MT748016
MBAE130MY		5 BB/Sultani	Manisa/Şehzadeler	MT740298	MT711176	MT748017
MBAE134MY		5 BB/Sultani	Manisa/Şehzadeler	MT740299	MT711177	MT748018
MBAE135MY	*D. torresensis*	5 BB/Sultani	Manisa/Şehzadeler	MW995963	MW979406	MW995966
MBAE155MY	*I.liriodendri*	5 BB/Sultani	Manisa/Yunusemre	MT740300	MT711178	MT748019
MBAE159MY		5 BB/Sultani	Manisa/Yunusemre	MT740301	MT711179	MT748020
MBAE160MY		5 BB/Sultani	Manisa/Yunusemre	MT740302	MT711180	MT748021
MBAE165MY		5 BB/Sultani	Manisa/Yunusemre	MT740303	MT711181	MT748022
MBAE205MY		Sultani	Manisa/Alaşehir	MT740304	MT711182	MT748023
MBAE214MY		Sultani	Manisa/Alaşehir	MT740305	MT711183	MT748024
MBAE255MY		1103P/Sultani	Manisa/Turgutlu	MT740306	MT711184	MT748025

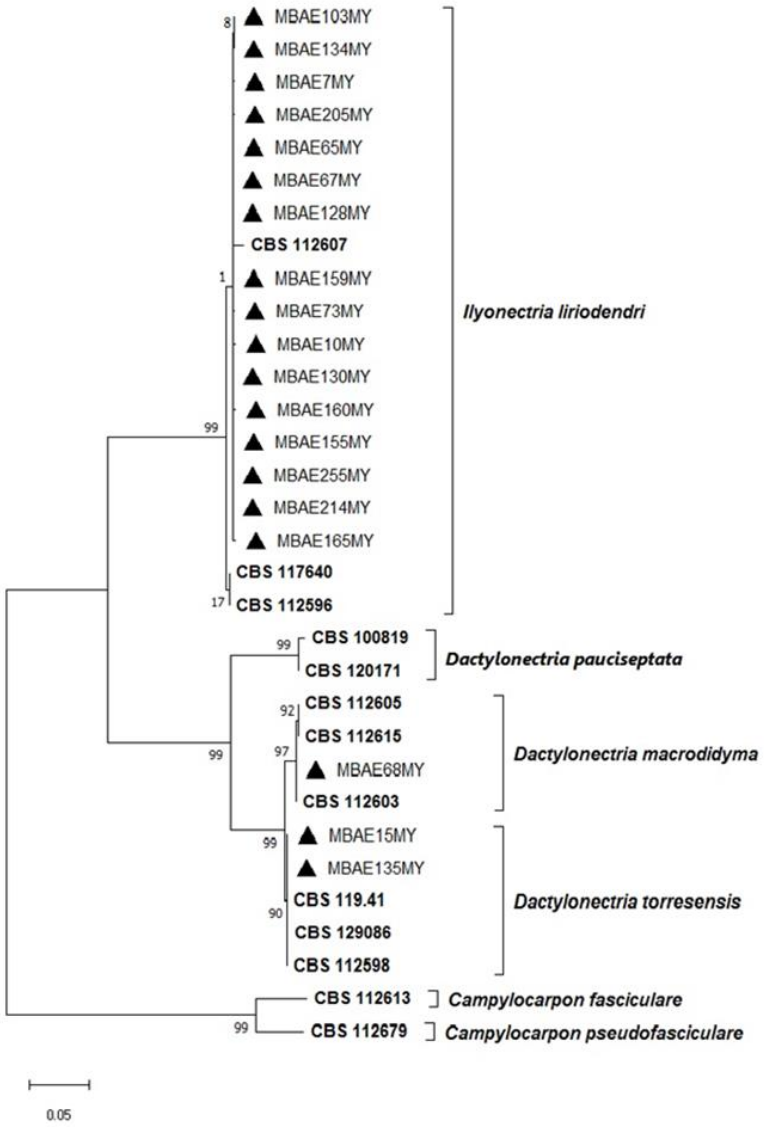

Fig. 3. Maximum Likelihood tree inferred from the combined *his3*, *tub2* and ITS sequence alignments. *Campylocarpon fasciculare* (CBS 112613) and *Campylocarpon pseudofasciculare* (112679) obtained from GenBank were treated as outgroups. Local isolates are indicated by the solid black triangles. Scale bar indicates the number of nucleotide variations.

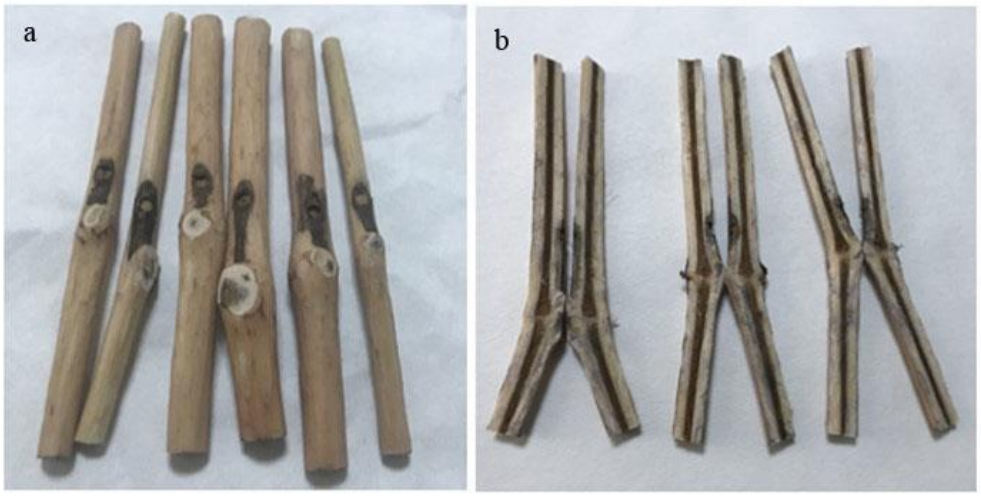

Fig. 4. Lesions caused by black foot disease isolates (a-b). a. Lesions under the bark and b. lesions in vascular tissues.

Table 8. Lesion size (mm) and reisolation rates (%) in inoculated grapevine cuttings.

Sample No	Isolate Number	Origin	Mean Lesion Length (mm)	Reisolation (%)
1	Control	-	6.0 ± 0.3 a*	0.0
2	MBAE5MY	Denizli/Çal	22.8 ± 2.2 b	77.1
3	MBAE7MY	Denizli/Çal	23.4 ± 1.7 b	60.0
4	MBAE10MY	Denizli/Çal	24.6 ± 1.1 bc	57.1
5	MBAE15MY	Manisa/Yunusemre	33.0 ± 0.7 fg	60.0
6	MBAE65MY	Manisa/Salihli	26.8 ± 1.2 cd	80.0
7	MBAE67MY	Manisa/Salihli	31.6 ± 0.7 fg	54.2
8	MBAE68MY	Manisa/Salihli	27.6 ± 1.0 cde	51.4
9	MBAE73MY	Manisa/Salihli	27.8 ± 0.9 cde	62.8
10	MBAE103MY	Manisa/Salihli	32.8 ± 0.9 fg	68.5
11	MBAE128MY	Manisa/Yunusemre	32.2 ± 1.1 fg	74.2
12	MBAE130MY	Manisa/Şehzadeler	34.4 ± 1.2 g	85.7
13	MBAE134MY	Manisa/Şehzadeler	34.6 ± 1.0 g	80.0
14	MBAE135MY	Manisa/Şehzadeler	30.6 ± 0.7 ef	62.8
15	MBAE155MY	Manisa/Yunusemre	30.0 ± 0.7 def	60.0
16	MBAE159MY	Manisa/Yunusemre	34.4 ± 0.7 g	74.2
17	MBAE160MY	Manisa/Yunusemre	45.4 ± 1.2 h	60.0
18	MBAE165MY	Manisa/Yunusemre	23.0 ± 0.5 b	45.7
19	MBAE205MY	Manisa/Alaşehir	43.6 ± 1.3 h	51.4
20	MBAE214MY	Manisa/Alaşehir	34.2 ± 1.6 g	62.8
21	MBAE255MY	Manisa/Turgutlu	28.2 ± 0.4 de	65.7

Pathogenicity test values of the isolates. Lesion size in inoculated grape cuttings. * Mean values within a column are significantly different based on Duncan's multiple range test (P = 0.05).

Table 9. EC_{50} and MIC values (µg/ml) of the used fungicides on *I. liriodendri* isolates.

Active ingredient (a.i)	Isolates			
	MBAE160MY		MBAE205MY	
	EC_{50}*	MIC** (µg/mL)	EC_{50}	MIC (µg/mL)
Fenhexamid 500 g/L	>100 D	>100	64.14 EF	>100
Cyprodinil %50	>100 D	>100	52.82 DE	50
8-hydroxyquinoline 500 g/L	54.47 C	>100	47.09 CDE	>100
Hymexazole 360 g/L	53.92 C	>100	96.32 F	>100
Azoxystrobin 200 g + Difenoconazole 125 g	46.56 C	>100	39.30 BCDE	50
Fluoxapyroxad 300 g/L	40.13 BC	>100	46.41 CDE	50
Promocarb Hydrochloride (HCL) 530 g/L + Fosetyl-Al 310 g/L	39.53 BC	>100	34.99 ABCDE	>100
Boscalid 200 g/L + Kresoxim-methyl 100 g/L	39.02 BC	50	20.24 ABCD	50
Fludioxonil 25 g/L + Metalaxyl -M 10 g/L	7.04 AB	50	7.6 AB	10
Azoxystrobin 75 g/L + Metalaxyl -M 37.5 g/L + Fludioxonil 12.5 g/L	6.36 AB	10	8.69 ABC	10
Thiabendazole 300 g/L + Fludioxonil 37.5 g/L + Metalaxyl-M 30 g/L + Azoxystrobin 15 g/L	3.01 A	5	16.41 ABCD	5
Cyprodinil %37.5 + Fludioxonil %25	0.26 A	0.5	0.38 A	0.1

*EC_{50}: dose that inhibits mycelium growth by 50%. **MIC: lowest dose that inhibits mycelium growth. Mean values within a column are significantly different based on Duncan's multiple range test (P = 0.05).

Table 10. Dual Culture of *T. harzianum* against *I. liriodendri* after 5 days.

Trichoderma species	Isolate	Antagonism Class (grade scores) *	Interpretation
Trichoderma harzianum Rifai KRL-AG2 strain	MBAE160MY	2	Highly Efficient Antagonism
	MBAE205MY	2	Highly Efficient Antagonism

* Proposed classification by Bell *et al.* (1982).

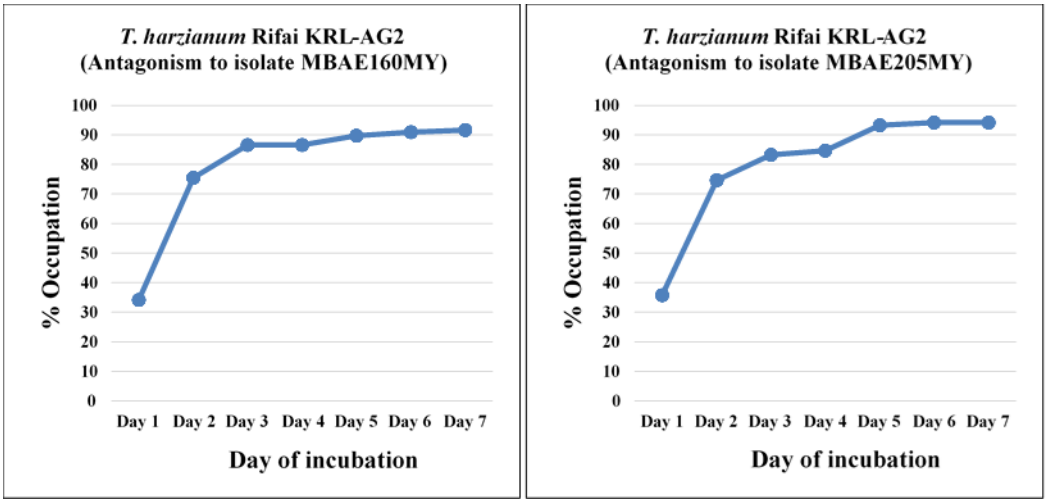

Fig. 5. Seven-day growth profile of *T. harzianum* against *I. liriodendri* isolates and percent occupancy in petri dish (%).

Table 11. Inhibition rate (%) of *I. liriodendri* isolates paired with *Trichoderma* species.

Trichoderma species	Isolate	Day 1	Day 2	Day 3	Day 4	Day 5
Trichoderma harzianum Rifai KRL-AG2	MBAE160MY	22.91	41.66	46.0	55.43	68.10
	MBAE205MY	32.14	38.75	40.0	52.58	80.60

In vitro efficacy of chemical preparations

As a result of the pathogenicity tests, the efficacy of chemical preparations was demonstrated according to the dose that inhibits mycelial growth by 50% (EC_{50}) and the lowest concentration (MIC) to inhibit mycelial growth, which was determined based on the diameter measurement values of two *I. liriodendri* isolates with the highest virulence (MBAE160MY and MBAE205MY).

Cyprodinil + fludioxonil, thiabendazole + fludioxonil + metalaxyl-m + azoxystrobin, azoxystrobin + metalaxyl-m + fludioxonil and fludioxonil + metalaxyl-m with EC_{50} values ranging from 0.26 to 16.41 µg/mL, were found to be the most effective fungicides on *I. liriodendri* isolates. All other fungicides were found to be significantly less effective with EC_{50} values ranging from 20.24 to >100 µg/mL. When fungicides were evaluated according to their MIC values, cyprodinil + fludioxonil was the most effective fungicide on both *I. liriodendri* isolates with values ranging from 0.1 to 0.5 µg/mL (Table 9).

In vitro efficacy of Trichoderma harzianum Rifai KRL-AG2 strain

Antagonism was evaluated following the method described by Bell et al. (1982). The *Trichoderma harzianum* KRL-AG2 strain had a very good average success score of ≤ 2 against both *I. liriodendri* isolates according to Bell's classification, and thus was accepted as a highly effective antagonist against *I. liriodendri* (Table 10).

There were significant increases in the percentage of occupation by *T. harzianum* against both *I. liriodendri* isolates until the third day. In addition, although the occupation percentage of *T. harzianum* in MBAE160MY isolate stopped on the fourth day, it then continued with a low increase in the following days. The increase in the occupancy percentage of *T. harzianum* in MBAE205MY was very low on the fourth day, then reached the highest occupancy percentage on the fifth day and stabilized with a low increase in the following days (Fig. 5).

At the end of the five-day period in which the experiment was evaluated, *T. harzianum* covered 91.6% and 94.1% of the media surface against MBAE160MY

and MBAE205MY isolates, respectively. The development of *I. liriodendri* isolates, which were cross-matched simultaneously with *T. harzianum* during the five-day incubation period, decreased at varying rates between isolates when compared with the control treatments. While *T. harzianum* achieved the highest inhibition rate with 80.6% in MBAE205MY isolate, it provided 68.1% inhibition in MBAE160MY (Table 11).

Determination of the inhibitory efficacy of the fungicides against black foot disease under controlled conditions

In order to determine the effect of the fungicides, that were found to be the most effective *in vitro* (Table 3), on infected vine cuttings, two separate pot trials were conducted *in vivo* in 2020 and 2021. Analysis of variance (ANOVA) was applied to all data obtained in both pot experiments four months after artificial inoculation. Statistical analysis results showed that these fungicides were significant ($P<0.05$) in all variables evaluated in pot experiments carried out in 2020 and 2021.

In the pot trials conducted in 2020, all fungicides significantly increased root biomass of the vines compared to untreated controls and decreased root disease severity by reducing the rate of necrosis covering the roots. In reducing the severity of root disease caused by *I. liriodendri* isolates, thiabendazole + fludioxonil + metalaxyl –m + azoxystrobin (69.9%-71.1%); cyprodinil + fludioxonil (63.4%-69.6%); azoxystrobin + metalaxyl-m + fludioxonil were the most effective fungicides with (60%-69.6%) rates, followed by *Trichoderma harzianum* Rifai KRL-AG2 strain with (50%-60.8%).

In the pot trial, carried out as the second replication of 2021, it was determined that all fungicide applications significantly increased the root biomass of the vines compared to the untreated controls and reduced the severity of root disease by reducing the rate of necrosis covering the roots. In reducing the severity of root disease in treatment applications inoculated with both isolates, cyprodinil + fludioxonil (72.7%-76.2%); thiabendazole + fludioxonil + metalaxyl–m + azoxystrobin (72.7%-74.6%); and azoxystrobin + metalaxyl-m + fludioxonil were the most effective fungicide applications with (63.6%-66.7%) rates, followed by *Trichoderma harzianum* Rifai KRL-AG2 strain with (54.6%-57.1%). Consequently, the mixtures of thiabendazole + fludioxonil + metalaxyl–m + azoxystrobin and cyprodinil + fludioxonil had the highest effectiveness of the sprayings performed after artificial inoculation in 2020 and 2021, followed by other fungicides (Table 12).

As stated earlier, there is no licensed fungicide against black foot disease of grapevine in Turkey. However, in previous studies performed in various countries, it was reported that dipping in suspensions of cyprodinil + fludioxonil mixtures reduced the incidence and severity of black foot pathogens in grapevine plants (Rego *et al.* 2006, 2009). In Turkey, in a study evaluating the *in vitro* effectiveness of some fungicides against *Diplodia seriata* in vineyards, cyprodinil + fludioxonil showed the highest efficiency (100%) at a concentration of 1 μL mL^{-1} and was the most effective fungicide (Güngör Savaş & Yıldız 2021).

Funguicide applications/treatments have provided significant increase on growth parameters (root-shoot dry weight and plant-root length) of vines compared to control group. In 2020 pot trials, mixture of thiabendazole + fludioxonil + metalaxyl -m + azoxystrobin have been effective on all growth parameters except the root length of vines inoculated with the MBAE205MY isolate compared to the control group (Fig. 6). In the same way, on the second repetition of 2021 pot trials, funguicide applications/ treatments were the most effective compared to the control groups. However, only the plants inoculated with the MBAE205MY isolate have been effective on all parameters except the plant length of the vines (Fig. 7).

Table 12. Effect of fungicide applications on disease severity in grapevine roots (%).

Isolate	Active ingredient (a.i)	2020		2021	
		D.S. (%)	Efficacy (%)	D.S. (%)	Efficacy (%)
MBAE160MY	Thia. + Flud. + Met. + Azoxy.	25.4 ± 0.39 a*	69.9	22.2 ± 0.21 b	74.6
	Azoxy. + Meta. + Flud.	33.3 ± 0.73 c	60.0	29.1 ± 0.23 c	66.7
	Cyp. + Flud.	30.6 ± 0.20 b	63.4	20.8 ± 0.26 a	76.2
	T. harzianum	41.6 ± 0.38 d	50.0	37.5 ± 0.23 d	57.1
	Untreated control	83.3 ± 0.92 e	-	87.5 ± 0.23 e	-
MBAE205MY	Thia. + Flud. + Met. + Azoxy.	27.7 ± 0.28 a	71.1	25.0 ± 0.04 a	72.7
	Azoxy. + Meta. + Flud.	29.1 ± 0.10 b	69.6	33.3 ± 0.18 b	63.6
	Cyp. + Flud.	29.1 ± 0.33 b	69.6	25.0 ± 0.13 a	72.7
	T. harzianum	37.5 ± 0.31 c	60.8	41.6 ± 0.26 c	54.6
	Untreated control	95.8 ± 0.68 d	-	91.6 ± 0.31 d	-

D.S.: Disease Severity, **Thia. + Flud. + Met. + Azoxy.**: Thiabendazole 300 g/L + Fludioxonil 37.5 g/L + Metalaxyl -M 30 g/L + Azoxystrobin 15 g/L, **Azoxy. + Meta. + Flud.**: Azoxystrobin 75 g/L + Metalaxyl -M 37.5 g/L + Fludioxonil 12.5 g/L, **Cyp. + Flud.**: Cyprodinil 37.5%+ Fludioxonil 25%, ***T. harzianum***: *Trichoderma harzianum* Rifai KRL-AG2. * Mean values within a column are significantly different based on Duncan's multiple range test (P = 0.05).

As a result, it was determined that these fungicides have reduced/declined severity of the root disease and significantly improved/enhanced the plant growth parameters. Therefore, it is predicted that the introduction of these fungicides to the grapevine industry and, their application during and after planting process of the grapevine saplings could be a crucial strategy to control and manage black foot disease.

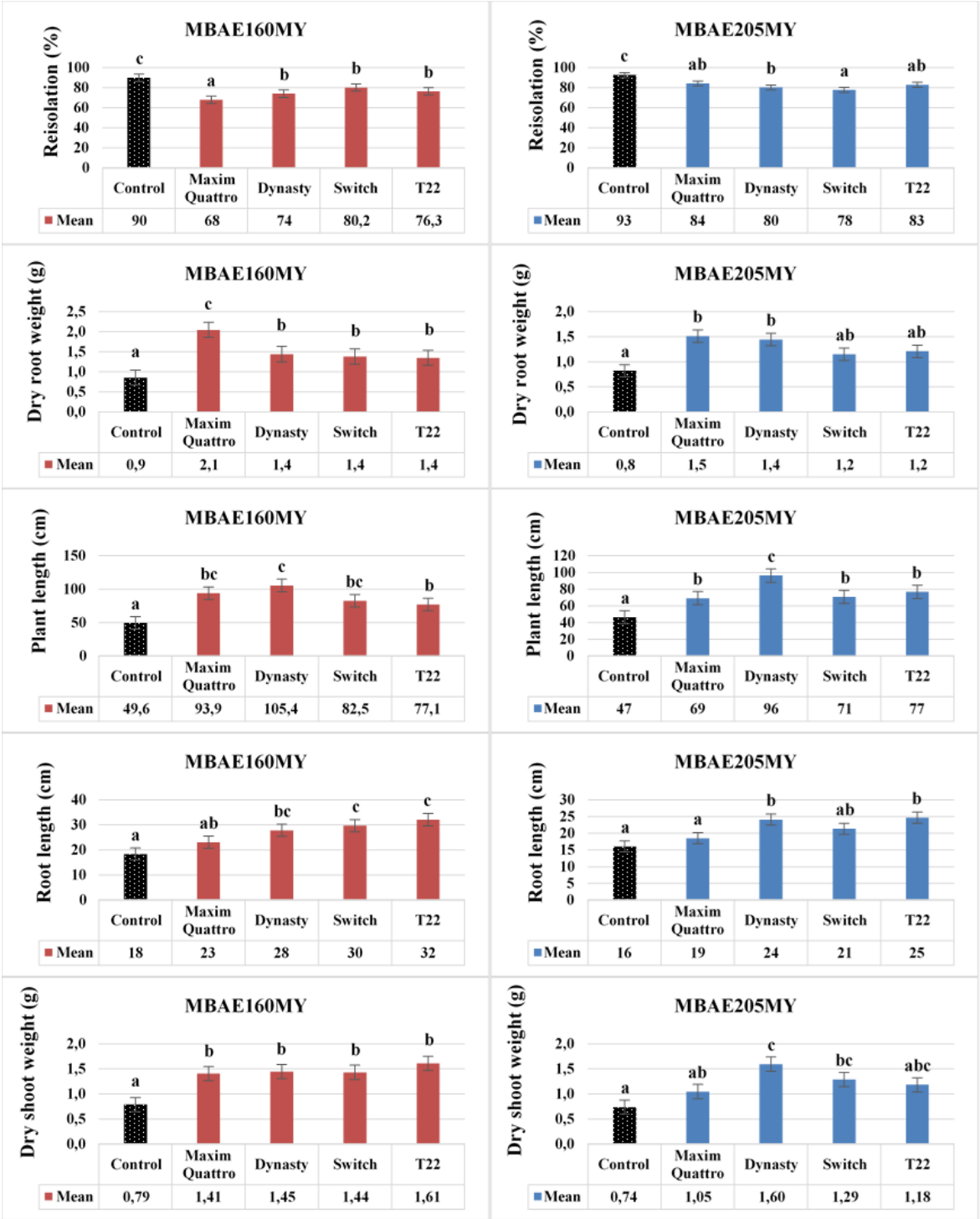

Fig. 6. *In vivo* efficacies of selected fungicides against *I. liriodendri* isolates (2020 trial data). **Maxim Quattro:** Thiabendazole 300 g/L + Fludioxonil 37.5 g/L + Metalaxyl -M 30 g/L + Azoxystrobin 15 g/L, **Dynasty:** Azoxystrobin 75 g/L + Metalaxyl -M 37.5 g/L + Fludioxonil 12.5 g/L, **Switch:** Cyprodinil 37.5%+ Fludioxonil 25%, **T22:** *Trichoderma harzianum* Rifai KRL-AG2.

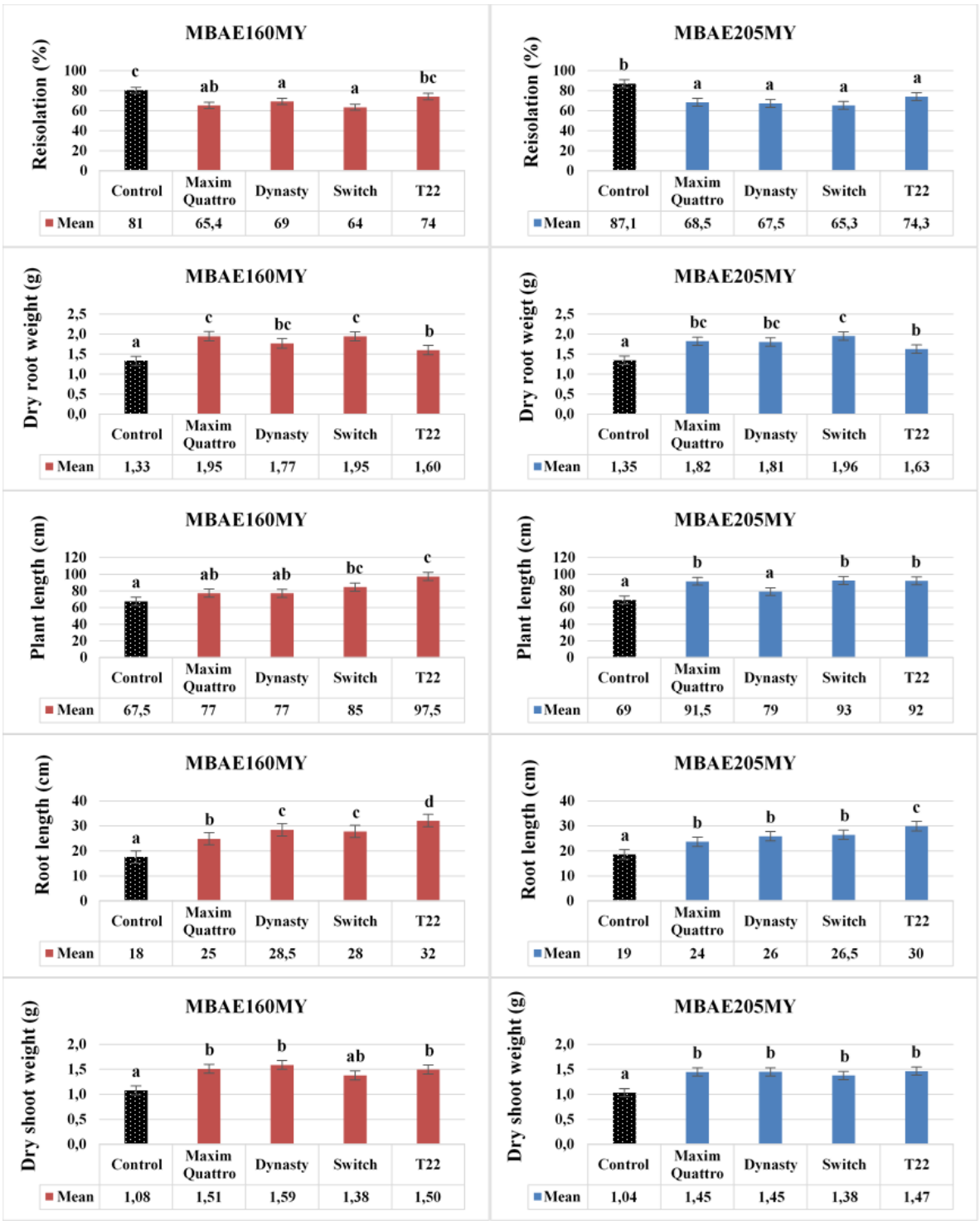

Fig. 7. *In vivo* efficacies of selected fungicides against *I. liriodendri* isolates (2021 trial data).

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