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Article

The Control of Fungal Pathogens in Grapevine Nurseries in Türkiye and Their Impact on Sapling Quality

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Running head: Control of fungal pathogens during grafted grapevine sapling production

Brief summary: Currently, we have limited information about which fungal pathogens are most common and the incidence of fungi during the production of grapevine seedlings. It is therefore unclear at which stage of seedling production fungicides have best control these fungal pathogens. In this study, it has been showed that, *Trichoderma harzianum* Rifai KRL-AG2 strain and active ingredients cyprodinil + fludioxanil applied to cuttings + sawdust and nursery soil reduced the incidence of fungal wood diseases such as *Botryosphaeria obtusa*, *Phomopsis viticola* and *Ilyonectria liriodendri* and increased the total yield of saplings.

Abstract: In the production of grafted vines, losses are caused by fungal pathogens during callus forming or after planting in the soil. To control or reduce natural fungal infections in nurseries, certain applications were conducted in sapling cultivation stage to analyse the efficacy of cyprodinil + fludioxonil, floupyram + tebuconazole active substances and *Trichoderma harzianum* biological preparation. 1103 Paulsen rootstock and *Vitis viniferea* L. Sultana cultivars were stored in fungicide suspensions for 60 minutes before and after grafting in the study. After grafting, the seedlings were divided into i) cutting + sawdust ii) cutting + sawdust + soil application groups and transferred to the callus room. Fungicides were applied 1 to 7 days after the callus development to wet the seedling roots 1 to 7 days after they were planted. After nine months in the nursery, the plants were uprooted, classified as diseased or healthy, and analysed for morphological and molecular diagnosis of fungal species, isolation incidence, and seedling quality and yield. After callus development, *Fusarium solani* was most frequently isolated pathogen in the roots (21.5%), cyprodinil + fludioxonil reduced the *Ilyonectria* sp. isolation rate in both shoots and roots. *Botryosphaeria obtusa* and *I. liriodendri* pathogens were not detected in sick and healthy cyprodinil + fludioxonil-treated saplings. The highest seedling yield was observed with fludioxonil + cyprodinil, cutting + sawdust + soil (78.75%) and cutting + sawdust (70.68%) applications. According to the results of this study, it was found that fungicide applications before and after grafting prevented soil-borne pathogen infections and improved sapling quality.

Keywords: vine; soil-borne pathogens; chemical control; bio-fungicide; seedling quality

INTRODUCTION

Vine (*Vitis vinifera* L.) is a commonly cultivated plant globally since it is not very sensitive to climate and soil, could be reproduced easily, employed in several industries, and is economically significant. In Turkey, 4.2 million tons of grapes were cultivated in about 4 million decares in 2020 and 246,000 tons were exported with a revenue of 514 million dollars [1]. Despite the established viticulture in Türkiye, there is a significant need for sapling nursing due to aging and fungal lignum diseases and the need for new vines. The economic life of a vineyard is about 40 years [2, 3]. Thus, several authors indicated that 7.5 million to 15.0 million saplings are required in Turkey every year [4].

As in every sector in Türkiye, there are some problems in the vineyard nursery sector. Sustainability of vine cultivation, which is the most cultivated fruit in Turkey, is possible with healthy sapling supply [5]. In the last few decades, the interest in the impact of dieback on vine seedlings and

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studies to develop control strategies against them have increased [6, 7]. Investigations are generally limited to the diagnosis of disease pathogens, and the detection of susceptibility of the nurseries, cultivars, and rootstock [7, 8]. Besides that, it is unclear when fungal pathogens started to be a problem in grapevine nurseries and which fungicides can be used to control at various cultivation stages.

Grafted vine sapling cultivation includes the stages of graft and cutting procurement, cold storage, grafting, post-grafting callus development in germination chamber, and planting. The 25°C temperature and 90% relative humidity in post-grafting germination chambers are suitable for pathogen development. Particularly at the pre-grafting stage, there might be problems in pathogen control with fungicides. It was reported that soil-borne fungal species and other known vineyard pathogens (i.e., *Diaporthe ampelina*, *Phoma exigua* var. *exigua*, *Botrytis cinerea*, etc.) were observed on healthy main branches in vineyards [9, 10, 11]. It was reported that the vine cuttings were mostly infected with soil-borne polyphagous pathogens such as *Cylindrocarpon* spp., *Fusarium oxysporum*, *Phytophthora* spp. and *Rhizoctonia solani* after planting in nurseries [6, 10]. In previous studies to control of fungal infections that cause to low seedling yield, fungicide was not applied before and during rooting. At the same time, practices to control both soil-borne and transmitted fungal lignum disease pathogens were not effective before and after planting the seedlings in the nursery. In addition, in such studies, pre- and post-grafting drug applications were found to be inadequate [11, 12].

In Türkiye, Aegean region seedling cultivators generally do not use fungicides before or after grafting, or a licensed fungicide is preferred for the control of vineyard diseases, since there are no licensed fungicides specific for grapevine nurseries. In this study, which was conducted to prevent losses caused by fungal pathogens during seedling cultivation, it was aimed to determine the effects of fungicidal and antagonist applications on the identification and control of fungal pathogens that cause to rot and death between callus development and planting stages of grafted vine cuttings. The effects of these applications on seedling yield, quality and root development were also analysed.

MATERIAL AND METHOD

Plant Material

The study was conducted at a commercial nursery in Muradiye in Manisa Province of the Aegean Region, where vine nurseries are a common occupation, between 2017 and 2018. The nursery where the experiments were conducted has cultivated vine saplings for several years. Crop rotation has been adopted by the nursery, where vine saplings have been produced in every two years, and barley has been planted for rotation in the remaining years.

Sultan 7 seedless grape cultivars grafted on 1103 Poulsen American vine rootstocks with deep root structure and good compatibility with grape varieties were employed in the experiment. Both rootstock and cultivar were considered susceptible to soil-borne pathogens. Sultan 7 seedless grape variety was selected since it was the most exported variety from the Aegean region for both fresh and dried consumption.

Fungicides Applications in the production of grapevine planting material

In the study, pre-grafting fungicides were applied on cuttings and grafts in two stages. The fungicides presented in Table 1 were mixed separately in plastic tanks that included 20 litres of water, based on manufacturer recommended doses. During pre-grafting storage, the cuttings were blunted except for the bottom buds, and the cuttings and rootstocks were kept in the fungicide solution for 60 minutes for them to regain the water lost. The experiments were conducted in 5 replicates, and each replicate included 50 cuttings and grafts, and 1800 cuttings and grafts were placed in polyethylene bags and stored at 0°C (Figure 1). No fungicide was applied to the control group, only water was used. The cuttings and grafts were removed from the cold storage and transferred into grafting chamber. Grafting was conducted with a foot pedal grafting machine with omega-type graft sections, and immediately afterwards, the grafted cuttings were paraffinized [13]. Two applications

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were conducted in parallel and in the same sequence (Figure 1). i) Cutting + sawdust application: The grafted cuttings were stored in water that included the same fungicide/bio-fungicide as treatment before folding for 60 minutes, and 1000 saplings were placed in plastic crates in 5 replicates (50 cuttings in each replicate) after folded with pine sawdust moistened with the fungicide/bio-fungicide. For the control group, only water was used in folding. The crates were stored in germination chambers at 25°C and 90% relative humidity for three weeks for callus development. Fungal isolations were conducted on 10 seedlings (shoots and roots) randomly selected from the crates treated with each fungicide/bio-fungicide. The remaining seedlings were planted in the nursery in early April with the random plot design, where the row spacing was 60 cm and the space between the seedlings was 8-10 cm, in the cutting + sawdust application. ii) The cutting + sawdust + soil application; The grafted cuttings were stored in water that included the same fungicide/bio-fungicide as treatment before folding for 60 minutes and folded in plastic boxes with pine shavings moistened with the same fungicide/bio-fungicide. Then, the saplings were removed from the chamber, and 800 saplings (40 grafted cutting in each replicate) were planted in 5 replicates with the random plots design in the nursery, with 60 cm row spacing and 8-10 cm space between the saplings in early April. One day after planting, each fungicide/bio-fungicide was diluted with 10 litres of water based on the application doses specified in Table 1, and 50 ml fungicide solution was applied to the soil at the root collar region. One week after the application, fungicide applications were repeated to the root and root collar areas. Only water was used in the control group. The isolation incidence was calculated by counting the fungal colony growth in wood chips placed in petri dishes, and the isolation frequencies of pathogenic fungi (%) were calculated based on the ratio of 100 tissues collected from 10 vines in each application.

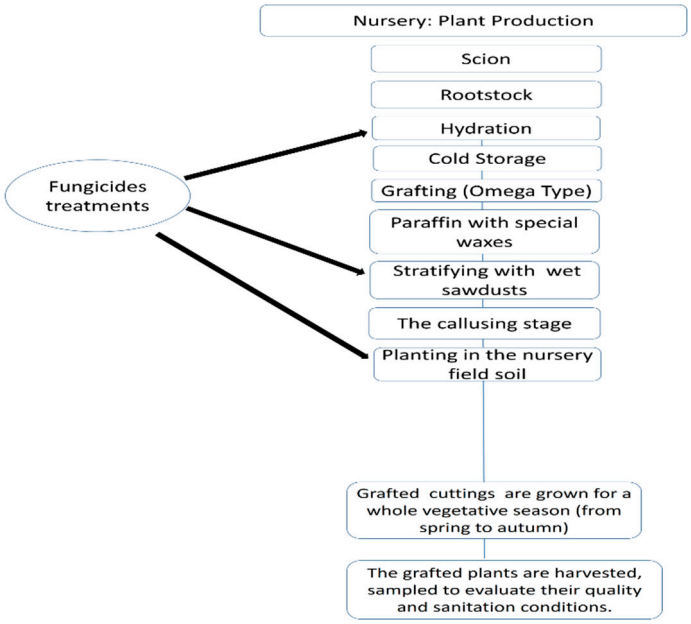

Figure 1. Main steps in vine sapling cultivation applications.

The grafted cuttings were irrigated once a week with a drip irrigation system until September in both applications and maintained based on regular maintenance schedule throughout the year. The seedlings developed for nine months.

Table 1. Employed fungicide and bio-fungicide active ingredients, commercial names, and doses.

Active ingredient	Short nomenclature given during the trial	Trade name/Company	Product rate (recommended dose) (100 L water)

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Floupyram 20 g/l + Tebuconazole 200 g/l	A	Luna Experience SC 400/Bayer	25 ml
Cyprodinil % 37.5 + Fludioxanil % 25	B	Switch 62.5/WG Syngenta	50 g
Trichoderma harzianum Rifai KRL-AG2	C	T-22 Planter Box/ Bioglobal	50 g
Control	D	Water	-

Evaluation of the Efficacy of the Fungicides Applied to Root Zones

After the saplings were uprooted in December, two methods were used in the analysis.

In the first methods, samples were collected from the sick and healthy saplings into plastic bags in 5 replicates (4 saplings in each replicate for each application). Fungi were isolated from the root and root collar regions of the samples. After surfaces were sterilized in the laboratory, the samples were planted in petri dishes that included PDA medium in 4 replicated, and each replicate included seven pieces. After the samples were incubated for 7 days, fungal development was identified and the isolation incidence of the fungi in naturally infected seedlings was determined as a percentage. Isolation incidence was calculated by counting the fungal colony growth on wood chips in petri dishes, and the fungal infection rate was expressed as the ratio of the total number of colonies for each fungus to the total wood chip area (coated on PDA) for each application.

In the second method, after the saplings were removed from the experimental area, they were counted and classified based on the TS 3981 standard. The number of saplings/planted cuttings was calculated as the total seedling yield (%), and the number of 1st size saplings/total seedlings was accepted as the 1st size sapling yield (%). Furthermore, the mean shoot length (cm), root length (cm) and callus development level (0-4 scale) were determined for the seedlings [14]. Considering the condition of the callus tissue that surrounded the graft site, the samples without callus formation were graded 0, unilateral callus formation 1, bilateral callus formation 2, three-sided callus formation 3, and circumferential (four-sided) callus formation 4. These figures are presented as ratios [14] and analyzed statistically.

Identification of Fungal Pathogens

The fungal pathogens were isolated from randomly selected 10 seedling roots and shoots in the fungicide/bio-fungicide application crates transferred from the grafting chamber and vine roots and shoots uprooted after 9 months with the method proposed by Rego et al. [15]. The morphological identification of the species was conducted based on the microscopic features and colony morphology specified in the literature [16, 17, 18, 19].

Molecular diagnoses were conducted on pure and morphologically diagnosed isolates. DNAs were extracted based on the method suggested by Cenis [20] with the 50 mg fresh mycelium collected from isolate colonies. The concentration and purity of the isolated DNAs were determined with the Multi-scan GO µ-drop plate (Thermo Scientific, USA). PCR was determined with internal transcribed spacers (ITS) rDNA region ITS1 (5' TCC TCC GCT TAT TGA TAT GC 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') primer pairs [21]. In real-time (RT)-PCR reactions, 0.3 µl 20 µM forward primer, 0.3 µl 20 µM reverse primer, 2 µl DNA, and 10 µl 2x FastStart Essential DNA Green Master Mix were added into sterile PCR tubes and the volume was completed to 20 µl with DNase/RNase distilled water. RT-PCR (Roche Light Cycler® Nano) amplification conditions were determined as follows: Initial denaturation at 95°C for 10 min and denaturation for 30 sec at 95°C, annealing temperature ITS 56°C, 72°C for 1 min and 35 cycles. PCR sequence data were obtained with bidirectional genome sequencing at a sanger sequencing laboratory (TiroGen, Turkey). The sequence data chromatogram files were analyzed with the ChromasPro 1.7.6 chromatogram analysis software, and consensus sequences were obtained by combining the forward and reverse sequence data. Blast analyzes of the fungi species and consensus sequences for each gene region were conducted with the National Center

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for Biotechnology Information (NCBI) Gene Bank database. Access numbers of the diagnosed isolates were obtained from the NCBI gene bank library.

Statistical analysis

All trials were set up based on random plot design, and post-callus development fungal isolation rate was determined GraphPad Prism (Version 8.01) (*P < 0.001) and two-way ANOVA. Seedling isolation finding analysis after nursery soil treatment and analysis of the impact of the treatments on seedling yield and analysis of variance for quality (ANOVA) were conducted pn SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) software and Duncan's multiple range test ($p < 0.05$).

RESULTS

Identification of Fungi that Cause Rot During Callus Development

The most frequently isolated fungus was *Fusarium* spp. (30.10%, 13.53%, 12.21%, and 9.84%), followed by *Alternaria alternata* in sapling shoots after application of two fungicides and one bio-fungicide and grafting (Figure 2). In application D, the most frequently isolated fungus in both shoots and roots ($p<0.0001$) was also *Fusarium* spp. (30.10% and 27.19%, respectively) (Figure 4). The most frequently isolated fungus was *Fusarium* spp. in roots (21.55%). It was determined that the reason for the high isolation of *Fusarium* spp. in vine seedlings after grafting and cutting and sawdust applications was the neutralization of the protective effects of fungicides by the grafting chamber temperature and humidity (25°C and 90% humidity) and almost provided control group conditions. *Botryosphaeria* spp. was isolated from shoots and roots (9.67%, 9.70%) and led to dieback in the control group (Figures 2 and 3). However, *Botryosphaeria* spp. was isolated in the B treatment group from the shoot (7.04%) and in the C treatment group from the root (4.87%) asymptomatic tissues (Figure 3). *Diaporthe ampelina* isolation rate was 3-5% from the shoots of the vine seedlings after the grafting chamber at 25°C and 90% relative humidity, and it was the highest dieback pathogen, and its isolation rate was 11.67% from the roots in the control group. In A, C and D applications, *Ilyonectria* spp. was isolated and led to black foot disease. In the B treatment callus stage, black foot development was controlled in shoots and roots. Eight fungal genera were isolated after callus development. Among these genera, *Botrytis, Botryosphaeria, Rhizoctonia, Ilyonectria* spp. and *Diaporthe* were known vineyard pathogens. *Phoma* spp. was detected in all applications including control (with isolation rate 7.78%), especially in shoots.

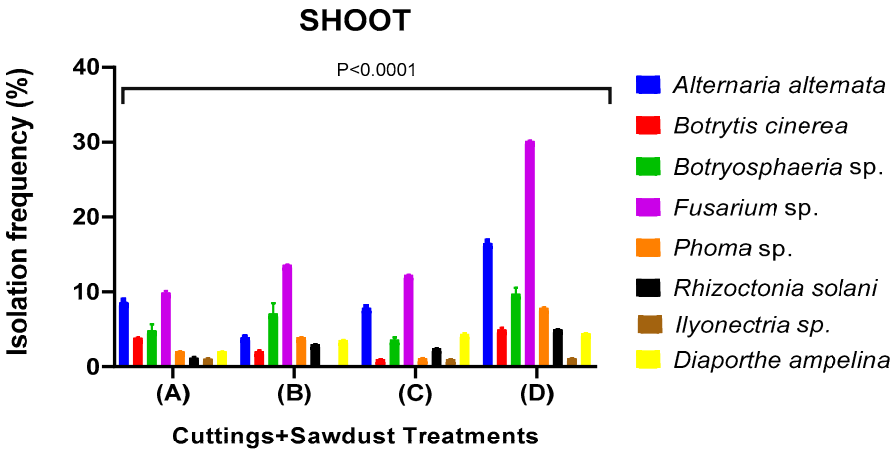

Figure 2. Fungal infection incidences in isolations from the shoots of the grafted vine cuttings, where fungicide was applied to the cuttings + sawdust, after the callus chamber. Error bars symbolize ± mean standard error (SE) (n=3).

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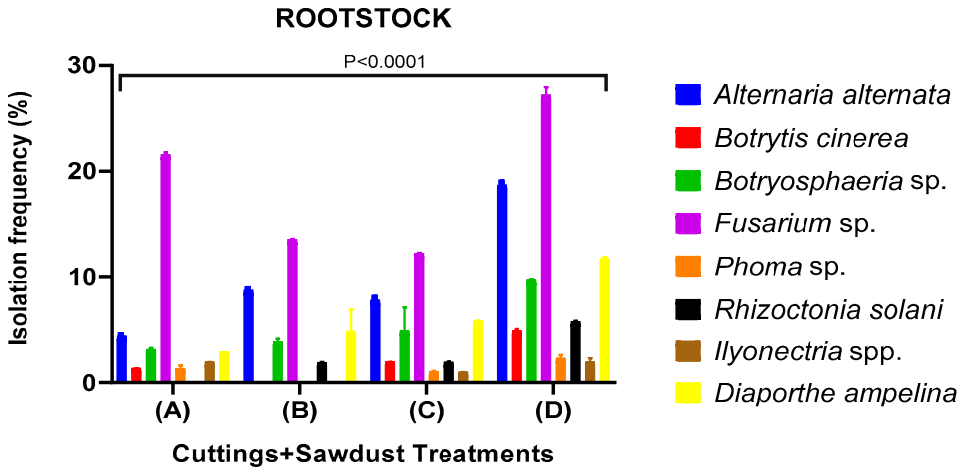

Figure 3. Fungal infection incidences in isolations from the roots of the grafted vine cuttings, where fungicide was applied to the cuttings + sawdust, after the callus chamber. Error bars symbolize ± mean standard error (SE) (n=3).

Morphological and molecular identification of fungal pathogens

The morphological character of post-isolation fungal pathogen infections was employed for the preliminary identification of fungi. 515 isolates were obtained from the treated seedling shoot and root samples. The most isolated soil-borne pathogen species were analyzed in the study. Morphological and molecular identifications of one or more of these isolates, which represented colony morphology and conidial structure that were determined under the microscope, were conducted at species level.

Real-time PCR was conducted with universal ITS primer and SYBR Green fluorescent dye. MBAE321, MBAE322, MBAE329, MBAE241, MBAE259, MBAE77 and MBAE249 isolated from the root zones peaked over the threshold (Ct) level the earliest, and the mean figures were 16.75, 17.14, 17.21, 18.20, 18.50, 19.67, and 22.20, respectively. This figure was 0.0 for the negative control reaction with sterile distilled water. The SYBR melting curve was conducted at the ramp rate of 0.2˚C/s at 56-95˚C temperatures to determine the amplification products of Real-Time PCR. The melting curve reaction continued at elevated temperatures after the completion of the normal Real-Time PCR reaction. MBAE322, MBAE329, MBAE241, MBAE259 and MBAE77 produced melting peaks at 80.44˚C, 83.34˚C, 84.22˚C, 86.65˚C and 89.34˚C, respectively. This confirmed the single fragment recorded in previous PCR tests.

The sequenced PCR products were aligned, and BLAST analyzes were conducted with the BLASTn software, and confirmed the 99%-100% of the species. NCBI GenBank access numbers were obtained for 11 isolates other than the *Fusarium solani* isolates, one for each genus, based on gene sequencing and nucleotide BLAST analysis findings (Table 3).

Table 3. Isolates for which Access numbers were obtained from the GenBank database based on ITS partial sequences.

Species	Isolate Code	Host	Origin	GenBank ITS Accession No
Phoma exigua var. *exigua*	MBAE77	*Vitis vinifera*	Manisa	OQ392363
Fusarium solani	MBAE236	*V. vinifera*	Manisa	OQ392364

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Fusarium solani	MBAE237	*V. vinifera*	Manisa	OQ392365
Fusarium solani	MBAE259	*V. vinifera*	Manisa	OQ392370
Diaporthe ampelina	MBAE239	*V. vinifera*	Manisa	OQ392366
Rhizoctonia solani	MBAE241	*V. vinifera*	Manisa	OQ392367
Botryosphaeria dothidea	MBAE242	*V. vinifera*	Manisa	OQ392368
Ilyonectria liriodendri	MBAE322	*V. vinifera*	Manisa	OQ392371
Alternaria alternata	MBAE329	*V. vinifera*	Manisa	OQ392372
Trichoderma gamsii	MBAE374	*V. vinifera*	Manisa	OQ359568
Trichoderma afroharzianum	MBAE377NS	*V. vinifera*	Manisa	ON819609

Fungicide Efficacy and Sapling Quality in Saplings Uprooted from Nursery

Soil-borne fungi are the main cause of loss in vine nurseries during the sapling cultivation stage. It was determined that there was a statistical difference between the isolation incidence of fungi based on the treatment applied to sick and healthy seedlings removed from the nursery soil (Table 4). *Botryosphaeria, Ilyonectria, Diaporthe, Alternaria, Rhizoctonia, Phoma,* and *Botrytis cinerea* were accepted as grapevine nursery pathogens. Furthermore, *Acremonium, Aspergillus, Aureobasidium, Clado-sporium, Clonostachys, Epicoccum, Penicillium* and *Trichoderma* species were also identified, and these fungi were considered endophytic species. Since *Trichoderma harzianum* sp. bio-preparate was included in the applications, this fungus was included in the analyses conducted after soil applications (Tables 4, 5).

The highest isolation incidence was observed in *Fusarium solani* (36.86%, 25.99%, 15.31%, 14.88%, 10.88%, 9.16%, 9.08%, and 4.79%) in sick saplings uprooted from the vine nursery in the control and all fungicide application groups, as presented in Table 3. *Botryosphaeria obtusa* pathogen incidence was 18.75% and 21.84% in A (cutting + sawdust + soil) and A (cutting + sawdust) applications, and these figures were higher when compared to D (cutting + sawdust + soil) and D (cutting + sawdust) applications (15%, 63 and 12.25%). *Ilyonectria liriodendri* isolations were low in B (cutting + sawdust + soil), C (steel+sawdust) and C (cutting + sawdust + soil) applications. It was observed that *Trichoderma* spp. isolation in all applications except B (cutting + sawdust + soil) from sick saplings demonstrated that the fungicides did not affect *Trichoderma* spp. It was determined that B (cutting + sawdust + soil) application is effective in the control of trunk decline and black foot disease pathogens.

Table 4. Isolation incidences of the fungi isolated from the roots of the sick saplings uprooted from the nursery (%).

Looking Disease	**Fungal İncidence %**						
Treatment	***Alternaria alternata***	***Botryospheria obtusa***	***Fusarium solani***	***Diaporthe ampelina***	***Rhizoctonia solani***	***Ilyonectria liriodendri***	***Trichoderma* spp.**

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A(Cuttings+Sawdust+Soil)	3,125 ab	6,51 bc	9,08 c	1,92 a	15,625 d	1,46 a	9,56 c
A(Cuttings+Sawdust)	0,00 a	5,08 ab	15,31 b	6,25 ab	0,00 a	3,125 a	15,54 b
B(Cuttings+Sawdust+Soil)	3,14 b	0,00 a	9,16 c	3,13 b	0,00 a	0,00 a	0,00 a
B(Cuttings+Sawdust)	12,53 c	0,00 a	4,79 ab	3,25 a	9,46 bc	0,00 a	6,25 ab
C(Cuttings+Sawdust+Soil)	0,00 a	0,00 a	10,88 b	9,43 b	15,625 c	0,00 a	18,28 c
C(Cuttings+Sawdust)	6,25 ab	3,16 ab	14,88 c	9,38 bc	6,25 ab	0,00 a	10,63 bc
D(Cuttings+Sawdust+Soil)	15,68 a	15,63 a	36,86 b	12,52 a	6,25 a	6,25 a	15,65 a
D(Cuttings+Sawdust)	6,25 a	12,25 a	25,99 b	12,50 a	6,50 a	3,13 a	6,25 ab

As seen in Table 3, the isolations conducted on healthy saplings uprooted from the nursery in autumn demonstrated the growth of 6 fungal pathogen colonies and biocontrol agent *Trichoderma* spp. *Fusarium solani* was isolated in all applications except for A (cutting + sawdust + soil). The least infected seedlings were observed in the healthy saplings in the A (cutting + sawdust + soil) application group. It could be suggested that the application of these two fungicides to the soil led to a significant decrease in soil-borne fungal pathogens. Pathogenic species *Fusarium solani* (12.67%), *Alternaria alternata* (15.63%), *Botryospheria obtusa* (12.25%), *Diaporthe ampelina* (6.25%) were isolated in the C (cutting + sawdust) application group, demonstrating that these species were asymptomatic in the inner tissues of healthy vines. The high *Trichoderma* spp. incidences in both bio-fungicide application groups (C: 56.25%, 34.36%, respectively) and control groups (D: 31.25%, 28.79%, respectively) demonstrated that its antagonistic effect was promoted in the soil over time, and it was effective in controlling soil-borne pathogens. This was explained by rapid colonization of *Trichoderma* bio-fungicide in the soil, limiting pathogen development due to antagonistic properties. It was determined that there was a positive correlation between the isolation incidences of *Fusarium solani* (36.86%), *Botriospheria obtusa* (15.63%), and *Diaporthe ampelina* (12.52%) pathogens in D (cutting + sawdust) application and the development of certain pathogens in the moisture generated by soaking the seedlings with water during initial planting.

Table 5. Fungal isolation incidences in the roots of the healthy saplings uprooted from the nursery after treatment (%).

Looking Healthy	**Fungal Incidence %**						
Treatment	***Alternaria alternata***	***Botryospheria obtusa***	***Fusarium solani***	***Diaporthe ampelina***	***Rhizoctonia solani***	***Ilyonectria liriodendri***	***Trichoderma* spp.**
A(Cuttings+Sawdust+Soil)	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a	3,125 b
A(Cuttings+Sawdust)	0,00 a	0,00 a	6,50 b	0,00 a	0,00 a	0,00 a	10,75 b

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B(Cuttings+Sawdust+Soil)	0,00 a	0,00 a	6,50 b	0,00 a	0,00 a	0,00 a	8,67 b
B(Cuttings+Sawdust)	0,00 a	0,00 a	3,75 a	0,00 a	0,00 a	0,00 a	18,13 c
C(Cuttings+Sawdust+Soil)	0,00 a	0,00 a	6,25 b	3,25 b	0,00 a	0,00 a	56,25 d
C(Cuttings+Sawdust)	15,63 c	12,25 c	12,65 c	6,25 b	0,00 a	0,00 a	34,36 c
D(Cuttings+Sawdust+Soil)	3,25 a	12,50 b	31,25 c	6,17 ab	0,00 a	0,00 a	31,25 c
D(Cuttings+Sawdust)	3,13 ab	8,49 c	18,68 d	6,25 bc	0,00 a	0,00 a	28,79 d

The impact of fungicide and biological preparate applications on seedling quality and yield was statistically significant at 1% and 5% confidence levels (Table 4). B (cutting + sawdust + soil) application was statistically significant in total seedling yield (78.75%) and first size grafted seedling yield (61.25%), and it was the most effective treatment. It was determined that the fungicides that included fluopyram + tebuconazole and cyprodinil + fludioxonil improved seedling yield and quality. It was determined that C (cutting + sawdust) application promoted callus development and shoot length by 3.90% and 28.70%, respectively. It was determined that bio-fungicide application during the seedling cultivation stage were effective on callus and shoot development.

Table 4. The impact of applications on total seedling yield, 1st size grafted vine seedling yield, callus development and shoot length. .

Treatments	**Total Sapling Yield (%)**	**I. Length Grafted Vine Sapling Yield (%)**	**Callus development level (scale 0-4)**	**Shoot length (cm)**
A(Cuttings+Sawdust+Soil)	69.38 ab**	40.625 ab*	3.75 ab**	23.60 ab*
B (Cuttings+Sawdust+Soil)	78.75 a	61.25 a	3.80 b	22.90 ab
C (Cuttings+Sawdust+Soil)	64.38 abc	44.25 ab	3.60 ab	16.40 bc
D (Cuttings+Sawdust+Soil)	57.50 bc	34. 38 b	3.45 ab	16.00 bc
A (Cuttings+Sawdust)	61.25 abc	43.75 ab	3.65 ab	21.00 ab
B (Cuttings+Sawdust)	70.63 ab	50.00 ab	3.85 b	20.70 ab
C (Cuttings+Sawdust)	60.00 abc	43.13 ab	3.90 a	28.70 a
D (Cuttings+Sawdust)	45.00 c	25.00 ab	3.60 ab	11.20 b

The data were analyzed with the Duncan multiple comparison test. *: Significant at 1%, **: Significant at 5%. Lower case letters on the same line indicate statistically significant differences between applications.

DISCUSSION

The present study demonstrated that *Botryosphaeria dothidea, Diaporthe ampelina* and *Ilyonectria liriodendri* species were a significant threat to grapevine nursery industry [22]. Furthermore, it

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demonstrated that *Fusarium solani* and *Alternaria alternata* species were the most frequently isolated fungi in grapevine nurseries; and thus, the main reason for the decline in young grapevine [11]. Certain studies reported *Alternaria alternata* as an endophyte and saprophyte species in grapevine saplings [7]; however, in the present study, isolations conducted in all stages demonstrated that, there is possible pathogenicity and a need for further studies. During the isolation steps, it was observed that *Fusarium solani* and *Alternaria alternata* fungi were the most common species in shoots and roots during callus development, *Botrytis cinerea* pathogen was found in low amounts during callus development, and was not detected after planting. Although *P. exigua* var. *exigua* was detected at the shoots and roots during callus development, it was not found in uprooted saplings. In a previous study, it was shown that *Phoma negriana* Thum. pathogen led to black rot and necrosis in the infected vine stems and leaves [23], demonstrating that it was pathogen remained effective during callus development under high humidity. A comparable study was conducted by Yıldız and Gursoy [24] on a grapevine nursery in the Aegean Region in Turkey, and *B. cinerea, Pythium* sp. and *Fusarium* sp. were reported as the most frequently isolated species. Numerous species in *Fusarium* genus have been isolated from seedling phyllosphere and *F. oxysporum* was reported to be responsible for the vineyard seedling decline [8, 10, 25]. In a study where the fungal species associated with healthy vine cuttings were identified in South African nurseries, *Alternaria, Epicoccum, Cladosporium* were the most frequently isolated fungal species during callus development stage and before planting. Although *Fusarium* spp. was isolated during callus development, it was the most frequently isolated species 3 months after planting the grapevine seedlings [6, 26]. In the present study, *Fusarium solani* was the most isolated species after fungicide and bio-fungicide applications and during folding. *Fusarium solani* was responsible for losses in seedling cultivation and isolated from seedlings with symptoms in transmission bundles such as the young grapevine decline disease. The high incidence of *Fusarium solani* in healthy saplings during seedling cultivation (Table 3) demonstrated its asymptomatic character. In the isolations conducted after folding, *Diaporthe ampelina,* the root and leaf spot disease pathogen, was isolated from shoots and roots. It was proved that the disease was transmitted to the vineyards due to the employment of *P. viticola*-contaminated cuttings and grafts in nurseries, increasing the incidence and early yield losses in vines, and evidencing the latency of the pathogen in vine shoots [10]. Similar findings that revealed the damages induced by *P. viticola* in grapevine cuttings in various parts of the world were reported previously [27, 28, 29]. One of the fungal lignum disease agents, *Botryosphaeria* spp., and the black foot disease agent *Ilyonectria* spp. were isolated from shoots and roots in certain applications, albeit with low incidence. It was claimed that *Cylindrocarpon* spp., the black foot disease pathogen, was rarely observed in propagation materials during this period in isolations from callused cuttings before planting [6]. In the study, *Ilyonectria liriodendri* isolation incidence was 1.93% in roots before planting, while the isolation incidences of *Ilyonectria liriodendri* were 6.25% and 3.13% in the roots of the diseased vines in the control group uprooted from the nursery. According to Halleen et al. [6], *Cylindrocarpon* spp. incidence was less than 1% before planting, and more than 50% in the planted seedlings at the end of the season (June). The spread of the black foot disease in nurseries indicates that the disease is a soil-born pathogen and could spread rapidly based on the soil and via water [30, 31].

In the triazoles group, certain active substances such as tebuconazole exhibited different behaviours in various grapevine stem diseases. It was reported that these differences were due to the differences between the tested vine tissues, pathogens, strains and vine varieties. Tebuconazole was reported to be effective against the cancer induced by *Botryosphaeria* sp. [32], and was effective against *Diplodia seriata, Eutypa lata, Inocutis* sp. and *P. chlamidospora* when rubbed on fresh pruning wounds [33, 34]. It was reported that floupyram, a SDHI (Succinate Dehydrogenase Inhibitor) fungicide, was effective against soil borne *Sclerotinia* spp., *Rhizoctonia* spp., *Fusarium* spp., and *Phoma* spp. and *Alternaria* solani fungi in certain plant tissues [35]. It was also reported that, when applied on pruning wounds, floupyram was successful against wood tissue cancers caused by various pathogens such as *D. seriata, D. mutila, Botryosphaeria* spp., *Lasiodiplodia theobromae* [36]. In the present study, the application of fungicide that included a mixture of fluazinam + tebuconazole solution to soil decreased the incidence of soil pathogens such as *R. solani, F. solani, A. alternata* and *I. liriodendri* in

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both sick and healthy seedlings when compared to controls. Also, it was determined that both callus and soil period applications of this fungicide were successful against *Botryosphaeria obtusa* and *Diaporthe ampelina*.

Cyprodinil + fludioxonil solution significantly reduced the incidence and latency of *B. obtusa, D. ampelina* and *I. liriodendri* in healthy plants after soil application under high and very high inoculum pressures. The incidences of *A. alternata* and *Rhizoctonia solani* fungi were also significantly reduced. This fungicide solution did not eradicate these pathogens in cuttings, but significantly reduced their incidence and severity when compared to the water-treated control. The spread of endophyte and root rot pathogens in transported grafted vine seedlings was reduced, preventing further contamination during grafting, callusing and storage, and positively affecting the health and quality of the vines [37]. It was determined that Cyprodinil + fludioxonil fungicide was effective against *Ilyonectria* spp. in vitro. It was confirmed that cyprodinil + fludioxonil, employed in controlled trials and pots against *I. liriodendri* during the rooting of 1103 Paulsen rootstock cuttings, reduced the incidence by 63.4% in 2020 and 69.6% in 2021 in the roots [38].

In the present study, *Trichoderma* was the second most frequently isolated genus in both isolation points (shoots and roots) after soil applications. It was observed that *T. afroharzianum* and *T. gamsii* species were beneficial endophytes in lignum and stimulated seedling growth after the applications. Selected *Trichoderma harzianum* strains were applied during layering, soil improvement and irrigation in the nursery on petri and blackfoot disease pathogens, and the findings were compared with (standard) grapevines treated with quintozene/procymidone fungicides. The bioactivity of *Trichoderma harzianum* Rifai KRL-AG2 strain was tested in pot trials to prevent *I. liriodendri* infections during rooting of 1103 Paulsen vine rootstock in Aegean Region nurseries in Turkey. In pot trials, *Trichoderma harzianum* Rifai KRL-AG2 strain significantly increased the vine root biomass when compared to the control and reduced the root disease severity by 60.8% by reducing necrosis in the roots [38]. In our study, the *Trichoderma harzianum* Rifai KRL-AG2 bio-preparate applied to cuttings + sawdust and nursery soil reduced fungal disease incidence and increased the total seedling yield as much as the fludioxonil + cyprodinil application.

It was observed that shoot growth was noticeably better in vines treated with Trichoderma when compared to the control. Significantly fewer fungi were isolated from the roots of the *Trichoderma*-treat*ed* vines. The Petri disease incidence was similar in the roots treated with Trichoderma and the standard application, but *Cylindrocarpon* spp. was isolated less in *Trichoderm*a-treated vines [39]. These findings demonstrated that *Trichoderma* treatment would contribute to strong vine growth with lower *Botryopshaeria* and *Ilyonectria* infection levels. *Trichoderma* treatment could produce varying findings based on the seedling cultivation stage. *Trichoderma* applications in rooting stage were the most effective, while the applications in callus development or the rooting and callus stages were inconsistent but generally produced negative results. *Trichoderma* treatment also reduced necrotic areas induced by *Botrytis cinerea* in leaves and the degree of cutting necrosis in plants inoculated with *Phaeomoniella chlamydospora*. These reductions in necrosis were significantly higher 15 months after inoculation [40].

Planting could stress vines in the nurseries and grafting could lead to detrimental changes in plant physiology [41]. Among the bench grafting techniques, omega grafting is the most common grafting method in grapevine nurseries due to its high success rate. Little research has been conducted on grafting and grafting quality to date. Further studies are required to confirm the significance of GTDs in the development of graft quality [42]. Soil fungicide applications and rooting and callus stages further improved the quantitative and qualitative properties of the root system and increased the rate of certified vine production. It was confirmed that *Trichoderma* application only during rooting had positive effects on the morphophysiological properties of the vine and increased the tolerance to stress-related diseases such as esca forms in nurseries [40].

Conclusion

This study was conducted with the aim of determining the effects of fungicidal and antagonist applications on the control of fungal pathogens during seedling cultivation. The effects of these

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applications on seedling yield, quality and root development were obtained. In the study, *Fusarium solani* and *Alternaria alternata* species were the most frequently isolated species in the vine nursery before and during rooting, and before and after planting. The isolation of *Botryosphaeria* spp., *Diaporthe ampelina* and, most importantly, *Ilyonectria* spp., the causal agent of black foot disease in some treatments, from roots and shoots before planting proved that the seedling production stages are the starting point of the infection source and that they can remain latent until planted in the soil. It could be suggested that these species were responsible for sapling loss during vine sapling cultivation. It was demonstrated that during callus development, the pre- and post-planting soil applications prevented the infection and colonization of soil-borne fungi. Thus, the overall reduction in the pathogens by cyprodinil + fludioxonil and fluazinam + tebuconazole clearly demonstrated that these fungicides applied in a nursery rotated for 3 years could be alternatives or complement to other strategies. In particular, it was determined that the *Trichoderma harzianum* bio-fungicide reduced fungal pathogen incidence to a certain extent during the seedling cultivation and led to stronger plant growth by promoting root and callus development. The employment of bio-fungicides that include *Trichoderma* spp. was recommended in every seedling cultivation stage to control plant stress. Bio-preparation of *Trichoderma harzianum* Rifai KRL-AG2 race and cyprodinil + fludioxanil active substances applied to cuttings+sawdusts and nursery soil both reduced the incidence of fungal trunk tissue and root rot diseases such as *B. obtusa*, *D. ampelina* and *I. lirio*dendri and increased the total yield of seedlings. Solarisation and fumigation employed to disinfect the nursery soil could limit pathogen inoculum, while reducing other beneficial organism content. It was determined that treatments of fungicides or bio-fungicides before the disease symptoms, during seedling production, or by wetting the seedling roots and planted seedling root collars would prevent root rot pathogens and would not prevent the growth of other beneficial fungi (i.e., *Trichoderma* spp.).

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