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Fungicide suspensions combined with hot-water treatments affect endogenous *Neofusicoccum parvum* infections and endophytic fungi in dormant grapevine canes

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Summary. *Neofusicoccum parvum* is an important opportunistic fungus causing Botryosphaeria dieback on grapevines. Because of its opportunistic nature, this pathogen spreads to many grape growing areas in a latent phase, and causes serious economic losses. The aims of the study were to examine hot-water treatments combined with fungicides in order to cure latent infections of artificially inoculated *N. parvum* in dormant grapevine canes and to assess the effects of these treatments on endophytic fungi. Artificially *N. parvum*-inoculated canes were dipped into cyprodinil + fludioxonil, tebuconazole or thiophanate-methyl suspensions under the following temperature-duration combinations; 30°C for 12 h, 35°C for 6 h, 40°C for 2 h or 50°C for 30 min (temperature at 50°C was not combined with fungicides). Treated canes were cooled in tap water (18°C) for 1 h, and pathogen re-isolations were immediately attempted from inner wood tissues. Extent to which these applications affected the presence of endophytic fungi were also determined by calculating pre- and post-treatment isolation rates. Hot-water treatments (without fungicides) below 52°C did not reduce *N. parvum* re-isolation rates, and were ineffective. However, these treatments combined with fungicides decreased pathogen incidence at 50°C and below. Maximum reduction (34%) was obtained with heated tebuconazole suspensions at 40°C for 2 h and 50°C for 30 min, and eradicative ability was superior to that of cyprodinil + fludioxonil or thiophanate-methyl. The hot-water treatments reduced incidence of endophytic fungi but greater reduction was observed with the hot-water and fungicide combinations. Fungicide penetration into wood tissues of propagation material could be enhanced by increasing water temperatures in hydration or hot-water treatment tanks, and this approach could be useful method for production of healthy grapevine plants in nurseries.

Keywords. *Vitis vinifera*, tebuconazole, cyprodinil, fludioxonil, thiophanate-methyl.

INTRODUCTION

Neofusicoccum parvum (Pennycook & Samuels) Crous, Slippers & Phillips is one of the most important pathogens causing Botryosphaeria dieback of grapevines. This fungus causes a variety of symptoms in grapevines, includ-

ing brown wedge-shaped wood cankers, xylem necroses, failure of spring growth and black dead arm (Úrbez-Torres, 2011). Although *N. parvum* pycniospores are the main inoculum sources for short distance dissemination, the pathogen is more likely to spread to different geographical areas through infected propagation materials. When the pathogen infects vines through wounds, natural openings or direct penetration of bark, it can progress systemically in cane vascular and wood tissues, and can cause decline of vines (Amponsah *et al*., 2012). Isolation of botryosphaeriaceous fungi from asymptomatic canes suggest the importance of latent infections on pathogen dissemination in vineyard areas (Slippers and Wingfield, 2007).

Hot-water treatment (HWT) is a promising and plausible method to reduce endogenous infections of all grapevine trunk disease (GTD) and other pathogens, for production of healthy propagation material (Fourie and Halleen 2004; Halleen *et al.,* 2007). Though some studies have reported that standard HWT (at 50°C for 30 min) can eliminate grapevine pests, some bacterial pathogens (such as *Xylella fastidiosa* and *Rhizobium vitis*) and phytoplasmas (Flavescense dorée) are not completely eradicated (Ophel *et al.,* 1990; Caudwell *et al.,* 1997; Crous *et al.,* 2001). There is also evidence that GTD fungi are not sufficiently eliminated from dormant grapevine canes by these treatments (Rooney and Gubler, 2001; Waite and May, 2005; Gramaje *et al.,* 2010). *Neofusicoccum parvum* is among the GTD fungi which were subjected to HWT studies both in laboratory and controlled conditions. *Diplodia seriata*, *Neofusicoccum luteum*, *N. parvum* and *Spencermartinsia viticola* were found to be the most susceptible species, while *Lasiodiplodia theobromae* and *N. vitifusiforme* were very tolerant to HWT in *in vitro* conditions. Mycelium survival of *N. parvum* was about 15% from HWT at 50°C for 30 min in test tubes containing sterile water (Elena *et al*., 2015). In this study, when the curative ability of HWT was tested with *N. parvum* inoculated grapevine canes, HWT (at 51°C for 30 min) eradicated the pathogen from dormant canes of 110-Richter rootstock. However, Billones-Baaijens *et al*. (2015) found controversial findings from their study, showing that HWT (at 50°C for 30 min) was less effective or ineffective, respectively, for eradication of *N. luteum* and *N. parvum* from infested cuttings of 101-14 Mgt rootstock. While this treatment reduced the isolation frequency of *N. luteum* by 50%, it did not reduce isolation of *N. parvum*. The expected performance from HWT was obtained just with 53°C for 30 min, but this combination was harmful for bud vitality of the treated rootstock cuttings. Billones-Baaijens *et al*. (2015) suggested that research should be carried out to improve hot-water treatments practicable for grapevine nursery industries. In Turkey, most nurseries avoid HWT because of detrimental effects on plants, such as delayed callusing, bud death, and delayed development and rooting.

Dipping of grapevine cuttings in fungicide suspensions is another control measure to produce healthy grapevine plants in nurseries. Rego *et al*. (2009) determined the efficacy of applications of cyprodinil + fludioxonil, metiram + pyraclostrobin, cyprodinil or fludioxonil suspensions, by hydration (tap water) of the rootstock cuttings prior to grafting. They found that the cyprodinil + fludioxonil reduced the incidence of black foot (by 47%) and botryosphaeriaceous fungi (by 60%) in plants grown in a field that was naturally infested with these pathogens. Halleen and Fourie (2016) developed a combined control strategy to reduce GTD pathogen infections South Africa, showing that benomyl and Sporekill® were useful chemicals for increasing healthy plant rates (up to 100%) in the grapevine nurseries. These studies were conducted in naturally infested fields or mother plants that were known to be infected with some GTD pathogens. Billones-Baaijens *et al*. (2015) showed that tebuconazole and carbendazim soakings (at tap water temperature) were effective for curing superficial bark infections by *N. parvum* or inoculated canes.

Curative performance of fungicide treatments against latent inner tissue infections of canes is important, because outer bark is a natural barrier preventing entry of pesticides into internal host tissues. Fungicide penetration via diffusion is known to be very inefficient (Waite and May, 2005). Therefore, there is a need to increase fungicide penetration into grapevine wood tissues during the soaking stage before grafting or cold storage for production of healthy propagation material.

The combination of HWTs with fungicides may be an effective approach for curing latent infections by GTD fungi in grapevine nurseries. This has previously been studied by many researchers who work on postharvest diseases of some fruits (Wells and Harvey, 1970; Barkai-Golan and Apelbaum, 1991). These studies showed that heat treatment in combinations with fungicides could increase penetration of the pesticides into fruit tissues. Schirra *et al*. (1996) treated lemon fruits with imazalil suspensions (1500 $mg \cdot L^{-1}$ at 20°C or 250 $mg \cdot L^{-1}$ at 50°C for 3 min) to cure *Penicillium* infections. The second of these treatments was as effective as the first for disease control, and four or five-fold increases in fungicide residues were recorded in albedo tissues of fruits from the treatment of 250 $mg \cdot L^{-1}$ imazalil at 50°C for 3 min.

Endophytic fungi live within plants without causing disease symptoms (Freeman, 1904). These fungi can contribute to plant adaption and survival in unfa-

vourable conditions. They can also produce secondary metabolites, trigger biochemical pathways or interact with other microorganisms (Aly *et al*., 2010; Kusari *et al*., 2012). Gonzalez and Tello (2011) assessed endophytic fungi from different organs of seven grape cultivars in central Spain, and obtained more than 500 isolates belonging to 68 species. While the endophytic composition differed according to vine cultivars, *Acremonium, Alternaria, Aureobasidium, Cladosporium, Epicoccum, Fusarium, Penicillium, Phoma*, and *Trichoderma* were isolated most frequently. Some of these fungi were reported to have antagonistic ability towards plant pathogenic fungi. Eichmeier *et al*. (2018) investigated effects of hot-water treatments (at 50°C or 53°C for 30 min) on endophytic fungi from dormant canes of Garnacha Tintorera/110 Richter and Sauvignon Blanc/SO4 grapevines. Although hot-water treatments decreased the incidence of endophytic fungi depending on temperature, HWTs did not sterilize dormant canes, and these fungi were recovered from hot-water treated plants grown for 8 months in nurseries. Although several studies have investigated curative effects of hot-water treatments (conducted generally at 50-54°C for 30, 45 or 60 min) on GTD pathogens (Habib *et al.,* 2009; Serra *et al.,* 2009), limited results are available where effects have been assessed for HWTs on endophytic fungi in dormant grapevine canes.

In evaluating current literature, we hypothesized that penetration of fungicides into dormant grapevine canes could be enhanced by heating fungicide suspensions (at low temperatures combined with extended durations) at the pre-grafting host stage. The aims of the study were: (i) to determine the efficacy of heated fungicide suspensions on latent infections of *N. parvum* and their effects on endophytic fungi in dormant grapevine canes; and (ii) to assess antagonistic abilities of some endophytic isolates against *N. parvum* in *in vitro*.

MATERIALS AND METHODS

Three fungicides (Table 1) with different modes of action were used to prepare hot-water treatment suspensions at 50°C and lower temperatures (30, 35 and 40°C). Dormant grapevine canes (*Vitis vinifera* 'Prima') were used as plant material in all experiments.

Isolation and molecular identification of endophytic and pathogenic fungi

The dormant canes were taken (in December 2017) from randomly selected vineyards in three different geographic regions of Turkey (Manisa, Tokat and Tarsus Cities; in, respectively, the Aegean, Central Anatolia and Mediterranean Regions). Fifty, one-year-old dormant canes (each with three to five buds and 50 cm long) were randomly taken from each vineyards (two vineyards per region, 25 canes from each vineyard). These canes were washed under running tap water for 10 min., and 10 cm-long cuttings were used for endophyte isolations. The cuttings were dipped into ethyl-alcohol (70%) for 1 min, sodium hypochlorite solution (2.5% NaOCl) for 3 min, and ethyl-alcohol (70%) (Schultz *et al*., 1993). Canes were then rinsed twice with sterile distilled water and bark tissues were removed with a sterile knife. Wood tissues were cut into 3 mm^2 pieces, and seven each were plated onto potato dextrose agar (PDA, Becton Dickinson) amended with streptomycin sulfate (150 $mg{\cdot}L^{-1}$). Petri dishes were incubated for 10 d at 24°C in the dark, and growing fungal colonies were sub-cultured to fresh PDA (without antibiotic) for further microscope examinations and molecular identification. After morphological discrimination and microscope examinations, the incidence of endophytic species was calculated by counting colonies (at genus or species level) growing around tissues and average isolation rates were determined from 25 Petri dishes for each vineyard. For each geographic region, average incidence of fungi was calculated as percent.

For molecular identification of fungi, 10-d-old mycelium mats of the isolates (growth from hyphal tip or single spore cultures) were harvested (50–100 mg), and DNA was extracted according to the protocol of Nejat *et al*. (2009). PCR amplification of the ITS1, 5.8S and ITS2 regions of rDNA was performed using ITS4 and ITS5 primers (White *et al*. 1990), and thermocycler (SimpliAmp A24811™; Applied Biosystems) conditions were adjusted as follow; 95°C for 3 min (initial denatura-

Table 1. Fungicides and application rates used in this study.

Active ingredients	Fungicide group	Application dose (product in water·L^{-1})	Trade name and formulation	Manufacturer
Cyprodinil (37.5%) + fludioxonil (25%)	Anilinopyrimidine + phenyl-pyrrole	5 g	Switch® 62.5 WG	Syngenta
Tebuconazole (250g·L^{-1})	DMI-triazole	4 mL	Orius® 20 EW	Adama
Thiophanate-methyl (70%)	Benzimidazole	5 g	Sumitop 70 WP	Sumitomo

tion), followed by 35 cycles each of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were purified and sequenced (Sanger dideoxy sequencing) by MedSanTek Co. (Istanbul, Turkey), and nucleotide sequences (in forward and reverse directions) of the isolates were contiged using Chromas Lite free software (Technelysium™). Consensus nucleotide sequences were compared with those deposited in the NCBI GenBank database using the BLAST program, and identification of the isolates was decided according to maximum identity results (with 99% or 100% rates). Sequences were deposited (except the well-known species, *Aspergillus niger, Aureobasidium pullulans, Epicoccum nigrum, Nigrospora oryzae, Penicillum* sp. *Rhizopus stolonifer*) to the NCBI database and accession numbers were obtained.

Inoculation and incubation of Neofusicoccum parvum

An electric drill method (Pouzoulet *et al.*, 2013) was used to inoculate *N. parvum* in all HWT experiments. The *N. parvum* isolate (MBAi27AG, GenBank accession no: KF182330), formerly shown to be highly virulent, was selected from the fungal library of Cukurova University, Department of Plant Protection. This isolate was grown on PDA at 25°C for 10 d in the dark. Dormant grapevine cuttings (each measuring 45 cm) were taken from a Prima vineyard (grafted on 1103 Paulsen, 10 years-old) in Tarsus city (Southern Turkey), and were transferred to the laboratory in December 2017. To prevent dehydration, they were immersed in clean tap water at ambient temperature (20°C) overnight. The cuttings were then trimmed to 35 cm, and the superficially sterilized in 2.5% NaOCl solution for 3 min and rinsed twice with sterile distilled water. Cane internodes were laterally drilled with a 2 mm-diam. drill bit and 2 mm-mycelium plugs were inserted into the drill holes which were then sealed with Parafilm®. In control plants, sterile agar pieces were inoculated into the wounds and the holes were sealed with Parafilm®. The inoculated canes were planted into plastic bags containing growth mixture (fine sawdust, perlite, sand, soil and peat, in equal volumes), and were then watered and maintained in a growth room (28°C. 90% relative humidity, 12 h dark/12 h light) for 2 months. For further experiments, each of the cuttings was numbered to compare initial and final endophytic flora after all treatments. The endophytic fungi were isolated from these cuttings before inoculation with *N. parvum*, and their isolation frequencies were recorded for further comparisons.

Hot-water treatment effects on Neofusicoccum parvum *infections and endophytic fungi*

The curative effects of hot-water treatments were investigated with and without fungicides. All HWT experiments were conducted in an adjustable 10 L capacity water bath device (Memmert WB 10). Air was pumped through the device using an aquarium pump to provide homogenity of fungicide suspensions. In fungicide-HWT treatment combinations, the fungicide suspensions were prepared at the application rates specified in Table 1, and at temperature/time treatments of 30°C for 12 h, 35°C for 6 h, 40°C for 2 h or 50°C for 30 min. The plants that were previously inoculated with *N. parvum* and grown for 2 months were uprooted from pots, and their roots and shoots were removed before HWTs. The cuttings (30 cm) were firstly washed and immersed in clean tap water at ambient temperature for 1 h. Bundles of cuttings, each comprising 12 cuttings, were then dipped into heated fungicide suspensions at the temperatures and for the durations specified above. The treated cuttings were cooled in clean tap water (at 18°C) for 1 h. Pathogen isolations were then carried out from the wood tissues from around inoculation points (4 cm from both sides of the inoculation holes), to investigate *N. parvum* survival, and from 10 cm from inoculation points to assess presence of endophytic fungi. Inner wood tissues were cut into 3 mm pieces with sterile secateurs and placed onto PDA amended with streptomycin-sulfate in Petri plates. The plates were then incubated at 25°C for 7 d in the dark. Control canes (inoculated but not treated with HWT-fungicide combinations) were subjected to the same isolation process. All the wood chips from a cane were placed on one plate, so 12 plates were used for each treatment (completely randomized design, four replicates and three plates from each replicate). Fungal colonies were counted and incidence was calculated as percentages.

The inoculated cuttings were also subjected to hot-water treatments (without fungicides) at temperature-time combinations of: 30°C for 12 h, 35°C for 6 h, 40°C for 2 h, or 50°C, 51°C, 52°C, or 53°C, each for 30 min. After HWTs, the cooling, isolation and incidence calculation procedures described above were carried out. This experiment was repeated once and average incidences were calculated.

In vitro *antagonistic effects of endophytic fungi against* Neofusicoccum parvum

Frequently isolated endophytic species (one isolate each) were selected to check antagonistic ability against *N. parvum*. Mycelial agar discs from 10-day-old cul-

tures of endophytic isolates were placed on one side of PDA plates. They were allowed to grow at 24°C for 72 h (because of slow growth), and *N. parvum* discs (from 10-d-old cultures) were placed on the other side of plates. These dual culture plates were incubated at 24°C for 10 d, with *N. parvum* growth observed daily and colony diameters (length-width) were measured to determine average colony diameters. Experimental control plate contained sterile agar discs opposite the *N. parvum* discs. Inhibition rates (%) were calculated according to Reyes-Chilpa *et al*. (1997) as cited by Felber *et al*. (2016). Interactions between endophytic isolates and *N. parvum* were grouped using the scale of Badalyan *et al*. (2002); where A = deadlock with mycelial contact, B = deadlock at a distance, and C = replacement, overgrowth with and without initial deadlock. Dual culture tests were arranged in a completely randomized design with five replications, and were repeated once.

Statistical analyses

Variance analyses were performed on data to reveal differences between means of the treatments, and the statistically similar groups were determined by Fisher's Least Significant Difference (LSD) test $P \leq 0.05$) in all experiments (Gomez and Gomez, 1984). The data of isolation percentages and inhibition rates were converted by Arc Sin transformations before variance analyses.

RESULTS

Endophytic and pathogenic fungi from apparently healthy cuttings

Twenty-three operational taxonomic units (OTUs) of fungi belonging to three phyla were obtained from grapevine cuttings from three regions in Turkey (Table 2). Wood pieces plated on PDA yielded more than one colony (average two) after 7-8 days of incubation. While most of the fungi (19 OTUs) were Ascomycota, the others were Basidiomycota (*Cerrena unicolor, Quambalaria cyanescens* and *Schizophyllum commune*) and Zygomycota (*Rhizopus stolonifer*). All of the OTUs were not regularly isolated from all the regions, but *Acremonium* sp. *Aureobasidium pullulans, Alternaria alternata, Aspergillus niger, Cladosporium cladosporioides, Penicil-*

Table 2. Endophytic and pathogenic fungi isolated from dormant grapevine canes in the study.

Order	Genus	Species	Isolate	GenBank Accession Number
Ascomycota	*Acremonium*	sp.	CUZFVG42	MK120286
Ascomycota	*Alternaria*	*alternata*	CUZFVG12	MK120281
Ascomycota	*Alternaria*	*tenuissima*	CUZFVG276	MK120296
Ascomycota	*Aspergillus*	*niger*	N/A	N/A
Ascomycota	*Aureobasidium*	*pullulans*	N/A	N/A
Basidiomycota	*Cerrena*	*unicolor*	CUZFVG176	MK120292
Ascomycota	*Chaetomium*	*globosum*	CUZFVG10	MK120280
Ascomycota	*Cladosporium*	*cladosporioides*	CUZFVG38	MK120283
Ascomycota	*Diaporthe*	*foeniculina*	CUZFVG125	MK120290
Ascomycota	*Diplodia*	*seriata*	CUZFVG4	MK120279
Ascomycota	*Epicoccum*	*nigrum*	N/A	N/A
Ascomycota	*Fusarium*	*equiseti*	CUZFVG87	MK120289
Ascomycota	*Geosmithia*	sp.	CUZFVG40	MK120285
Ascomycota	*Gnomonia*	*idaeicola*	CUZFVG36	MK120282
Ascomycota	*Lasiodiplodia*	sp.	CUZFVG250	MK120294
Ascomycota	*Neofusicoccum*	*parvum*	CUZFVG2	MK120278
Ascomycota	*Nigrospora*	sp.	N/A	N/A
Ascomycota	*Penicillium*	sp.	N/A	N/A
Ascomycota	*Phoma*	*glomerata*	CUZFVG212	MK120298
Zygomycota	*Rhizopus*	*stolonifer*	N/A	N/A
Basidiomycota	*Quambalaria*	*cyanescens*	CUZFVG39	MK120284
Basidiomycota	*Schizophyllum*	*commune*	CUZFVG173	MK120291
Ascomycota	*Trichoderma*	*atroviride*	CUZFVG243	MK120297

Table 3. Mean incidences of frequently isolated endophytic fungi in grapevine cuttings from three regions of Turkey.

Endophytic fungi	Incidence (%)		
	Manisa	Tarsus	Tokat
Acremonium sp.	2.0	3.6	17.1[a]
Aureobasidium pullulans	22.3	1.2	88.0
Alternaria spp.	14.3	16.3	12.6
Aspergillus niger	0.9	1.2	0.6
Cladosporium sp.	51.4	32.8	16.6
Penicillium sp.	0.9	5.2	0.8
Quambalaria cyanescens	0.1	9.9	0.1

[a]Means were for two vineyards for each location, and 25 grapevine cuttings from each vineyard were used for the isolations.

lium sp. and *Quambalaria cyanescens* were endophytic fungi isolated from all the regions and vineyards. The isolation percentages of endophytic fungi showed differences by geographical region. When compared to mean incidence rates, *Cladosporium* sp. was predominant in Manisa (51% recovery) and Tarsus (33%), but *A. pullulans* was predominant in Tokat (88%) and Manisa (22%). *Alternaria* sp. was the second most frequently isolated species in Manisa (14%) and Tarsus (16%). *Quambalaria cyanescens* was the third most common species in Tarsus (10%), but incidence of this species was very low (0.1%) in the other regions (Table 3). In apparently healthy canes, some pathogenic species (Botryosphaeriaceae,) were also isolated at low incidence rates, and only one isolate each of *S. commune* and *D. foeniculina* were obtained (Table 2).

Curative effects of hot-water treatments on Neofusicoccum parvum *infections*

No fungicide was added in the dipping tank for temperatures above 50°C due to risks of chemical degradation. The hot-water treatments below 50°C with extended durations (without fungicide), and 30 min durations at 50 or 51°C), had no curative effects on *N. parvum* infections. The 2 month incubation period ensured good establishment of the pathogen in plants, and the pathogen was re-isolated (100%) from all inoculated plants (hot-water treated and untreated). When water temperature was 52°C, the pathogen re-isolation frequency was 97%, or at 53°C was 74 % (Table 4).

Some of the hot-water treatments combined with fungicides significantly decreased fungus re-isolation rates. When comparing all treatments, the most effective combinations were tebuconazole suspensions at 40°C for 2 h and 50°C for 30 min, giving pathogen re-isolation of 66% from the cutting inoculation points. The curative effects of these combinations was less (34% recovery) than for HWT alone (26% recovery) at 53°C for 30 min, though these recovery rates were not significantly different. The efficacy of other combinations ranged from 3 to 34% (Table 4). Tebuconazole and thiophanate-methyl suspensions at 40°C reduced the incidence of *N. parvum* to some extent, but the efficacy of cyprodinil + fludioxonil decreased above 30°C (Figure 1). The 50°C for 30 min treatment did not reduce curative performance of tebuconazole but reduced the activity of thiophanate-methyl (Figure 1).

Table 4. Mean incidences and eradication rates (%) of *Neofusicoccum parvum* after different hot-water treatments and fungicide combinations.

Hot-water treatments (with and without fungicides)	Incidence of *N. parvum* (%) ± SEs	Eradication of *N. parvum* (%) ± SEs
40°C for 2 h, tebuconazole	65.7 ± 3.5 a [a]	34.3 ± 3.8 a
50°C for 30 min, tebuconazole	65.7 ± 3.5 a	34.3 ± 3.2 a
53°C for 30 min, water	74.3 ± 5.4 ab	25.7 ± 4.8 ab
40°C for 2 h, thiophanate-methyl	88.6 ± 2.9 bc	11.4 ± 2.5 bc
35°C for 6 h, tebuconazole	88.6 ± 5.4 bc	11.4 ± 2.9 bc
30°C for 12 h, cyprodinil + fludioxonil	88.6 ± 5.4 bc	11.4 ± 4.1 bc
35°C for 6 h, thiophanate-methyl	91.4 ± 3.5 cd	8.6 ± 3.1 cd
50°C for 30 min, thiophanate-methyl	94.3 ± 3.5 cde	5.7 ± 2.9 cde
35°C for 6 h, cyprodinil,+,fludioxonil	94.3 ± 5.7 cde	5.7 ± 4.2 cde
30°C for 12 h, tebuconazole	97.1 ± 2.9 de	2.9 ± 3.1 de
40°C for 2 h, cyprodinil + fludioxonil	97.1 ± 3.5 de	2.9 ± 3.0 de
52°C for 30 min, water	97.1 ± 2.9 de	2.9 ± 2.7 de
30°C for 12 h, thiophanate-methyl	100 ± 0.0 e	0 ± 0.0 e
51°C for 30 min, water	100 ± 0.0 e	0 ± 0.0 e
50°C for 30 min, water	100 ± 0.0 e	0 ± 0.0 e
50°C for 30 min, cyprodinil + fludioxonil	100 ± 0.0 e	0 ± 0.0 e
40°C for 2 h, water	100 ± 0.0 e	0 ± 0.0 e
35°C for 6 h, water	100 ± 0.0 e	0 ± 0.0 e
30°C for 12 h, water	100 ± 0.0 e	0 ± 0.0 e
Untreated control	100 ± 0.0 e	0 ± 0.0 e

[a] Mean values within each column are significantly different ($P \leq 0.05$; LSD tests).
LSD (incidence) = 14.1, F Value: 4.53. LSD (eradication) = 10.7, F value: 6.39.
SEs: Standard error values.
The experiment was repeated once and the results were calculated by averaging incidence and eradication rates obtained from both experiments.

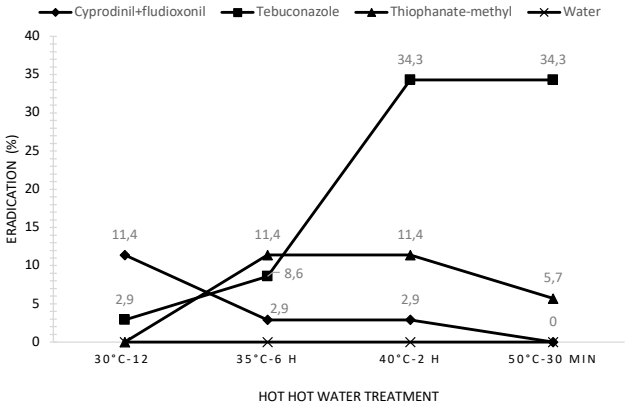

Figure 1. Mean eradication proportions (%) for *Neofusicoccum parvum* from grapevine cuttings by different fungicide suspensions and different hot-water treatments.

Effects of hot-water treatments on endophytic fungi

In the untreated control, most of the species had increased incidences during plant growth (Figure 2 a). However, with increased temperatures in the HWTs, there was progressive reductions in re-isolation rates of endophytic fungi in dormant canes (Figure 3 b-g). The 53°C for 30 min HWT almost eradicated all entophytic fungi except *Acremonium* sp. and *Alternaria alternata* (Figure 2h). *Acremonium* sp. was very tolerant to hot-water treatments, with increased re-isolation rates at from 30 to 52°C. In general, the HWTs decreased the other fungi. *Cladosporium* sp. also showed tolerance to HWTs. Compared with the initial isolation rates, final incidence of these fungi decreased for most of the treatments, but these fungi could not be fully eradicated, except at 53°C for 30 min. *Quambalaria cyanescens* was the most susceptible species, because this species was completely eradicated from dormant cuttings by temperatures of 50 to 53°C.

On the other hand, HWTs at 50°C and below, combined with fungicides gave greater eradication when compared to HWTs alone. Following these combined treatments, re-isolation rates of endophytic fungi (except *Acremonium* sp.) decreased, for almost in all of the combinations tested. Among the fungicides, tebuconazole was the most eradicative, at temperatures of 30°C to 50°C. At 30°C for 12 h dipping, most of the endophytic fungi were not be isolated after 10 d on PDA. All the tebuconazole suspension treatments completely suppressed endophytic fungi from wood pieces on isolation plates, however *Acremonium* sp. was observed at with low rates after 10 d (3 to 14%; Figure 3 b, e, h, k). For thiophanate-methyl, only *Penicillium* was eradicated at 40°C for 2h. Although incidences of *Alternaria* and

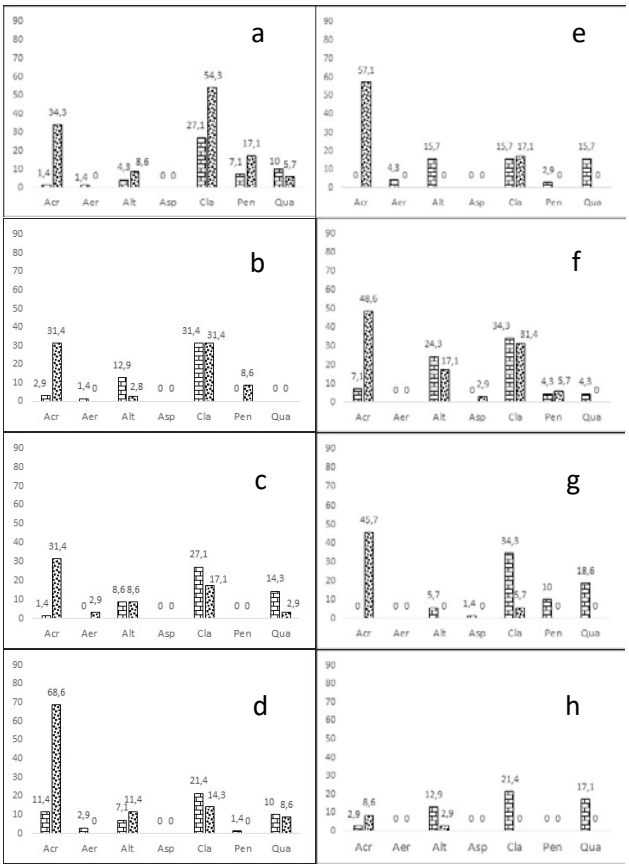

Figure 2. Mean incidence (%) of endophytic fungi in dormant grapevine cuttings after different hot-water treatments (without fungicides). The first and second columns in each histogram indicate, respectively, the re-isolation rates of endophytic fungi before and after the treatments. a) un-treated control, b) 30°C for 12 h, c) 35°C for 6 h, d) 40°C for 2 h, e) 50°C for 30 min, f) 51°C for 30 min, g) 52°C for 30 min, and h) 53°C for30 min.

Cladosporium were substantially decreased, they were not be eradicated with this treatment (Figure 3 i). The other combinations of thiophanate-methyl (30°C for 12h or 35°C for 6h) did not eradicate the other endophytic fungi (Figure 3 c, f). Cyprodinil + fludioxonil markedly suppressed occurrence of *Cladosporium* but did not affect *Acremonium* and *Alternaria* development (Figure 3 a, d, g, j).

Antagonistic effects of endophytes against Neofusicoccum parvum

The endophytic isolates gave inhibitive effects in the PDA dual culture experiment, and the inhibition interactions were of different types. *Trichoderma atroviride* gave the greatest inhibition of *N. parvum*.

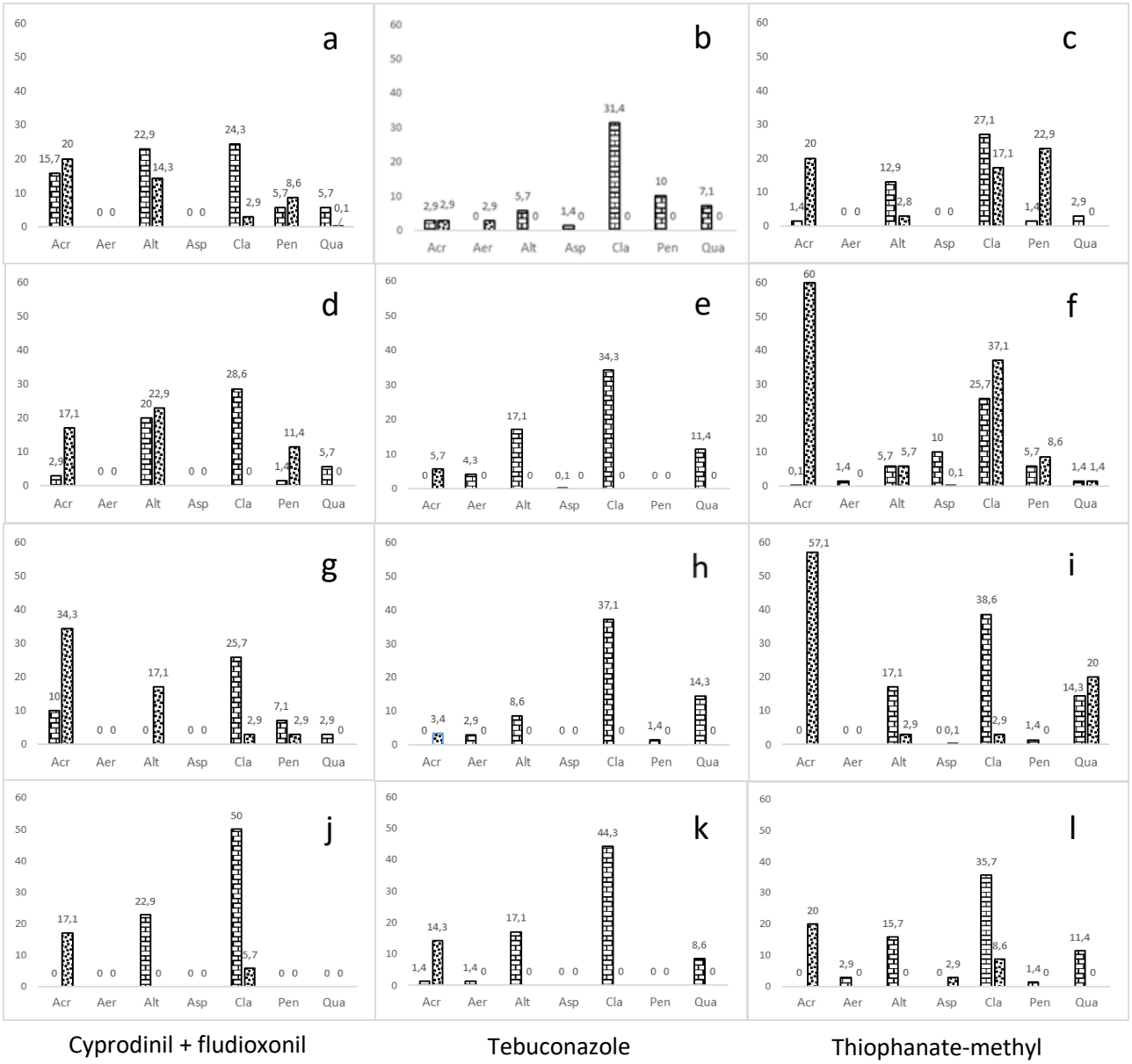

Figure 3. Mean incidence (%) of endophytic fungi in dormant grapevine cuttings after different hot-water treatments (combined with fungicides). The first and second columns in each histogram indicate, respectively, the re-isolation rates of endophytic species before or after the treatments were applied. a) b) c) 30°C for 12 h, d) e) f) 35°C for 6 h, g) h) i) 40°C for 2 h, j) k) l) 50°C for 30 min.

While *T. atroviride* (CUZFVG243) inhibited *N. parvum* growth by 69% (interaction class C), the inhibition percentages of *Acremonium* sp. (CUZFVG42), *E. nigrum* (CUZFVG88), *Geosmithia* sp. (CUZFVG40), and *Q. cyanescens* (CUZFVG39) were from 9 to 23% (class A). *Alternaria alternata* and *C. cladosporioides* moderately inhibited the pathogen, by, respectively, 31% and 30% (class B) (Table 5).

DISCUSSION

In this study, a variety of endophytic and pathogenic fungi were isolated from dormant and apparently healthy grapevine cuttings, taken from different geographical regions in Turkey. *Acremonium* sp., *Alternaria* spp., *Aureobasidium pullulans*, *Cladosporium* spp., and *Quambalaria cyanescens* were the most frequently isolated endophytic fungi, and *Diplodia seriata* was the

Table 5. Mean inhibition percentages, and competitive interactions with *N. parvum* in dual culture tests, for different isolates of grapevine endophytic fungi.

Endophytic isolates	Inhibition (%)	Interaction Class
Alternaria alternata (CUZFVG12)	31.1 ± 0.9 d [a]	B [b]
Acremonium sp. (CUZFVG42)	9.6 ± 1.0 a	A
Cladosporium cladosporioides (CUZFVG38)	29.5 ± 0.3 d	B
Epicoccum nigrum (CUZFVG88)	22.6 ± 1.1 c	A
Geosmithia sp. (CUZFVG40)	9.0 ± 1.4 a	A
Quambalaria cyanescens (CUZFVG39)	14.5 ± 1.0 b	A
Trichoderma atroviride (CUZFVG243)	69.0 ± 1.0 e	C

[a] Means values within the column are significantly different ($P \leq 0.05$), based on LSD tests.
LSD = 2.37, F value: 277.6, SEs: Standard error values
[b] Classification using the Badalyan scale (Badalyan *et al.*, 2002). A: deadlock with mycelial contact, B: deadlock at a distance, C: replacement, overgrowth with and without initial deadlock.

predominant pathogenic species from all of the regions. Halleen *et al.* (2003), screened fungi associated with healthy grapevine plants from nurseries in South Africa, and they obtained endophytic and pathogenic fungi from roots, and rootstock, grafting union and scion tissues. *Acremonium* spp. *Phoma* spp. *Alternaria* spp. *Aspergillus* spp. *Clonostachys* spp., and *Cladosporium* sp. were considered to be endophytic, but *Phaeomoniella chlamydospora, Phaeoacremonium* spp. *Botryosphaeria* spp. *Cylindrocarpon* spp. and *Phomopsis viticola* were found to be pathogenic. Pancher *et al.* (2012) investigated endophytic fungal communities from organic and Integrated Pest Management (IPM) vineyards at seven locations in Italy. Dormant canes of 'Cabernet Sauvignon' and 'Merlot' cultivars were examined for fungi using classical and molecular (ITS sequencing). Their results showed that organic vineyards had richer endophyte communities than IPM vineyards, and that *Alternaria* sp., *Epicoccum nigrum*, *Aureobasidium pullulans* and *Cladosporium* sp. were the most frequently isolated fungi from both types of vineyards. Kraus *et al.* (2019) investigated early development of endophytic fungi in healthy grapevines (from 2 months to 8 years old), and determined genera and species of fungi. Fast-growing fungi, such as *Alternaria* spp., *Aureobasidium pullulans*, *Cladosporium* spp. and *Epicoccum nigrum*, were isolated from vines that were less than 1-year-old, while many grapevine trunk diseases associated pathogens such as *D. seriata* or *Eutypa lata*, along with endophytic fungi, were obtained from perennial branches. *Alternaria* spp., *Aureobasidium pullulans*, *Cladosporium* spp. and *Epicoccum nigrum* made up 81% of the total fungal flora. Casieri *et al.* (2009) assessed vine wood samples taken from different regions and cultivars in Switzerland for endophytic and pathogenic fungi. They found that the composition of endophytes was different for each grape cultivar, and suggested that biochemical composition of the grape cultivars may have played an important role in fungal endophyte diversity. Endophytic and pathogenic fungi may vary according to plant age, geography, cultivar, type and age of tissues (Sieber *et al.*, 1991; Rodrigues, 1994). The results of these studies corroborate our isolation results, from 1-year-old dormant grapevine canes taken from three regions in Turkey.

The hot water treatments (below 50°C with extended duration) were not effective for eradication of *N. parvum* from inoculated dormant grapevine cuttings, but these treatments reduced re-isolation rates of the pathogen, when they were combined with fungicides. This indicates that increased water temperatures probably enhanced penetration of the fungicides into the inner wood tissues. We have been unable to find similar reported results for grapevine, but there are supportive studies conducted on postharvest disease management for fruits. Positive synergistic effects of hot-water and fungicide combinations have been reported Barkai-Golan and Appelbaum (1991), McGuire and Campbell (1993) and Smilanick *et al.* (1995). Cabras *et al.* (1999) have also demonstrated that the cuticle barrier of fruits was weakened with hot water, thus increasing the diffusion of fungicides into fruit rinds. Schirra *et al.* (1996) investigated curative effects of imazalil against blue mould of lemon (caused by *Penicillium italicum*) at the rates from 250 mg·L^{-1} to 1500 mg·L^{-1}, and with water temperatures of 20°C to 50°C for 3 min. Imazalil at 250 mg·L^{-1} and 50°C gave the same curative effect as 1500 mg·L^{-1} at 20°C, Imazalil residue in fruit rinds was 4-5 time greater for the low rate high temperature treatment than for high rate low temperature treatment. Waite *et al.* (2018) reported that dipping grapevine propagation material in hydration tanks for more than 30 min had caused softening of bark tissues which became prone to increased fungal infections. These results indicate that plant tissue softening caused by heated water may also enhance fungicide penetration. It is possible that fungicides may transport from the tips of grapevine cuttings to further parts, by passive diffusion, but this would only be possible if there was one-way flow of water during hydration. There would likely be very little water influx from the opposite ends of cuttings allowing passive infusion of fungicides in hydration tanks.

The heated suspensions of tebuconazole were more effective for eradication of *N. parvum* from dormant grapevine cuttings than those of cyprodinil + fludiox-

onil or thiophanate-methyl. Similar effects were also observed on endophytic fungi, so that most of the endophytes could not be isolated from the cuttings treated with hot-water and tebuconazole combinations. The fungicides assessed in the present study have been reported to be effective for preventing wound infections of vines in field conditions, or contamination of cuttings by many GTD pathogens in hydration tanks (Rolshausen *et al*., 2010; Amponsah *et al*., 2012; Pitt *et al*., 2012). However, in our experiments only tebuconazole showed high efficacy for reducing of colonization by *N. parvum*. We suggest that the differences in fungicide formulations may be responsible for these results. While tebuconazole was applied as an emulsion oil formulation in water, cyprodinil + fludioxonil and thiophanate-methyl were, respectively, wettable granule and wettable powder formulations. Active ingredients in powder or granule formulations may not have passed through grapevine barriers to reach inner tissues. Reports in PubChem indicate that these fungicides are stable in water, soil and air for long periods, so maximum duration in hot-water treatments (12 h) is unlikely to degrade the fungicides (Anonymous, 2019).

The present study investigated whether some endophytic fungi obtained from dormant grapevine cuttings had antagonistic effects against *N. parvum* in dual culture tests. All the endophyte isolates used in these tests were antagonistic to the pathogen. It has been previously shown that secondary metabolites secreted by endophytic fungi affect host plant physiology and other fungi (Schulz and Boyle, 2005). In an *in vitro* study by Wang *et al*. (2013), *Cladosporium cladosporioides* produced four different secondary metabolites (cladosporin, isocladosporin, 3,5'hydroxasperentin and cladosporin-8-methyl ether) in potato dextrose broth. Of these, cladosporin was the most inhibitory to mycelium growth of *Colletotrichum acutatum, Co. fragariae, Co. gloeosporioides* and *Phomopsis viticola* in a micro dilution broth assay reducing growth of these fungi by 80 to 93%. Springer *et al*. (1981), reported cladosporin to be a plant growth regulator, suppressing growth of etiolated coleoptiles of wheat. In our results, *Cladosporium* was a predominant endophytic genus isolated from vineyards in Manisa and Tarsus cities (located in warm climates), but antagonism by *Cladosporium* was moderate to *N. parvum* mycelium growth in dual culture tests. Musetti *et al*. (2007) extracted diketopiperazine compounds from the culture filtrates of *Alternaria alternata*, and tested inhibitory effects of these against downy mildew of grapes in greenhouse conditions. Spray applications of these secondary metabolites reduced (by up to 100%) disease severity, when vine leaves were treated with the compounds 2 or 24 h after inoculation with *Plasmopara viticola*. *Acremonium* has also been reported to produce biologically active metabolites, including β-lactam and cephalosporin antibiotics, and tremorgenic indole-diterpenoids. *Acremonium* also triggers some physiological pathways related to resistance of *Gramineae* plants (Gatenbay *et al*., 1999; Moussaif *et al*., 1997; Lindsey *et al*., 2002; Adinaryana *et al*., 2003). Assante *et al*. (2005) determined secondary metabolite profiles of endophytic *Acremonium* isolates (from grapevine leaves, cv. Reginabianca), and detected four different Acremines (A, B, C, D) in an *in vitro* study. These compounds have been found to inhibit sporangium germination of *P. viticola*, and maximum inhibition (99.8%) was obtained with "Acremin C" at 1 mM concentration. *Trichoderma atroviride* is a well-known endophytic and soil-borne fungus with antagonistic ability to many fungal pathogens, and some of the *Trichoderma* isolates are widely used in the control of GTD pathogens in Europe. Kotze *et al*. (2011) reported that *T. atroviride* was effective as a pruning wound protectant against GTD pathogens, including *N. parvum*, in South Africa. Their isolates provided satisfactory protection of grapevine pruning wounds against *Phompsis viticola*, *E. lata*, *P. chlamydospora*, *N. australe*, *N. parvum*, *D. seriata* and *L. theobromae*, reducing disease incidence by 69-92%. In Portugal, another strain of *T. atroviride* was reported to be highly suppressive to incidence and severity of disease caused by *P. chlamydospora* and *N. parvum* (Reis *et al*., 2017).

In the present study, the *T. atroviride* isolate (CUZ-FVG243) exhibited antagonistic performance (69% reduction) against *N. parvum* in PDA dual cultures competing for space and hyperparasitism. This isolate needs to be tested under field conditions for useful biocontrol effects. The endophytic *Q. cyanescens* was frequently isolated as an endophyte in Tarsus vineyards, but did not show strong antagonism to *N. parvum* (14.5%). This endophyte has been reported to affect grapevine physiology by producing secondary metabolites such as resveratrol (Srivastava, 2015).

CONCLUSIONS

Hot-water treatment is an effective strategy which should be used in grapevine nurseries to produce healthy plants. In the present study, hot-water treatments using fungicide suspensions, at 40°C and for 2 h durations, facilitated fungicide penetration into inner tissues of dormant grapevine canes. Although maximum reduction of *N. parvum* infections was 34%, this reduction could be practically significant because the propagation material

used was severely colonized by the pathogen. Reduction rates could be greater where infections were less severe. Hot-water treatment also eradicates endophytic fungi, and this effect could be negative for plant health. However, due to rapid proliferation and recovery by endophytes, these negative effect may be compensated by additional measures such as using antagonistic *Trichoderma* biocontrol products in nurseries. Additionally, fungicides need to be further evaluated for curative effects on latent infections by *N. parvum* in plant material, when applied in hydration and hot-water treatment systems.

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