Determination of antifungal efficiency of some fungicides and secondary metabolites of *Trichoderma* species against *Botrytis cinerea*

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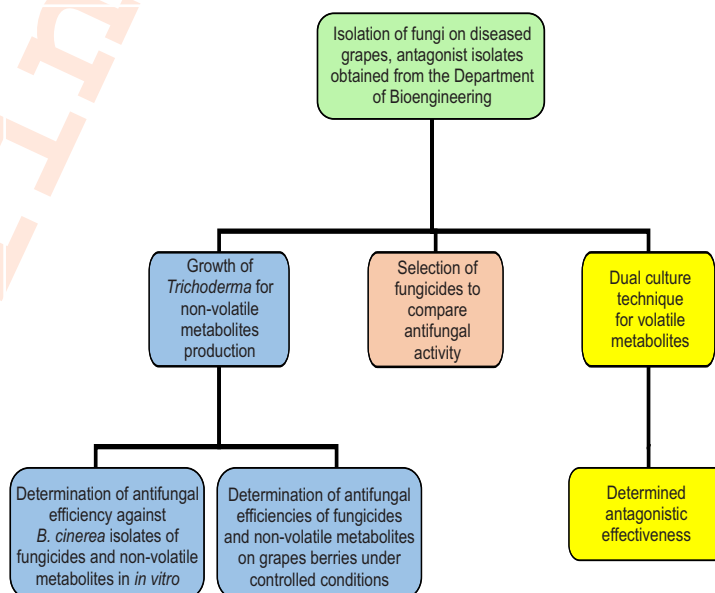*Corresponding Author Email : nurdangungor79@hotmail.com

Abstract

Aim: The present study aimed to investigate the antifungal effects of fenhexamid, cyprodinil+fludioxanil, *Bacillus subtilis* QST 713, *Trichoderma* secondary metabolites (EGE-K-38 and EGE-K-71) on 6 isolates of necrotrophic fungi (*Botrytis cinerea*) under *in- vitro* conditions.

Methodology: A dual culture technique was used to evaluate the effects of volatile compounds produced by *Trichoderma* in culture media against *Botrytis cinerea* isolates. For non-volatile and volatile metabolites, measurements for growth inhibition were made with the mixture of filter sterilized liquid medium of *Trichoderma* and potato dextrose agar. The fungicidal effects of these non-volatile metabolites and fungicides were first evaluated according to the effective concentration (EC$_{50}$) data of growing mycelium inhibition of disease agents.

Results: According to the results obtained, the drug/agent with the lowest fungicidal activity was determined as fenhexamide, which seems to have the lowest EC$_{50}$ value of 0.05 ug m l^{-1}. Both non-volatile metabolites and volatile metabolites of *Trichoderma* strains showed strong inhibition against *B. cinerea* isolates under experimental conditions. The highest growth inhibition percentage was determined with volatile metabolites of *Trihoderma atroviride* EGE-K-71 strain as 71.8%.

Interpretation: The study suggest that filtrates that contain secondary metabolites and volatile compounds of *Trichoderma* strains produced in both liquid culture and dual culture conditions can be used as an effective fungal control agents against pre and postharvest contamination of grapes with *Botrytis cinerea*.

Key words: *Botrytis cinerea*, Fungicides, Non-volatile compounds, *Trichoderma*

Isolation of fungi on diseased grapes, antagonist isolates obtained from the Department of Bioengineering

Growth of *Trichoderma* for non-volatile metabolites production

Selection of fungicides to compare antifungal activity

Dual culture technique for volatile metabolites

Determination of antifungal efficiency against *B. cinerea* isolates of fungicides and non-volatile metabolites in *in vitro*

Determination of antifungal efficiencies of fungicides and non-volatile metabolites on grapes berries under controlled conditions

Determined antagonistic effectiveness

Introduction

The production of grapes in Turkey was 4.1 million metric tons in 2018 which constitutes 6.8% of the total world production and, is one of most important export fruit products of Turkey. Turkey is the world's largest raisin producer with 1.2 million metric tons of raisin production (FAO, 2018). Turkey exports 85% of the raisin to European Union countries (Akgül *et al.,* 2015). *Botrytis cinerea* Pers ex Fr is a major global pathogen that causes gray mold disease (also known as *Botrytis bunch* rot) and results in severe loss in more than 200 host species (Elmer and Michailides, 2007). *B. cinerea* is responsible for pre- and post-harvest losses in grapevines also. The estimated loss caused by *B. cinerea* in vineyards is reported to be about $2 billion per year (Elad *et al.,* 2007). *Botrytis cinerea* is difficult to control due to its different attacking modes, survival under less favourable conditions for extended periods as sclerotia in crop debris and development of fungicide-resistant strains (Williamson *et al.,* 2007).

Chemical control of noble mold caused by *Botrytis cinerea* has always been very important, however, continuous use of same fungicide against the same pathogen causes the development of fungicide-resistant strains (Kumar and Dubey, 2001; Mamgain *et al.,* 2013). In addition, chemical fungicides not only leads to the development of fungicide-resistant species but also accumulates as residues in food and groundwater. Due to the environmental hazards and harmful side effects on humans and animals, *B. cinerea* is well known as being a "high risk" fungus that quickly develops resistance (Amiri *et al.,* 2014). In many studies, it has been reported that *B. cinerea* isolates have developed resistance against many fungicide groups such as benzimidazole, dicarboximide, phenylpyrroles, anilinopyrimidine, hydroxyanilides and SDHI (Mamiev *et al.,* 2013; Leroux *et al.,* 2002; Leroux, 2004). Studies conducted in the Aegean and Marmara regions of Turkey, resistance against gray mold isolates on grapes have also been reported (Koplay *et al.,* 2004). Different reports on the development of fungicide resistant strains of pathogens have also been reported earlier (Kumar and Dubey, 2001; Mamgain *et al.,* 2013).

A recent worldwide trend on disease/plant control management is the use of environmentally friendly methods such as biological control (Hajieghrari *et al.,* 2010). Biological control includes the use of antagonistic microorganisms that occur naturally in soil (Karkachi *et al.,* 2010; Abano and Sam-Amoah, 2012). Alternative methods have been developed to protect vineyards against the negative effects of fungicides and to counter resistance. Biological control plays an important role in the fight against *B. cinerea*. Along with the scope of the European Union (EU) integration process, biological control has gained importance by allowing at least five active substances in the range of maximum residue limit (MRL) values in grapes exported in recent years (Yanar *et al.,* 2015). *Trichoderma* is a genus of fungi containing anamorphic fungi that have been isolated mainly from soil and decay organic matter (Harman *et al.,* 2004). *Trichoderma* species have antagonistic effects against various soil phytopathogens, including fungi such as *Fusarium oxysporum, Rhizoctonia solani, Sclerotium rolfsii*, and *Verticillium dahliae* (Spiegel and Chet, 1998; Jabnoun-Khiareddine, *et al.,* 2009). The antagonistic activity shown by *Trichoderma* species has been linked to mycoparasitism, competition for nutrients and niche, production of antibiotics, and enzymes (Verma *et al.,* 2007). The antagonism of *Trichoderma* spp. was observed in both *in-vitro* conditions (Mishra *et al.,* 2011) as well as in greenhouse and field trials (Kexiang *et al.,* 2002). Some species of *Trichoderma* also promote plant growth and improve nutrient uptake, resulting in better production of plant hormones and vitamins (Joshi *et al.,* 2010). *Trichoderma* species are efficient in terms of hyperparasitism and nutritional competition in addition to their abilities to produce antibiotics, and they can be used as good antagonists against both soil pathogens and pathogens that can infect leaves and fruits (Gezgin *et al.,* 2019).

It has been determined that *T. harzianum* is capable of controlling pathogens *B. cinerea*, *Altrenaria alternata* and *Fusarium acuminatum* in *in-vitro* studies (Mokhtar and Aid, 2013). Trichodex (*T. harzianum*) is used against diseases of *B. cinerea*, *Sclerotinia sclerotiorum, Cladosporium fulvum* in greenhouse-grown tomatoes and cucumbers and vineyards (Freeman *et al.,* 2004). Binab T (*T. harzianum* and *T. polysporum*) controls wound rot and trunk diseases (Mehrotra and Aggarwal, 2003), and might stimulate Supresivit (*T. harzianum*) *Phytophthora* spp. and *Pythium ultimum* and the growth of plants (Brožová, 2004). There are limited studies on *Trichoderma* spp., which causes cluster rotting in vineyards, or on the biological control of post-harvest pathogen *B. cinerea*. The objective of the present study was to investigate the antifungal effects of fenhexamid, cyprodinil +fludioxanil, *Bacillus subtilis* QST 713, *Trichoderma* secondary metabolites (EGE-K-38 and EGE-K-71) on six isolates of necrotrophic fungus (*Botrytis cinerea*) obtained from grapes in the aegean region of Turkey under *in-vitro* and control conditions.

Materials and Methods

Fungal species: *Trichoderma harzianum* EGE-K-38 and *Trichoderma atroviride* EGE-K-71 isolates identified as morphological and molecular were obtained from the Department of Bioengineering, Faculty of Engineering, Ege University. Morphological traits of *Trichoderma* spp. were evaluated according to Hoyos- Carvajal et al. (2009). Their genomic DNAs were isolated by the EurX geneMatrix DNA Isolation Kit according to the manufacturer's instructions. Amplification conditions for rRNA regions of nuclear internally transcribed spacers (ITS 1 & 2) and tef 1 gene primers were used (Hoyos-Carvajal *et al.,* 2009). Amplicons were purified and sequenced by TrioGene firm. For species identification, sequences were subjected to analysis by BlastN National Center for Biotechnology Information (NCBI).

B. cinerea isolates were collected from diseased clusters in different commercial vineyards in the Aegean region in 2018-2019. Conidiophore and spore masses were transferred directly to the PDA (potato dextrose agar, Oxoid) media and incubated at

22 ± 2°C for 5-7 days. Six of them were selected and kept for later use in Eppendorf tubes containing water with 20% glycerol at -20°C in the Plant Health Laboratory Fungal Culture Collection of the Manisa Viticultural Research Institute.

Fungicides: Three commercial fungicides were used in this study to compare antifungal activity: Teldor® SC 500 (fenhexamid 500 g l⁻¹, Bayer), Switch 62.5 WG (cyprodinil 37.5% + fludioxonil 25%, Syngenta) and Serenade® SC (1.34% *Bacillus subtilis* QST 713 strain, min. 1.0x10⁹ cfu.ml⁻¹, Bayer).

Dual culture technique: The dual culture technique, defined by Morton and Stroube (1955), was used to test the antagonistic efficiencies of volatile metabolites (VM) of *T. harzianum* and *T. atroviride* against *B. cinerea* isolates. *B. cinerea* and *Trichoderma* strains were grown at room temperature (25°C) in PDA for a week. Agar discs with diameters of 5 mm were cut from around the developing colony of *B. cinerea* isolates and were later transferred to the petri dishes containing PDA. After the 2-day development of the isolates, the 5-mm agar discs of *Trichoderma* species were cultured on the opposite end of the isolates in the petri dish and incubated at room temperature with a light-dark regimen of 8-12 hr, and were observed periodically. The experiment was replicated four times according to the design of the randomized plot, and incubated at 28°C. Radial growth of the antagonists and pathogen was measured at 24-hr intervals until day 7, and the percentage of inhibition over control for each treatment was calculated according to Hajieghrari (2010).

Non-volatile metabolites: To obtain the liquid phase (filtrate) of non-volatile metabolites (non-VM), all *Trichoderma* test strain were cultured with 100 ml sterilized potato dextrose broth medium (PDB) in 250 ml Erlenmeyer flasks over an orbital shaker at 150 rpm at 26°C for 25 days. Later, the liquid phase was collected by filtering through sterile filter paper (or gauze) and centrifuged to remove fungal spores. The liquid phase was filtered through cellulose membranes with a diameter of 0.2 µm and was prepared for use in fungicidal susceptibility tests at a concentration of 25% (v/v) and included in PDA medium (45°C) (Marques *et al.,* 2018). The experiment was carried out with three replications according to the design of the randomized plot.

Determination of antifungal efficiency against *B. cinerea* isolates of fungicides and non-volatile metabolites *in-vitro*: In fungicidal efficiency tests, the commercial fungicide formulations of cyprodinil + fludioxonil, fenhexamide, 1.34% *Bacillus subtilis* QST 713 strain (min. 1.0 x10⁹ cfu.ml⁻¹) and non-volatile filtrates of *T. harzianum* and *T. atroviride* of 25% (v.v⁻¹) were used. In the autoclaved PDA media, 0.05, 0.5, 5, 50 and 100 µg ml⁻¹ concentrations were used for each fungicide. In the control was added the same amounts of sterile pure water. Pathogen agar disc (5 mm) from a 3-day-old culture was inoculated at the centre of each PDA plate. Three discs were used for each concentration. All treatments were set up in three replications. Colony diameters were measured in a dark environment after four days of incubation at 25±1°C as reported by Delen *et al.* (1984).

Effective concentrations (EC₅₀) that inhibit mycelium growing at different concentrations of fungicides at the level of 50% were determined by using Probit analysis with the help of SPSS statistics program (Version 17.0, SPSS Inc., Chicago, IL, USA) using the values obtained in different concentrations for each chemical.

Determination of antifungal efficiencies of fungicides and non-volatile metabolites on grapes berries in controlled conditions: The antifungal efficiencies were also determined by grape tests in mature grapes in accordance with the method of Koplay *et al.* (2004). In berries tests, two isolates were selected considering the highest and lowest value of EC₅₀ based on pathogens for each fungicide. Healthy grape berries were drilled once with the help of sterile injectors to avoid wound on grapes. The full (1/1) and half (1/2) doses recommended for each fungicide and 25% non-VM antagonists were sprayed separately on drilled grapes, and the berries were fixed on styrofoam molds with the help of adhesive tape. Control grapes were sprayed only with water. Styrofoam molds were then placed in plastic boxes, and sterile pure water was added to provide moisture. Berries were then inoculated with *B. cinerea* spore suspension at 1.0x10⁶ spor ml⁻¹ (Zahavi *et al.,* 2000). The grapes held at 25±1°C in the climate room were evaluated after 7 days of storage. The research was established with four replications, and 5 of them were used in each replication. The diameter of lesions formed due to infection in grapes was measured to best illustrate the differences in the effectiveness of fungicides and their doses on pathogens in grapes.

Statistical analysis: Statistical analyses was done using SPSS software (Versiyon 17, SPSS Inc., Chicago, IL, USA) by applying Duncan Multi Comparison Test at P<0.01 significance level, to determine difference among per treatment and lesion growing in control conditions.

Results and Discussion

In this study, *T. harzianum* and *T. atroviride* antagonists that developed under the same culture conditions limited the development of *B. cinerea* isolates. Table 1 shows the inhibition rates of isolates studied according to the dual culture technique and the average colony diameters. *T. atroviridae* EGE-K-71 antagonist was found to be effective on *B. cinerea* 338 MBAEM with an average colony diameter of 39.33 mm with 71.83% inhibition rate. *T. harzianum* EGE-K-38 antagonist was found to form a low zone with 47.62% inhibition rate developed against the isolates 362MBAEM, which was statistically significant from the others. The antagonistic activity of *Trichoderma* depends on multiple mechanisms that have a tendency to show a stronger or completely different effect than the obvious effect it has alone (Nallathambi *et al.,* 2009; Howell, 2003). Various mechanisms include antibiosis, parasitism, inducing host-plant resistance, competition, and secretion of chitinolytic enzymes, mycoparasitism, and the production of inhibitory compounds (Harman *et al.,* 2004). It has also been found that there is a wide

Table 1: The average colony diameter and mycelial inhibition percentage of six *Botrytis cinerea* isolates by two *Trichoderma* strains EGE-K-38 and EGE-K-71 in dual culture test

| *Trichoderma* species	Botrytis İsolate Numbers	Average colony diameter (mm)[a]	Percentage of mycelial inhibition (%)
	228MBAEM	36.67 ± 3.21^{abc}	56.00[b]
	230MBAEM	41.00 ± 3.21^{ab}	51.38
	301MBAEM	26.67 ± 4.17^{bc}	50.00
Trichoderma harzianum EGE-K-38	302MBAEM	30.33 ± 7.21^{abc}	63.89
	338MBAEM	42.00 ± 1.15^{ab}	67.87
	362MBAEM	44.00 ± 3.60^{a}	47.62
	228MBAEM	38.00 ± 5.50^{abc}	54.40
	230MBAEM	40.00 ± 3.60^{abc}	52.57
Trihoderma atroviridae EGE-K-71	301MBAEM	31.33 ± 5.48^{abc}	62.25
	302MBAEM	27.33 ± 4.40^{bc}	67.46
	338MBAEM	39.33 ± 4.48^{abc}	71.83
	362MBAEM	23.67 ± 13.54^{c}	53.17

[a]Each value is an average of three replicates. Average were grouped by Duncan's Multiple Comparison Test (P≤0.05), so the same letters how the same statistical group. [b]The Inhibition rates for growth were calculated in the equation

range of volatile secondary metabolites produced by *Trichoderma*, such as ethylene, hydrogen cyanide, aldehydes, and ketones, which play an important role in the control of plant pathogens (Vey *et al.,* 2001). Volatile and non-volatile compounds were associated with their ability to control pathogens (Harman, 2006). Dennis and Webster (1971) observed that *Trichoderma* spp. produced a volatile that inhibited the mycelium growth of *R. solani* and other test fungi. Volatile and non-volatile metabolites of *Trichoderma* spp. were also reported to reduce mycelial growth and sclerotium germination in sclerotia-forming fungi through various studies (Srinivasa *et al.,* 2014; Kumar *et al.,* 2017). Under the influence of two salts (CaCl$_2$ and NaCl), the tolerance of *T. harzianum* and its antagonistic activity against *B. cinerea* were examined. In the absence of salt, the volatile and non-volatile secondary metabolites of *T. harzianum* showed inhibitions of *B. cinerea* by 52%, 79% and 81%. Volatile metabolites produced by *T. harzianum* in the presence of a high concentration of calcium chloride (300 ppm) inhibited the mycelium growth of isolate *B. cinerea* no. B27 by 90.037% (Boualem *et al.,* 2008). The effect of volatile and non-volatile metabolites of *Trichoderma* spp. against phytopathogens was determined previously by Rauti *et al.* (2014).

The volatile metabolites secreted by the *T. atroviride* strain were found to have the highest inhibition rates against *Fusarium graminearum, Rhizoctonia solani* and *Pythium ultimum* pathogens, with 45%, 40% and 37.5% inhibition rates, respectively. Chaudhary *et al.* (2020), a total of 31 *Trichoderma* spp. were studied for their antagonistic potentials against *Rhizoctonia solani* in dual culture, effect of volatile and nonvolatile metabolites on the growth inhibition of test pathogen under *in-vitro* condition. The most *T. harzianum* isolates showed higher growth inhibition of *R. solani* compared to the other isolates. Among the *T. harzianum* isolates, percent of growth

inhibition was highest in SVPRT- THLi6 (90.9%) isolate whereas the minimum growth inhibition rate (41.9%) was observed in *T. asperellum* isolates SVPP-5. An isolate of *T. reesei* studied by El-Naggar *et al.* (2008) showed only a 30% reduction in the growth of *B. cinerea*, 40.2% in the growth of *B. fabae* and only 4% in the growth of *B. allii* after five days of incubation. Fiume and Fiume (2006) observed the antagonistic activity of *T. harzianum* against gray mold from 4.7% after three days of incubation and up to 75.76% after seven days of incubation. Furthermore, *B. cinerea* and *T. harzianum* reported any state of no inhibition zone between colonies. In a study in Poland the antagonistic activities of 52 isolates of *Trichoderma* spp. against *Botrytis cinerea* were tested in vitro conditions using the dual culture technique. All of the tested *Trichoderma* isolates grew rapidly and covered the entire surface of the Petri dishes intensively after 10 days. The most effective *T. atroviride* (T43 and T52) strains revealed more than 70% of the growth inhibition of B. cinerea (Bogumił *et al.,* 2013). In the present study, the *T. atroviride* strain had the highest inhibition rate (71.83%) against the isolate 338MBAEM of *B. cinerea* phytopathogen and as per above similar results were obtained.

In the examination of antifungal effects of different fungicides and 25% non-VM concentration of *Trichoderma* spp., on prevention mycelial growth of *B. cinerea*, it was observed that 228MBAEM, 302MBAEM, and 362MBAEM isolates were highly sensitive than other fungicides except for EGE-K-71 *Trichoderma* isolate. Among the fungicides with different chemical structures included in the experiment, fenhexamide displayed the highest efficacy in the concentration range of 0.05 - 5.18 µg ml^{-1}. Effective concentration values required for each fungicide in the experiments to reference were determined through Probit analysis. According to the results obtained, the drug which showed the highest fungicidal effect with the lowest EC_{50} value of
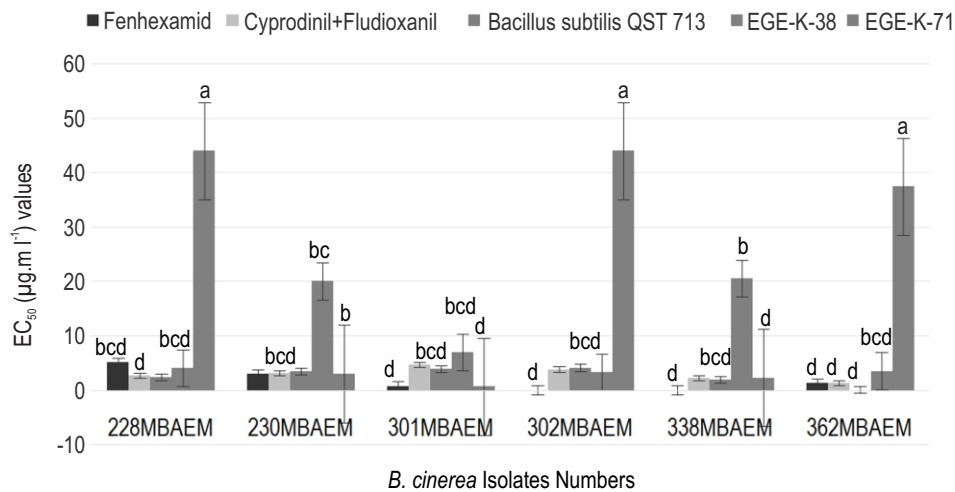
Fig.1: EC_{50} (µg ml⁻¹) values of Fenhexamide, cyprodinil + fludioxanil, *Bacillus subtilis* QST 713 fungicides, and *Trichoderma* (EGE-K-38 and EGE-K-71) of 25% filtrate concentration against six *B. cinerea* isolate in *in-vitro*; * Different lower-case letters indicate significant differences according to Duncan Multiple Comparison Test (P≤0.05).

0.05 µg ml⁻¹ was determined to be fenhexamide, and this fungicide was followed by Bacillus subtilis QST 713 with 0.11 µg ml⁻¹, cyprodinil + fludioxonil with 1.39 µg ml⁻¹, and *Trichoderma harzianum* EGE-K-38 active ingredient fungicides with 3.32 µg ml⁻¹ and metabolites. Among the applications, the lowest efficacy was shown against 228MBAEM and 302MBAEM isolates by *Trichoderma* atroviride EGE-K-71 that had the highest EC_{50} value with 44.00 µg ml⁻¹, while a low EC50 value (2.4 µg ml⁻¹) was obtained against 338MBAEM isolate (Fig. 1).

As a result, among the fungicides used in the study, fenhexamide (0.05 µg ml⁻¹) against 338MBAEM and 302MBAEM isolates and against 301MBAE isolate, *Trichoderma atroviride* EGE-K-71 (0.73 µg ml⁻¹) with low EC_{50} values were the most effective fungicide and metabolite. Fig. 1. Effective concentration (EC_{50}) values which are necessary for 25% non-volatile metabolites of *Trichoderma* spp. to prevent the mycelial growth of *B. cinerea* isolates by 50% was determined for the first time in this study. The bioactive compound trichodermin being a non-volatile metabolite was isolated from the endophytic fungus *T. brevicompactum*. It displayed significant inhibitory activity on *R. solani* and *B. cinerea*, with an EC_{50} of 0.25 µg ml⁻¹ and 2.02 µg ml⁻¹, respectively. However, a relatively poor inhibitory effect was shown against *Colletotrichum lindemuthianum* (EC_{50} = 25.60 µg ml⁻¹) (Shentu *et al.,* 2014). In the study a total of 11 metabolites were obtained in the main secondary metabolites of two *T. harzianum* strains (T-4 and T-5). Of these metabolites, Harzianopyridone was the most effective and had an antifungal effect that inhibited the growth of *R. solani, F. oxysporum* and *Sclerotium rolfsii* by more than 90% (EC_{50} 35.9– 50.2 µg ml⁻¹) (Ahluwalia et al., 2015). Harzianopyridone is a powerful inhibitor of complex II (NADH-ubikinon reductase) enzymes in the mitochondrial respiratory chain in fungus (Lv *et al.,* 2015). Four

toxins (gliotoxin, dimethyl gliotoxin, viridin and viridiol) were isolated from *T. virens*. Of these toxins, Gliotoxin, *Rhizoctonia Bataticola* (ED_{50} 0.03 µg ml⁻¹), *Macrophominia phaseolina* (ED_{50} 1.76 µg ml⁻¹), *Pythium deharyanum* (ED_{50} 29.38 µg ml⁻¹) were effective by preventing mycelium development by 50% (Shyamli *et al.,* 2005). The antagonistic effect of the metabolites of some isolates of *Trichoderma* genus in different concentrations (10%, 20%, 25%, 50%) on *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum, Sclerotium* spp., *Pythium ultimum*, *Phytophthora* spp., *Armillaria* spp., *Fusarium oxysporum*, *Alternaria solani* and *Gauemannomyces graminis* pathogens was determined by the percentage inhibition of mycelial growth (Barakat *et al.,* 2014; Brewer and Larkin, 2005; Woo *et al.,* 2014). However, effective concentration values that inhibit the mycelial growth used to determine the fungicidal effect of metabolites by 50% was not investigated. In fact, fungal secondary metabolites that have a chemically low molecular weight (< 3kDa) are used effectively in human treatment and the food industry. It is believed that this study will form a basis for the utilization of non-volatile secondary metabolites as bio fungicides in agriculture.

Cyprodinil is included among the fungicides in anilopryminide group, and they have the risk of forming a moderate resistance. The maximum number of cyprodinil use with or without fludioxonil in a year was determined as two (Leroux, 2004; Saito *et al.,* 2014). EC_{50} value of cyprodinil *in vitro* against nine *B. cinerea* isolate obtained from vineyards in Chile was determined as <0.001-4.89 µg ml⁻¹. It has been reported that new strategies should be developed in vineyards and should be applied in a combination with different groups in order to prevent resilience formation. 55 single-spore isolates of *Botrytis cinerea* were collected at the end of the season from vegetable crops grown in 18 greenhouses on the island of Crete, Greece by

Table 2: The effect of full and half dose applications of fenhexamide, cyprodinil + fludioxanil, *Bacillus subtilis* QST 713, and *Trihoderma* (EGE-K-38 and EGE-K-71) on fruits under the controlled conditions of two *Botrytis cinerea* isolates selected according to EC_{50}.

Fungicide	Isolates	EC_{50} values ($\mu g\ m\ l^{-1}$)	Doses ($\mu g\ m\ l^{-1}$ e.m)[a]	Lesion Diameter (mm)	Impact (%)
Fenhexamide	228	5.18	250	0.67±b 0.06[klc]	86.30
			125	0.93±0.29[kl]	80.82
			0	4.87±0.94[defgh]	0.00
	338	0.05	250	0.47±0.24[i]	87.27
			125	0.80±0.46[kl]	78.18
			0	3.67±1.07[efghij]	0.00
Cyprodinil+fludioxanil	228	2.80	125	1.67±0.176[ijkl]	70.93
			62,5	2.67±0.98[ghijkl]	53.49
			0	5.60±0.30[bcde]	0.00
	338	2.04	125	0.73±0.54[kl]	85.71
			62,5	1.53±0.64[ijkl]	70.13
			0	5.13±0.89[cdefg]	0.00
Bacillus subtilis QST 713	228	3.54	3750	2.60±0.80[hijkl]	73.47
			1875	5.33±1.23[bcdef]	53.74
			0	9.80±1.42[a]	0.00
	338	2.51	3750	1.27±0.29[jkl]	80.81
			1875	3.07±0.70[fghijk]	53.54
			0	6.60±1.00[bcd]	0.00
Trihoderma harzianum EGE-K-38	228	44.00	6250	2.20±0.30[ijkl]	71.79
			3125	3.93±2.07[efghi]	49.57
			0	7.80±0,34[ab]	0.00
	338	2.07	6250	1.40±0.50[jkl]	80.37
			3125	2.40±0.70[hijkl]	66.36
			0	7.13±0.96[bcd]	0.00
Trihoderma atroviride EGE-K-71	228	35.60	6250	1.13±0.06[kl]	86.54
			3125	2.45±0.61[hijkl]	66.83
			0	7.33±1.39[abcd]	0.00
	338	0.05	6250	1.83±1.16[ijkl]	82.24
			312,5	2.38±0.49[hijkl]	69.16
			0	7.45±1.57[abc]	0.00

[a]Values represent mean of three replications of full and half doses of field recommended rate of fungicides and 25% non-VM of *Trichoderma* species used for two isolates of *B. cinerea*; [b]Mean values followed by standard error of the mean; [c]Averages were divided by Duncan test (P≤0.05), so the same letters show statistically the same impacts

Myresiotis *et al.* (2007). They were tested for sensitivity to the anilinopyrimidine fungicides pyrimethanil and cyprodinil, the hydroxyanilide fungicide fenhexamid, the phenylpyrrole fungicide fludioxonil, the dicarboximide fungicide iprodione, and the benzimidazole fungicide carbendazim.

The EC_{50} values of the isolates ranged from 0.003 to 25.2 $\mu g\ ml^{-1}$ and from 0.03 to 75.0 $\mu g\ ml^{-1}$ for cyprodinil and pyrimethanil, respectively. In this study, the EC50 values of the isolates were obtained at the rate of 2.29-4.77 $\mu g\ ml^{-1}$ for cyprodinil + fludioxanil. It was determined that these EC_{50} values were realized between the values obtained for cyprodinil stated in the study by Myresiotis *et al.* (2007). The effectivenesses of non-volatile metabolites and three fungicides, against *B. cinerea* isolates were shown with the grape test. Considering the results of the statistical analysis, it was determined that two doses of fenhexamide (250 $\mu g\ ml^{-1}$, 125 $\mu g\ ml^{-1}$) were effective on grape

against isolates according to the highest and lowest EC_{50} values (Table 2). The full dose (6250 $\mu g\ ml^{-1}$) of *T. atroviride* EGE-K-71 was effective against isolates 338MBAEM with 228MBAEM (Table 2). This antagonist is followed by cyprodinil + fludioxanil fungicide, *B. subtilis* QST 713 and *T. harzianum* EGE-K-38 antagonist, respectively. The full dose (6250 $\mu g\ ml^{-1}$) of *T. harzianum* EGE-K-38 antagonist was determined to be effective at a rate of 71.79% against 228MBAEM isolates with a high EC_{50} value. *T. atroviride* EGE-K-71 was found to significantly inhibit lesion formation on grapes. Similar results were reported by Al-Saeedi and Al-Ani, (2014).

Inhibition effects of 25, 50, 75%, and 100% non-volatile filtrate concentrations of *T. harzianum* T1 and T2 strains to prevent incubation periods of *Alternaria* spp. and *Pythium* spp. pathogens were evaluated. The 25% filtrate concentration of *T. harzianum* had a high percentage of inhibition of 63.53% and

67.78% at the 3rd and 6th day incubation period, respectively. Table 2. As a result of the test on grapes under controlled conditions, the isolates were found to be effective against fenhexamide, but the effectiveness (70.93% at full dose, 53.49% at half dose) of cyprodinil + fludioxanil against 228MBAEM isolate with the high EC$_{50}$ value was a little lower when compared to fenhexamide (86.30% at full dose, 80.82% at half dose). *B. cinerea* isolates are considered to hinder the development of *B. cinerea* isolates where *B. subtilis* QST 713 biological fungicide has good efficacy. The usage of Bacillus species, including *Bacillus circulans*, *B. brevis* and *B. subtilis*, as antagonists against *B. cinerea* was documented in laboratory and field conditions (Ben Maachia *et al.,* 2015). *B. subtilis* strain QST 713, formulated as Serenade (Agra Quest, USA), and provided good gray mold control under field conditions in Chile. In *B. subtilis*, cyclic lipopeptides (surfactants, iturins, and fengicins) play a role in activating plant defenses; some of these cyclic lipopeptides may act directly against *B. cinerea* (Farace *et al.,* 2015).

In our study, full dose (3750 µg ml^{-1}) administration of Serenade (*B. subtilis* strain QST 713) under controlled conditions had 73.47% and 80.81% effect on grapes in controlled *B. cinerea* isolates No. 228 and 338, respectively. The EC$_{50}$ value of 31 of the 638 *B. cinerea* isolates derived from fenhexamide-administered vineyards is ≥0.1 µg ml^{-1}, thus they are considered resistant. In the tests performed on grapes with 4 isolates of different endurance levels, 25% and 100% of the recommended dose of fenhexamide (150-600 µg ml^{-1}) were administered as a preservative and therapeutic. It was determined that isolates with EC$_{50}$ values of 0.03 and 0.15 µg ml^{-1} could prevent disease with their recommended doses and that isolates with the EC$_{50}$ value of 0.32 and 62.5 µg ml^{-1} could not. In ERG 27 gene of the isolates with an EC$_{50}$ value of 62.5 µg ml^{-1}, F 427 V mutation was detected (Fournier *et al.,* 2005). Because fenhexamide is a specialized fungicide in terms of the binding site, there is a risk of creating certain durability. In laboratory conditions, isolates improve endurance as they can find continuous, long and optimal growth conditions to metabolize fenhexamide, and because optimal conditions in nature do not occur exactly as desired, very few durable isolates may be found (Esterio *et al.,* 2007; Suty and Stenzel, 1999).

In conclusion, this study showed that *T. atroviride* species is a new type of antagonistic fungus that can be used for controlling *B. cinerea*, the pathogen of bunch rot disease. The fungicidal effect of non-VM at the concentration of 25% of the *T. harzianum* and *T. atroviride* species was calculated for the first time using EC$_{50}$ values in *in vitro*. It has been established that 25% of the non-VM and VM of the *T. atroviride* is as effective fungicides in inhibition the spore and mycelium production of *B. cinerea* as on grapes in post-harvesting. The study showed that secondary metabolites, which are alternative to fungicides as an environmentally friendly product, can be evaluated at pre and post-harvest against *B. cinerea* in the agents' bunch rots.

Add-on Information

Authors' contribution: N.G. Savas: Conceived of presented idea, developed the theory and performed computations, writing manuscript; **M. Yıldız:** Carried out experiment, **R. Eltem:** Provided consultancy; **and E. Ozkale:** Help in research and writing manuscript.

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