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Phenolic compounds inhibit viability and infectivity of the grapevine pathogens *Diplodia seriata*, *Eutypa lata*, *Fomitiporia mediterranea*, and *Neofusicoccum parvum*

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Summary. Many fungal pathogens are associated with grapevine trunk diseases (GTDs), which cause important yield and economic losses in grape production. There are no effective control methods against GTDs once plants are infected, so research is aimed at preventive measures to avoid infections in nurseries and vineyards. Inhibitory activities of the phenolic compounds eugenol, epigallocatechin–3–O–gallate (EGCG) and thymol against the GTD fungi *Diplodia seriata, Eutypa lata, Fomitiporia mediterranea* and *Neofusicoccum parvum* were assessed *in vitro*, and *in planta* as grapevine pruning wound treatments. Greatest inhibition of pathogen mycelium growth was observed with eugenol (fungistatic at 1,500 μg mL^{-1}, fungicidal at 2,500 μg mL^{-1}). No inhibitory activity against GTD fungi was observed with EGCG. Minimum concentrations with *in vitro* inhibitory effects on *D. seriata* and *N. parvum* spore germination were 360 μg mL^{-1} for thymol and 750 μg mL^{-1} for eugenol. In the grapevine wound protection tests, thymol was effective against *N. parvum* at 360 μg mL^{-1}, but eugenol was not.

Keyword. Grapevine trunk diseases, thymol, eugenol, epigallocatechin-3-O-gallate, mycelium growth, spore germination, wound protection.

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INTRODUCTION

Grapevine trunk diseases (GTDs) are major threats to the sustainability of vineyards, and are widespread in all wine-producing countries (Songy *et al.*, 2019). As no curative treatments for these diseases is available, infected grapevine plants must be replaced, resulting in financial losses of approx. $1.5 billion annually (Fischer *et al.*, 2019). In the Czech Republic, damage caused by GTDs is estimated at CZK 150 million p.a. (approx. € 6 million) (Baránek *et al.*, 2017). In the 2000s, GTDs reduced potential wine produc-

tion in France by 13% (Bruez *et al*., 2013). More than 130 species of fungi in 34 genera have been associated with GTDs, and are the largest group of pathogenic fungi that infect one host species (Gramaje *et al*., 2018).

GTDs include the six diseases, the ESCA complex, Eutypa dieback, Botryosphaeria dieback, and Phomopsis dieback occurring especially in mature vineyards, and Petri disease and black foot in young grapevines (Fussler *et al*., 2008; Bertsch *et al*., 2009; Hofstetter *et al*., 2012; Fontaine *et al*., 2016; Gramaje *et al*., 2018; Mondello *et al*., 2018a). Effective management of GTDs is difficult, especially after sodium arsenite was banned, due to its human and animal toxicity (Mondello *et al*., 2018b). Because complete eradication is not possible, GTD control is primarily focused on disease prevention and alleviation (Úrbez–Torres, 2011).

In addition to chemical control attempts (Mondello *et al*., 2018a), recent studies have focused on developing sustainable management strategies against GTD fungi (Compant and Mathieu, 2016). Many organic extracts, such as chitosan, *Evernia prunastri* lichen extract, garlic extract, lemon peel extract, vanillin, and propolis, have been shown to inhibit GTD pathogens such as *Botryosphaeria dothidea*, *Diaporthe ampelina Diplodia seriata*, *Eutypa lata, Ilyonectria macrodidyma, Phaeoacremonium minimum*, and *Phaeomoniella chlamydospora* (Cobos *et al*., 2015, Mondello *et al*., 2018b).

With the increasing implementation of integrated pest management (IPM) systems, natural products are of considerable importance. These substances are obtained from biological sources and include essential oils (phenolic compounds), and natural plant protection products are considered more environmentally friendly than synthetic pesticides (Raveau *et al*., 2020). The use of biological agents instead of synthetic pesticides is strongly encouraged in Europe by Directive 2009/128/EC, which aims to reduce the pesticide use and thus bring agriculture in line with sustainable development.

The present study aimed to evaluate the potential of phenolic compounds for natural resource protection of grapevines. Phenolic compounds are secondary metabolites ubiquitous in most plant tissues (de la Rosa *et al*., 2019). Eugenol ($C_{10}H_{12}O_2$; 2-methoxy-4-(2-propenyl) phenol) is a major component of clove oil (*Syzygium aromaticum, Myrtaceae*), and is also occurs basil, cinnamon, lemon balm, and nutmeg (Dable-Tupas and Egbuna, 2022). It is an allyl chain–substituted guaiacol, clear, light yellow, and greasy liquid, that is slightly soluble in water and soluble in organic solvents (Pramod *et al*., 2010). The antifungal effects of eugenol have been demonstrated on *Aspergillus* spp. and *Cladosporium* spp. (Abbaszadeh *et al*., 2014), and inhibition of the pathogens *Botrytis cinerea* (Hastoy *et al*., 2023; Wang *et al*., 2010), *Fusarium oxysporum* f. sp. *vasinfectum* (Abd–Elsalam and Khokhlov, 2015), and *Rhizoctonia solani* (Zhao *et al*., 2021).

The polyphenolic compound (-)-epigallocatechin-3-O-gallate, $C_{22}H_{18}O_{11}$ (EGCG), is the main catechin detected in green tea (*Camellia sinensis* (L.) Kunze). This compound is an ester of epigallocatechin and gallic acid (Nagle *et al*., 2006), and has antiviral (Calland *et al*., 2012; Steinmann *et al*., 2013; Kaihatsu *et al*., 2018), antibacterial (Kanagaratnam *et al*., 2017; Lee *et al*., 2017), and antifungal effects (Li *et al*., 1999; Navarro-Martinez *et al*., 2006; Park *et al*., 2006) which have been demonstrated in *in vitro* studies.

Thymol ($C_{10}H_{14}O$; 2-isopropyl-5-methylphenol) is the main monoterpene phenol present in essential oils isolated from plants in *Lamiaceae* (*Thymus, Ocimum, Origanum,* and *Monarda*). Thymol can also be isolated from other plants in *Verbenaceae, Scrophulariaceae, Ranunculaceae* and *Apiaceae* (Marchese *et al*., 2016). Thymol had marked *in vitro* inhibitory activity against the phytopathogens *Alternaria* spp., *Botrytis sp., Fusarium* spp., *Phytophora capsici* (Kordali *et al*., 2008), *Colletotrichum acutatum*, *Lasiodiplodia theobromae* (Numpaque *et al*., 2011), and *Rhizoctonia solani*. In *in planta* tests, thymol promoted the emergence of cucumber seedlings (Chauhan *et al*., 2017).

Based on current knowledge of antifungal activities of the phenolic substances, the compounds eugenol, thymol, and EGCG were tested *in vitro* and *in planta* (eugenol and thymol) against GTD fungi. *Diplodia seriata, Eutypa lata, Fomitiporia mediterranea,* and *Neofusicoccum parvum* were selected for these experiments. The aim of this study was to determine whether selected phenolic substances have inhibitory effects against these selected GTD fungi that could be utilized for sustainable management of GTDs in nurseries and vineyards. Inhibitory effects on mycelium growth and spore germination of the three phenolic compounds were tested *in vitro*, and then *in planta* as pruning wound treatments of grapevine cuttings.

MATERIALS AND METHODS

Phenolic substances and chemicals

The phenolic compounds and chemicals used in this study are listed in Table 1.

In the *in vitro* tests where the effects of phenolic substances on the growth of fungal mycelia were assessed, eugenol was used at concentrations of 1.5 μL mL^{-1} (effective concentration (e.c.) 1,500 μg mL^{-1}) and 2.5 μL mL^{-1}

Table 1. Phenolic compounds and chemicals used in this study.

Name	Form	Chemical formula	Stock solution concentration (mg mL^{-1})	Concentration used (µg mL^{-1})	Supplier
Ethanol absolute	liquid	C_2H_6O	-	2,250.0 13,500.0 18,000.0	Charbonneaux Bradant, France
Eugenol (100%)	liquid	$C_{10}H_{12}O_2$	-	375.0 750.0 1,500.0 2,500.0	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Epigallocatechin-3-O-gallate (EGCG)	powder	$C_{22}H_{18}O_{11}$	1	7.5 45.0	Merck KGaA, Darmstadt, Germany
Thymol	powder	$C_{10}H_{14}O$	1	7.5 45.0 90.0 180.0 360.0	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Benomyl. 1-(butylcarbamoyl)-1*H*-1,3-benzimidazol-2-yl methylcarbamate	powder	$C_{14}H_{18}N_4O_3$	10	20.0	Sigma Aldrich, Saint Louis, MO, USA

Table 2. Details of the four selected grapevine trunk pathogens examined in this study.

Pathogen	Isolate	Geographical origin	Sampling date	Grape cultivar	Accession number	Literature source
Diplodia seriata	Ds 99-7	Perpignan, France	1998	Syrah	MSZU00000000	Robert–Siegwald *et al*., 2017
Eutypa lata	Bx1.10	Gironde, Bordeaux, France	1990	Cabernet-Sauvignon	–	Péros and Berger, 1994
Fomitiporia mediterranea	CO36	Saint–Preuil, France	1996	Ugni-Blanc	–	Laveau *et al*., 2009
Neofusicoccum parvum	Bt 67	Portugal	–	Aragonez	CBS140888	Rego *et al*., 2009

(e.c. 2,500 µg mL^{-1}), according to Chauhan *et al*. (2017). For thymol, a stock solution of 1.0 mg mL^{-1} was first prepared using 70:30 water:absolute ethanol solvent (Scoralik *et al*., 2012). Thymol was used at concentrations of 7.5 µL mL^{-1} (e.c. 7.5 µg mL^{-1}) and 45 µL mL^{-1} (e.c. 45 µg mL^{-1}) (Chauhan *et al*., 2017). Effects of ethanol on fungal growth were assessed at of 2.25 µL mL^{-1} (e.c. 2,500 µg mL^{-1}) and 13.5 µL mL^{-1} (e.c. 13,500 µg mL^{-1}). An EGCG stock solution of 1.0 mg mL^{-1} was prepared in water (Bartosikova and Necas, 2018). The tested EGCG concentrations were 7.5 µL mL^{-1} (e.c. 7.5 µg mL^{-1}) and 45 µL mL^{-1} (e.c. 45 µg mL^{-1}) (Chauhan *et al*., 2017).

In the *in vitro* tests for effects on spore germination, thymol solution was prepared at 1.0 mg mL^{-1} in 5% ethanol and then diluted with sterile deionized water to obtain final concentrations of 7.5, 45, 90, 180, or 360 µg mL^{-1}. Eugenol solutions were prepared in 1% DMSO at final concentrations of 375, 750, 1,500, or 2,500 µg mL^{-1}. To exclude inhibitory effects of ethanol and DMSO on spore germination, concentrations of 18,000 µg mL^{-1} of ethanol and 1% of DMSO were tested. Benomyl (1-(butylcarbamoyl)-1*H*-1,3-benzimidazol-2-yl methylcarbamate; Sigma Aldrich) at 20 µg mL^{-1} was used as a synthetic fungicide for comparisons.

The lowest concentrations that inhibited spore germination were selected to evaluate the potential protective effects of the phenolic compounds on pruning wounds. Thymol solution was used at 360 µg mL^{-1}, and eugenol solution was used at 750 µg mL^{-1} with the addition of 1% DMSO.

Fungal isolates

The selected pathogens are shown in Table 2. These fungi were isolated from grapevine wood, and the isolates are available at the collection at Unité Résistance Induite et Bioprotection des Plantes, Université de Reims Champagne-Ardenne, France. Four GTD fungi were tested. These were *D. seriata* Ds 99-7 (Larignon *et al*.,

Table 3. Details of the phenolic compounds assessed in this study for their effects on grapevine trunk diseases pathogens. The tests were carried out in PDA culture plates.

Phenolic compound	Concentration (µL mL^{-1})	Amounts of stock solution per 20 mL PDA	Effective concentration (µg mL^{-1})	Pathogen	Number of repetitions	Total
Ethanol	2.25	45 µL	2,250.0	*Diplodia seriata*	4	8
				Eutypa lata	4	8
	13.5	270 µL	13,500.0	*Fomitiporia mediterranea*	4	8
				Neofusicoccum parvum	4	8
Eugenol	1.5	30 µL	1,500.0	*Diplodia seriata*	4	8
				Eutypa lata	4	8
	2.5	50 µL	2,500.0	*Fomitiporia mediterranea*	4	8
				Neofusicoccum parvum	4	8
EGCG	7.5	150 µL	7.5	*Diplodia seriata*	4	8
				Eutypa lata	4	8
	45.0	900 µL	45.0	*Fomitiporia mediterranea*	4	8
				Neofusicoccum parvum	4	8
Thymol	7.5	150 µL	7.5	*Diplodia seriata*	4	8
				Eutypa lata	4	8
	45.0	900 µL	45.0	*Fomitiporia mediterranea*	4	8
				Neofusicoccum parvum	4	8
Control	–	–	–	*Diplodia seriata*	4	4
				Eutypa lata	4	4
				Fomitiporia mediterranea	4	4
				Neofusicoccum parvum	4	4
Total Petri dishes						140

2001; Robert-Siegwald *et al*., 2017), *E. lata* Bx1.10 (Péros and Berger, 1994; Laveau *et al*., 2009), *F. mediterranea* CO36 (Laveau *et al*., 2009) and *N. parvum* Np Bt 67 (Rego *et al*., 2009; Reis *et al*., 2016).

In vitro *assessments of inhibition of mycelium growth*

Inhibitory effects of the selected substances against the four GTD fungi were tested *in vitro* using the potato dextrose agar (PDA; Difco™, Becton, Dickinson and Company) supplemented with different concentrations of phenolic compounds (Table 3). The enriched media were pipetted onto Petri dishes (20 mL per dish). After solidification, a mycelium plug (5 mm diam.) taken from the edge of a 7-d-old colony of the assessed GTD fungus was placed into the centre of the prepared Petri plate. The plates (four replicates per treatment) were then incubated at 25°C in the dark and observed until the mycelium of the control treatment plates reached the plate edges, or for a maximum of 9 days post inoculation (dpi). Two orthogonal diameters of each fungi colony were measured, and the mean colony diameter was calculated. The mycelial plugs that did not show any growth in the enriched PDA were transferred onto nonamended PDA to verify fungistatic or fungicidal effects of the tested phenolic compounds. The same method was used to test effects of ethanol on the growth of *D. seriata* and *N. parvum*, because ethanol was used to prepare the thymol stock solutions.

Statistical analyses of the data obtained were carried out using Statistica 12 CZ StatSoft Prague CZ. Analysis of variance (ANOVA) was performed (at P = 0.01), and post hoc tests (Tukey's HSD tests: P = 0.01) were then applied. The percentage inhibition was calculated according to the formula $I=[(r_c-0.5)-(r_t-0.5)]/[(r_c-0.5)\times100]$, where I = percentage of inhibition, r_c = average of the measured values for the experimental controls, and r_t = average of the measured values for the treated condition. The value 0.5 cm was subtracted as the disc size (Chauhan *et al*., 2017).

The EC_{50} were determined for the phenolic compounds that gave statistically significant inhibition. Concentrations and percentage inhibition values were used for linear regression analyses. Regression equations were determined, from which the EC_{50} value for each compound was calculated. This experiment was carried out twice.

In vitro *assessments of inhibition of spore germination*

Based on the results from the mycelium growth assessments, thymol and eugenol were selected for *in vitro* treatments of *D. seriata* and *N. parvum* spore suspensions. *Eutypa lata* and *F. mediterranea* were discarded due to the lack of sporulation of both fungi in laboratory conditions, difficulty in collecting *E. lata* ascospores from infected grapevine wood, and the low germination rates of *F. mediterranea* basidiospores (Živković *et al*., 2012; Moretti *et al*., 2021).

Isolates of *D. seriata* Ds 99-7 and *N. parvum* Bt 67 were maintained on PDA at 25°C. Pycnidia of *D. seriata* were observed after an approx. 4 week incubation period. For *N. parvum,* conidia were obtained according to Úrbez-Torres and Gubler (2009). Mycelial plugs from 7- to 10-day-old cultures were placed on 2% water agar containing sterile pine needles. Petri dishes were incubated at 25°C in a 12 h light:12 h dark cycle, until pycnidia were formed. Pycnidia of the two isolates were collected separately, and were then each crushed in sterile demineralized water in a 1.5 mL sterile microcentrifuge tube, using a sterile plastic pestle. Each spore suspension was filtered through sterile cheesecloth, and the spore concentrations were adjusted with potato dextrose broth (PDB, Difco Laboratories) to 2×10^4 conidia mL^{-1} using haemacytometer spore counts.

In the first phase of testing, inhibitory effects of thymol and eugenol on *D. seriata* spore germination were assessed at the same concentrations that were used in the *in vitro* tests on mycelium growth, thymol at 7.5 and 45 μg mL^{-1}, and eugenol at 1,500 and 2,500 μg mL^{-1}. The fungicide benomyl at 20.0 μg mL^{-1}, and water with 1% DMSO were also tested, respectively, as positive and negative experimental controls. In the second phase of testing against *D. seriata*, double concentrations of thymol (90, 180 and 360 μg mL^{-1}) and half doses of eugenol (375 and 750 μg mL^{-1}) were used. The effects of ethanol (at 18,000 μg mL^{-1}), which was used for the preparation of thymol solutions, and 1% DMSO, which was used to increase the dispersion of eugenol in water, were also assessed. For *N. parvum*, the two greatest concentrations of thymol (180 and 360 μg mL^{-1}), and the two lowest concentrations of eugenol (375 and 750 μg mL^{-1}) were tested. Effects of benomyl (20.0 μg mL^{-1}) were also assessed. Spore suspension (100 μL) was mixed with 100 μL of each tested solution in each well of 96-well microplates, and these were incubated at 25°C with shaking (180 rpm) in the dark. Optical density measurements were carried out at 0 and 72 h at 600 nm wavelength, using a spectrophotometer (MP96 Safas). This experiment was carried out twice.

In planta *treatment of grapevine wounds with phenolic compounds*

Evaluation of the potential protective effects of phenolic compounds on grapevine wounds was carried out using the method described by Lecomte and Dewasme (2004), with some modifications. Cuttings from *Vitis vinifera* 'Chardonnay' plants were collected from 15-year-old pruned canes growing in established vineyards, after winter pruning (January). The three-node long segments were cut and kept in a cold chamber at 4°C for 1 month. These segments were then surface sterilized with 0.05% cryptonol (8-hydroxyquinoline sulfate), rinsed with tap water, and placed back at 4°C until used. The cuttings were cut into 1-node segments, surface sterilized by wiping with cotton wool swabs of 70% ethanol, and the tips were each trimmed to 1 cm lengths with scissors, to produce fresh wounds. The cuttings were then placed vertically in plastic boxes containing moistened sand to maintain high humidity. On each fresh wound spray applications were made with approx. 650 μL of thymol solution (360.0 μg mL^{-1}) or eugenol solution (750 μg mL^{-1} with 1% DMSO). For experimental controls, the same volumes of water containing 1% DMSO were used. Each plastic box was covered with a lid, and the samples were incubated at 25°C for 24 h. Subsequently, 50 μL of spore suspension of *N. parvum* or *D. seriata* in sterile deionized water (prepared as described above), containing approx. 200 conidia were pipetted onto each wound, and the samples were left at room temperature until the conidium suspension drops were absorbed. Plastic boxes (one for each tested condition) were covered with lids and incubated at 25°C for 7 d. Ten cuttings were used for each treatment.

After incubation, each cutting was removed from the sand, and the surface was sterilized by cotton wool wiping with 70% ethanol. The cutting tip was briefly sterilized in the flame, and the bark was removed using a sterile scalpel. Five wood chips (approx. 2 mm thick) were cut using sterilized scissors that were flamed before each cut. The five wood chips (levels) cut from the same cane were placed onto one Petri dish containing PDA, noting their position with regard to the inoculated wound zone, and the dishes were then incubated at 25°C for 5 d.

Development of *N. parvum* mycelium was evaluated on every wood chip, to determine how deeply the germinating conidia of the pathogens were able to colonize the woody tissues of each cane, and, inversely, the efficacy of phenolic compound treatment to prevent pathogen colonization. The percentage of colonized chips was calculated using the formulae $\%_{col.} = x \times 100/n$, and the percentage of treatment efficiency was calculated as $\%_{ef.} = y \times 100/n$, where $\%_{col.}$ = colonization percentage, x = number of chips in which pathogen mycelium growth

was observed, $\%_{ef.}$ = efficiency percentage, y = number of chips in which no mycelial growth was observed, and n = 10 (the number of replicates). The efficiency percentages were calculated for each level of wood section (1 to 5). The test results were considered valid if the experimental controls gave at least 60% colonized chips in the first two wood levels. The treatments were considered efficient if the colonized chips did not exceed 30% in the first two wood levels (= efficiency at > 70%).

RESULTS

In vitro *inhibitory effects on mycelium growth*

The results of the *in vitro* assays are reported in Table 4. Among the studied phenolic compounds, eugenol and thymol inhibited all of the tested GTD fungi, while no significant inhibition was detected from the EGCG treatments.

Eugenol at both concentrations had strong antifungal activity. Complete growth inhibition (100%) was observed for all four GTD pathogens after treatment with 2,500 μg mL^{-1}, due to an ascertained fungistatic effect, and only a slightly lower effect was observed at 1,500 μL mL^{-1}, where *D. seriata* growth was reduced by 98%, *E. lata* by 99%, *F. mediterranea* by 100%, and *N. parvum* by 99%.

Thymol at a concentration of 45 μg mL^{-1} inhibited the growth of all the tested pathogens, with greatest activity (89%) against *D. seriata* and least (67%) against *E. lata*. Concentration of 7.5 μg mL^{-1} thymol was less effective and reduced the mycelium growth of three of the four pathogens, by 23% for *F. mediterranea,* 16% for *N. parvum* and 12% for *D. seriata*. Ethanol, regardless of concentration, did not affect ($P > 0.01$) growth of *D. seriata* or *N. parvum* growth, so ethanol did not affect the efficacy of thymol solution. For *E. lata* and *F. mediterranea*, the effect of ethanol was not tested due to the low percentages of thymol inhibition found for the four fungi.

EC_{50}s for phenolic compounds with statistically significant inhibitory activity were calculated using linear regression analyses (Table 5). For eugenol, the EC_{50} were

Table 4. Mean percent (%) inhibition of mycelial growth of four grapevine trunk disease pathogens measured in *in vitro* tests of phenolic compounds. (* indicates treatments giving differences ($P = 0.01$) from experimental controls).

Substance	Concentration (μL mL^{-1})	Effective concentration (μg mL^{-1})	*Diplodia seriata*	*Eutypa lata*	*Fomitiporia mediterranea*	*Neofusicoccum parvum*
Ethanol	2.25	2,250.0	0%	-	-	2%
	13.5	13,500.0	0%			19%
Eugenol	1.5	1,500.0	98%*	99%*	100%*	99%*
	2.5	2,500.0	100%*	100%*	100%*	100%*
EGCG	7.5	7.5	0%	9%	11%	8%
	45.0	45.0	0%	3%	0%	6%
Thymol	7.5	7.5	12%*	4%	23%*	16%*
	45.0	45.0	89%*	67%*	74%*	88%*

Table 5. Linear regression analyses, and determination of the EC_{50} concentrations, for two phenolic compounds (eugenol or thymol) with statistically significant inhibitory activities when tested against four grapevine trunk pathogens.

Treatment	Pathogen	Regression equations	R^2	EC_{50} (μg mL^{-1})
Eugenol	*Diplodia seriata*	$y = 41.707x + 10.139$	0.8492	960.0
	Eutypa lata	$y = 41.758x + 10.379$	0.8435	950.0
	Fomitiporia mediterranea	$y = 42.061x + 10.438$	0.8440	940.0
	Neofusicoccum parvum	$y = 41.571x + 8.544$	0.8659	1,000.0
Thymol	*Diplodia seriata*	$y = 2.0062x - 1.0627$	0.9977	25.45
	Eutypa lata	$y = 1.5565x - 3.3607$	0.9469	34.28
	Fomitiporia mediterranea	$y = 1.5472x + 5.0982$	0.9728	29.02
	Neofusicoccum parvum	$y = 1.9466x + 0.6071$	0.9961	25.37

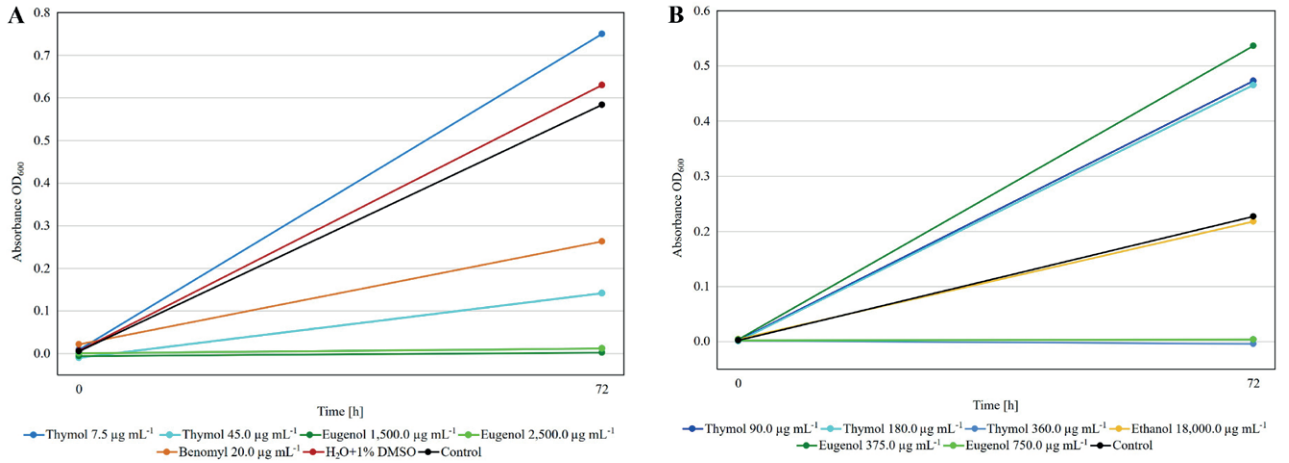

Figure 1. Absorbances measured for *Diplodia seriata* conidium suspensions treated with different phenolic compounds or chemicals (benomyl or DMSO). A) Tests of different concentrations of thymol and eugenol in *in vitro* tests for effects on conidium germination. B) Tests of doubled concentrations of thymol and halved concentrations of eugenol.

from 940 to 1,000 µg mL^{-1}, and for thymol, were from 25.4 to 34.3 µg mL^{-1}, depending on the pathogen.

In vitro *effects of phenolic compounds on conidium germination*

Thymol and eugenol, at the same concentrations as those for the mycelium inhibition assays, were used to evaluate their activities against conidia of *D. seriata*. Eugenol at 1,500 or 2,500 µg mL^{-1} prevented germination of conidia (Figure 1A). Thymol at 7.5 µg mL^{-1} did not inhibit spore germination, while at 45 µg mL^{-1} conidium germination was reduced. Similar results were obtained for the fungicide benomyl at 20 µg mL^{-1}, which also suppressed *D. seriata* conidium germination.

To determine the minimum effective concentrations against *D. seriata* conidia, the eugenol concentration was decreased, and the thymol concentration increased. Eugenol completely inhibited spore germination at 750 µg mL^{-1}, while thymol was similarly efficient at 375 µg mL^{-1} (Figure 1B). Conidia treated with ethanol at 18,000 µg mL^{-1} germinated at a similar rate as the control, showing that ethanol did not affect the thymol assay, even at the greatest thymol concentration used.

For treatments of *N. parvum* conidia, thymol and eugenol concentrations were selected based on results obtained for *D. seriata*. No increased absorbance was observed for thymol at 360 µg mL^{-1}, or eugenol at 375 or 750 µg mL^{-1}, indicating inhibition of conidium germination (Figure 2). For *N. parvum*, the fungicide benomyl completely inhibited conidium germination.

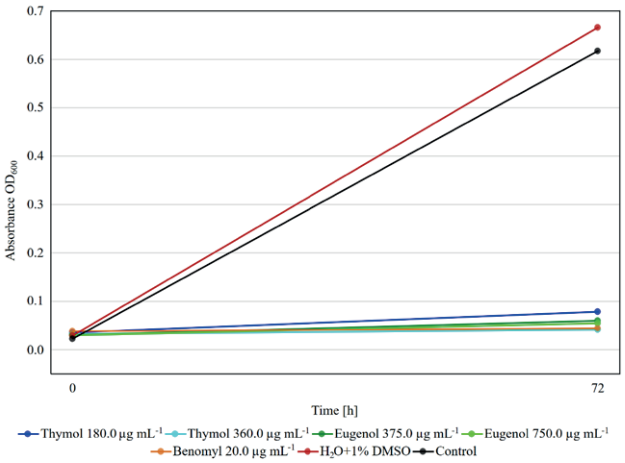

Figure 2. Absorbances measured for *Neofusicoccum parvum* spore suspensions treated with different phenolic compounds each at two concentrations, or chemicals (benomyl or DMSO).

Water plus 1% DMSO had no effect on the germination of *D. seriata* or *N. parvum* conidia, indicating that use of DMSO to facilitate formation eugenol emulsions did not affect the results (Figure 1 A and B, and Figure 2).

In planta *treatments of grapevine wounds with phenolic compounds*

Thymol at 360 µg mL^{-1} or eugenol at 750 µg mL^{-1} were assessed on grapevine canes as wound protection agents against *N. parvum* and *D. seriata*. The results for *N. parvum* can be considered valid, as the colonization

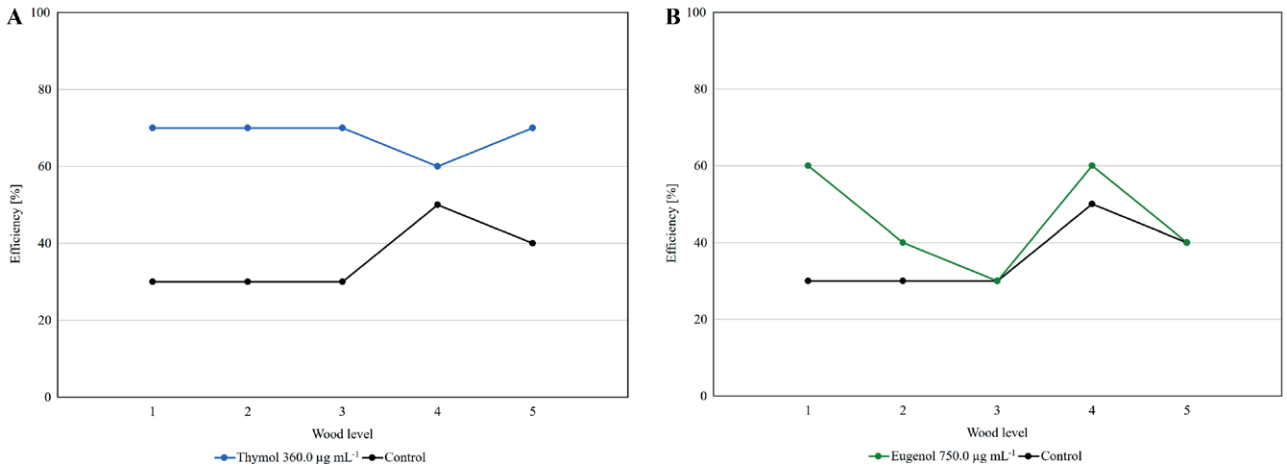

Figure 3. Efficiency (%) of grapevine wound protection from *Neofusicoccum parvum* infection, with: A) thymol, and B) eugenol.

of wood at the first two levels exceeded 60% in the controls (Figure 3 A and B). In contrast, *D. seriata* did not reach the minimum of colonized fragments in untreated wood under the same conditions as *N. parvum*, despite repetition of the experiment (observed colonization rates were 35% in the first experiment and 40% in the second). The thymol treatment reduced *N. parvum* colonization to 30% in the first three wood chips, which confirms efficiency of this compound against *N. parvum*. In contrast, eugenol showed maximum efficacy of 60%; so it is unlikely to be an effective grapevine wound treatment for against *N. parvum*.

DISCUSSION

Previous studies have confirmed the antimicrobial effects of phenolic compounds produced and purified from plants. Substances that inhibit fungi, bacteria, or viruses include eugenol, thymol, and EGCG (Li *et al.*, 1999; Kordali *et al.*, 2008; Wang *et al.*, 2010; Calland *et al.*, 2012; Kanagaratnam *et al.*, 2017; Hakalová *et al.* 2022; Hastoy *et al.,* 2023), and these were selected for the present study. The *in vitro* tests demonstrated the antifungal activity of thymol and eugenol towards mycelium growth and conidium germination of the tested GTD fungi. In addition, thymol was also effective for pruning wound protection against *N. parvum*.

These results confirm previous reports demonstrating that eugenol and thymol reduced mycelium growth of fungal plant pathogens. Eugenol limited *in vitro* growth of the postharvest apple pathogens *Phlyctema vagabunda, Penicillium expansum, Botrytis cinerea* and *Monilinia fructigena* by 73% to 90% at a concentration of 1,000 µg mL^{-1}, depending on the pathogen species and incubation temperature, and 100% at 2,000 µg mL^{-1} for all these fungi and at 4 and 20°C (Amiri *et al.*, 2008). This is similar to the present study results where greater than 98% growth decreases were obtained after treatment with 1,500 µg mL^{-1} eugenol, and complete inhibition with 2,500 µg mL^{-1}. Lower concentration (30 µg mL^{-1}) of eugenol added to liquid medium inhibited growth of *Botrytis fabae* by 73% (Oxenham *et al.*, 2005).

The efficiency of thymol for suppression of GTD pathogen mycelium growth in the present study was comparable to or greater than that of other pathogenic fungi, according to some reports. Numpaque *et al.* (2011) showed that thymol, at 50 µg mL^{-1}, reduced mycelium growth of *Lasiodiplodia theobromae*, a pathogen associated with grapevine dieback, by approx. 25%. The present study measured approx. 90% decreases for *D. seriata* and *N. parvum* at 45 µg mL^{-1} of thymol. Complete inhibition of *L. theobromae* growth was achieved only after treatment with 150 µg mL^{-1}. The same concentration completely inhibited the mycelium growth of *Fusarium moniliforme, Rhizoctonia solani,* and *Sclerotinia sclerotiorum* (Müeller-Riebau *et al.*, 1995). Zhang *et al.* (2019) reported 100% effectiveness of thymol at 65 µg mL^{-1} against *B. cinerea*.

For EGCG (concentrations 7.5 and 45.0 µg mL^{-1}), no inhibitory effects against the tested GTD fungi were measured. EGCG has exhibited antifungal activity in other studies, at concentrations similar to those used in the present study experiment. Li *et al.* (1999) measured an EGCG MIC of 12.5 µg mL^{-1} against the human pathogen *Cryptococcus neoformans*. This result was different from the MIC determined by Matsumoto *et al.* (2012) (64 µg mL^{-1}) for the same species. Only 0.31 µg

mL^{-1} EGCG was sufficient to inhibit the growth of *Candida glabrata*, while for *C. albicans* and *C. parapsilosis*, 5 μg mL^{-1} was necessary (Chen *et al*., 2015). Hirasawa and Takada (2004) reported that antifungal activity of catechin was pH dependent. pH strongly influenced EGCG efficiency against *C. albicans* strains, as demonstrated by yeast growth reduction of 90% from 2,000 μg mL^{-1} at pH 6.0, and from 15.6 to 250 μg mL^{-1} at pH 7.0.

The EC_{50} determined for eugenol in the present study was greater than those established for other pathogens. The EC_{50}s ranged from 940 μg mL^{-1} for *F. mediterranea* to 1,000 μg mL^{-1} for *N. parvum,* which contrast with the published EC_{50} of 3.6 μg mL−1 for *Rhizoctonia solani* (Zhao *et al*., 2021). Lower EC_{50} values than those for the fungi examined in the present study were also reported for *B. cinerea*, at 36.62 μg mL^{-1}) by Wang *et al.* (2010) and 240 and 263 μg mL^{-1} by Hastoy *et al*. (2023), depending on the isolate. Other *Botryosphaeriaceae* pathogens were found to be sensitive to lower eugenol concentrations than *D. seriata* and *N. parvum*, such as *Botryosphaeria kuwatsukai* (EC_{50} = 65.07 μg mL^{-1}; Wang *et al*., 2010) and *B. dothidea* (EC_{50} = 114.43 μL mL^{-1}/120.08 μg mL^{-1}; Li *et al*., 2021).

The EC_{50} values determined for thymol in the present study were 40 times less than those for eugenol, and are similar to those reported in other studies. Ding *et al.* (2023) evaluated thymol activity against the postharvest blueberry pathogens *Aspergillus niger*, *Neopestalotiopsis* sp., *Alternaria alternata*, *Penicillium* sp., *Cladosporium xylophilum* and *B. cinerea*, which indicated a mean EC_{50} of 38.52 μg mL^{-1} for the six fungi. This is similar to the EC_{50} observed here for *E. lata* (34.28 μg mL^{-1}). The thymol EC_{50} for the remaining pathogens tested in the present study was from 25.37 μg mL^{-1} for *N. parvum* to 29.02 μg mL^{-1} for *F. mediteranea*, which correspond to reports for *Fusarium graminearum* (26.3 μg mL^{-1}; Gao *et al*., 2016) and for *F. oxysporum* (26.4 μg mL^{-1}; Liu *et al*., 2022).

Compared to other research, the present study indicates that eugenol can exhibit diverse efficacy for suppressing conidium germination, depending on the fungal species. Conidium germination of *Diplodia seriata* and *N. parvum* was completely inhibited by eugenol at concentration of 750 μg mL^{-1}. Abbaszadeh *et al.* (2014) tested eugenol against propagules of important plant and food pathogens, and determined MIC values from 350 μg mL^{-1} for *Aspergillus ochraceus* to 500 μg mL^{-1} for *Alternaria alternata*, while Wang *et al.* (2010) indicated that 40,000 μg mL^{-1} did not inhibit conidium germination of *B. cinerea*. The concentration of eugenol was greater (2,000 μg mL^{-1}) than the EC_{50}s determined in the present study for *D. seriata* and *N. parvum*, and reduced germination of *Phytophthora vagabunda* and *P. expansum* by less than 60% (Amiri *et al.,* 2008). Thymol had greater efficacy than eugenol, and at 360.0 μg mL^{-1}, thymol completely inhibited germination of *N. parvum* and *D. seriata* conidia. A lower thymol concentration (100.0 μg mL^{-1}) was sufficient to reduce *F. oxysporum* spore germination by 80% (Liu *et al*., 2022), and 100% inhibition was achieved by Oh *et al.* (2022) for *Botrytis aclada* conidia. Their study also reported that *Aspergillus awamori*s conidia were resistant to thymol, and only treatment with 500 μg mL^{-1} completely inhibited germination. These results indicate that the two tested GTD fungi are susceptible to thymol at similar concentrations to those of other investigated fungi.

Of the two pathogens inoculated onto grapevine wounds, only *N. parvum* colonized the wood of control canes at sufficient depths (70% in the first two wood levels) to give valid results. *Diplodia seriata* colonization was only 40% at maximum, which could be due to reduced aggressiveness of the isolate assessed, or to low conidium viability. Grapevine wound treatments with eugenol against *N. parvum* were not effective.

No previous studies have focused on the application of eugenol to wood wounds for management of fungal pathogens. Thymol treatment (360 μg mL^{-1}) was efficient against *N. parvum*. According to Jankura (2012), thymol at 500 μg mL^{-1} inhibited *in planta Phaeomoniella chlamydospora* on the ends of woody grapevine segments.

Commercial antifungal preparations containing phenolic compounds are approved in some European countries. For example, BIOXEDA (clove (*Syzygium aromaticum*) essential oil), which can be used as a fungicide or bactericide for apple and pear storage pathogens. PREV-AM and ESSEN' CIEL (sweet orange (*Citrus sinensis*) essential oil) is approved for use against mealybug, potato blight, powdery mildew and tobacco thrips on vegetables, fruit, ornamental crops, and tobacco. BIOXEDA has certified approval in Cyprus, France, Greece, and Spain. PREV-AM is approved in Belgium, France, German, Italy, Romania, and Spain. ESSEN' CIEL is approved for use in France, Italy, and Spain. The use of these products can therefore lead to reductions in the use of chemical pesticides in agriculture (Raveau *et al*., 2020). According to Torre *et al*. (2014), BIOXEDA can also be used in vineyards to protect against downy mildew (*Plasmopara viticola*). Application of the product to grapevine leaves reduced symptom incidence by 30%. According to Lasorello *et al*. (2018), applying PREV-AM in vineyards reduced incidence of powdery mildew (*Erysiphe necator*) by 92% on leaves and 79% on grapes. Applications of preparations based on phenolic substances that inhibit grapevine pathogens is therefore likely to reduce application of chemical pesticides, especially in vineyards with ecological management systems.

CONCLUSIONS

This study examined the antifungal effects of three phenolic compounds on the GTD fungi *D. seriata, E. lata, F. mediterranea*, and *N. parvum*. In the *in vitro* mycelial growth tests, the inhibitory effects were demonstrated for eugenol and thymol, but no impacts of EGCG on mycelium growth were detected. Based on the inhibitory effects observed for eugenol and thymol against GTD fungi, their efficacy was further evaluated by treating conidium suspensions and grapevine pruning wounds under controlled conditions. Protection with thymol at 360 µg mL^{-1} was effective against conidium germination of these fungi *in vitro*, and against *N. parvum* development on pruning wounds. Eugenol at 750 µg mL^{-1}, prevented conidium germination *in vitro* but did not prevent development of *N. parvum* on pruning wounds. These results show that thymol has potential for use in vineyards as a natural agent for use against the *Botryosphaeriaceae* pathogen *N. parvum*.

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