Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Antifungal efficacy of four plant-derived essential oils against *Botrytis cinerea*: chemical profiles and biological activities

Faiçal AOUJIL[1,2], Loubna DRA[1], Chaimae EL GHDAICH[1], Sarah TOUFIQ[3], Hiba YAHYAOUI[1,2], Majida HAFIDI[2], Aziz AZIZ[4], Khaoula HABBADI[2,*]

[1] *Phytobacteriolgy and Biological Control Laboratory, Regional Center of Agricultural Research of Meknes, National Institute of Agricultural Research, Avenue Ennasr, BP 415 Rabat Principal, Rabat 10090, Morocco*
[2] *Laboratory of Biotechnology and Bio-Resources Valorization, Faculty of Sciences, Moulay Ismail University, Meknes 50000, Morocco*
[3] *Faculty of Agricultural Science, University of Hohenheim, Stuttgart 70593, Germany*
[4] *Research Unit "Induced Resistance and Plant Bioprotection", RIBP-USC INRAe 1488, University of Reims Champagne-Ardenne, 51100 Reims, France*
* Corresponding author. E-mail: khaoula.habbadi@inra.ma

Summary. Chemical compositions and the antifungal efficacy of essential oils derived from *Origanum elongatum, Mentha pulegium, Thymus vulgaris*, or *Corymbia citriodora* were assessed against the grapevine gray mold pathogen *Botrytis cinerea*, isolated from Moroccan vineyards. Gas chromatography-mass spectrometry (GC-MS) analyses identified the major constituents of these oils as carvacrol (61.8%) from *O. elongatum*, pulegone (91.2%) from *M. pulegium*, thymol (47.8%) from *T. vulgaris*, and cineol (78.11%) from *C. citriodora*. All these essential oils had antifungal activity, inhibiting *in vitro* colony radial growth and conidium germination of *B. cinerea*. Among the essential oils, that from *O. elongatum* exhibited the greatest inhibition of mycelium growth, with minimum inhibitory concentrations (MICs) and effective concentrations (EC_{50}), respectively, of 252.5 µL L^{-1} and 33.27 µL L^{-1} in direct contact, and 56.17 µL L^{-1} and 12.75 µL L^{-1} in fumigation. At 125 µL L^{-1}, origanum essential oil completely inhibited *B. cinerea* conidium germination. *In vivo* tests with detached leaves of two grapevine cultivars and grape berries showed that essential oils from *M. pulegium* and *O. elongatum* reduced the lesion diameters by, respectively, 78% and 72% on the leaves, and by 58% and 50% on grape berries. The results indicate the potential of using these essential oils as natural and effective alternatives to chemical fungicides for control of *B. cinerea*, offering a promising strategy for sustainable and environmentally friendly disease management practices.

Keywords. *Vitis vinifera,* plant extracts, chemical composition, sustainable agriculture.

OPEN ACCESS

Citation: Aoujil, F., Dra, L., El Ghdaich, C., Toufiq, S., Yahyaoui, H., Hafidi, M., Aziz, A. & Habbadi, K. (2025). Antifungal efficacy of four plant-derived essential oils against *Botrytis cinerea*: chemical profiles and biological activities. *Phytopathologia Mediterranea* 64(1): 109-127. doi: 10.36253/phyto-15566

Accepted: February 2, 2025

Published: May 15, 2025

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Antonio Evidente, University of Naples Federico II, Italy.

ORCID:
FA: 0009-0004-3763-9841
CEG: 0009-0005-0469-8105
ST: 0009-0008-2293-9007
MH: 0000-0002-6207-2822
AA: 0000-0003-1602-2506
KH: 0000-0002-1890-4601

INTRODUCTION

Gray mold, caused by the necrotrophic fungus *Botrytis cinerea*, adversely affects a wide range of host plants, both pre- and post-harvest, including important horticultural crops in temperate and subtropical regions (Wil-

liamson *et al*., 2007). This widespread impact makes *B. cinerea* the second most important phytopathogenic fungus (Bi *et al*., 2023). Annual economic impacts of *B. cinerea* have been estimated to exceed 10 billion dollars (Boddy, 2016). Additionally, the annual expenditure for botrytis control exceeds 1 billion euros (Hua *et al*., 2018). In grapevine, gray mold is an important disease, causing 10 to 70% of losses (Orozco-Mosqueda *et al*., 2023), reducing the quality of fresh grapes (Xueuan *et al*., 2018) and posing major postharvest challenges (Xu *et al*., 2007; Adaskaveg *et al*., 2022).

In recent decades, synthetic fungicides have been used to prevent the onset of fungal diseases (Yu *et al*., 2020), and synthetic fungicides (e.g. fludioxonil, fenhexamid, iprodione, boscalid) are utilized manage diseases caused by *B. cinerea* (Notte *et al*., 2021).However, application of these chemicals has led to development and prevalence of resistant pathogen strains (Shao *et al*., 2021). There are also risks of food contamination with pesticide residues, which can pose threats to human health and the environment due to their high toxicity and persistence (Aoujil *et al*., 2024). Consequently, there is need to develop fungicides that are safe, biodegradable, and derived from natural sources for managing *B. cinerea*. Essential oils derived from plants (EOs) have promise for protecting plants against fungal pathogens, and mitigating the harmful effects of chemical fungicides, due to their biocompatibility, biodegradability, and cost-effectiveness (Chang *et al*., 2022).

EOs, characterized by their hydrophobic composition and volatility, are natural secondary metabolites produced by aromatic plants (Nazzaro *et al*., 2017). They have diverse chemical structures, including phenylpropanoids, aliphatic hydrocarbons, terpenoids, and phenolic compounds (Maurya *et al*., 2021), and these phytochemicals are responsible for EO inhibitory effects (Moghaddam and Mehdizadeh, 2016).

EOs inhibit fungal pathogens by directly reducing mycelium growth and spore germination, and by modifying cellular metabolism, mainly through metabolic disruptions and cellular damage (Tang *et al*., 2018). They can also affect cellular pH and electrochemical gradients of plant plasma membranes (Li *et al*., 2017; Yang *et al*., 2022), and can interfere with metabolic processes associated with changes in gene expression (Singh *et al*., 2024). Numerous studies have shown that EOs have antifungal properties that are effective against *B. cinerea* infections in different fruits, including tomatoes, apples, strawberries, and grapes (Šernaitė *et al*., 2020; Almasaudi *et al*., 2022; Di Francesco *et al*., 2022; Hong *et al*., 2023). However, the effectiveness of EOs in reducing gray mold on grapevine fruit and vegetative organs has not been assessed.

The present study aimed to evaluate the antifungal properties of EOs derived from the aerial parts of three *Lamiaceae* plants (*Origanum elongatum*, *Mentha pulegium*, *Thymus vulgaris*) and one *Myrtaceae* plant (*Corymbia citriodora*). These EOs were selected from an initial screening of 18 EOs (see supplementary file S1), against *B. cinerea*. The chemical compositions of the four EOs were first characterized using GC/MS analysis. Their effects on *B. cinerea* conidium germination and hyphal growth were assessed, through direct and vapour contact assays. *In vivo* efficacy of these EOs for managing gray mold infections was also evaluated on detached grapevine leaves and grape berries.

MATERIALS AND METHODS

Fungal isolate

Botrytis cinerea strain BC53 used in this study was obtained from the mycotheque collection of the National Institute of Agronomic Research, Unit of Plant Protection (CRRAM) Laboratory. This strain was isolated from infected grape berries during the 2021–2022 agricultural season. After cultivation on potato dextrose agar (PDA) and monoconidium subculture on water agar, the isolate was identified based on morphological characteristics and molecular analysis using the primer pairs G3PDHfor and G3PDHrev, which amplify partial fragments of glyceraldehyde-3-phosphate dehydrogenase. The nucleotide sequences obtained were compared with those available from the National Center for Biotechnology Information (NCBI) database, using the Basic Local Alignment Search Tool (BLAST) (Aoujil *et al*., unpublished data). For long-term preservation, mycelia of this isolate were stored at -80°C, in cryovials containing 25% glycerol.

Plant material and extraction of essential oil

Aerial parts of the four medicinal and aromatic plants (MAPs) under study were collected from locations in Morocco between February and April 2024 (Table 1). The plant samples were each cleaned, air-dried in the shade (except for eucalyptus, which was used fresh), and were then stored in the dark at 4°C until further use. EOs were each extracted from the plant samples by hydrodistillation for 2 h using a Clevenger-type apparatus. The extracted EOs were then each separated by decantation, dried over anhydrous sodium sulfate, and then stored in a dark glass bottle at 4°C. The extraction yields were calculated as the ratio of the mass of the EOs to the mass of the original plant material.

Table 1. Information on the classifications and origins of the medicinal and aromatic plants used in this study.

Common name	Botanical name	Plant family	Phenological stage	Sampling site
Oregano	*Origanum elongatum*	*Lamiaceae*	Flowering stage	Bouiblane
Pennyroyal	*Mentha pulegium*	*Lamiaceae*	Vegetative stage	Meknes
Thyme	*Thymus vulgaris*	*Lamiaceae*	Flowering stage	Sefrou
Lemon Eucalyptus	*Corymbia citriodora*	*Myrtaceae*	Vegetative stage	Sidi yahya gharb

Gas chromatography-mass spectrometry analyses of essential oils

Gas chromatography-mass spectrometry (GC-MS) analyses were carried out to determine the chemical composition of each EO. These analyses were performed using a Hewlett Packard model HP6890 gas chromatograph (Agilent Technologies) equipped with a DB-5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm; Agilent Technologies) coupled to an HP model 5973 mass selective detector. The chromatographic conditions involved an initial oven temperature of 50°C, which was ramped up at 7°C min^{-1} until reaching 300°C. The injector temperature was maintained at 290°C. Helium gas served as the carrier with a flow rate of 1 mL min^{-1}, and a split ratio of 60:1 was employed. Mass spectra were obtained in electron ionization (EI) mode at 70eV ionization energy, covering a mass range from m/z 35 to 400. For each EO sample, 10 µL was diluted in 990 µL of pure hexane, and 1 µL of this solution was injected into the GC-MS system for analysis. Instrument control and data processing were carried out using "HP ChemStation Software" G1701BA, version B.01.00. To aid compound identification, Kovats retention indices (RI) were calculated using a standard mixture of n-alkanes (C8 to C26, Sigma-Aldrich Co.) analyzed under identical conditions. Identification of EO constituents was achieved by comparing their RI values and mass spectra with literature data (Adams, 2007), and by computer matching against standard reference databases (NIST98, Wiley275, and CNRS libraries).

Effects of essential oils on in vitro *mycelium growth of* Botrytis cinerea

Direct contact assay Antifungal activity of the four EOs against *B. cinerea* was determined according to the method of Fontana *et al.* (2021). Sterile molten PDA, cooled to 45°C, was supplemented with the EOs to obtain the following concentrations: 0.97, 1.91, 3.93, 7.88, 15.77, 31.56, 63.12, 126.25, 252.5, 505, 1010 or 2020 µL L^{-1}, in Tween 80 (1%) to increase the solubility of the EOs (Figure 1) (López-Meneses *et al.*, 2017). PDA containing Tween 80 (1%) was used as an experimental control. EO amended and non-amended agar plates were each inoculated with a 6 mm diam. *B. cinerea* mycelium plug taken from a 3-day old culture. The agar plates were then closed, sealed with Parafilm and incubated at 21 ± 1°C.

Vapour phase assay to determine the effects of volatile phase, 90 mm Petri dishes were filled with 20 mL of PDA, resulting in a 40 mL air space after adding the agar medium. The middle of each Petri dish was inoculated as previously described. The EOs were pipetted onto sterile filter paper discs (8 mm diameter) to achieve final concentrations of 5.61, 11.23, 22.47, 44.94, 56.17, 67.41, 89.88, and 112.35 µL L^{-1} of the Petri dish clearance volume (Figure 1) (Zhao *et al.*, 2021). The treated discs were attached to the Petri dish lids (one disc per lid). The dishes were then inverted, immediately sealed with parafilm to prevent the loss of volatile compounds and incubated in the dark at 21 ± 1°C.

For both assays, the mean radial mycelium growth of the pathogen was assessed by measuring colony diameters in two perpendicularly opposite directions, once the surfaces of control Petri dishes were completely covered by the fungus. The inhibition rate of mycelium growth was calculated using the equation (Chen and Dai, 2012):

$$\%\text{inhibition} = \frac{D1-D2}{D2} \times 100$$

where (%) is the percentage inhibition of mycelial growth; D1 is the mean value of the colony radius (mm) grown in the PDA-Tween control; and D2 is the colony diameter of the treated fungi.

The fungal growth observed on the last day of cultivation was used to determine the minimum inhibitory concentrations (MIC) and the effective concentrations (EC_{50} and EC_{90}) of the four essential oils. The MIC was defined as the lowest concentration of EO that completely inhibited mycelium growth of *B. cinerea* (Parikh *et al.*, 2021). The EC_{50} and EC_{90}, the concentrations, respectively, at which mycelium growth

was inhibited by 50% and 90%. were calculated with dose-response analyses, using R statistical software (Ritz *et al*., 2015). Three replicates of each treatment were performed in these experiments, and the experiment was carried out twice, with each Petri dish considered as the experimental unit.

Conidium germination assays

Botrytis cinerea was cultured on PDA Petri dishes for 14 d to produce conidia. The conidia were then collected to prepare a conidium suspension, which was adjusted to contain 10^5 conidia mL^{-1} using a hemocytometer. Petri dishes were prepared containing water agar and different concentrations of EO (2500, 2000, 1500, 1000, 500, 250, 125, 62.5, and 31.25 µL L^{-1}) dissolved in 1.0% Tween 80 (1%). An aliquot of 40 µL of the *B. cinerea* conidia solution was added to the agar surface of each dish (Figure 1). Experimental control dishes contained conidia exposed only to Tween 80 (1%). Dishes were then sealed with parafilm to prevent evaporation, and were incubated at 21 ± 1°C for 20 h. Conidium germination was assessed by counting at least 100 randomly selected conidia per replicate using an optical microscope. Conidia were considered to have germinated if the lengths of the germ tubes were equal to, or greater than, the diameter of the conidia (Parikh *et al*., 2021). The results were expressed as the percentage of conidium germination, which was calculated as follows:

$$\text{Conidium germination rate } (\%) = \frac{\text{Number of germinated conidia}}{\text{Total number of conidia}} \times 100$$

Antifungal efficacy of EOs on detached grapevine leaves

The effectiveness of the four EOs for protecting *Vitis vinifera* L. 'Chardonnay' and 'Cabernet Sauvignon' leaves was assessed using inoculation tests with *B. cinerea* mycelium agar plugs. Young leaves from commercial vineyards, treated with 3 kg ha^{-1} copper at the time of collection and free of disease lesions, were thoroughly washed with water, then immersed in 1% sodium hypochlorite for 2 min, rinsed three times with sterile distilled water, and were then dried on filter paper. EO treatments were applied by immersing leaves for 1 min in the appropriate solutions, following the experimental design detailed in Table 2 and Figure 1. After treatment, the leaves were each wounded with a sterile needle (four wounds per leaf). Subsequently, 5 mm agar plugs containing fresh mycelium from the edge of actively growing *B. cinerea* fungal colonies on PDA plates were placed on the wounds, mycelium side down. The inoculated leaves were incubated in closed plastic boxes at 21°C and with a 16 h photoperiod, with filter paper soaked in water to maintain humidity. The effects of the treatments were evaluated by measuring lesion diameters at 120 h post-inoculation (hpi).

Table 2. Experimental design to assess *in vivo* effects of essential oils on disease development on the grapevine leaves and berries.

Code	Treatment
T-	Treated with sterile distilled water (SDW) + Tween 80 (1%)
T+	Treated with fungicide (fenhexamid)
EC_{50}	Treated simultaneously with the fungus and the EO at EC_{50}
EC_{90}	Treated simultaneously with the fungus and the EO at EC_{90}
EC_{50}+1	Treated with EO at EC_{50} 24 h before fungal inoculation
EC_{90}+1	Treated with EO at EC_{90} 1 24 h before fungal inoculation

Antifungal efficacy of EOs on grape berries

The effects of the four EOs on grape berries was investigated using the methods outlined by Fontana *et al.* (2021), with slight modifications. Healthy table grapes, *Vitis vinifera* L. 'Muscat of Italy', from contaminants or injuries, were randomly selected and purchased in Meknes City, Morocco. These berries were washed under running water, surface-sterilized with a 1% sodium hypochlorite solution, rinsed three times with distilled water, and then air-dried in a laminar flow hood. (Table 2), the berries were placed in 90 mm diam. Petri dishes without lids, and were secured using double-sided tape, with 12 fruits per treatment. Inoculations were carried out injecting a 10 µL aliquot of a *B. cinerea* conidium suspension containing 10^5 conidia mL^{-1} into the equatorial region of each fruit (Figure 1). The inoculated fruits were then incubated in closed plastic boxes (to maintain high humidity conditions) at 22°C with a 16 h photoperiod. On the final day of evaluation, disease incidence and lesion diameters were analysed. Incidence was determined as presence or absence of typical disease symptoms. Lesion diameter was calculated from the average length and width (cm) of each lesion.

Statistical analyses

All calculations were performed using R statistical software (version 4.3.1), and Data were arcsine-transformed where necessary before statistical analysis. Analyses of variance (ANOVA) were carried out at $P \leq$

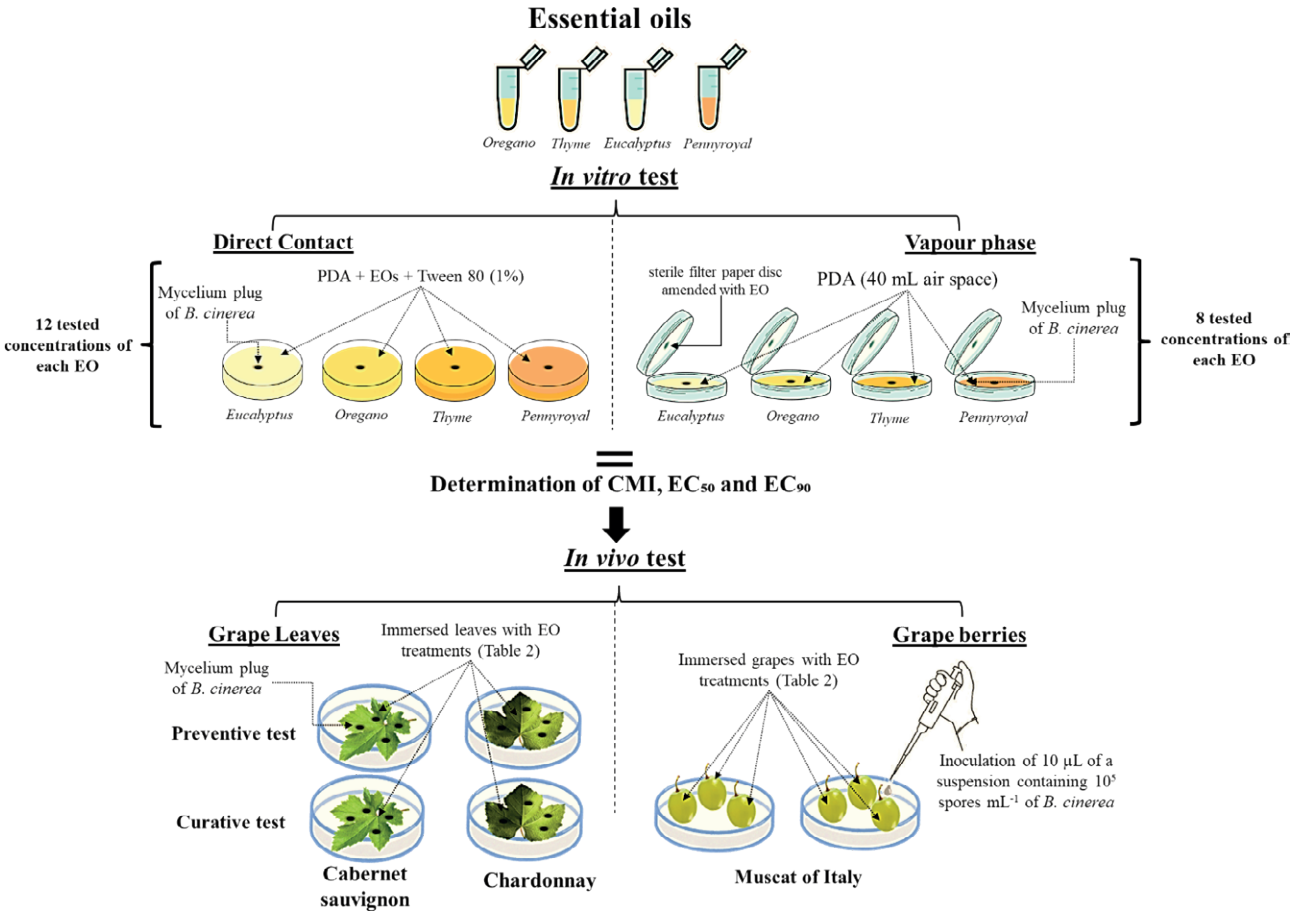

Figure 1. Experiment setup for the *in vitro* and *in vivo* tests to assess effects on *Botrytis cinerea* of treatments of grapevine leaves or berries with four Eos.

0.05. Tukey's HSD test was used to separate means when appropriate ($P \leq 0.05$). The EC_{50} and EC_{90} values for each assessed EO were calculated using the 'drc' package, based on percentage inhibition of mycelium growth. All the histograms presented below were generated using the 'ggplot2' package.

RESULTS

Yields and chemical compositions of essential oils

The EOs, extracted through hydrodistillation, ranged in colour from light yellow to brown, with each possessing a distinctive aroma. The yields of these oils varied depending on the plant species. *Oreganum elongatum* produced the greatest yield (3%), followed by *C. citriodora* (2.7%, *M. pulegium* at 3.2, and *T. vulgaris* (0.7%). The main components of the different EOs assessed in this study are presented in Table 3. Gas chromatography-mass spectrometry (GC-MS) analyses revealed distinct chemical compositions among the EOs. Forty-eight compounds were identified, with 35, 26, 11 and nine compounds detected, respectively, from *T. vulgaris*, *O. elongatum*, *M. pulegium*, and *C. citriodora*, representing, respectively, 98.4%, 98.9%, 98.67%, and 98.7% of the total oil for *T. vulgaris*, *O. elongatum*, *M. pulegium*, and *C. citriodora*. The primary constituents of the *T. vulgaris* EO were thymol (47.8%), p-cymene (24.1%), γ-terpinene (8.2%), and carvacrol (5%). The EO of *O. elongatum* was primarily composed of carvacrol (61.8%), γ-terpinene (12.5%), p-cymene (8.2%), and thymol (7%). The *M. pulegium* EO was predominantly pulegone (91.2%), while that from *C. citriodora* consisted mainly of cineole (78.1%) and α-pinene (12.2%). The main chemical classes identified in the analyses were monoterpenoid phenols (thymol, carvacrol), monoterpenes (γ-terpinene, p-cymene, and α-pinene), monoterpenoid ketone (pulegone), and monoterpenoid ether (cineole).

Table 3. Chemical compositions (%) and yields of essential oils analysed by GC-MS.

Compound	Essential oil				Identification
	Origanum elongatum	*Mentha pulegium*	*Thymus vulgaris*	*Corymbia citriodora*	
Thujene	0.92	-	0.57	-	RI and MS
α-Pinene	0.59	0.19	0.54	**12.21**	RI and MS
Camphene	0.08	-	0.16	-	RI and MS
β-Pinene	0.12	0.21	0.11	0.58	RI and MS
Octen-3-ol	1.17	-	0.32	-	RI and MS
3-Octanone	0.10	-	0.06	-	RI and MS
Myrcene	**1.43**	-	1.34	0.4	RI and MS
3-Octanol	-	0.41	0.06	-	RI and MS
α-Phellandrene	0.22	-	0.16	-	RI and MS
δ-3-Carene	0.08	-	0.07	-	RI and MS
Cineole (Eucalyptol)	-	-	-	**78.11**	RI and MS
Cis-β-Ocimene	0.13	-	-	-	RI and MS
α-Terpinene	**1.85**	0.53	1.72	-	RI and MS
γ-Terpinene	**12.46**	-	**8.19**	-	RI and MS
Terpinolene	0.08	-	0.10	-	RI and MS
cyclohexanone	-	0.30	-	-	RI and MS
Isomaylisovalerate	-	-	-	-	RI and MS
trans-isopulegone	-	1.43	-	-	RI and MS
1-oxaspiro-2,5-octane-4-one	-	0.94	-	-	RI and MS
Pulegone	-	**91.18**	-	-	RI and MS
Pipertenone	-	1.41	-	-	RI and MS
Cyclopentane	-	0.42	-	-	RI and MS
ρ-Cymene	**8.21**	-	**24.13**	**0.79**	RI and MS
Trans-Ocimene	-	-	-	-	RI and MS
Limonene	0.23	1.55	0.38	-	RI and MS
Cis-Linalool Oxide	-	-	0.09	-	RI and MS
Linalool	0.86	-	**3.06**	-	RI and MS
p-Cymenene	-	-	0.07	-	RI and MS
1,8-Cineole	-	-	0.12	-	RI and MS
Camphor	-	-	0.05	-	RI and MS
Borneol	0.14	-	0.45	-	RI and MS
Terpinen-4-ol	0.42	-	0.59	-	RI and MS
2-β-pinene	-	-	-	**5.27**	RI and MS
Cis-Dishydro Carvone	0.06	-	0.06	-	RI and MS
Thymol Methyl Ether	-	-	0.04	-	RI and MS
Carvacrol Methyl Ether	-	-	0.05	-	RI and MS
Carvacrol	**61.78**	-	**5.03**	-	RI and MS
Thymol	**7.01**	-	**47.79**	-	RI and MS
(E)Caryophyllene	**1.47**	-	1.76	-	RI and MS
α-Humulene	0.06	-	0.06	-	RI and MS
α-Selinene	-	-	0.04	-	RI and MS
α-Murolene	-	-	0.01	-	RI and MS
β-Bisabolene	0.13	-	0.05	-	RI and MS
γ-Cadinene	0.03	-	0.07	-	RI and MS
δ-Cadinene	0.06	-	0.16	-	RI and MS
Caryophyllene Oxide	-	-	0.80	-	RI and MS
Isoaromadendrene	-	-	-	0.33	RI and MS

(Continued)

Table 3. (Continued).

Compound	Essential oil				Identification
	Origanum elongatum	*Mentha pulegium*	*Thymus vulgaris*	*Corymbia citriodora*	
Aromadendrene	-	-	-	1	RI and MS
δ- gurjunene	-	-	-	0.35	RI and MS
Total	98.89	98.57	98.35	98.69	RI and MS
Yield (%)	3.00	2.17	0.7	2.67	

RI: Retention index. MS: Comparison of the mass spectra with the NIST98 NIST98, Wiley275, and CNRS libraries.

Effects of essential oils on in vitro mycelium growth of Botrytis cinerea

The *in vitro* direct contact (DC) antifungal activities of EOs from *O. elongatum, M. pulegium, T. vulgaris,* and *C. citriodora* at different concentrations against *B. cinerea* are presented in Figure 2. The minimum inhibitory concentration (MIC), defined as the lowest concentration that inhibited 100% of fungal growth, was 253 μL L^{-1} for the oils from oregano and thyme, and 1010 μL L^{-1} for those from pennyroyal and eucalyptus. Mycelium growth (colony diameter) was reduced ($P < 0.05$) with increasing concentrations of EOs, indicating dose-dependent activities. The mean inhibition zone diameters obtained from determinations of the EO MICs against on *B. cinerea* are presented in Table 4.

The negative control (0 μL L^{-1} of essential oil) gave no inhibition zone formation, suggesting that the diluent (Tween 80) had no antifungal activity, and did not interfere with the MIC analysis. The EO of *O. elongatum* was the most suppressive of *B. cinerea*, reducing growth of *B. cinerea* at low concentrations. At 7.8 μL L^{-1}, differences ($P < 0.05$) were observed, with 21.1% inhibition for the oil of *O. elongatum*, whereas oils from *M. pulegium*, *T. vulgaris*, and *C. citriodora*, gave inhibition rates, respectively, of 0.8, 0.5, and 3.5%. At 31.56 μL L^{-1}, the oregano essential oil gave 50.5% inhibition of the fungus, while the inhibition rates from oils of *M. pulegium*, *T. vulgaris*, and *C. citriodora* were, respectively, 11.1, 19.5, and 18.5%.

The mean essential oil EC_{50} and EC_{90} for inhibition of colony size of *B. cinerea* on PDA after 4 d of incubation are shown in Table 4. These values were derived from dose-response curve regression equations, and are expressed in μL L^{-1}. Oregano oil gave the greatest inhibition of the fungus (mean EC_{50} = 33.27 μL L^{-1}, mean EC_{90} = 139.17 μL L^{-1}. The greatest values were from the pennyroyal oil (mean EC_{50} = 221.74 μL L^{-1}; mean EC_{90} = 828.26 μL L^{-1}.

In the vapour contact (VC) assays, the fumigant activities of the EOs from *O. elongatum*, *M. pulegium, T. vulgaris,* and *C. citriodora* against *B. cinerea* were also determined (Figure 3). This activity is different from that assessed in traditional DC assays. Although the antifungal activity trends in the VC assays were similar to those in the DC assays, the inhibitory effects of the oils in the VC assays were significantly more potent. All the assessed EOs inhibited mycelium growth of *B. cinerea* in dose-dependent ways. Oregano and thyme EOs were more inhibitory effects than those from pennyroyal or Eucalyptus at all the assessed *in vitro* concentrations.

At 44.94 μL L^{-1}, the inhibitory effect of oregano oil was 98.43%, and that of thyme oil was 84.82%, whereas the inhibitory effects of oils from eucalyptus and pennyroyal were, respectively, 48.97% and 26.81%. The mean MICs were 56.17 μL L^{-1} for oregano EO, 89.88 μL L^{-1} for thyme EO, and 112.35 μL L^{-1} for eucalyptus and pennyroyal EOs.

The mean EC_{50} were estimated using a regression model (Table 4). In VC assays, the mean EC_{50} were lower than those obtained in the DC assays. The lowest mean EC_{50} (12.85 μL L^{-1}) resulted from oregano oil, followed by those from thyme EO (19.08 μL L^{-1}), eucalyptus EO (48.11 μL L^{-1}), and pennyroyal EO (61.35 μL L^{-1}).

Effects of essential oils on Botrytis cinerea *conidium germination*

The effects of different concentrations of the four EOs on the *B. cinerea* conidium germination of were assessed using direct contact assays. The results presented in Figure 4 indicate that among the EOs tested, only that of oregano completely inhibited conidium germination at 125 μL L^{-1}. In contrast, at the same concentration, the EOs from *M. pulegium, T. vulgaris*, and C. *citriodora* gave mean conidium germination rates not exceeding, respectively 29.0%, 47.4%, and 28.7%. At 250 μL L^{-1} and greater, toxicity of the EOs to conidia was high, as the germination percentages were 0% for all the EOs except that from *C. citriodora*, which gave 12.5% germination. It was only at a concentration of 2000 μL L^{-1} that the *C. citriodora* EO completely inhibited conidium germination.

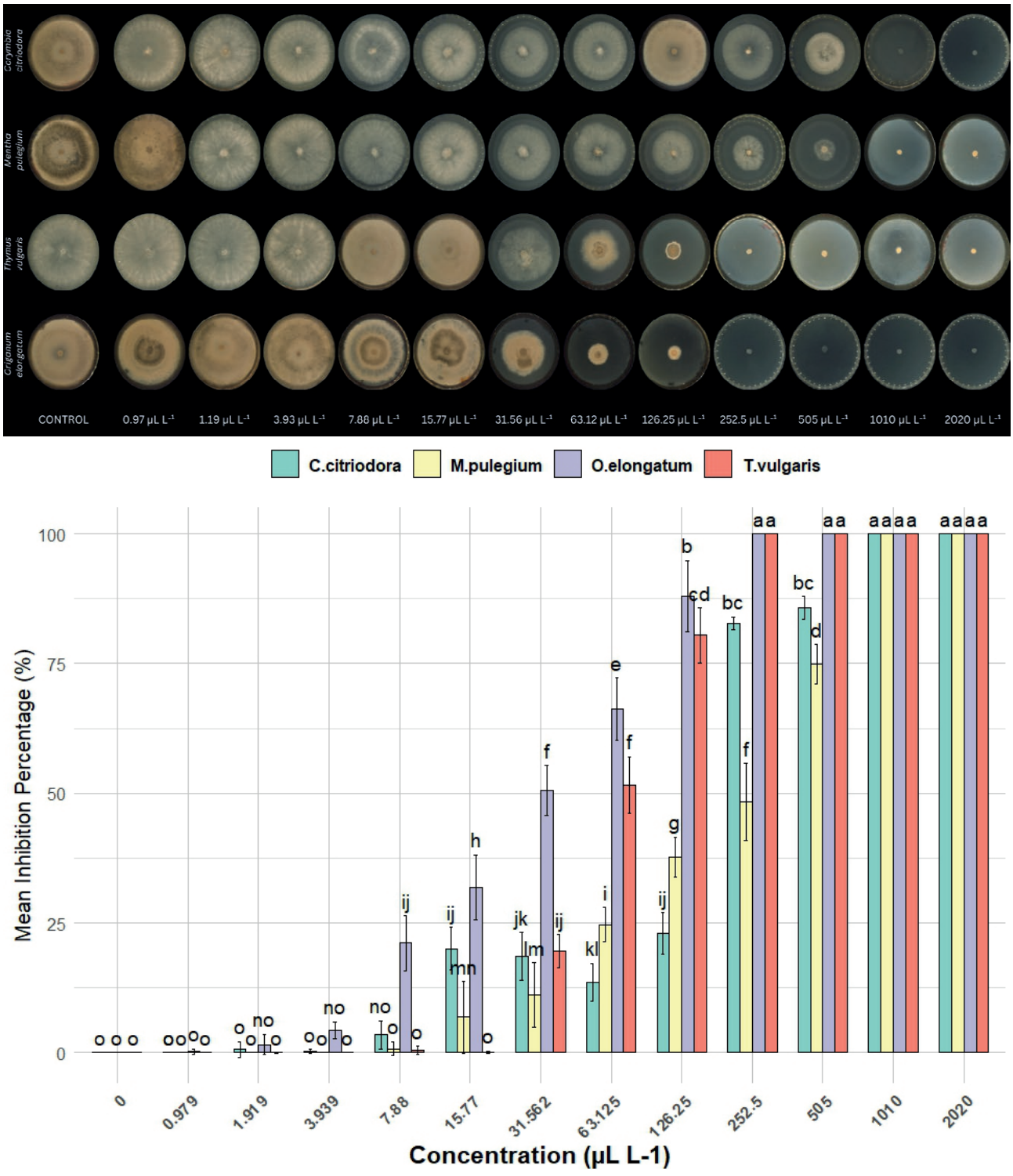

Figure 2. *In vitro* antifungal activities of the four essential oils (EOs) indicated from direct contact assays against mycelium growth of *Botrytis cinerea*, after 4 d of incubation at 24 ± 1 °C. Top: Mycelium growth in Petri dishes containing different concentrations of each EO in agars plates, in the direct contact assay. Bottom: Mean percentage inhibition of mycelium growth at different concentrations of the tested EOs. Different letters above bars indicate differences ($P < 0.05$), according to Tukey's high significance difference (HSD) test. All the data are means ± SD.

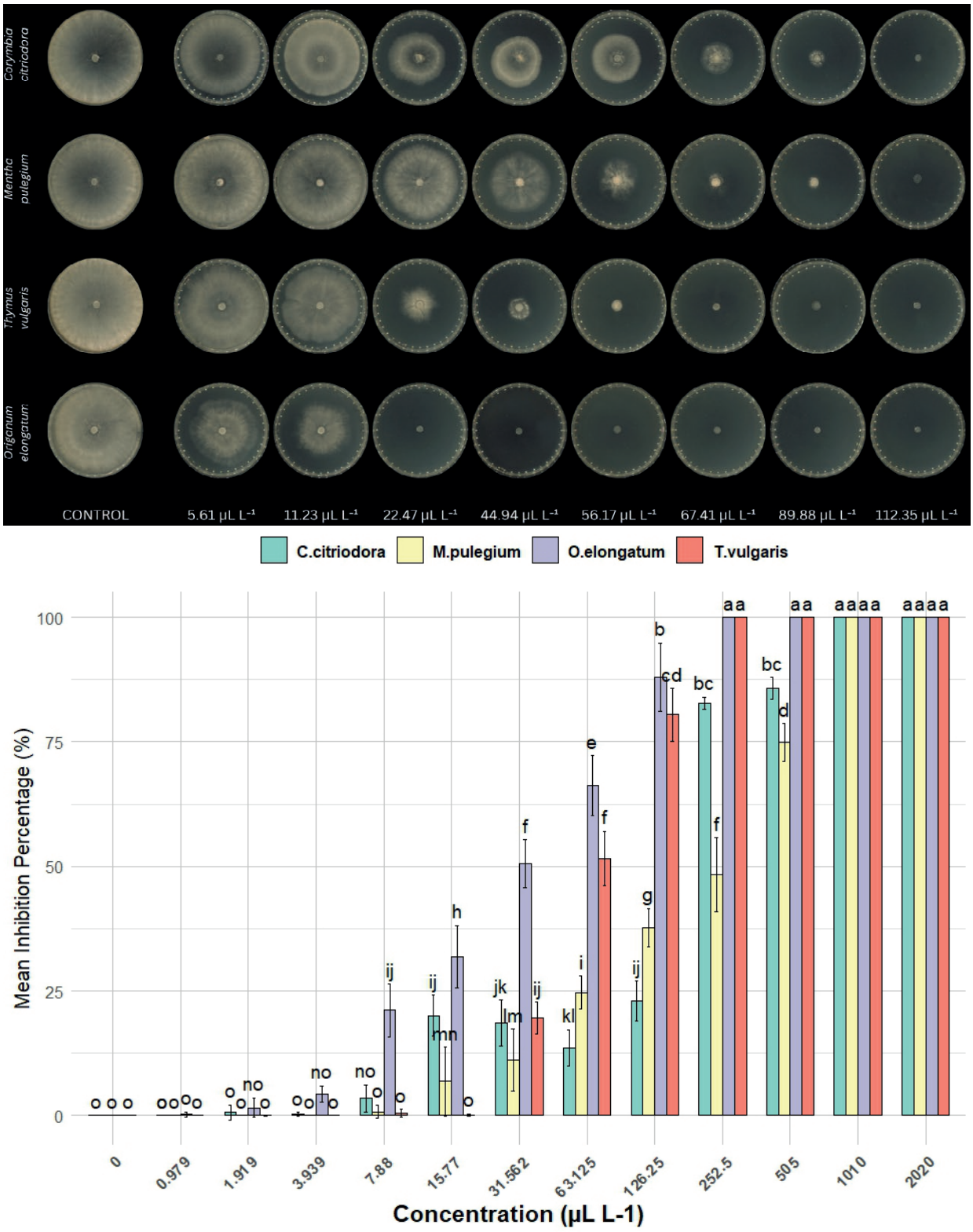

Figure 3. *In vitro* antifungal activities of the four essential oils (EOs) indicated from vapor-phase assays against mycelium growth of *Botrytis cinerea*, after 4 d incubation at 24 ± 1 °C. Top: Mycelium growth in Petri dishes containing different concentrations of each EO in the direct contact assay. Bottom: Mean percentages of inhibition of mycelium growth at different concentrations of the tested EOs. Different letters above bars indicate differences ($P < 0.05$), according to Tukey's high significance difference (HSD) test. All data are means ± SD.

Table 4. mean minimum inhibitory concentrations (MIC) and mean effective concentrations (EC_{50} and EC_{90}) from essential oils from of *Oreganum elongatum*, *Mentha pulegium*, *Thymus vulgaris* and *Corymbia citriodora* against mycelium growth of *Botrytis cinerea*.

Essential oil	Methods	R^{2*}	MIC (µL L^{-1})	EC_{50} (µL L^{-1})	95%Confidence Limits (µL L^{-1})	EC_{90} (µL L^{-1})	95% Confidence Limits (µL L^{-1})
O. elongatum	DC*	0.99	252.5	33.27	[30.13-36.41]	139.17	[121.46-156.88]
	VC*	0.95	56.17	12.75	[11.07-14.44]	26.56	[21.76-31.35]
M. pulegium	DC	0.97	1010	223.867	[194.62-253.11]	816.763	[644.44-986.08]
	VC	0.97	112.35	61.347	[55.30-67.39]	99.5098	[81.59-117.42]
T. vulgaris	DC	0.99	252.5	59.799	[53.90-65.69]	142.040	[132.68-151.39]
	VC	0.96	89.88	18.495	[1652-20.47]	42.995	[30.38-55.60]
C. citriodora	DC	0.94	1010	176.455	[137.73-215.17]	258.78	[238.71-278.84]
	VC	0.97	112.35	53.708	[41.48-65.93]	99.90	[64.28-135.51]

*R^2: Coefficient of determination. DC = Direct contact assay. VC = Vapor contact assay.

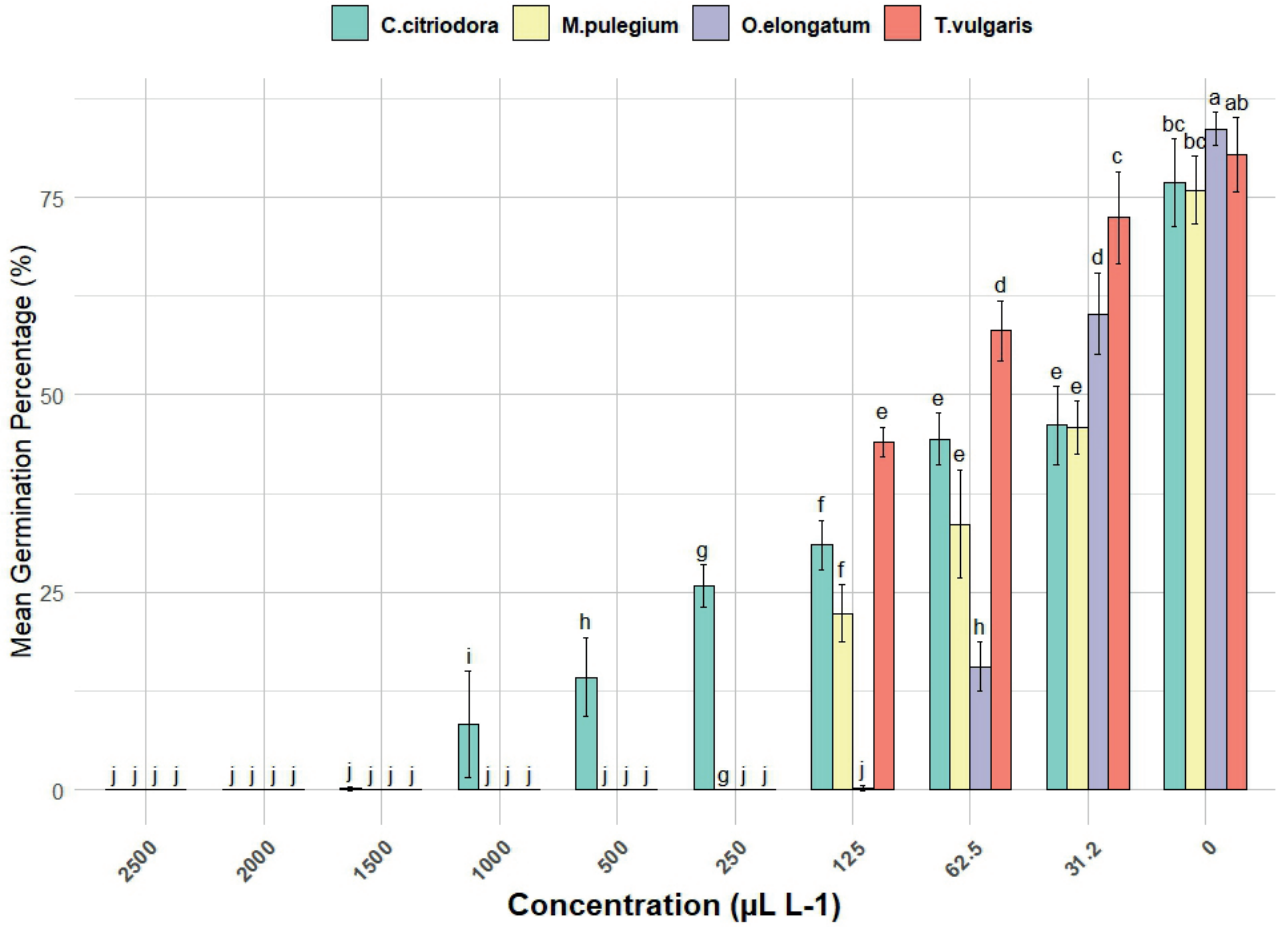

Figure 4. Effect of different concentrations of essential oils on conidial germination in contact phases. Letters (a, b, c, d, e, f, g, h, i, j) indicate homogeneous groups of means based on the high significant difference (HSD) test for each variable ($P < 0.05$). Arcsine transformation was performed prior to statistical analyses.

These results also showed that the EO of *C. citriodora* did not prevent, or only slightly reduced, *B. cinerea* conidium germination, even at high concentrations (1500 µL L^{-1}). In contrast, the oregano EO was the most effective against the fungus, even at low concentrations. For all control treatments, which contained only 1% Tween 80, conidium germination exceeded 75%.

In vivo *Antifungal assays*

Protection assays on detached grapevine leaves

Disease development was also monitored *in vivo* using the EC_{50} and EC_{90} concentrations of each EO that was determined from the *in vitro* assessments. The test was conducted on 'Chardonnay' and 'Cabernet Sauvignon' grapevines, which are, respectively, highly susceptible and less susceptible to *Botrytis cinerea* (Jeandet et al., 1992).

Illustration of the *in vivo* fungicidal activity of different concentrations of the four EOs against *B. cinerea* is presented in Figure 5. The *B. cinerea* isolate used in this experiment causes disease in grape berries. In the direct contact treatment trial, all four EOs applied preventively or simultaneously showed strong antifungal activity compared to untreated leaves, and significantly ($P < 0.05$) reduced lesion areas.

For all treatments, the effectiveness of the EOs was greatest for 'Cabernet Sauvignon', with mean lesion size reductions of 84.2% for *M. pulegium* essential oil, 72.4% for that from *T. vulgaris*, 68.9% for that from *C. citriodora*, and 68.6% for the EO from *O. elongatum* EOs, compared to their respective negative controls. For 'Chardonnay', the reductions in mean lesion sizes were 74.6% for the oil from *O. elongatum*, 72.7% for that from *M. pulegium*, 68.2% for the oil from *T. vulgaris*, and 62.6% for the EO from *C. citriodora*. Ranking of the EOs according to their impacts on leaf lesion sizes in the two grapevine varieties was as follows: The *M. pulegium* EO (78.5%) was the most effective, followed by that from *O. elongatum* (71.6%), from *T. vulgaris* (70.3%) and from *C. citriodora* (65.7%).

Protection assays on berry grapevine

The effects of EOs from *O. elongatum, M. pulegium, T. vulgaris* and *C. citriodora* on development of *Botrytis cinerea* in wound-inoculated grape berries of 'Muscat of Italy' are shown in Figure 6. All treatments reduced the incidence and development of gray mold at 22°C over a 6 d storage period. All the grape berries not treated with the EOs were almost completely infected by the fungus. The results showed that the four EOs inhibited fungal growth, but their effectiveness varied. The most effective EO was from *M. pulegium* causing a 57.7% reduction in mean lesion diameter, followed by the EO from *O. elongatum* (49.7% reduction), then *T. vulgaris* (40.1%) and *C. citriodora* with 31.7% reduction, compared with the negative experimental controls. The most effective treatment was the preventive application of Eucalyptus, pennyroyal and thymus EOs, which reduced average lesion diameters in grape berries. For the oregano EO, the treatment resulted in the smallest lesions. The EOs showed no phytotoxic effects on the grape fruit tissues at all studied concentrations.

DISCUSSION

Essential oils from plants and their chemical components have long been studied for their antifungal activities, particularly within sustainable environmental practices to enhance agricultural yields and food storage durability (Carrubba and Catalano, 2009; El-Mohamedy, 2017; Habbadi *et al*., 2018; Cheng *et al*., 2024; Nunes *et al*., 2024; Wike *et al*., 2024). Grapevine has also been the subject of research into alternative treatments using EOs to treat fungal infections, particularly gray mold, which is a major cause of fruit losses (Antonov *et al*., 1997; Walter *et al*., 2001; Tripathi *et al*., 2008; Burggraf and Rienth, 2020). Although numerous studies have reported inhibition of *B. cinerea* by EOs *in vitro*, none have assessed the *in vivo* protective effects of EOs on grape berries and vegetative organs of the grape varieties, 'Cabernet Sauvignon' and 'Chardonnay', which have, respectively, intermediate resistance and susceptibility to *B. cinerea* (Jeandet *et al*., 1992). The present study investigated the chemical composition and *in vitro* antifungal activity of EOs from *O. elongatum*, *M. pulegium*, *T. vulgaris* and *C. citriodora* in direct contact and vapour contact *B. cinerea* mycelium growth and spore germination assays. The study also evaluated effects of these EOs on detached grapevine leaves of two grapevine varieties and on grape berries *in vivo*.

The main constituents of the four EOs found in the present study (Table 2) corroborate those reported in the literature. Lemon eucalyptus EO was contained cineole and α-pinene (Low *et al*., 1974; Ramezani *et al*., 2002), while carvacrol was prominent in *O. elongatum* EO, and thymol in *T. vulgaris* EO. These results are comparable with previous research (Sivropoulou *et al*., 1996; Dorman *et al*., 2000; Aligiannis *et al*., 2001; Burt, 2004). However, other studies have shown that the chemical composition of thyme and oregano EOs can vary greatly depending on the plant chemotype assessed, and that the antifungal activity of the EO can vary significantly (Daferera *et al*., 2003; Della Pepa *et al*., 2019; Drioiche *et al*., 2022). Pennyroyal is associated with pulegone (see figure S2). This high concentration of pulegone is supported by numerous studies, which also report pulegone as the main constituent (Kokkini *et al*., 2002; Stoyanova *et al*., 2005), while other studies have reported lower pulegone levels but high amounts of menthone/isomen-

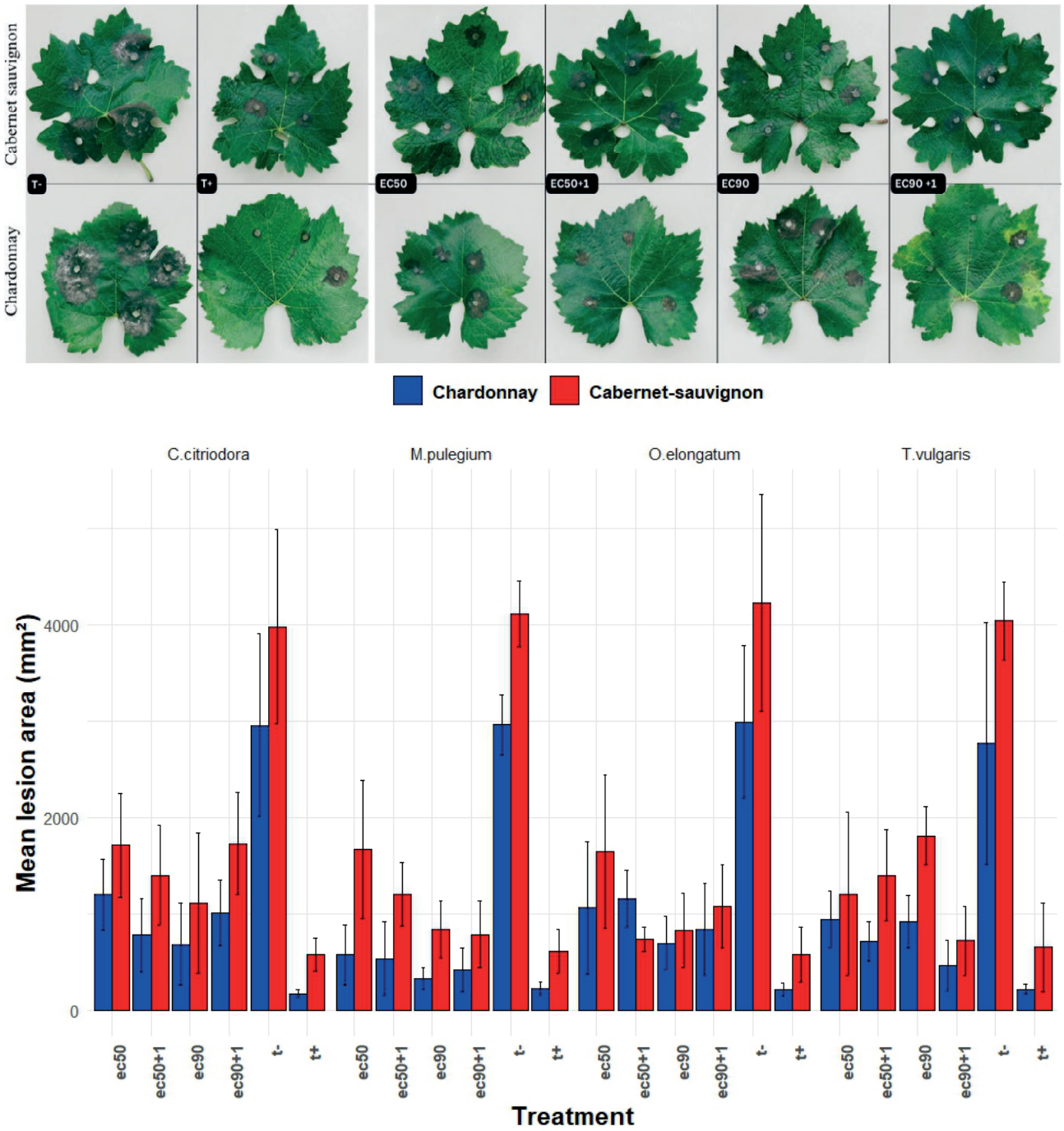

Figure 5. Top: Representative grapevine leaves that had been inoculated with *Botrytis cinerea* and treated with two concentrations (EC_{50} or EC_{90}) of essential oils from four different plants, in preventive and simultaneous applications, with fungicide fenhexamid as the positive experimental control. Bottom: Mean lesion surface areas caused by *B. cinerea* on two grapevine varieties treated with the essential oils *in vivo* by direct contact. All the data are expressed as means ± SD.

thone, piperitenone/piperitone, or piperitone (Lorenzo *et al.*, 2002; El-Ghorab, 2006; Ait-Ouazzou *et al.*, 2012; Abdelli *et al.*, 2016; Brahmi *et al.*, 2016). Variabilities in chemical composition could be related to effects of geographical location, genetic factors, collection period, and extraction techniques (Mechergui *et al.*, 2016; Elansary *et al.*, 2018). Data from the present study indicate qualitative and quantitative variations in the EO com-

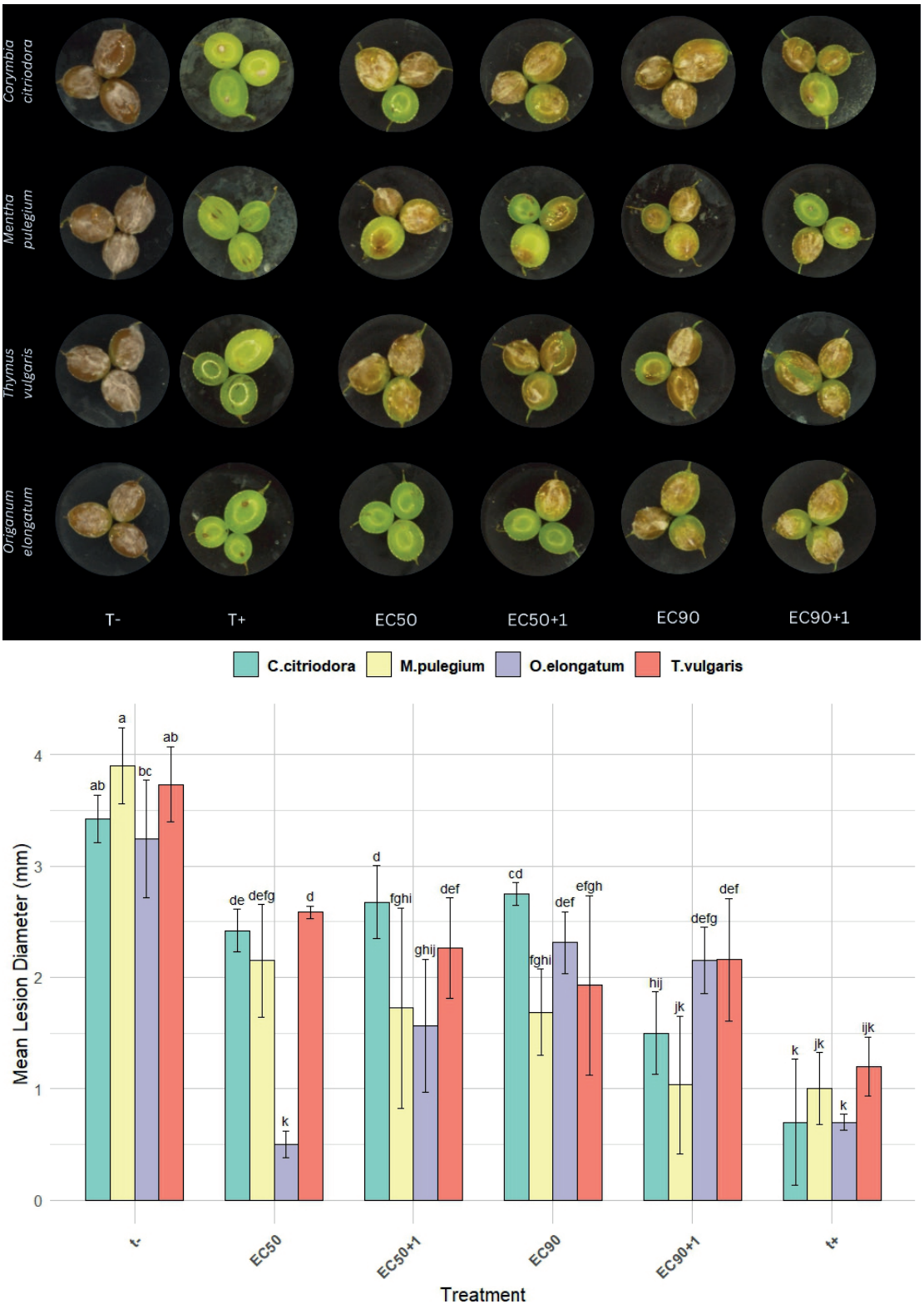

Figure 6. Top: Visual differences in *Botrytis cinerea* mycelium growth and fruit desiccation of detached 'Muscat of Italy' berries after 6 d of treatment. These images were captured at the end of the *in vivo* trial. Bottom: Mean *in vivo* antifungal activities of four essential oils against *B. cinerea* on detached grapes berries. Letters accompanying the means indicate homogeneous groups based on the Tukey HSD test for each variable at ($P < 0.05$).

positions of the four investigated plant species. However, GC-MS analyses have shown that terpenoids and terpenes predominate in all the four oils examined.

The present study presents *in vitro* inhibition proportions of *B. cinerea* through direct contact of the pathogen with different EOs at various concentrations. The MIC at which no fungal growth was observed for the oregano oil was is 252.5 µL L^{-1}, greater than 125 µL L^{-1} reported by Zhao *et al.* (2021), and that obtained by Yilmaz *et al.* (2016), but less than the 0.5 mg mL^{-1} obtained by Hou *et al.* (2020) for *O. vulgare* EO. For thyme EO, the MIC was also 252.5 µL L^{-1}, which is less than the 800 µL L^{-1} obtained by Abdolahi *et al.* (2010) , the 400 µL L^{-1} obtained by Fathi *et al.* (2012), but similar to the 200 µg mL^{-1} reported by Daferera *et al.* (2003). The MIC for pennyroyal is 1010 µL L^{-1}, which is less than the 2660 µL L^{-1} obtained by Aouadi *et al.* (2022), but greater than the 250 ppm obtained by Singh and Pandey (2018). Similarly, the MIC for lemon Eucalyptus oil was 1010 µL L^{-1}, which is greater than the MIC reported by Tripathi *et al.* (2008).

The present study results show that oregano and thyme EOs were effective against *B. cinerea*, even at very low concentrations. This efficacy may be due to the presence of thymol and carvacrol (see figure S2) as major components in these two EOs. This observation is supported by the results of Zhang *et al.* (2019), who showed that thymol and carvacrol had antifungal activity against *B. cinerea* at low concentrations compared to crude EO (Hou *et al*., 2020), inducing clear morphological changes in fungus hyphae. Zhao *et al.* (2021) also obtained similar results, where thymol and carvacrol at 125 mg L^{-1} completely suppressed gray mold. In addition, p-cymene, present as a major component in the two EOs, completely inhibited *B. cinerea* growth at 80.0 µg L^{-1}, as observed in the study by Pinto *et al.* (2020). Efficacy of Eucalyptus and pennyroyal EOs requires high concentrations to maintain significant *in vitro* inhibition of *B. cinerea*.

In the vapour contact assays, oregano EO gave an MIC of 44.9 µL L^{-1}, differing from the value of 31.25 mg L^{-1} obtained by Zhao *et al.* (2021). For thyme EO, the MIC was 89.88 µL L^{-1}, greater than the 45.45 µL L^{-1} reported by Álvarez-García *et al.* (2023). These results confirm those obtained through the direct contact method, demonstrating that, of the four oils studied, that from oregano EO was the most inhibitory of *B. cinerea*, followed by the EO from thyme. This efficacy is likely to be due to thymol and carvacrol, which induce morphological changes by disrupting the *B. cinerea* mycelium and hyphal structure (Abbaszadeh *et al*., 2014; Elshafie *et al*., 2015), whereas the oils from pennyroyal and eucalyptus had reduced efficacy at low concentrations. However, the inhibitory effects of the oils in the vapor contact assays were stronger. To explain this toxicity difference, the pathogen may have been more exposed to the volatile substances released in the closed environment of the VC assay, whereas in a DC assay, the mycelia are only exposed to a small portion of a substance on the surface of the agar in assay plates. Consequently, inhibitory effects on the mycelium growth of *B. cinerea* in the VC assays was greater than in the DC assays, as was confirmed by Soylu *et al.* (2010).

Inhibition of conidium germination is crucial for evaluating the efficacy of EOs, as conidium germination is the starting point for fungal infection (Agrios, 2008). Additionally, germination leads to eventual multiplication and dissemination of pathogenic fungi. Previous studies have shown that *T. vulgaris* oil suppresses the germination of *B. cinerea* at 1000 µL L^{-1} (Fincheira *et al*., 2023), a concentration greater than the 250 µL L^{-1} obtained in the present study with the same EO. For the oregano oil, the values obtained were 250 µL L^{-1}, which is less than the 3.2 µg mL^{-1} reported by Soylu *et al.* (2010) for oregano oil. For the inhibitory effect of Eucalyptus EO on conidium germination, Aguiar *et al.* (2014) found that at 150 mg L^{-1}, spore germination of three assessed fungi was completely inhibited. Inhibition of fungal spore germination strongly depends on the applied EO and its concentration (Fincheira *et al*., 2023). Therefore, it is likely that suppression of spore germination by EO treatments could contribute to limiting the spread of *B. cinerea* by reducing the conidium load.

Grapevine leaves were chosen in the present study for the *in vivo* test because young leaves emerging at bud burst are the primary sources of *B. cinerea* inoculum (Balasubramaniam, 1997). The objective of the test was to evaluate whether the potential of EOs depends on the grape variety tested and the mode of application (simultaneous or preventive). The experiment showed that 'Cabernet Sauvignon' was better protected by the direct application of EOs, regardless of the treatment, compared to 'Chardonnay'.

The treatments applied during the tests did not inhibit fungal growth in a dose-dependent manner. For treatments with the EC_{50} doses, the preventive treatments (EC_{50}+1) were more effective than the concomitant treatments. However, for the EC_{90} doses, the concomitant treatments were more effective. Although all EOs were effective in reducing fungal infections, the *M. pulegium* EO was the most effective in controlling infections caused by *B. cinerea* on detached leaves of both grapevine varieties. Aouadi *et al.* (2022) reported in a subsequent study that, during *in vivo* tests, *M. pulegium* EO completely suppressed, by direct contact, gray mold

on strawberries previously inoculated with *B. cinerea* conidia. Oumzil *et al.* (2002) demonstrated that pulegone inhibited all tested microorganisms and was the most effective against three assessed fungi. De Sousa Barros *et al.* (2015) studied the functional properties of EOs from different *Mentha* species, demonstrating that oils rich in pulegone exhibited significant antifungal activity against *Microsporum canis* and *Trichophyton rubrum*. Pulegone, a monoterpene ketone, is the principal component of *Mentha pulegium* EO. This compound has an important role in the antimicrobial activity of the EO (Areco *et al.*, 2024). The second *in vivo* experiment of the present study was carried out on detached grape berries, to determine effectiveness of direct contact treatments. All the applied treatments resulted in reductions in *B. cinerea* lesion diameter. Inhibition of fungal growth on the berries varied according to the different EOs and treatments. These results are consistent with those from other studies conducted on harvested fruits and vegetables from growing plants. Soylu *et al.*, (2010) demonstrated the ability of EOs from oregano, lavender and rosemary, applied in contact and vapour forms, to reduce disease on tomatoes infected by *B. cinerea*.

The present study did not assess *in vivo* fumigation effectiveness of EOs against *B. cinerea* on leaves and grapes berries. Further research is therefore required to investigate the vapour contact effects against *B. cinerea* of EOs on grape berries. It would be particularly interesting to conduct *in vivo* fumigation tests to avoid direct contact of the EOs with food commodities, therefore maintaining the organoleptic features of these oils and avoiding the alterations that occur during soaking treatments. Increasing resistance of pathogens to fungicides, and awareness of the dangers associated with the intensive use of pesticides indicate that EOs from oregano, thyme, pennyroyal and lemon Eucalyptus have potential to be effective and sustainable alternatives to conventional phytosanitary products. The present study confirms the antifungal activity of the EOs tested, which at low doses effectively protected grapevine organs against gray mold under controlled conditions. These results pave the way for more extensive studies to evaluate their long-term effectiveness of these oils under field conditions.

CONCLUSIONS

In this study, the essential oils of *Origanum elongatum*, *Mentha pulegium*, *Thymus vulgaris*, and *Corymbia citriodora* exhibited strong antifungal activity against *Botrytis cinerea*, a phytopathogenic fungus of major importance. *In vitro* and *in vivo* tests both confirmed effectiveness of these oils, suggesting that they could serve as viable alternatives to synthetic chemical fungicides for disease management. However, further innovation is needed to develop practical application techniques for the use of essential oils in agriculture and food production industries.

ACKNOWLEDGMENTS

This research was funded by PRIMA-MiDiVine project 1564 coordinated by Aziz Aziz, 2021/2025 (https://www.univ-reims.fr/MiDiVine/, accessed on 1 January 2025) (Innovative Approaches Promoting Functional Microbial Diversity for Sustainable Grapevine Health and Productivity in Vineyard Systems of Mediterranean Areas), supported by the European Union, with co-funding by MESRSI (Morocco).

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