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Origanum vulgare essential oil vapour impedes *Botrytis cinerea* development on grapevine (*Vitis vinifera*) fruit

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Summary. *Botrytis cinerea* infections of *Vitis* spp. fruits cause major economic losses, and grape producers rely on synthetic and copper-based fungicides for control of this pathogen. These pesticides present risks for human health and the environment. Implementation of low-impact disease management solutions is important for improving sustainability of viticulture industries. This study investigated the effects of *Origanum vulgare* (oregano) essential oil (EO) as an antifungal agent. *In vitro* and *in vivo* experiments with *B. cinerea* were carried out using a vaporization system to circumvent drawbacks of direct EO application. *In vitro* experiments confirmed the effectiveness of EO vapour treatments, which gave 100% inhibition of *B. cinerea* growth. Treatment of *V. vinifera* cv. Chasselas berries resulted in a 73% reduction in fungal growth, confirming the efficacy of the oregano EO vapour for control of grey mould caused by *B. cinerea*. This study has demonstrated the efficacy of EOs in the vapour phase on grape berries, which provides new possibilities for development of in-field or greenhouse vaporization systems that can reduce the use of synthetic and copper-based fungicides.

Keywords. Postharvest disease, grape grey mould, volatile organic compounds, sustainable viticulture, biopesticides, essential oils.

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INTRODUCTION

Fungicides represent 49% of the pesticides utilized in the European Union (European Commission Eurostat, 2007). The viticulture industry contributes to this high fungicide use via efforts to control vineyard diseases caused by fungi, including *Botrytis cinerea, Plasmopara viticola* and *Erysiphe necator*. This is a growing problem, due to the possible negative impacts on consumer and producer health and on the surrounding ecosystems and soils (Komárek *et al*., 2010; Aminifard and Mohammadi, 2012). *Botrytis cinerea* is an important fungal pathogen in viticulture and in other crops. This pathogen causes grey mould (Naegele, 2018) and Botrytis bunch rot. *Botrytis cinerea* infections cause major economic losses (Elmer and Michailides, 2007) due to reductions in grape yields and quality (Jacometti *et al*., 2010; Paňitrur-De La Fuente *et al*., 2018). Due to the high frequency of usage and possible detrimental health

and environmental impacts of pesticides, alternative disease management strategies are required to reduce pesticide use for sustainable agriculture.

Alternatives to pesticides include measures to increase plant and fruit resistance to *B. cinerea* and other fungal diseases, and cultural practices to encourage the maintenance of unfavourable habitats for pathogen development. Cultural practices in viticulture include maintenance of good vine canopy structure, the growing of cover crops and mulching to reduce excessive vine vigour. However, these practices can be challenging and only marginally effective. Other alternatives include the use of biological control agents and plant extracts, all of which have shown some efficacy for control of *B. cinerea*, and are typically applied in conjunction with other control methods (Jacometti *et al.*, 2010). Interspecific crossbreeding of naturally disease-resistant varieties is also a very cost-effective, environmentally-friendly solution for the control of fungal diseases. However, challenges regarding the marketing of disease-resistant varieties, such as labelling concerns and varietal reputation, are important when assessing the economic viability and future of new varieties (Fuller *et al.*, 2014). Recent research suggests that essential oils (EOs), with antifungal capacities and low environmental impacts, could be alternatives for control of fungal fruit pathogens. Development of EO treatments for fungal disease management could solve the environmental and human health issues caused by pesticide-based disease management.

EOs naturally present in plants can protect against infections by pathogenic microorganisms (Mohammadi *et al.*, 2013; Nazzaro *et al.*, 2017). These materials are generally recognized as safe (GRAS) by the United States of America Food and Drug Administration (FDA), and are widely accepted as alternatives to synthetic chemicals because of their natural origins (Nazzaro *et al.*, 2017).

The antifungal properties of EOs are primarily linked to terpenes (monoterpenes and sesquiterpenes), terpenoids (isoprenoids), alipathic and aromatic compounds such as aldehydes and phenols. Terpenes are naturally occurring hydrocarbons with various chemical and biological properties, and constitute up to 90% of most EO components (Sakkas and Papadopoulou, 2017).

The active antifungal properties of EOs are mainly attributed to disruption of cell wall formation and interference with phospholipid bilayers of cell membranes. Their high lipophilicity allows absorption by fungal mycelium (Soylu *et al.*, 2007). They also have low molecular weights, allowing cell death and inhibition of fungal sporulation and germination (Nazzaro *et al.*, 2017), or deformation of cell structure and functional disruption (Mohammadi *et al.*, 2013; Sakkas and Papadopoulou, 2017). Furthermore, the antifungal compounds in EOs have been shown to prevent fungal pectinases from hydrolyzation and invasion of host plant cells (Soylu *et al.*, 2010; Aminifard and Mohammadi, 2012).

EOs can also debilitate mitochondria of fungal pathogens (Nazzaro *et al.*, 2017). Effects of EOs on mitochondria and fungal plasma membranes cause inhibition of the synthesis of ergosterol and activities of mitochondrial ATPase, malate dehydrogenase, and succinate dehydrogenase (Hu *et al.*, 2017). Carvacrol, a monoterpene present in many EOs, including those from oregano and thyme, may also inhibit fungal growth by stimulating pathogen responses similar to calcium stress and inhibition of the target of rapamycin (TOR) pathway. This suggests that antifungal properties of carvacrol include activation of specific signalling pathways within fungi, causing debilitation (Rao *et al.*, 2010).

Numerous studies have confirmed the efficacy of EOs for inhibiting growth of fungal pathogens, including *B. cinerea, Rhizopus stolonifer, Fusarium* spp., *Clavibacter michiganensis, P. viticola*, and *Sclerotinia sclerotiorum*. Mohammadi *et al.* (2013) found through gas chromatography-mass spectrometry (GC/MS) analyses of black caraway, fennel, peppermint and thyme oils that they were mainly composed of monoterpenes and terpenes. They confirmed that all four EOs inhibited *B. cinerea* and *R. stolonifer* under *in vitro* conditions, with black caraway and fennel oils showing the greatest efficacy for reducing growth of the fungi. In a study of the efficacy of EOs for inhibition of *in vitro* and *in vivo* growth of *B. cinerea*, Aminifard and Mohammadi (2012) found that the EO of black caraway and fennel completely inhibited the growth of the fungus at, respectively, 400 and 600 µL L^{-1}. Daferera *et al.* (2003) demonstrated, under *in vivo* conditions, that black caraway, fennel, and peppermint oils inhibited the growth of *B. cinerea*. EOs of oregano, thyme, dictamnus and marjoram completely inhibited the growth of *B. cinerea, Fusarium* sp. and *C. michiganesis* at 85 to 300 µg mL^{-1}, and the main antifungal components in the EOs were identified as thymol and carvacrol.

The antifungal effects of the EOs of oregano and fennel were studied for inhibitory effects on *S. sclerotiorum* by Soylu *et al.* (2007). Similar to *B. cinerea, S. sclerotiorum* produces overwintering sclerotia, which make the fungus particularly difficult to control. Their results showed that both EOs in volatile and liquid phases inhibited fungal growth, reducing mycelium growth and germination of sclerotia. Soylu *et al.* (2010) evaluated the effectiveness of oregano, lavender, and rosemary (Lamiaceae), and showed that the EO of oregano was the most effective at low concentrations in vapour (0.2 µg mL^{-1} air) and contact (12.8 µg mL^{-1}) phases under *in vitro* and *in vivo* conditions for

inhibiting the growth of *B. cinerea*. Oregano oil was also shown to possess strong antifungal properties by Vitoratos *et al.* (2013), who observed complete inhibition of fungal growth by oregano EO at 0.30 μL mL^{-1}. Lemon oil was also shown to reduce growth of *B. cinerea*. *Salvia officinalis* (sage) EO was studied for efficacy in control of *P. viticola* by Dagostin *et al.* (2010). The EO had inhibition potential similar to copper hydroxide, showing that sage extract was another promising alternative to copper fungicides in viticulture. However, the rainfastness of the EO was very low, indicating potential complications associated with its practical efficacy (Daferera *et al.*, 2003; Soylu *et al.*, 2010; Mohammadi *et al.*, 2013).

Several studies have shown that application of EOs can be very effective in vapour phase at lower concentrations than in liquid phase (Edris and Farrag, 2003; Soylu *et al.*, 2010; Babalık *et al.*, 2020). In addition to being more effective at lower doses than contact applications, vapour treatments could be efficient due to lack of direct contact between EOs and host plants, reducing phytotoxicity and circumventing the drawbacks of direct applications (low rainfastness, UV degradation, poor mixability with water and/or other products) (Edris and Farrag, 2003). Drawing upon results from previous studies, the present research aimed to investigate whether continuous fumigation with EO vapour from *Origanum vulgare* could reduce *B. cinerea* development *in vitro* and *in vivo* on grape berries, thereby circumventing the drawbacks of direct applications.

MATERIALS AND METHODS

Isolation and culture of Botrytis cinerea

Botrytis cinerea conidia used for *in vivo* and *in vitro* tests were harvested from decaying strawberries. Conidia were removed from berries with forceps and plated on potato dextrose agar (PDA) in Petri dishes, using a sterile inoculation loop. The Petri dishes were incubated at 22.5°C under continuous light for 14 d to encourage conidium production. Actively growing *B. cinerea* cultures on PDA were maintained through conidium transfer onto freshly prepared Petri dishes every 7–14 d throughout experimentation.

After successful growth of *B. cinerea* on PDA, conidia were collected from cultures and suspended in Ringer's solution. The solution was added to the dishes and the culture surfaces were then rubbed with an inoculation loop to dislodge conidia. Solutions and spores were removed from the Petri dishes and passed through 100 mm diam. filter paper (Schleicher & Schuell; pore size 11 μm) to remove agar pieces and mycelia. The suspensions were then vortexed before quantification using a vortex mixer (Bender & Hobein Vortex Genie 2, 1410). Conidia concentration was calculated using a microscope (Carl Zeiss, Lab A1 AXIO) with an A-Plan 10x/0.25 Ph1 lens, and a 0.100 mm depth 0.0025 mm^2 Thoma counting chamber. The conidial suspension concentrations were adjusted to the range of 5×10^5 to 1×10^6 conidia mL^{-1} recommended by Guetsky *et al.* (2001) throughout the experiment.

In vitro *fumigation with oregano essential oil*

Petri dishes were assigned to treatment and control groups and were placed inside a climate chamber (Percival Scientific; Intellus Ultra C8), which was customized to allow simultaneous treatment and control fumigation to be carried out inside airtight plexiglass chambers (dimensions: 67 × 67 × 108 cm). The customized two-chamber pump mechanism included two plastic boxes, one treatment box containing EO and one empty box assigned to the control group. EO vapour and air (control) treatments were transferred by a pump system to the climate chamber containing the petri dishes. These were left inside the chamber with the lids off, facing upward during treatment. Mycelium growth in the dishes was quantified until fungus colonies in a majority of the control dishes had reached full capacity. The increases in the diameter of *B. cinerea* colonies in the Petri dishes were determined by tracing the radial growth on the underside of each dish. The proportion of mycelium growth inhibition was calculated using the following equation:

$$\left(\frac{DC-DT}{DC}\right) \times 100$$

where DC = the average colony diameter on the control dishes, and DT = the average colony diameter on the treatment dishes (Badawy and Abdelgaleil, 2014).

Three different experiments were carried out. A diurnal climate chamber programme was run during each experiment, with a daily regime of 12 h light, 20°C, 70% relative humidity (RH) / 12 h dark, 15°C, 50% RH. Each Petri dish was inoculated at the centre with 10 μL of *B. cinerea* conidial suspension, which was allowed to dry before measuring the initial colony diameter. EO of oregano (Origan Vert, Companie des Sens, France), was placed in a glass dish inside the treatment chamber of the customized fumigation system.

Specifications for the individual experiments are shown in Table 1. EO composition was determined by GC with flame ionization detection (GC-FID; Agilent GC system 7890B/7010 Agilent GC-TQ).

Table 1. Specifications for three *in vitro* experiments (T = Treatment, C = Control).

Experiment	Sample size	Conidial solution concentration (conidia mL^{-1})	Dosage and parameters	Duration
Trial I	11 (T) 11 (C)	9.11×10^5	4 mL EO inside pump chamber	4 d
Trial II	10 (T) 10 (C)	7.49×10^5	5 mL EO inside pump chamber	3 d
Trial III	10 (T) 10 (C)	7.71×10^5	5 mL EO inside pump chamber 5 mL EO inside climate chamber	3 d

In vivo *treatment of grape berries with oregano essential oil*

Four trials were carried out with detached *Vitis vinifera* cv. Chasselas berries. The different parameters of the experiments are summarized in Table 2. Ripe grape berries were collected from the vineyards at Changins, Haute Ecole de Viticulture et Oenologie (Nyon, Switzerland). Total soluble solids (TSS, expressed in °Brix) were measured with a Wine Line HI96811 0-50°Brix wine refractometer. Thirty healthy berries were each punctured once using a 0.5 mm diam. sterile pin with a puncture depth of 3 mm. All berries were inoculated with *B. cinerea* by individual submersion for 5 s in a conidial suspension prepared as described above.

The grape berries were placed into 90 mm diam. sterile Petri dishes, which were placed inside the climate chamber for treatment. A diurnal climate chamber programme was run during each experiment, with a daily regime of 12 h light, 25°C, 90% RH / 12 h dark, 17°C, 50% RH. Five milliliters of EO were placed in a glass dish on top of a heating pad inside the treatment chamber of the fumigation system. The air pump was turned on at maximum air flow intensity, transferring air into the treatment and control chambers.

Quantification of grey mould severity on cv. Chasselas berries

At the end of each treatment, three replicates each of five berries from the treatment and control groups were placed into 50 mL capacity sterile centrifuge tubes each containing 25 mL of reverse osmosis water. Each centrifuge tube was agitated for 2 min with a vortex (Bender & Hobein Vortex Genie 2) to dislodge conidia from berries. After vortexing, the berries were removed from the solution by filtering through a 100 mm diam. filter paper (Schleicher & Schuell). Conidia concentrations in the solutions were quantified with a microscope with an A-Plan ×10/0.25 Ph1 lens (Carl Zeiss Lab A1 AXIO), using a 0.100 mm depth 0.0025 mm^2 Thoma counting chamber.

Table 2. Specifications for four fumigation treatment trials applied to detached cv. Chasselas grape berries.

Experiment	Berry collection site	°Brix	Spore suspension concentration (conidia mL^{-1})	Duration
Trial I	Changins Vineyard	20.7	9.98×10^5	10 d
Trial II	Changins Vineyard	17.4	1.96×10^6	7 d
Trial III	Supermarket	25.5	1.05×10^6	7 d
Trial IV	Changins Vineyard	17.6	1.02×10^6	7 d

Quantification and identification of the active compounds present during treatment

Analysis was carried out using the saturation point of activated charcoal (Supelco Activated Coconut Charcoal; 20-40 mesh) to capture oil vapours for analysis of vapour concentrations during treatment. This was used to determine the appropriate quantity to be used in subsequent experiments. Tissue bags filled with 500 mg, 1 g, or 1.5 g of the charcoal were hung in the treatment and control chambers at the beginning of treatment. At the end of the 7-d treatment, samples were analyzed with GC-FID on a gas chromatograph (Agilent 7890B) with an autosampler (Agilent 7693). The charcoal samples in which the volatile compounds were trapped were eluted by dichloromethane. Two milliliters of dichloromethane was used for extraction of the 500 mg and 1 g samples. Four milliliters of dichloromethane were used for extraction of the 1.5 g sample. Additionally, an internal standard of 10 µL of 1,6-heptendiol at 50 mg L^{-1} was added during sample preparation. Samples were incubated and mixed for 1 h at room temperature. The supernatant from each sample was then collected and transferred into a sterile vial for analysis. The sample was injected directly for component determination by GC using hydrogen as the carrier gas at a constant flow of 4 mL min^{-1}. Separation of the compounds was performed using a capillary column (60 m, 0.25 mm ID, 1.4 µm; Rtx®-1301) and detection was carried out by flame ionization. P-cymene, thymol, and carvacrol were identified, quantified and expressed in mg L^{-1}. The limit of quantification (LOQ) for all three molecules was set at 3.50 mg L^{-1}, and the limit of detection

Table 3. Saturation point analysis of activated charcoal.

Sample	Charcoal control 0.5 g	Charcoal control 1.0 g	Charcoal control 1.5 g	Charcoal treatment 0.5 g	Charcoal treatment 1.0 g	Charcoal treatment 1.5 g
P-cymene (mg L^{-1})	31.513	48.059	60.22	471.85	834.55	816.73
Thymol (mg L^{-1})	3.825	4.274	13.49	32.27	34.62	38.21
Carvacrol (mg L^{-1})	5.358	4.821	8.60	665.26	770.89	1012.94
Extraction measurement	2 mL	2 mL	4 mL	2 mL	2 mL	4 mL

Table 4. Dose and treatment parameters for individual trials with corresponding conidial suspension solution.

Parameter	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
EO dose parameters	CONTROL No EO Treatment	2 mL EO in pump chamber No heat application	2 mL EO in pump chamber Heat application	3 mL EO in pump chamber Heat application	3 mL EO in pump chamber 3 mL EO in treatment chamber Heat application
Conidium suspension concentration	9.5×10^5	9.33×10^5	9.33×10^5	9.75×10^5	9.75×10^5

(LOD) for all three molecules was 2.5 mg L^{-1}. Five hundred milligrams was shown to be the saturation point during the treatment period. Saturation was not reached in the 1 g samples; therefore, 1 g was determined to be the appropriate quantity to be used for vapour quantification in subsequent experiments (Table 3).

Analysis of essential oil residual surface contamination on berry skins and in berry flesh

Evaluation of p-cymene, thymol, and carvacrol present on the surfaces of berry skins and in berry flesh after treatment with EO of oregano was carried out twice during each fumigation experiment. Nine berries were assigned to each treatment group and nine berries were assigned to each control group. Healthy and intact berries were placed on standard 90 mm diam. sterile Petri dishes inside of the treatment and control fumigation chambers. After 7 d of treatment, the berries were placed into 50 mL capacity sterile centrifuge tubes, each containing 25 mL of reverse osmosis water, and these were each vortexed for 1 min. The berries remained in each solution until GC-FID analysis and the solution was prepared for GC-FID analysis following the procedure outlined above using 2 mL of dichloromethane for extraction. For berry flesh analyses, a second experiment was carried out, and at the end of 7 d of treatment, berries were rinsed under lukewarm water for 2 min to remove surface residues. The berries were left to dry and then placed inside 50 mL capacity sterile centrifuge tubes for storage until quantification. GC-FID was carried out on the berries by pressing the whole berries to extract juice from the flesh, which was then prepared as described above for GC-FID using 2 mL of dichloromethane for each extraction.

Conidium suspension preparation and essential oil dose parameters for dose-dependent in vitro *treatments*

Conidial suspensions were prepared as described above. One suspension was individually prepared for the control trial I harvesting conidia from actively growing *B. cinerea* colonies. One suspension was prepared for experimental treatment trials 2 and 3, which were carried out simultaneously. One suspension was prepared for experiments 4 and 5, which were also carried out simultaneously. Conidial suspension concentrations had little variation during the three phases of experimentation. The five trials were carried out using different concentrations, placements, and modes of diffusion of oregano oil (Table 4). The conidial suspension concentrations and the corresponding EO dosage parameters are outlined in Table 4.

During each treatment, eight Petri dishes were placed in the climate chamber and each was inoculated with 10 µL of conidial suspension. Fumigation parameters were the same for all five trials. A diurnal climate chamber programme was run during each experiment, with a daily regime of 12 h light, 25°C, 90% RH / 12 h dark, 17°C, 50% RH. Treatments were carried out over 3 d.

Statistical analysis and data presentation

Statistical analyses (Welch's two sample t-test and two-way ANOVA) were carried out using R Studio. Graphical presentation of data was performed with MS Excel and OriginPro.

RESULTS

Chemical composition of essential oils

Chemical composition as determined by GC-FID showed that the oregano EO used in this study contained 68.60% carvacrol, 11.27% p-cymene, 5.91% γ-terpinene, and 1.93% thymol (Table 5). This composition is similar to that from other studies, which have reported 58.1% carvacrol and 11.4% p-cymene as the composition of *O. vulgare* EO (Bouchra *et al.*, 2003; Daferera *et al.*, 2003; Teixeira *et al.*, 2013; Rienth *et al.*, 2019). Variation in active compounds and the presence of additional components can be attributed to source plant genotype and nutritional status, and to environmental conditions and geographical location (Badawy and Abdelgaleil, 2014).

Table 5. Composition of the oregano essential oil determined by GC-FID.

Retention time (min)	Composition	Percent probability	Percent measured	Theoretical percentage
18.43	Carvacrol	93.2	68.60	21–63%
7.59	Paracymène	96.6	11.27	6–20%
8.72	Gamma-terpinene	94.8	5.91	9-26%
23.11	Caryophyllène	96.6	2.46	
18.10	Thymol	95.3	1.93	3–28%
7.34	Alpha-terpinene	85.4	1.44	
6.58	Beta-myrcene	90.4	0.94	
5.11	Alpha-pinene	93.5	0.90	
10.28	Linalool	82.5	0.61	≤2%
7.73	D-limonene	80.8	0.58	≤1%
4.94	Alpha-thujene	89.4	0.40	
5.47	Camphene	86.3	0.21	

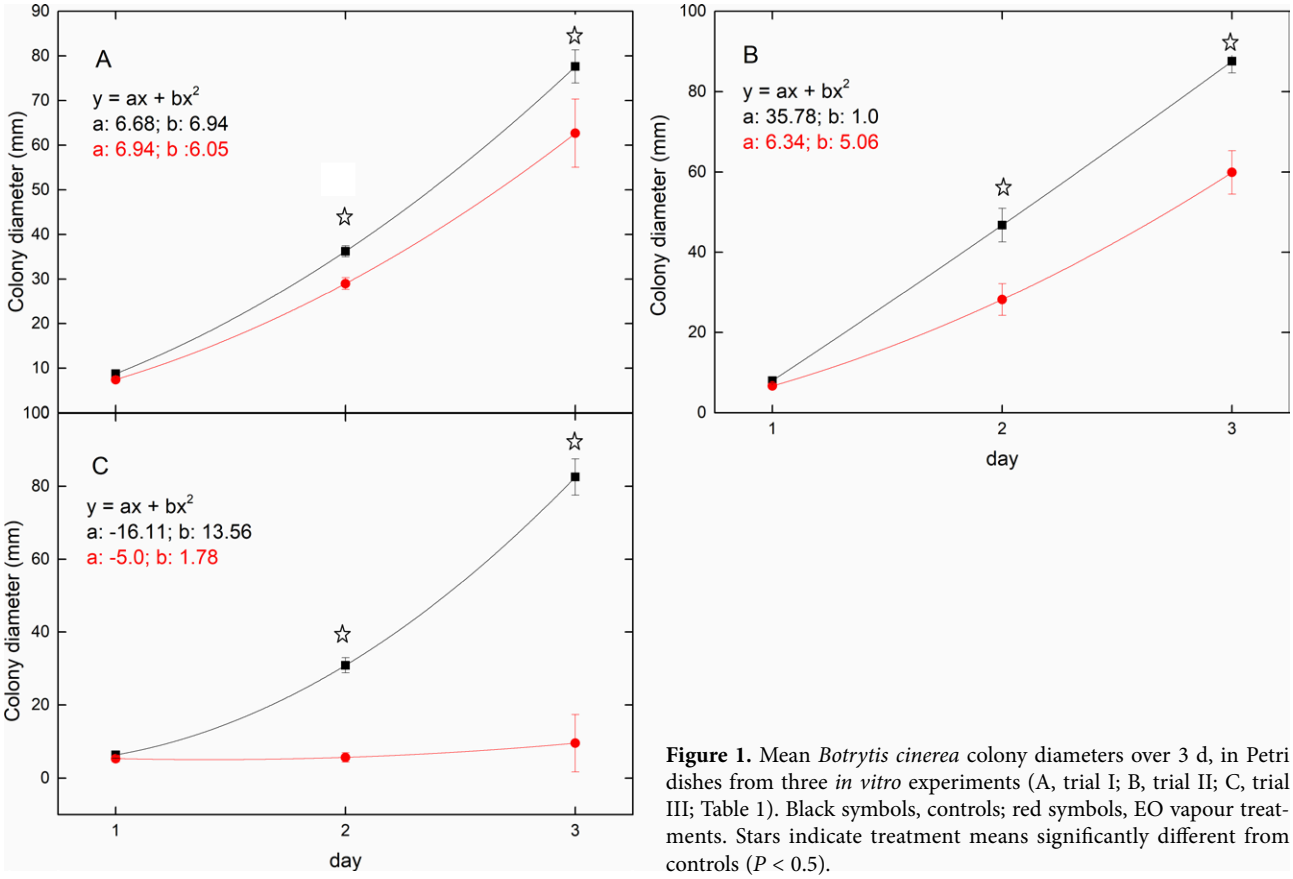

Figure 1. Mean *Botrytis cinerea* colony diameters over 3 d, in Petri dishes from three *in vitro* experiments (A, trial I; B, trial II; C, trial III; Table 1). Black symbols, controls; red symbols, EO vapour treatments. Stars indicate treatment means significantly different from controls ($P < 0.5$).

Effects of in vitro *fumigation with essential oil on* Botrytis cinerea

The average colony radial growth difference between *B. cinerea*-inoculated Petri plates dishes treated with EO vapour and control samples is illustrated in Figure 1. Treatment with EO vapour inhibited the growth of *B. cinerea* in all three experiments.

Effects of essential oil vapour on Botrytis cinerea *growth on grape berries*

Treatment with oregano oil vapour on detached cv. Chasselas berries reduced *B. cinerea* infections in all four trials, with the results from trials II and IV showing the greatest proportional inhibition, as indicated by the conidial suspension concentrations from the control and treatments (Figure 2).

An example of cv. Chasselas berries before and after treatment for the control and essential oil treatment samples is shown in Figure 3. EO vapour-treated berries developed browning due to the puncture wounds made at inoculation, but low levels of *B. cinerea* conidia were present on the berries. Control berries browned significantly after the 7 d treatments and showed significant amounts of mycelial growth and decay of berry flesh.

GC-FID analyses of fumigation residues in grape berries

GC-FID analyses of berries that underwent EO treatments identified the oil components p-cymene, thymol, and carvacrol (Table 6 and Table 7). Thymol and carvacrol were shown to be absorbed by berries during treatments, with greatest absorption of carvacrol in the treated samples. For the berry surface residues, p-cymene was the least persistent of the active compounds, which was less than the LOQ in trial III, compared to thymol and carvacrol. Thymol was present in the surface residue samples of both the treatment and control samples during both rounds of GC-FID analyses, demonstrat-

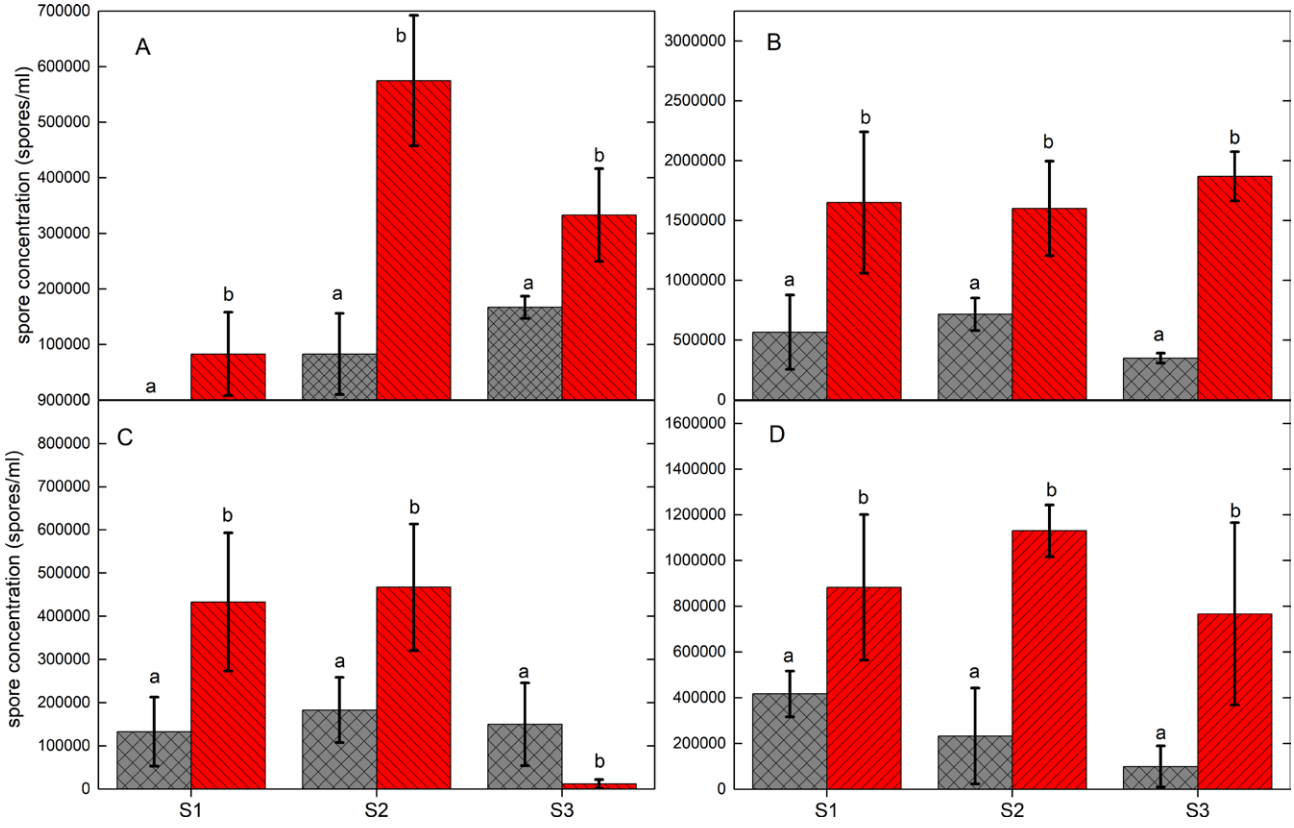

Figure 2. Mean numbers of *Botrytis cinerea* conidia from controls (gray histograms) and essential oil treatments (red histograms) for samples (S1, S2, S3) in four *in vivo* experiments (Table 2); trial I (A), trial II (B), trial III (C) and trial IV (D). Different letters for each experiment indicate significant differences between treatments and controls ($P < 0.5$), and bars indicate standard errors of the means.

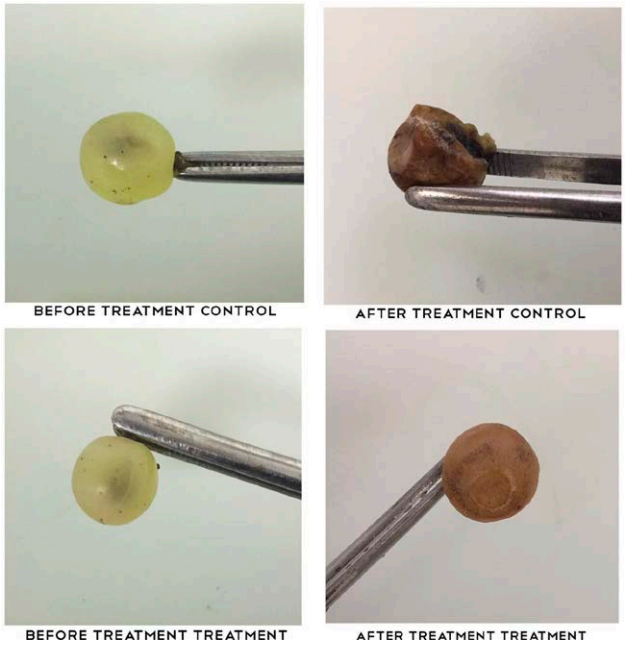

Figure 3. Visual differences in mycelium growth and fruit desiccation of detached cv. Chasselas berries for control berries (top), and treatment berries (bottom) after 7 d of treatment. These images were captured at the end of *in vivo* trial IV.

ing its strong persistence after treatment. Thymol was not quantifiable in the berry absorption analyses of the control group during the trial II GC-FID analysis, but was present in trial III, where carvacrol and p-cymene were not measurable. Carvacrol was not measurable in the surface residue samples for the control groups during both rounds of GC-FID analysis and was present at concentrations less than those of thymol during both rounds of analysis in the treatment samples. For berry absorption, carvacrol was at substantially different concentrations in the treatment group during trials II and III. For p-cymene, average berry absorption in the treatment sample was less than the LOQ and the measured surface residue concentration was 4.54 mg L^{-1}. For thymol, the average berry absorption for the treated berries was 7.57 mg L^{-1} and the surface residue concentration was 11.36 mg L^{-1}. For carvacrol, the average berry absorption level was 11.06 mg L^{-1} and the surface residue concentration was 6.81 mg L^{-1}.

Table 6. Mean inhibition (%) of *Botrytis cinerea* radial colony growth after treatment with essential oil vapour. In each trial, radial colony growth was reduced ($P < 0.05$) by treatment.

	Inhibition after 36 h	Inhibition after 48 h
Trial I	19.3%	20.2%
Trial II	31.8%	39.9%
Trial III	89.0%	81.7%

Effects essential oil dose on in vitro *growth inhibition of* Botrytis cinerea

Treatment parameters and dosages of EO vapour were modified during five *in vitro* trials to assess the impacts of different concentrations of the active components on growth inhibition of *B. cinerea*. The inhibition

Table 7. Essential oil component residues in grape berries and on berry surfaces in trials I and II. C1, C2, and C3 are three experimental controls: T1, T2 and T3 are three treatments with essential oil.

Component	Berry absorption Trial I						Surface residue Trial I					
	C1	C2	C3	T1	T2	T3	C1	C2	C3	T1	T2	T3
p-cymene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	5.4	6.3	4.73	4.94	6.31	5.47
Thymol (mg L^{-1})	<LOQ	<LOQ	<LOQ	5.90	9.38	5.74	10.9	11.29	11.69	9.76	11.29	11.95
Carvacrol (mg L^{1})	<LOQ	<LOQ	<LOQ	20.29	35.68	12.23	<LOQ	<LOQ	<LOQ	4.44	6.65	6.26
	Berry absorption Trial II						Surface residue Trial II					
	C1	C2	C3	T1	T2	T3	C1	C2	C3	T1	T2	T3
p-cymene	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Thymol (mg L^{-1})	8.37	8.18	7.63	6.54	6.89	10.95	11.39	9.57	9.915	11.98	12.33	10.82
Carvacrol (mg L^{1})	<LOD	<LOD	<LOD	5.06	4.78	6.28	<LOD	<LOD	<LOD	7.95	6.39	9.17

LOQ = limit of quantification
LOD = Limit of detection

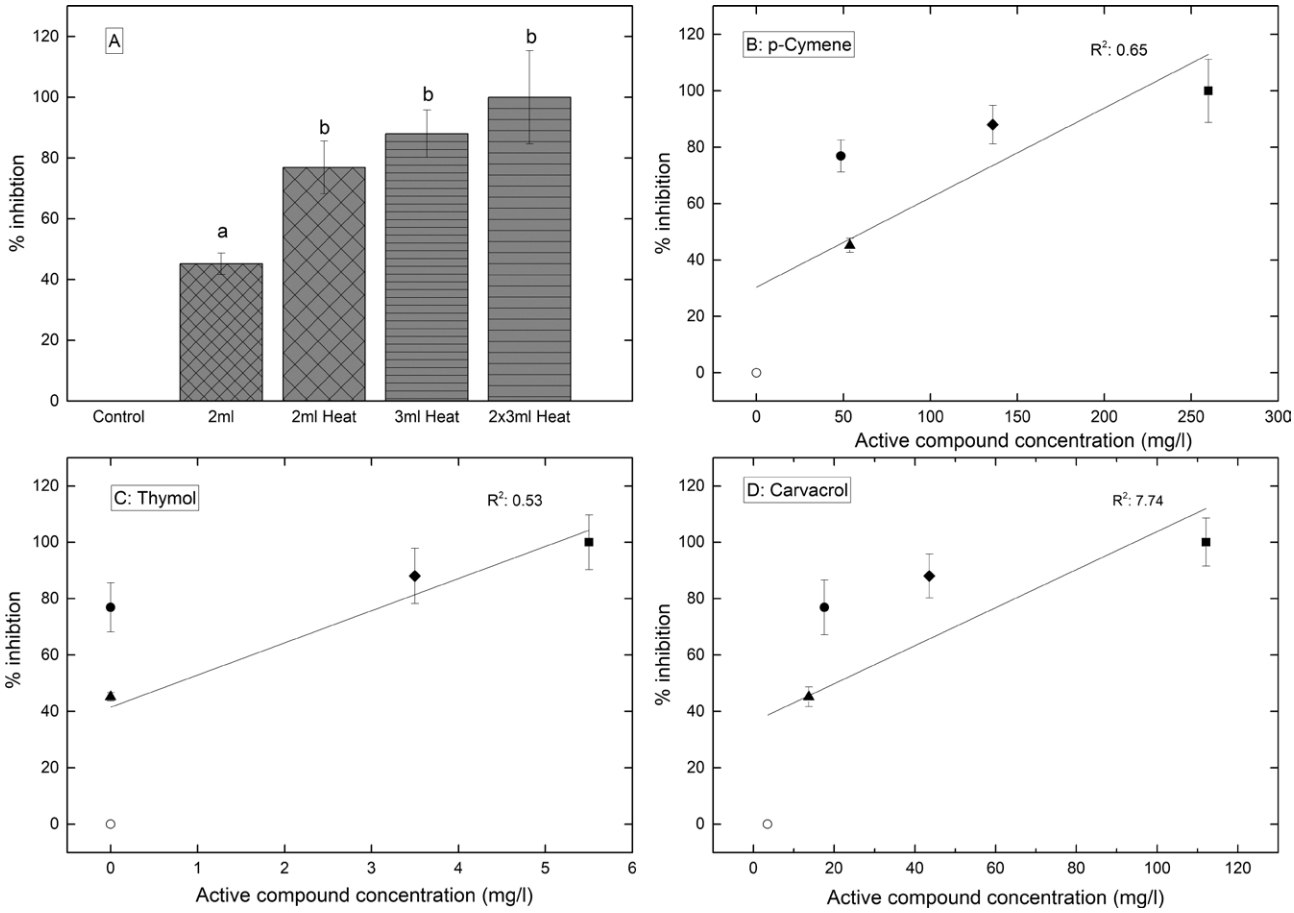

Figure 4. A) Mean growth inhibition proportions (%) for *Botrytis cinerea* on individual grape berries after 48 h exposure to different oregano EO exposure treatments. B), C) and D), mean growth inhibition of *B. cinerea* colonies exposed for 48 h to different concentrations of three EO components at different concentrations. Individual analyses of the relationships between each active component and percent growth inhibition are indicated. Scatter plot point shapes correspond to the same shape shown below each treatment parameter on the bar graph. Different letters indicate significant differences ($P < 0.05$) between treatments and controls.

proportions for the treatment parameters are shown in Table 8. For the controls (no oil treatment) and for treatments including heat, the dish containing oil was placed on top of a heating pad. In the treatment including two oil dishes, one was placed in the pump chamber with a heating pad, and the other was placed in the treatment chamber directly under the Petri dishes, resulting in an 100% inhibition of growth (Figure 4).

EO component compounds measured with GC-FID are indicated in Figure 4, which shows the concentrations of the active compounds p-cymene, thymol, and carvacrol in relation to the different dosage parameters. Components with concentrations less than the LOQ (3.50 mg L^{-1}) and LOD (2.50 mg L^{-1}) were plotted with a baseline value of zero.

The active component p-cymene was present at the greatest concentrations in all the trials, with concentrations of 53.7 mg L^{-1}, 48.5 mg L^{-1}, 135.9 mg L^{-1}, and 259.9 mg L^{-1}, where 259.9 mg L^{-1} corresponded to 100% inhibition of *B. cinerea* growth. Carvacrol was present at the second greatest concentrations throughout all the experimental trials, with concentrations of 13.7 mg L^{-1}, 17.6 mg L^{-1}, 43.6 mg L^{-1}, and 112.1 mg L^{-1}, where 112.1 mg L^{-1} corresponded to 100% inhibition. Thymol was present at low concentrations during all the trials, with concentrations of <LOQ, <LOQ, 3.5 mg L^{-1}, and 5.2 mg L^{-1}, where 5.2 mg L^{-1} corresponded to 100% inhibition. In this case, the concentrations of active compounds giving 50% inhibition of mycelial growth (EC_{50}; Badawy and Abdelgaleil, 2014), could be estimated using the concentrations obtained by GC-FID analysis after trial I, where 45.2% inhibition of mycelium growth inhibition was achieved. These estimated EC_{50} values in the vapour states were: for p-cymene, 53.7 mg

Table 8. Percent *Botrytis cinerea* colony growth inhibition and corresponding treatment parameters and EO dosages.

	Control	2 mL no heat	2 mL heat	3 mL heat	2 × 3 mL heat
Mycelium growth inhibition (%)	0	45.2	76.9	88	100

L^{-1}; for carvacrol, 13.70 mg L^{-1}; and for thymol, <3.50 mg L^{-1}.

Two-way ANOVA of the relationship between concentration of active compounds and percent inhibition of radial growth of *B. cinerea* showed significant relationships ($P < 0.05$) for all three compounds.

DISCUSSION

There is urgent need in global food production for natural alternatives to synthetic fungicides. The results of the present study confirm the findings of previous studies which have reported effectiveness of EOs and their major components, specifically that of oregano and particularly in the vapour state, as alternatives for management of fungal diseases (Dagostin *et al.*, 2010; Soylu *et al.*, 2010; Matusinsky *et al.*, 2015; Rienth *et al.*, 2019; Babalık *et al.*, 2020). However, direct treatment with EOs has several drawbacks, which probably explains why the observed inhibition of fungal development is often inconsistent in field experiments (Dagostin *et al.*, 2010). In the present study, we developed an innovative experimental approach that enabled applications of EO vapour continuously to Petri dishes and to grape berries inoculated with *B. cinerea*.

In vitro *treatment with essential oil vapour against* Botrytis cinerea

Three experiments were carried out to evaluate the effectiveness of vapour from the oregano EO for inhibition of radial colony growth of *B. cinerea*. All treatments reduced the growth of the fungus, with the greatest inhibition in trial III, where an additional dish containing the EO was placed directly under inoculated Petri dishes inside the fumigation treatment chamber. These results are consistent with previous findings (Daferera *et al.*, 2003; Aminifard and Mohammadi, 2012; Vitoratos *et al.*, 2013), where oregano EO and EOs of thyme, dictamnus, marjoram, lemon, and black caraway, completely inhibited the growth of *B. cinerea* under *in vitro* conditions. The present results highlight that the vapour phase of EOs has direct antifungal effects against *B. cinerea*, in a dose-dependent manner, and is not only through stimulation of innate plant immunity as shown in previous studies (Rienth *et al.*, 2019).

In vivo *treatment of grape berries with essential oil vapour*

In vivo experimentation was carried out on detached grape berries to determine the effectiveness of vapour treatments. Treatments were applied in four trials, each of which resulted in inhibition of *B. cinerea* growth. The extent of fungal growth in cultures and inhibition of fungal growth on berries varied during the different trials, with the most consistent growth and inhibition patterns observed in trials II and IV. These results are consistent with the findings of other studies carried out on harvested fruits and vegetables, as well as on growing plants. For example, in *V. vinifera* cv. Chasselas vines, Rienth *et al.* (2019) found 95% inhibition of the growth of *P. viticola* after treatment with vapour of oregano EO. Efficacy was mainly associated with stimulation of grapevine innate immunity by the EO. Aminifard and Mohammadi (2012) demonstrated reduced decay caused by *B. cinerea* on plum fruit treated with EO of black caraway. Soylu *et al.* (2010) demonstrated the ability of EOs of oregano, lavender and rosemary, applied as contact and vapour phases, to reduce disease on *B. cinerea*-infected tomatoes. These EOs inhibited fungal growth in dose-dependent manners, with the vapour phase treatments being more effective at low dosages than the contact phase treatments. Soylu *et al.* (2007) showed the effectiveness of soil amendments with the EOs of oregano and fennel for protection of tomato seedlings against *S. sclerotiorum*. The antifungal effects of oregano EO were also observed by Vitoratos *et al.* (2013), where treatments with the EO completely inhibited the growth of *B. cinerea* in tomato plants. The present study highlighted the effective inhibition of fungal growth and emphasized the efficacy of the volatile phase against fungal pathogens, which is of interest for development of sustainable treatment strategies. These could rely on EO vapour diffusion systems or encapsulated EOs to circumvent commonly encountered drawbacks of direct application and decrease phytotoxicity. The present results confirm the direct effects of EO vapour on the fungus hyphae since treatments were applied to detached fruits. This was also shown with the scanning electron microscope analysis carried out in a similar experiment by Soylu *et al.* (2010).

Residues on berry skins and in berry flesh after treatment with essential oil vapour

An important question regarding the persistence of aroma compounds on fruit after treatment has not been reported in previous research. In the present study, consideration of the impacts of residual active molecules on cv. Chasselas berries was a fundamental question, because of the importance of aroma compounds in winegrapes. GC-FID analyses of the active compounds present in the residues left on berry skins, and those absorbed into berry flesh, showed consistently low concentrations of p-cymene in both trials. Carvacrol was present at high concentrations in the berry flesh, but at low concentrations on berry skins during trial II, but during trial III, this compound was at low concentrations on berry flesh and surfaces. Thymol was consistently present at slightly greater concentrations than the other two compounds on the berry surfaces and in berry flesh during both rounds of treatment.

The persistence of the different active molecules can be determined by comparing the total concentrations of the molecules present during treatment in the chamber with the residual concentrations left on the berries after treatment. P-cymene had the greatest concentration in the chamber during treatment but the lowest residual concentrations after treatment, showing its persistence to be low. Marchese *et al.* (2017) reported a short half-life for p-cymene, resulting in rapid absorption and rapid *in vivo* elimination. Carvacrol was also present at high concentrations in the chamber during treatment and at low residual concentrations, with the exception of the berry absorption levels, in trial II. This also showed that carvacrol had low persistence on berries after treatment. Thymol showed very high persistence, with high residual concentrations on berry skins and in berry flesh after treatment. The concentrations of thymol were also greater, in some cases, in the control samples than in the treatment samples for both surface residues and berry absorption. This could be due to the presence of residual concentrations of thymol left in the chamber from previous trials that involved EO treatments. As demonstrated in a previous study, lemons treated with carvacrol and thymol for postharvest fungal protection exhibited persistence of these compounds directly after application, which disappeared shortly thereafter, leaving aroma and taste of the fruit unaffected (Castillo *et al.*, 2014).

Continued research on the concentrations of active compounds present in wine grapes treated with EOs is important before moving forward with the next research steps, primarily using sensory analyses. It must be determined that the berries are safe for human consumption. According to the National Cancer Institute, most EOs are considered to be safe and without adverse side effects. Only natural substances with EC_{50} > 20 µg m L^{-1} are considered toxic to human cells (Puškárová *et al.*, 2017). In the present study, all of the total concentrations of p-cymene, thymol, and carvacrol left on the berries after treatment were below this threshold, with the exception of the concentration of carvacrol in berry flesh after the trial II treatments. The concentrations used for comparisons correspond to the total concentrations present during treatment, not specifically to the EC_{50} dosages. For the samples in question, the total concentration of carvacrol (35.68 mg L^{-1}) present in berry flesh after treatment gave 70.9% inhibition of mycelium growth. Although sensory analysis was not carried out in this experiment, compared to the average sensory threshold reported for panelists by Bitar *et al.* (2008), we observed that the average concentration of carvacrol in berry flesh was 11.06 mg L^{-1}, which was greater than the sensory threshold of 3.1 mg L^{-1} for this compound. The equivalent thymol concentration in berry flesh was 7.57 mg L^{-1}, which is less than the sensory threshold of 12.4 mg L^{-1}. The p-cymene concentration was <LOQ and was considerably less than the sensory threshold of 79.4 mg L^{-1} reported by Bitar *et al.* (2008). Because of the probability of sensory detection of carvacrol in treated berries as well as the potential for damage to berries after treatment, it is important that the EO dosages are applied at quantities that are efficient at impeding fungal growth without causing undesirable secondary effects.

Impacts of vapour concentrations on Botrytis cinerea *growth inhibition*

Five experiments were carried out to assess the relationships between the concentration of active EO compounds and inhibition of *B. cinerea* mycelium growth. Doses and treatment parameters were adjusted for each trial to increase the concentration of vapour in the fumigation chamber. Fumigation with the oregano EO inhibited the radial growth of *B. cinerea* in a dose-dependent manner.

After the end of the 3-d treatment period, test dishes were observed for an additional 4-d period, during which no fumigation was applied. For dishes treated with p-cymene at 135.9 mg L^{-1} or 259.9 mg L^{-1}, carvacrol at 43.6 mg L^{-1} or 112.1 mg L^{-1} or thymol at 3.5 or 5.2 mg L^{-1}, no additional growth was seen during the post-treatment observations. This indicated that the greater dosages killed *B. cinerea* conidia. Dishes treated with p-cymene (53.7 mg L^{-1}, 48.5 mg L^{-1}), carvacrol (13.7 mg L^{-1}, 17.6 mg L^{-1}), and thymol (<LOQ, <LOQ), exhibited slow, minimal growth after the 3-d treatment period, but

the growth never reached the total Petri plate diameter of 90 mm. All of the control sample plates that were not treated with any EO had reached this diameter after the 3 d treatment period, and continued vigorous growth was observed throughout the 4-d observation period. These observations indicate that the three analyzed terpenic components of oregano EO interacted with fundamental *B. cinerea* cell functions, rendering the pathogen incapable of growing. Prediction of the antifungal mechanisms of EOs and interpretation of the results in the present study indicated that damage to the cell structure of *B. cinerea* had occurred, resulting in inhibition of vigorous growth after treatments with low dosages of the oil, and total inhibition of growth after treatment with greater dosages.

The EC_{50} values for p-cymene (53.7 mg L^{-1}), carvacrol (13.7 mg L^{-1}) and thymol (<3.50 mg L^{-1}) were similar to those reported in previous studies, where the EO of oregano was used for control of fungal pathogens (Badawy and Abdelgaleil, 2014). The oregano EO gave the lowest EC_{50} compared to the EOs of *P. graveolens, S. cumini* and *V. agnus-castus*. This was the reason for focusing on the oregano EO in the present experiments. The EC_{50} values for the oregano EO against *B. cinerea* (44 mg L^{-1} for the vapour phase and 241 mg L^{-1} for the contact phase) reported by Soylu *et al.* (2010) also correspond to those reported in the present study, and demonstrate the improved effectiveness of EO treatments in volatile phases. Figure 4 shows the low concentration of thymol compared to the concentrations of carvacrol and p-cymene. However, it cannot be concluded how the different terpenes contributed individually to the antifungal activity of EO vapour due to the importance of their synergistic effects.

Although significant alterations to hyphal morphology and cell structure have been observed in numerous studies, the specific mechanisms of antifungal activity of EOs have not been fully elucidated (Soylu *et al.*, 2010). Recent research indicates that inhibition of fungal pathogens can be partly due to immune responses triggered within host plants after treatment with EOs, which activate genes responsible for syntheses of compounds involved in plant immune systems and defense response pathways (Rienth *et al.*, 2019). Priming of these defense response mechanisms in plants after treatment with oregano EO has been shown in *V. vinifera* cv. Chasselas (Rienth *et al.*, 2019), in apples after treatment with the EO of thyme (Banani *et al.*, 2018), in strawberries after treatment with tea tree and thyme EOs (Liu *et al.*, 2016), and in *Arabidopsis thaliana* after treatment with the EO of *Gaultheria procumbens* (Vergnes *et al.*, 2014).

CONCLUSION

The present study has highlighted the remarkable capability of EOs, specifically that of *O. vulgare*, as effective antifungal agents. The study confirmed the ability of the EO to completely inhibit the *in vitro* growth of *B. cinerea*, and to inhibit the pathogen development on grapevine berries. The study also demonstrated the efficacy of the EO in vapour phase using innovative experimental procedures. These results emphasize that EOs are promising alternatives to synthetic and copper-based fungicides currently being used in agriculture and food industries. The results of vapour phase efficacy indicate potential for development of disease management systems using vapor diffusion in crops. These could be more efficient than direct applications since they could circumvent commonly encountered problems due to poor rain fastness, mixability or degradation of fungicide compounds.

Botrytis cinerea causes considerable economic losses in agriculture, and is one of the most damaging pathogens in vineyards. Finding environmentally sustainable treatments against this pathogen is, therefore, very important.

Further research is required to fully understand the effects of EOs on fungal diseases and the mechanisms of the antifungal activity. To fully implement the practical usage of EOs as viable alternatives to the fungicides on which agriculture heavily relies, further research is also required to determine the impacts of these materials on living plants and particularly on harvested fruits.

AUTHOR CONTRIBUTIONS

AB and MR designed the experiments. AB carried out the experiments. MR and AB analyzed the data. AB and MR wrote the paper.

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