

Antifungal Studies of Selected Essential Oils and a Commercial Formulation against *Botrytis Cinerea*

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Abstract

Growing concerns about food safety and environmental protection have created a need for new and safe plant disease control strategies. The aim of this study was to find an alternative to synthetic fungicides currently used in the control of the devastating fungal pathogen *Botrytis cinerea* Pers., the causal agent of grey mould disease of strawberry (*Fragaria ananassa* Duch). The antifungal activity of the essential oils of *Origanum vulgare* L., *Monarda didyma* L. and of a commercial formulation of thyme oil (Gloves Off®) was investigated against *B. cinerea* and compared with controls. Contact phase effects of different concentrations of the essential oils and commercial formulation were found to inhibit the growth of *B. cinerea* in a dose dependent manner. Complete growth inhibition of the pathogen was recorded at 200 µg/ml of 'Gloves Off®'. The mycelial growth of the pathogen was significantly reduced at the highest concentration of the essential oils of *O. vulgare* and *M. didyma* tested, which was 51.2 µg/ml. Spore germination and germ tube elongation were also inhibited by the essential oils and Gloves Off®. Light microscopic observations revealed that the essential oils caused morphological degenerations, such as cytoplasmic coagulation, hyphal shrivelling and protoplast leakage of the fungal hyphae. The essential oils of *O. vulgare* L. and *M. didyma* L. are promising, antifungal agents against *B. cinerea* similar to the commercial formulation 'Gloves Off®'.

Keywords: antifungal activity, *Botrytis cinerea*, *Monarda didyma*, *Origanum vulgare*, strawberry

1. Introduction

Gray mould disease caused by *Botrytis cinerea* Pers. is a major constraint to cultivation of strawberry (*Fragaria ananassa* Duch) globally (Elad, Williamson, Tudzynski, & Delen, 2004). It infects the plant both during growing season and during storage and causes up to 55% loss during post-harvest storage (Martinez-Romero et al., 2007). Chemical control was the main control measure used until the mid-1990s. Site specific fungicides belonging to the benzimidazole, dicarboximide and N-phenylcarbamate families were used alone or in rotation with multi specific inhibitors, such as chlorothalonil, iminoctadine, captan and dichlofluanid (Leroux et al., 2002). Although these synthetic chemicals are quick-acting and effective, their continued use has led to resistance to various types of chemicals (Beever, Laracy, & Pak, 1989; Elad, Yunis, & Katan, 1992; Brent & Hollomon, 1998), residues in food, toxicity to non-target organisms and environmental problems. Growing concerns about food safety and environmental protection have created the need for the development of new and safe control strategies.

Essential oil bearing plants constitute a rich source of bioactive chemicals, which have been reported to have various antifungal properties (Daferera, Ziogas, & Polissiou, 2003; Kalemba & Kunicka, 2003). These chemicals are often active against a limited number of species, including the specific target species. They are also biodegradable and non-toxic. The antifungal and antibacterial activity of oregano essential oil against a number of plant pathogens has been reported, such as *Aspergillus niger* Tieghem, *Aspergillus flavus* Link, *Aspergillus ochraceus* Wilhelm (Paster, Menasherov, Ravid, & Juven, 1995), *A. niger*, *Fusarium oxysporum* Snyder and Hansen, *Penicillium sps* L. (Daouk, Dagher, & Sattout, 1995), *Pseudomonas aeruginosa* Schroter ATCC 2730 and *Staphylococcus aureus* Rosenbach ATCC 6538 (Lambert, Skandamis, Coote, & Nychas, 2001), *Fusarium solani* var. *coeruleum* (Martius) Saccardo and *Clavibacter michiganensis* S. (Daferera et al., 2003), *Phytophthora infestans* Mont. (E. Soylu, Soylu, & Kurt, 2006), *Sclerotinia sclerotiorum* Lib. (S. Soylu, Yigitbas,

Soylu, & Kurt, 2007) and *Xanthomonasvesicatoria* D. (Vansinauskiene, Radusiene, Zitikaite, & Surviliene, 2006). The sensitivity of *B. cinerea* to the essential oil of different species of Oregano like *Origanumvulgare* L. (Daferera et al., 2003) and *Origanumsyriacum* L. (E. Soyly, Kurt, & Soyly, 2010), has also been reported.

Similarly, plants in the genus *Monarda* produce complex essential oils that contain antifungal compounds and are reported to inhibit the growth of *Rhizoctoniasolani* K. (Fraternale et al., 2006; Gwinn et al., 2010) and *Sclerotiniasclerotium* (Gwinn, Greene, Trently, & Ownley, 2006). However to date, no study has been done to investigate the effect of essential oils of *Monarda* on *B. cinerea*.

Although several essential oils have been reported to have antifungal properties, few have been developed as commercial formulations for use in plant disease control. Biomor Israel Ltd. developed a new organic formulation BM-608 containing 23.8% of *Melaleucaalternifolia* oil, which is effective against a broad spectrum of plant diseases in various crops. Reuveni, Neifeld, Dayan and Kotzer (2009) reported its use for the control of fungal diseases in tomato with no phytotoxicity to plant foliage.

In this study, we examined the effects of essential oil of two species, oregano and monarda, as well as that of 'Gloves Off®', a commercial formulation presently sold in Quebec, Canada. Gloves Off® contains essential oils, with thymol and carvacrol as its active ingredients, which inhibits mycelium growth and spore germination of *B. cinerea*. Results from the study will provide information on effective essential oils for the control of gray mould of strawberry.

2. Materials and Methods

2.1 Chemicals

Potato dextrose agar (PDA) was purchased from Sigma-Aldrich (St. Louis, MO, USA), while Tween 20 was the product of Agdia. Gloves Off®, is a commercial disinfectant produced by Planet People and Laboratoire M², INC (Sherbrooke, QC, Canada).

2.2 Plant Material and Characterization of Essential Oils

For the extraction of essential oils, plants of (*O. vulgare* L., and *Monardadidyma* L.) were collected from field accessions growing at the L'Acadie (45°17'N; 73°20'W), the experimental station of the AAFC's Horticultural Research and Development Centre in Saint-Jean-sur-Richelieu, Canada. The composition of the essential oils was determined using gas chromatography and mass spectrometry (GC-MS) analysis.

2.3 Test Fungi

Botrytis cinerea previously isolated from diseased strawberry was provided by the Phytopathology Laboratory of AAFC's Horticultural Research and Development Centre in Saint-Jean-sur-Richelieu. Stock cultures were maintained on potato dextrose agar slants and sub cultured once a month

2.4 Determination of Antifungal Activities

The antifungal properties of the essential oils and commercial formulation were evaluated for their contact effect on the mycelium growth of *B. cinerea* using the method of E. Soyly et al. (2010). Different concentrations were prepared by dissolving various amounts of oregano and monarda essential oils in 1 ml of ethanol (0.5%) and Tween 20 (0.1%). The commercial formulation was used without any emulsifier; 20 ml of the amended medium was transferred to each sterile glass petri dish (90x20mm in diameter) at a temperature of 40-45°C. The essential oils were tested at 0.8, 3.2, 12.8 and 51.2 µg/ml, while the commercial formulation was tested at 50, 100, 150 and 200 µg/ml. The controls received the same quantity of ethanol and Tween 20 mixed with and PDA and only sterile distilled water. Potato dextrose agar discs (7 mm diameter) from the edge of a 7 day old *B. cinerea* culture were used to inoculate the centre of each plate. Plates were incubated in the dark at 22°C for 7 days. For each treatment and concentration, 5 replicate plates were used. The mean radial mycelium growth was measured with a Verniercaliper by measuring the diameter of the colony in two directions at right angles.

2.5 Effect on *Conidia* Germination and Germ Tube Elongation

The effects of the essential oil and commercial formulation on spore germination and germ tube elongation of *B. cinerea* were as described by E. Soyly et al. (2010). A spore suspension (10⁴ spores ml⁻¹) of *B. cinerea* was prepared from actively growing culture (7-8 days old) in sterile distilled water using a haemocytometer. Three different 50 µl aliquots of the spore suspension drops were spread on PDA medium supplemented with different concentrations of the essential oils and commercial formulation. Sterile distilled water alone and sterile distilled water with ethanol and Tween 20 were used as controls. Plates were incubated at 22°C for 10-12 hours, after which germination was stopped by applying a drop of lacto phenol-cotton blue to the inoculation sites on plates. The percentage of spore germination and the lengths of germ tubes were estimated under a microscope. The

percent inhibition was calculated in relation to the respective control. Three replicates of each treatment were performed and the experiments were repeated twice.

2.6 Determination of Treatments Effects on Hyphal Morphology

Thin layers (1 mm) of agar blocks were removed for examination under light microscopy. The block cuts from growing edges were placed in a drop of lacto phenol on microscope glass slides, covered with cover slips and examined using a phase contrast light microscope (Olympus BX41, Tokyo, Japan).

2.7 Statistical Analysis

Each experiment was analysed separately and the two sets of data were pooled together after testing the homogeneity of the two experimental errors. The efficacy of the selected levels was tested and a least significant difference (LSD) test was used to separate means for each time interval tested using SAS, GLM procedure (SAS Institute, 1989).

3. Results

3.1 Composition of the Essential Oils

The chemical composition of the essential oils used, as determined by GC-MS analysis is shown in Table 1. The essential oils were characterized by the presence of major compounds such as thymol, carvacrol, para-myrcene, γ -terpinene, α -terpinene, α -thujene, β -myrcene. Thymol (41.17%) was the main compound found in the monardaessential oil, while carvacrol (37.03%) was the main compound found in the oregano essential oil.

Table 1. Chemical composition for the essential oils from Oregano and Monarda

Component	Retention time (min)	Composition (%)	
		<i>Origanumvulgare</i>	<i>Monardadidyma</i>
α -Thujene	5.55	4.07	2.62
α -Pinene	5.67	1.91	0.73
β -myrcene	7.07	4.58	2.489
α -phellandrene	7.41	0.60	0.47
α -Terpinene	7.73	3.87	4.58
para-myrcene	7.95	14.26	12.58
Limonène	8.06	1.47	1.53
γ -terpinène	8.88	18.14	15.88
Sabinene	9.11	0.00	0.57
1-terpinene-4-ol	12.17	0.81	0.64
Thymylméthyléther	14.0	0.00	1.18
Thymol	15.40	10.40	41.17
Carvacrol	15.65	37.03	15.20
caryophyllène	18.44	2.88	0.000
germacrene-D	19.62	0.00	0.37
Total		100.0	100.0

3.2 Antifungal Activities of Essential Oils on Mycelial Growth

The effects of different concentrations of the essential oils on mycelial growth of *B. cinerea* are shown in Table 2. All of the essential oils inhibited the growth of *B. cinerea* in a dose dependent manner. 'Gloves Off®' achieved complete growth inhibition at higher concentrations of 150 and 200 $\mu\text{g/ml}$. Concentrations of 'Gloves Off®' of 50 and 100 $\mu\text{g/ml}$ inhibited growth for 24 and 48 hours respectively, after which continued mycelial growth occurred. Although growth in essential oils amended plates had a significantly lower mycelial diameter, complete growth inhibition was achieved at the highest concentration tested (51.2 $\mu\text{g/ml}$) only for 24 hours (Table 2).

Table 2. Effect of oregano, monarda essential oil and 'Gloves Off®' on mycelial growth of *Botrytis cinerea*

Concentration ($\mu\text{g/ml}$)	Mycelial Growth Diameter (cm)				
	24h	48h	72h	96h	120h
Oregano					
0	1.69	3.26	4.36	6.04	7.09
3.2	1.66	3.23	4.16	5.65	6.97
12.8	1.51	3.13	4.03	5.58	6.93
51.2	0	1.48	2.04	2.86	3.78
LSD	0.14	0.32	0.50	0.45	0.42
Monarda					
0	1.76	3.26	4.45	6.07	6.94
3.2	1.40	2.92	4.26	5.34	6.70
12.8	1.20	2.73	4.08	5.24	6.31
51.2	0	1.03	1.83	2.53	3.31
LSD	0.19	0.33	0.32	0.52	0.37
Gloves Off®					
0	1.95	4.40	7.01	7.67	8.2
50	0	1.02	1.58	2.48	3.21
100	0	0	0.95	1.48	1.91
150	0	0	0	0	0
200	0	0	0	0	0
LSD	0.06	0.07	0.22	0.31	0.27

3.3 Effect of Essential Oil on *Conidia* Germination and Germ Tube Elongation

The effects of the various concentrations of the essential oils and commercial formulation on conidia germination and germ tube elongation are shown in Figures 1-3. The trend was similar to that recorded with mycelial growth. Higher inhibitory activities were recorded with the commercial formulation. Although percentage germination and germ tube length were significantly reduced with each tested concentration, complete germination inhibition was achieved at higher concentrations of 150 and 200 $\mu\text{g/ml}$. Complete germination inhibition was not achieved with the essential oils although significantly lower percentage germination and germ tube lengths were associated with increased concentrations of the essential oils.

3.4 Effects of Oils on Hyphal Morphology

Microscopic observation of hyphae grown on PDA amended with the essential oils and Gloves Off® showed degenerative changes in hyphal morphology compared with the control (Figure 4). Healthy mycelium grown on PDA medium presented a normal morphology with smooth, linear, homogeneous cell wall hypha. However, these normal morphological structures varied in the presence of the essential oils and commercial formulation. Shrivelled hyphal cells with cytoplasmic coagulation or even no cytoplasm were observed. In some cases cell wall disruption and consequent cell death were recorded (Figure 4).

4. Discussion

In recent years, interest has been generated in the development of safer antifungal agents such as plant-based essential oils and extracts to control phytopathogens in agriculture (Costa et al., 2000). As a matter of fact, volatile essential oils from several medicinal plants have been reported.

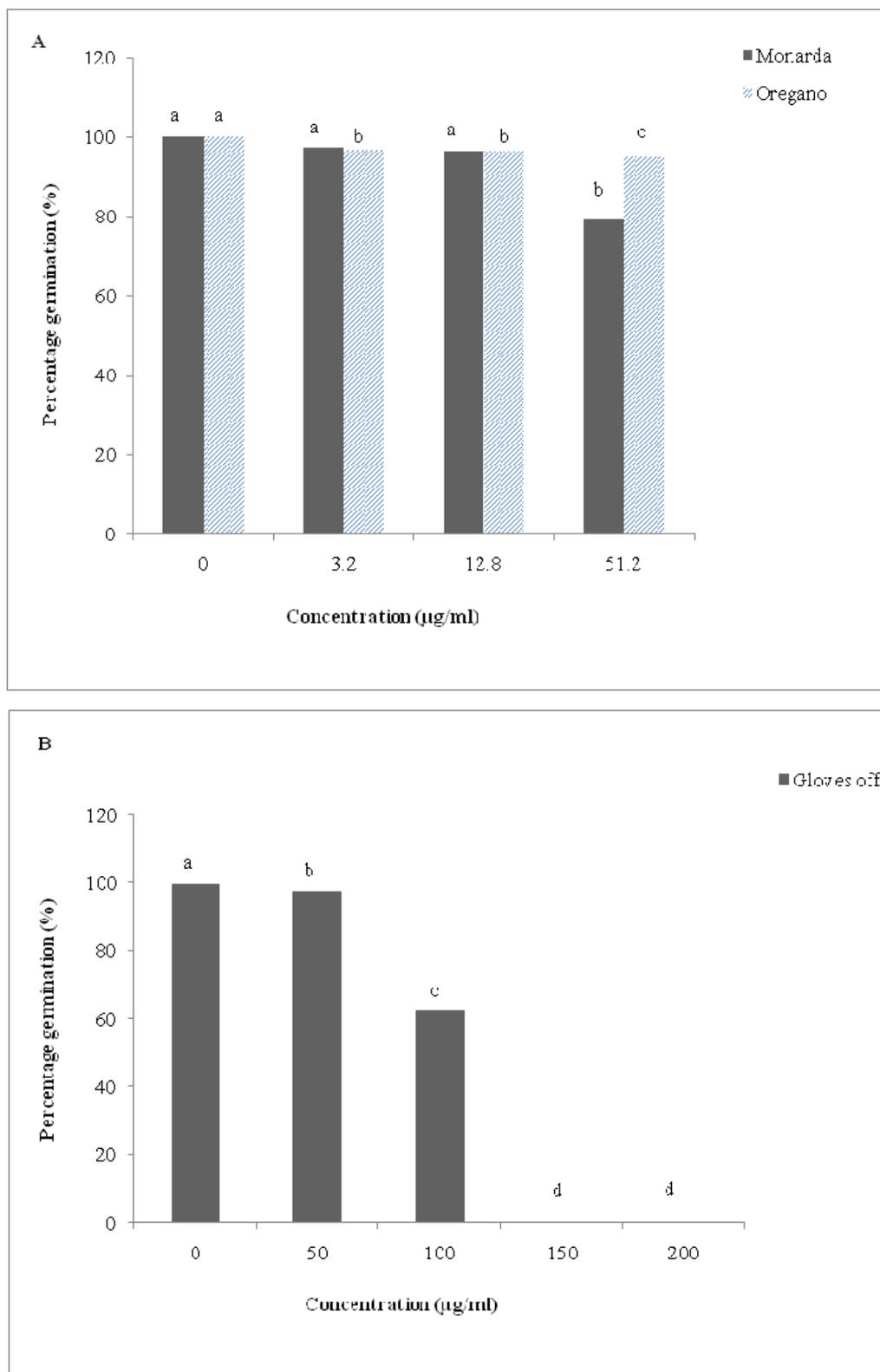


Figure 1. Spore germination in cultures amended with (A) Monarda and Oregano (B) Gloves Off®
Bars of each essential oil with the same letters represent values which are not significantly different according to t test ($P \leq 0.05$).

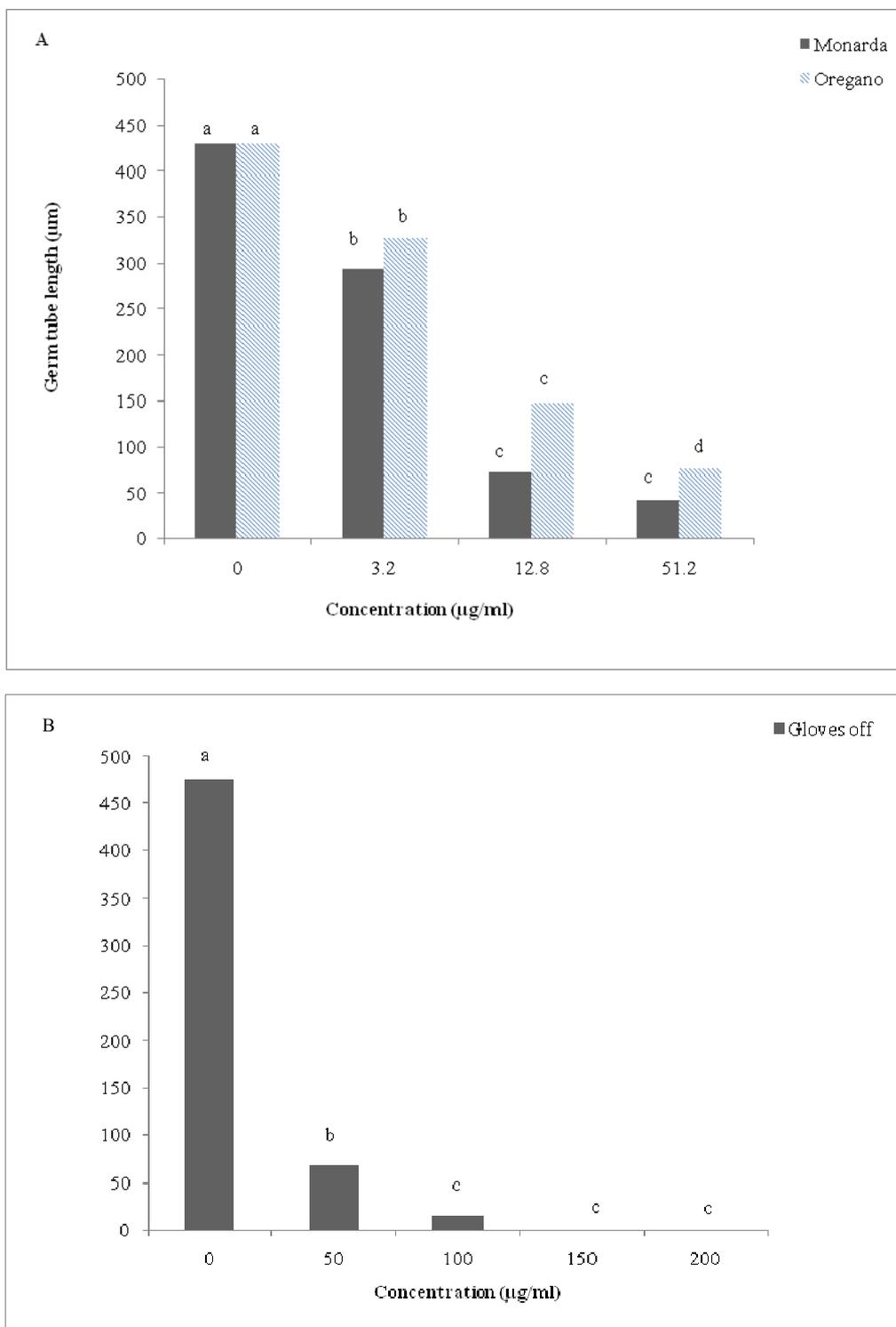


Figure 2. Germ tube length in cultures amended with (A) Monarda and Oregano (B) Gloves Off®

Bars of each essential oil with the same letters represent values which are not significantly different according to t test ($P \leq 0.05$).

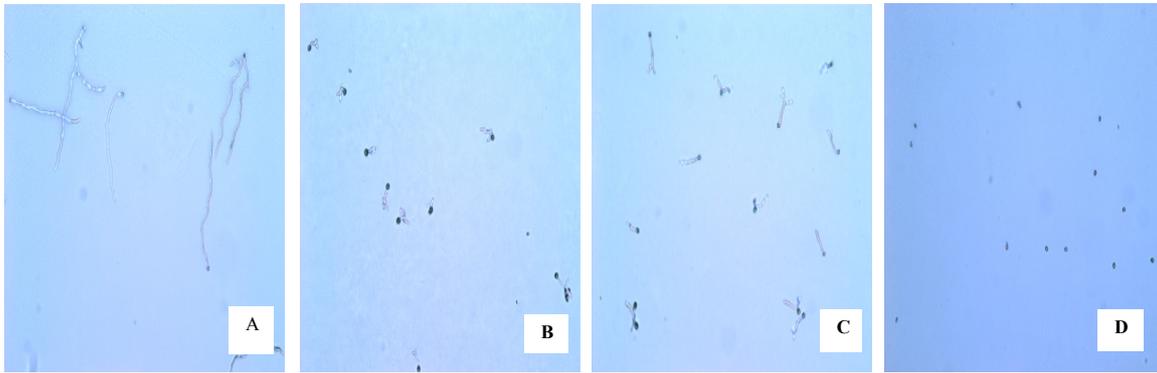


Figure 3. Light micrographs of conidia germination and germ tube length of *Botrytis cinerea* grown in (A) Control (B) Monarda EO (C) Oregano EO (D) 'Gloves Off®' with EO as active ingredient

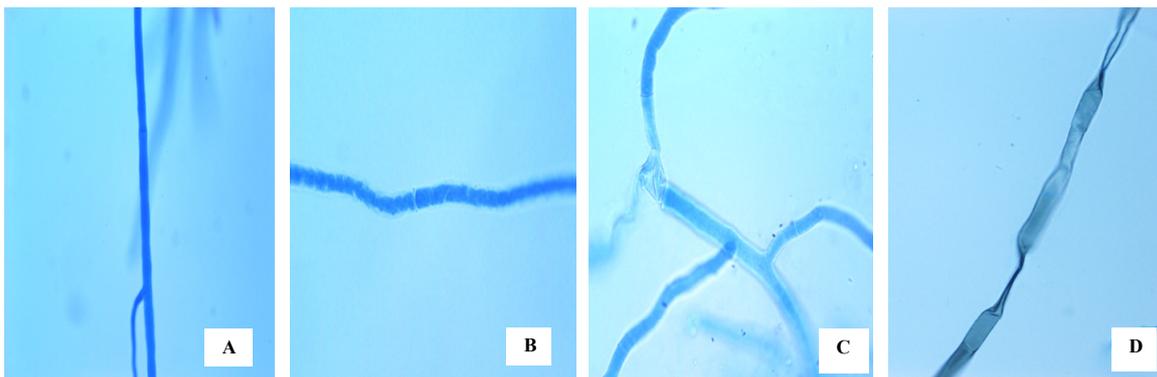


Figure 4. Hyphal morphology of *Botrytis cinerea* grown in (A) control (B) Monarda EO (C) Oregano EO (D) 'Gloves Off®' with EO as active ingredient

In this study, we found that 'Gloves off®' and essential oils from oregano and monarda inhibited spore germination and mycelial growth of *B. cinerea*. The efficacy was positively correlated with the concentration of the solution (Table 1, Figures 1 and 2). These results confirm the literature data about the effectiveness of plant essential oils extracted by hydro-distillation from aromatic plants on the growth of a wide range of plant pathogenic fungi and bacteria (Daferera et al., 2003). It also suggests the inhibitory efficacy of commercial products derived from such plants against the growth and spore germination of *B. cinerea*. These inhibitory activities may be linked to the chemical composition of both Gloves Off® and the essential oils of oregano and monarda. In our study, the major constituents of the oregano and monarda essential oils and the 'Gloves off®' were carvacrol and thymol. This indicated that the two plants tested can be good sources of carvacrol and thymol for the development of safe fungicides. Various studies concerning the genus *Origanum* have shown that its oils possess strong antimicrobial activity, which is attributed to their high percentage of phenolic compounds, specifically carvacrol, thymol, p-cymene and their precursor α -terpinene (Sivropoulou et al., 1996; Davidson & Naidu, 2000). Specific studies have linked these compounds to antifungal activities against *B. cinerea*. Bouchra, Achouri, Hassani and Hmamouchi (2003) reported that the essential oils of seven Moroccan Labiatae consists mainly of carvacrol, linalyl acetate and thymol as major components, and exhibited a complete mycelial inhibition effect on the growth of *B. cinerea*. In another study, carvacrol, thymol and citral were again reported to show complete inhibition of *B. cinerea* (Plotto, Roberts, & Roberts, 2003).

Data for typical essential oil contents and composition of *O. vulgare* are abundant with variable results in terms of chemical composition. Ferreira et al. (1998) reported that the essential oil of *O. vulgare* subsp. *Virens* is not rich in carvacrol. However, Salgueiro et al. (2003) studied a carvacrol-rich *O. vulgare* subsp. *Virens* and its activity on *Candida albicans*. Figueredo, Cabassu, Chalchat and Pasquier (2006) reported that there are significant differences in the yield and composition of essential oils from populations of *O. vulgare* arising from environmental factors, the most important of which is altitude, relating this to lack of water and short growing

periods. We report here in our study the composition of *O. vulgare*, with carvacrol (37%) as its major constituent, as well as an appreciable amount of thymol (10.4%).

Essential oils collected from *M. didyma* have also been reported to be effective antifungal agents in previous studies, the primary constituent of the essential oils was thymol (Fraternale et al., 2006), which is consistent with what we found in our study.

Apart from the positive value of each of the chemical components of the essential oils identified, there seems to be a synergy between some of the chemical constituents of the oregano and monarda essential oils tested in our study, making them more effective and low risk in terms of the development of resistance by plant pathogenic fungi. In fact, Daferera et al. (2003) reported that it is very difficult for the fungi to develop resistance to such a mixture of oil components with different mechanisms of antimicrobial activity. For instance the biological precursors of carvacrol, γ -terpinene and p-cymene, are not effective antibacterial agent when used alone; however, when combined with carvacrol, synergism has been observed (Utree, Bennink, & Moezelaar, 2002). The greater efficiency of p-cymene was reported to facilitate the transport of carvacrol across the cytoplasmic membrane of fungi (Morcia et al., 2011). They also concluded that thymol, eugenol and carvone are highly active in the in vitro control of fungal species responsible for mycotoxin contamination of economically valuable crops (Morcia et al., 2011).

Light observations of hyphae of *B. cinerea* exposed to essential oils revealed alterations in hyphal morphology. Shrivelled hyphal aggregates reduce hyphal diameters and lyses of hyphal wall were commonly observed in mycelium treated with oregano and monarda essential oils and Gloves Off®, compared with thick, elongated, normal mycelial growth in controls. E. Soylu et al. (2007, 2010) suggested that such modifications may be related to the effect of the essential oil as enzymatic reactions regulating wall synthesis. The lipophilic properties of essential oil components might have also aided its ability to penetrate the plasma membrane (Knobloch, Pauli, Iberl, Weigand, & Weiss, 1989). The observations made with light microscopy are in accordance with previous studies in which essential oils of aromatic plants caused morphological alterations on the fungal hyphae (Bianchi et al., 1997; Fiori et al., 2000; Romagnoli et al., 2005; E. Soylu et al., 2005; E. Soylu et al., 2006; E. Soylu et al., 2010).

In future work, it will be crucial to determine the concentration and timing of application of these essential oils and the commercial formulation for successful gray mould disease control in detailed field evaluations. There will also be need for phytotoxic test on these products.

In conclusion, oregano and monarda mediated essential oil and Gloves Off® could be applied as an alternative to synthetic fungicides for the control of *B. cinerea*. They could also be screened to develop such novel types of selective and natural fungicides in the safe control of many agricultural plant pathogens causing drastic crop losses.

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