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Investigation of in vitro and in vivo antifungal activities of different plant essential oils against postharvest apple rot diseases – *Colletotrichum gloeosporioides, Botrytis cinerea* and *Penicillium expansum*

In vitro und in vivo Untersuchungen zur antimykotischen Aktivität verschiedener pflanzlicher ätherischer Öle gegenüber Nacherntekrankenheiten – Colletotrichum gloeosporioides, Botrytis cinerea und Penicillium expansum

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Summary

The essential oils (EOs) extracted from sage *(Salvia officinalis),* rosemary *(Rosmarinus officinalis),* oregano *(Origanum vulgare),* eucalyptus sp. and fennel *(Foeniculum vulgare)* were tested for their antifungal capacities against *Penicillium (P.) expansum, Colletotrichum (C.) gloeosporioides* and *Botrytis (B.) cinerea in vitro* and *in vivo* using golden delicious apples. The main components found in the essential oils of rosemary, sage, fennel, eucalyptus and oregano were camphor (26.25 %), α-thujone (14.95 %) along with 1.8-cineol (13.40 %), trans anethole (80.73 %), cymene (24.90 %) and carvacrol (81.25 %) respectively. The percentage of mycelial growth inhibition was determined using fumigation bioassay and contact bioassay techniques. Oregano EO was found to be the most effective essential oil against fungi species in *in vitro* and *in vivo* trials. The descending order of inhibition power of other EOs was noted as eucalyptus, fennel, sage and rosemary. Oregano EO did not stop the fungal growth completely at selected concentrations. However, it was able to limit the growth of fungi (fungistatic) in *in vitro* bioassays from approximately 80 to 15 mm, from 19 to 4 mm and from 50 to 9 mm in diameter for *B. cinerea, P. expansum* and *C. gloeosporioides* respectively.

Keywords: Plant essential oil, golden delicious apple, *C. gloeosporioides, B. cinerea, P. expansum,* fungal deseases

Zusammenfassung

Ätherische Öle extrahiert aus Salbei *(Salvia officinalis),* Rosmarin *(Rosmarinus officinalis),* Oregano *(Origanum vulgare),* Eukalyptus und Fenchel *(Foeniculum vulgare)* wurden auf ihre antimykotische Eigenschaften gegenüber *Penicillium (P.) expansum, Colletotrichum (C.) gleosporioides* und *Botrytis (B.) cinerea* in vitro und in vivo (Golden Delicious Äpfel) getestet. Die Hauptkomponenten in den ätherischen Ölen waren Kampfer (26,25 %), α-Thujon (14,95 %), 1,8-Cineol (13,40 %), trans-Anethol (80,73 %), Cymol (24,90 %) und Carvacrol (81,25 %). Die Hemmung des Myzelwachstums wurde mit Begasungs- und Kontakttechniken bestimmt. Das aus Oregano extrahierte ätherische Öl zeigte den stärksten Effekt gegenüber den Pilzen sowohl in vitro als auch in vivo. Gefolgt von den ätherische Ölen von Eukalyptus, Fenchel, Salbei und Rosmarin. Das ätherische Öl von Oregano konnte bei den ausgewählten Konzentrationen das Pilzwachstum nicht vollständig stoppen. Es war aber in-vitro in der Lage, den Wachstumsdurchmesser der Pilze von ca. 80 mm auf 15 mm *(B. cinerea),* von ca. 19 mm auf 4 mm *(P. expansum)* und von ca. 50 mm auf 9 mm *(C. gleosporioides)* zu begrenzen.

Schlüsselwörter: Ätherische Öle, Golden Delicious, *C. gloeosporioides, B. cinerea, P. expansum,* Pilzerkrankungen

1 Introduction

Food safety is an important issue from the consumers' and food industry's point of view, especially due to the shifting knowledge level of consumers about food and health. Growth of fungi on food material leads to food spoilage and in most cases plant disease, which leads to significant economic losses (Da Cruz Cabral et al., 2013; Jing et al., 2014). During postharvest storage, fruits and vegetables are often subjected to varying levels of microbial activity, mainly caused by pathogenic fungi (Chang et al., 2008). These mycelial microorganisms usually contaminate the food material through cuts and wounds maintained during postharvest handling or processing. It has been estimated that around 20–25 % of fruits and vegetables are subjected to fungal activity during postharvest operations worldwide (Agrios, 1997; Da Cruz Cabral et al., 2013).

Fruits and vegetables are highly prone to fungal spoilage due to their low pH and therefore susceptible to rot deseases both in the field and during storage (Camele et al, 2012). Moulds are widely distributed microorganisms that are able to contaminate foods and grow easily due to their ability to synthesize a wide variety of enzymes. Some of them may cause deseases in plants leading to economic losses for food producers and some species may excrete toxic substances which were reported as potential carcinogen to human body (Da Cruz Cabral et al., 2013; Nanosombat et al., 2011). Many fungi including *B. cinerea, P. expansum* and *C. gloeosporioides* reduce the market values and deteriorate the quality of fruits, especially apples, and make them unsuitable for human consumption and may cause adverse effects on human health (Lopez-Reyes et al., 2010; Sellamuthu et al., 2013; Znini et al., 2011). Among them, *B. cinerea* has been known as a resistant fungi against fungicides (Leroch et al, 2013).

For many years, physical agents, such as refrigeration and modified atmosphere, synthetic pesticides and biological control agents (BCAs) have been extensively used to control the growth of the phytopathogenic fungi (Gebel and Magurno, 2014). However, the synthetic chemicals may be toxic to human body and possess risks to health and environment. In addition, microorganisms may develop resistance to commonly used fungicides, which can also become a significant problem (Gumus et al., 2010). Due to the risks associated with the use of these synthetic compounds, the researchers have been trying to develop natural antifungal agents derived from plant material such as extracts and essential oils to control fungal growth without using any synthetic chemicals (Cakir et al, 2004; Chuang et al, 2007; Prasad et al, 2004)

In the last decade, some researchers have studied the growth inhibition capacities of plant essential oils to control the growth of various fungal species in *in vitro* and *in vivo* tests (Camele et al, 2012; Hossain et al., 2016; Prabuseenivasan et al, 2006; Tian et al., 2011). Various studies have evaluated and shown the capacities of EOs to inhibit or control the growth of selected fungi species. It has been reported that the antifungal effects are highly depending on not only the concentration and chemical structure of the active compound tested, but also the type and number of microorganisms studied (Lee et al. 2007; 2010). Even though there are numerous *in vitro* studies to evaluate the antifungal effects of different EOs, research in *in vivo* tests using apples are limited. Therefore, determination of the antifungal capacities of different EO compounds on rot desease causing fungi species on apples will provide quantitative information on how to prevent economic losses during storage and processing in a natural way. The growth inhibition capacities of individual compounds found in EOs to the overall antimicrobial activity also need to be further studied. It has been reported that the antimicrobial activity of an essential oil is linked to its chemical composition (Cimanga et al. 2002; Inouye et al. 2006). The functional groups exist in EOs such as phenols, alcohol, ketones and terpenes are reported to be responsible for the antimicrobial effects. EOs also consist of compounds such as cymene, pinene, carvacrol, thymol, linalool and α-terpinly acetate responsible for their antimicrobial characteristics (Cimanga et al, 2002; Knobloch et al, 1985). Some findings revealed that major active compounds exist in EOs show stronger antimicrobial effects when they are together with minor compounds of EOs due to the synergistic effect (Cimanga et al, 2002; Hossain et al, 2016).

Essential oils are mix of volatile aromatic hydrophobic compounds (mono and sesquiterpenes including aliphatic hydrocarbons, phenols, terpenoids, alcohols, ethers, coumarins, aldehydes, ketones, fatty acids, acyclic esters or lactones, and homologues of phenylpropanoids) (Nazzaro et al., 2013) which can be extracted from plant parts such as seeds, leaves, flowers, buds, fruits and roots (Tabassum And Vidyasagar, 2013). Essential oils of plants have been reported to be predominantly a defence mechanism against pathogenic fungi (Vasantha Rupasinghe et al., 2006). Implementing this approach would result in using less or no synthetic fungicide at all, and therefore improving consumer satisfaction and reducing environmental pollution as well (Sokmen et al. 1999).

In this work the application of essential oils (EOs) extracted from plants to control the growth of fungi which is responsible to spoil fresh products, such as apples, has been evaluated. The antifungal capacities of 5 EOs from different plants have been identified using *in vitro* and *in vivo* tests against *B. cinerea, P. expansum* and *C. gloeosporioides.* Furthermore, the potential use of EOs in the protection of apple from postharvest fungal deterioration has been discussed.

2 Materials and methods

2.1 Materials

Leafy parts of eucalyptus plant were procured from Izmir, Turkey. Leaf and stem parts of sage *(Salvia officinalis),* rosemary *(Rosmarinus officinalis)* and oregano *(Origanum vulgare),* as well as seeds of fennel *(Foeniculum vulgare)* were harvested from fields of Medical and Aromatic Plants Research Center of Selcuk University, Konya, Turkey. The plant materials were harvested between June and September since they accumulate more oil in summer time (Verma et al, 2015). The plant materials were dried naturally in the shade and stored in a cool and dry place until conducting experiments.

C. gloeosporioides was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellekulturen GmbH, Mascheroder Weg, Braunschweig, Germany). *B. cinerea* and *P. expansum* were isolated from rotten apples and identified using appropriate techniques, as will be mentioned later.

The fruit material to be used as substrate in *in vivo* experiments in this study was the apple cultivar, 'Golden

Delicious' *(Malus domestica)* harvested from the Egirdir region (Isparta, Turkey) and was obtained from Konya fruit and vegetable market. The apples were harvested and the samples used were selected based on their uniform color, size, maturity and absence of injury. Additionally, they were manually sorted to remove those with blemishes and were stored at –0.5 ºC for a period of 2 months after harvest. Before analysis, the fruits were removed from the cold storage and were stored overnight at room temperature.The selected fruits were randomized before being used for the treatments.

2.2 Extraction of essential oils (EOs)

The hydrodistillation technique was used to extract of EOs from herbal plants using a Clevenger-type apparatus (ldam Cam, Ankara, Turkey). 100 g of dried plants was weighed and distilled in distilled water for 3 hours. The EOs were separated and dried over anhydrous sodium sulfate before placing in sealed vials. The EOs were kept in dark bottles at 4 °C for short term and at –20 °C for long term storage.

2.3 Chemical identification by GC/MS analysis

Essential oil compositions of the plant materials of EOs were determined by using a gas chromatography/mass spectrometry (GC/MS) system consisting of a GC instrument (Agilent 6890N) equipped with a mass selective detector (Agilent 5973N). HP Innowax Capillary Column (60.0 m x 0.25 mm x 0.25 µm film thickness) (Agilent 19091S-433) was used to differentiate the components. Identification of separated chemical compounds by GC was done by comparison of mass spectra of each peak with the data provided by National Institute of Standards and Technologies (NIST) Mass Spectra Library.

GC/MS operating conditions were as followed: Injection volume: 1 µL, Split 50:1, Helium flow rate: 1.2 mL/min, Column 60 °C, 10 min hold, ramp to 220 °C at 10°C/min, 10 min hold. MS: 250 °C Interface temperature: 200 °C Scan Range EI (70 eV): 35–450 amu.

2.4 Isolation of microorganisms and preparation of spore suspensions

Two fungal strains were isolated from naturally rotten apples and tested in terms of their virulency by inoculating on artificially wounded apples (hyphal tip). The isolated fungal strains (*B. cinerea* and *P. expansum*) were identified using micro and macro techniques given by Barnett and Hunter (1972) and Domsch et al. (1980). *C. gloeosporioides* was obtained from DSMZ, as mentioned above. Both *B. cinerea* and *P. expansum* were kept in slants at 4 °C on potato dextrose agar (Merck, Darmstadt, Germany) having streptomycin sulphate (Merck, Darmstadt, Germany) at a concentration of 50 mg/L.

The fungal strains were cultured on potato dextrose agar (PDA, pH 5.6, Merck, Darmstadt, Germany), containing streptomycin sulphate (50 mg/L) at 27 ºC for 10 days. The spores formed were harvested using sterile sand (Sigma-Aldrich, Taufkirchen, Germany) by gently agitating the plate to dislodge spores and to help them better adhere on the sand particles. The sand-spore mix was taken into sterile Ringer's solution (Merck, Darmstadt, Germany) to prepare a conidial suspension. The mixture was then filtered through eight layers of sterile cheesecloth and centrifugated. The spores were counted by using a haemocytometer (Hausser Scientific Horsham, PA, USA) to adjust the suspension to a final concentration of 10^5 spores/mL for each fungi. Prior to inoculation, the prepared spore suspensions were shaken using a vortex mixer (Grant Instruments, Cambridge, England) for 30 s.

2.5 *In vitro* assays

2.5.1 Contact bioassay (agar dilution method)

The experiments were conducted based on the method reported by Soliman and Badeaa (2002). PDA was dissolved in water in 6 seperate jars, autoclaved and cooled down to 45 °C in water bath. EOs were added at concentrations of 0 (control), 100, 200, 300, 400 and 500 µl/L in each jar respectively. The PDA medium with EOs was poured as 15 ml/plate into 90 mm Petri plates (Fisher Scientific, Pittsburgh, PA, USA) before inoculation. Plates were incubated for 6 days at 27 °C after inoculation with using 7-days-old cultures, previously grown in PDA. The diameter of colonies was measured using a digital caliper on 3rd, 4th, 5th and 6th days of incubation. The % growth inhibition was calculated using formula given in 2.5.2. Each of the essential oils applied at different concentrations were evaluated as fungicidal or fungistatic. No growth means fungicidal, whereas temporary inhibition of fungal growth means fungistatic. The Petri plates were prepared in triplicates and each set of experiments was repeated three times (n = 3).

2.5.2 Fumigation bioassay (disk diffusion method)

Fumigation bioassay was performed on solid media using disk diffusion method, as described by Duru et al (2003). Petri plates (90 mm diameter) containing sterile PDA (15 ml/plate) were inoculated with 6 mm agar plugs from previous cultures (7 days old). The filter paper disks (Fisher Scientific, Pittsburgh, PA, USA) (6 mm diameter) were sterilized before being placed in the center of the Petri lids and varying volumes (1, 3, 5, 7, 9 µl/plate) of EOs were pipetted onto paper discs. Petri plates without EOs served as control plates. Parafin film was used to seal the Petri plates with lids and were kept in upside down position. They were incubated for 6 days at 27 °C. The growth of fungi was recorded after 3, 4, 5, 6 days using a digital caliper (Westward, USA). Growth inhibition was calculated using the data obtained from the experiments and reported as percent reduction in radial growth relative to the control. The plates were prepared in triplicate for each set of experiments and each set of experiments was repeated three times (n = 3).

The relative % growth inhibition of treated plates compared to the control plates were calculated using the following formula (Al-Reza et al, 2010):

$$\% \text{ growth inhibition} = ([dc - dt]/dc) \times 100 \quad (1)$$

where dc and dt correspond to mean diameter of growth (mm) of fungi colonies in control and treated plates, respectively.

2.6 In vivo experimentation

2.6.1 Preparation of EOs stock solutions

Stock solutions [10 % EO, 88% distilled water, 2 % Tween 20 (Merck, Darmstadt, Germany)] were prepared using eucalyptus and oregano EOs which were identified as the most effective against test fungi, among others, in *in vitro* experimentation. Tween 20 was used to obtain a well mix of water and EOs. The stock solutions were diluted to specified concentrations (1 %, 3 % and 5 %) to apply on apple substrates as part of *in vivo* tests. The diluted solu-

tions were mixed well using a vortex mixer (Grant Instruments, Cambridge, England) right before application to ensure that the mixture was homogeneous (Lopez-Reyes et al., 2010).

2.6.2 Inocluation to apples

Twenty apples which had no noticeable bruise and cuts were used for each set of experiments. Apples were washed using tapwater after being dipped into 1 % sodium hypochlorite solution (Merck, Darmstadt, Germany) for two minutes. They were dried at room temperature and wounded with a sterile stainless steel scalpel at the equatorial region (3 mm deep × 3 mm wide; 2 wounds per fruit) as described by Lopez-Reyes et al. (2010). 10 µl aliquots of spore suspension (1×10^5 spores/mL) was dripped into the wounds. The inoculated apples were stored at 23 ± 1 °C for 12 hours to help the adaptation of the test microorganisms. Then 10 µl from EO stock solution at specified concentrations (1 %, 3 % and 5 %) were dropped into each inoculated wounds. Control samples without any EOs were also prepared. The inoculated samples were incubated at room temperature (23 ± 1 °C, 75 % relative humidity) for 6 days to assess rot desease formation as a result of fungal growth. The diameter of the rot established by fungi species was measured using a digital caliper during and after 6 days of icubation. Each test was performed using 20 samples simultaneously and each set of experients was repeated two times (n = 2).

2.7 Statistical analysis

The statistical analysis was performed by one-way analysis of variance (ANOVA), using SPSS software after pooling the data from the repetitions. Duncan's multiple range test was also employed ($p < 0.05$ was considered significant).

3 Results and Discussion

3.1 Chemical composition of EOs extracted

The EO yields were found to be 2.8 % *(Origanum vulgare),* 1.6 % *(Salvia officinalis),* 1.8 % (*Eucalyptus* sp.), 1.2 % *(Foeniculum vulgare)* and 0.7 % *(Rosmarinus officinalis).*

Gas chromatography coupled with mass spectrometry (GC/MS) system was employed to analyse chemical components of EOs. The results are outlined in Table 1. The major compounds found in the EOs of rosemary, sage, fennel, eucalyptus and oregano were camphor (26.25 %), α-thujone (14.95 %) together with 1.8-cineol (13.40 %), trans anethole (80.73 %), cymene (24.90 %) and carvacrol (81.25 %) respectively. Carvacrol is reported to be the major component of oregano (Arnold et al., 2000; Veres et al, 2003) and antifungal properties of oregano is mostly linked to this compound. According to Adams et al. (1996), phenol components may hamper the activities of cell wall enzymes such as chitin synthase, chitinase, α- and β-glucanases of the fungus. Therefore, the high content of phenolic substances may be responsible for antifungal activity of EOs (Adam et al, 1998). Some EO components might also help oil molecules to penetrate into the plasma membrane due to their lipophilic structure (Knobloch et al., 1989).

The chemical composition of different plant materials were investigated in the previous research works (Arras and Usai, 2001; Baratta et al, 1998; Kulevanova, 1996; Sokovi et al., 2009; Soylu et al., 2010; Soylu et al., 2006). The data provided in literature are in agreement with the data obtained from GC/MS analysis of this work except some variations in percent values of the chemical components. These variations might be due to the location where the plant materials was collected and seasonal conditions as the EO composition may vary according to geographycal and seasonal changes.

3.2 *In vitro* experimentation

In vitro antifungal capacities of selected EOs were tested through two bioassays i. e., fumigation bioassay and contact bioassay. Data obtained from these bioassays for *B. cinerea, P. expansum* and *C. gloeosporioides* are shown in Table 2, Table 3 and Table 4 respectively. Similar results were observed from the two bioassays for the test fungi studied. It can be derived from the tables that EOs exhibited different inhibitory effects on the growth of each of three fungi species and the level of antifungal action was proportional to the concentrations of EOs tested. In both bioassays oregano oil showed the strongest antifungal effect on the mycelial growth of fungal species as its lowest concen-

TABLE 1: *Major components detected in the essential oil samples used in this study (n = 3, % oil, wt/wt).*

Components	EO type* Rosemary	Sage	Fennel	Eucalyptus	Oregano
α-Pinene	13.35 ± 0,87	4.21 ± 0,31	–	–	–
Camphene	8.34 ± 0,52	2.71 ± 0,24	–	–	–
β-Pinene	3.90 ± 0,20	6.87 ± 0,42	–	–	–
α-phellandrene	5.37 ± 0,27	–	–	3.90 ± 0,87	–
Limonene	3.95 ± 0,14	–	8.03 ± 0,67	–	–
β-phellandrene	–	–	–	5.25 ± 0,46	–
1.8-cineol	17.95 ± 0,47	13.40 ± 0,87	–	–	–
γ-terpinene	–	–	–	2.46 ± 0,12	6.03 ± 0,30
Ortho-cymene	–	–	–	–	2.81 ± 0,16
Cymene	–	–	–	24.90 ± 0,83	–
Terpinen-4-ol	–	–	–	4.46 ± 0,27	–
Aromadendrene	–	–	–	4.80 ± 0,32	–
Cryptone	–	–	–	8.64 ± 0,48	–
α-thujone	–	14.95 ± 0,77	–	–	–
β-thujone	–	4.72 ± 0,32	–	–	–
Camphor	26.25 ± 0,67	8.84 ± 0,44	–	–	–
Estragole	–	–	5.18 ± 0,47	–	–
Caryophyllene	–	5.92 ± 0,30	–	–	2.20 ± 0,11
ρ-menth-1-en-7-ol	–	–	–	2.69 ± 0,15	–
cumin aldehyde	–	–	–	2.05 ± 0,13	–
Borneol	3.65 ± 0,18	8.44 ± 0,52	–	–	–
β-selinene	–	5.55 ± 0,25	–	–	–
Trans Anethole	–	–	80.73 ± 1,87	–	–
Bornyl acetate	3.09 ± 0,16	–	–	–	–
Viridiflorol	–	9.44 ± 0,46	–	–	–
Spathulerol	–	–	–	12.40 ± 0,65	–
Carvacrol	–	–	–	–	81.25 ± 2,07
Zerumbone	–	–	–	4.56 ± 0,24	–
Manool	–	5.02 ± 0,26	–	–	–

* For all essential oils, compounds > 2 % are indicated.

TABLE 2: *Contact and fumigation bioassays to determine inhibitory effects of essential oil type and level on in vitro mycelial growth of B. cinerea at different incubation times (n = 9).*

	Mycelial growth diameter (mm)											
	Contact bioassay					Fumigation bioassay					
	EO level	EO type Oregano	Fennel	Sage	Rosemary	Eucalyptus	EO level	EO type Oregano	Fennel	Sage	Rosemary	Eucalyptus
3rd day	Control	63.1 ± 2.5^{b,A}	62.9 ± 2.7^{b,A}	62.5 ± 2.9^{b,A}	74.1 ± 2.6^{a,A}	53.6 ± 2.6^{c,A}	Control	79.6 ± 0.6^{a,A}	74.5 ± 1.7^{b,A}	69.3 ± 0.8^{d,A}	71.5 ± 1.1^{c,A}	60.5 ± 2.9^{e,A}
	100 µL/L	0.0 ± 0.0^{e,B}	45.5 ± 4.0^{b,B}	37.0 ± 1.8^{c,B}	51.0 ± 4.0^{a,B}	26.9 ± 3.7^{d,B}	1 µl/Petri	0.0 ± 0.0^{e,B}	50.1 ± 5.1^{a,B}	32.0 ± 3.3^{c,B}	45.7 ± 1.3^{b,B}	18.5 ± 5.5^{d,B}
	200 µL/L	0.0 ± 0.0^{e,B}	42.9 ± 3.3^{b,B}	31.1 ± 2.8^{c,C}	46.9 ± 2.8^{a,C}	18.7 ± 4.7^{d,C}	3 µl/Petri	0.0 ± 0.0^{e,B}	38.9 ± 1.8^{a,C}	21.8 ± 2.8^{c,C}	32.2 ± 3.1^{b,C}	7.6 ± 1.4^{d,C}
	300 µL/L	0.0 ± 0.0^{d}	36.2 ± 7.2^{a,C}	30.2 ± 2.3^{b,C}	36.3 ± 4.7^{a,D}	14.5 ± 1.3^{c,D}	5 µl/Petri	0.0 ± 0.0^{d,B}	23.2 ± 1.7^{b,D}	17.0 ± 2.4^{c,D}	24.9 ± 1.9^{a,D}	0.0 ± 0.0^{d,D}
	400 µL/L	0.0 ± 0.0^{e,B}	36.5 ± 4.5^{b,C}	27.6 ± 1.6^{c,D}	44.7 ± 3.6^{a,C}	10.4 ± 1.0^{d,E}	7 µl/Petri	0.0 ± 0.0^{c,B}	16.7 ± 1.4^{a,E}	11.9 ± 0.3^{b,E}	15.8 ± 2.3^{a,E}	0.0 ± 0.0^{c,D}
	500 µL/L	0.0 ± 0.0^{e,B}	28.0 ± 6.0^{a,D}	18.4 ± 1.5^{b,E}	29.2 ± 4.6^{a,E}	8.7 ± 0.6^{c,E}	9 µl/Petri	0.0 ± 0.0^{c,B}	11.2 ± 0.2^{a,F}	8.4 ± 0.3^{b,F}	10.7 ± 0.7^{a,F}	0.0 ± 0.0^{c,D}
4th day	Control	75.5 ± 1.5^{c,A}	74.4 ± 2.5b^{c,A}	78.3 ± 2.8^{b,A}	90.0 ± 0.0^{a,A}	58.2 ± 1.5^{d,A}	Control	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	73.9 ± 4.8^{b,A}
	100 µL/L	0.0 ± 0.0^{e,B}	65.1 ± 5.3^{b,B}	53.6 ± 1.5^{c,B}	68.2 ± 3.4^{a,B}	41.4 ± 1.2^{d,B}	1 µl/Petri	0.0 ± 0.0^{d,B}	64.8 ± 6.1^{a,B}	59.4 ± 4.7^{b,B}	65.7 ± 2.4^{a,B}	29.3 ± 4.9^{c,B}
	200 µL/L	0.0 ± 0.0^{e,B}	59.7 ± 3.4^{b,C}	48.5 ± 3.4^{c,C}	66.3 ± 2.7^{a,B}	30.3 ± 5.9^{d,C}	3 µl/Petri	0.0 ± 0.0^{e,B}	58.1 ± 4.7^{a,C}	44.4 ± 4.3^{c,C}	53.6 ± 2.0^{b,C}	11.0 ± 2.8^{d,C}
	300 µL/L	0.0 ± 0.0^{e,B}	54.4 ± 7.1^{a,D}	47.4 ± 2.0^{b,C}	55.9 ± 6.4^{a,C}	40.5 ± 5.0^{c,B}	5 µl/Petri	0.0 ± 0.0^{d,B}	42.1 ± 3.6^{b,D}	38.6 ± 3.1^{c,D}	48.0 ± 3.3^{a,D}	0.0 ± 0.0^{d,D}
	400 µL/L	0.0 ± 0.0^{e,B}	52.9 ± 6.9^{b,D}	43.0 ± 2.9^{c,D}	57.4 ± 1.3^{a,C}	17.4 ± 4.5^{d,D}	7 µl/Petri	0.0 ± 0.0^{c,B}	35.3 ± 2.6^{a,E}	24.7 ± 0.3^{b,E}	34.0 ± 3.9^{a,E}	0.0 ± 0.0^{c,D}
	500 µL/L	0.0 ± 0.0^{d,B}	46.1 ± 3.0^{a,E}	32.3 ± 3.0^{b,E}	46.4 ± 5.7^{a,D}	11.3 ± 2.4^{c,E}	9 µl/Petri	0.0 ± 0.0^{c,B}	23.1 ± 3.6^{a,F}	15.1 ± 0.8^{b,F}	21.5 ± 2.5^{a,F}	0.0 ± 0.0^{c,D}
5th day	Control	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	69.5 ± 1.2^{b,A}	Control	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	84.5 ± 4.4^{b,A}
	100 µL/L	0.0 ± 0.0^{d,B}	79.0 ± 1.0^{a,B}	64.4 ± 1.6^{b,B}	79.5 ± 1.1^{a,B}	54.1 ± 1.8^{c,B}	1 µl/Petri	0.0 ± 0.0^{c,B}	75.6 ± 3.1^{a,B}	77.3 ± 3.2^{a,B}	76.1 ± 2.3^{a,B}	51.9 ± 8.5^{b,B}
	200 µL/L	0.0 ± 0.0^{d,B}	77.3 ± 2.0^{a,B}	61.0 ± 1.4^{b,C}	76.7 ± 5.5^{a,B,C}	45.6 ± 2.9^{c,C}	3 µl/Petri	0.0 ± 0.0^{d,B}	75.8 ± 2.3^{a,B}	67.8 ± 3.8^{b,C}	70.2 ± 2.6^{b,C}	19.6 ± 5.8^{c,C}
	300 µL/L	0.0 ± 0.0^{e,B}	76.7 ± 2.8^{a,B}	58.0 ± 2.6^{c,D}	71.5 ± 6.1^{b,D}	54.4 ± 3.6^{d,B}	5 µl/Petri	0.0 ± 0.0^{c,B}	65.3 ± 4.1^{a,C}	62.7 ± 4.0^{a,D}	64.9 ± 3.4^{a,D}	3.9 ± 4.2^{b,D}
	400 µL/L	0.0 ± 0.0^{e,B}	67.2 ± 8.4^{b,C}	54.3 ± 2.2^{c,E}	75.4 ± 2.7^{a,C,D}	25.5 ± 8.3^{d,D}	7 µl/Petri	0.0 ± 0.0^{d,B}	58.7 ± 3.8^{a,D}	24.5 ± 0.7^{c,E}	50.5 ± 3.7^{b,E}	0.0 ± 0.0^{d,D}
	500 µL/L	0.0 ± 0.0^{d,B}	67.4 ± 7.3^{a,C}	51.4 ± 1.5^{b,F}	62.8 ± 5.5^{a,E}	23.0 ± 7.2^{c,D}	9 µl/Petri	0.0 ± 0.0^{d,B}	45.8 ± 5.5^{a,E}	19.9 ± 1.6^{c,F}	34.3 ± 3.6^{b,F}	0.0 ± 0.0^{d,D}
6th day	Control	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	Control	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}	90.0 ± 0.0^{a,A}
	100 µL/L	0.0 ± 0.0^{d,B}	90.0 ± 0.0^{a,A}	77.5 ± 0.8^{b,C}	90.0 ± 0.0^{a,A}	63.2 ± 4.1^{c,B}	1 µl/Petri	0.0 ± 0.0^{d,B}	80.2 ± 4.2^{b,B}	90.0 ± 0.0^{a,B}	90.0 ± 0.0^{a,B}	65.7 ± 5.7^{c,B}
	200 µL/L	0.0 ± 0.0^{d,B}	90.0 ± 0.0^{a,A}	72.8 ± 2.6^{b,C}	90.0 ± 0.0^{a,A}	57.7 ± 5.1^{c,C}	3 µl/Petri	0.0 ± 0.0^{d,B}	81.5 ± 3.6^{b,B}	80.4 ± 3.9^{b,C}	90.0 ± 0.0^{a,C}	30.5 ± 7.3^{c,C}
	300 µL/L	0.0 ± 0.0^{e,B}	79.6 ± 1.6^{b,B}	71.0 ± 1.6^{c,C}	83.6 ± 5.5^{a,C}	60.2 ± 2.1^{d,B,C}	5 µl/Petri	0.0 ± 0.0^{d,B}	74.6 ± 7.1^{b,C}	79.3 ± 1.5^{a,D}	78.8 ± 1.6^{a,D}	5.2 ± 5.7^{c,D}
	400 µL/L	0.0 ± 0.0^{d,B}	67.2 ± 9.7^{b,C}	67.1 ± 3.5^{b,D}	87.0 ± 3.6^{a,B}	33.7 ± 5.5^{c,D}	7 µl/Petri	0.0 ± 0.0^{d,B}	74.5 ± 3.9^{a,D}	52.8 ± 6.0^{c,E}	69.9 ± 2.2^{b,E}	0.0 ± 0.0^{d,D}
	500 µL/L	0.0 ± 0.0^{d,B}	64.6 ± 4.8^{b,C}	63.0 ± 1.2^{b,E}	77.0 ± 2.6^{a,D}	27.1 ± 5.7^{c,E}	9 µl/Petri	0.0 ± 0.0^{d,B}	65.2 ± 5.4^{a,E}	34.3 ± 1.2^{c,F}	55.4 ± 3.9^{b,F}	0.0 ± 0.0^{d,D}

$^{a-f}$: Within each row, different superscript lowercase letters show differences between the essential oil types within each concentration ($p < 0.05$).
$^{A-F}$: Within each column, different superscript uppercase letters show differences between the essential oil concentrations within each storage period ($p < 0.05$).

tration of 100 µl/L gave approximately 100 % inhibition. Increasing the concentration of other EOs resulted in weaker growth and 500 µl/L concentration exhibited roughly from 30 % up to 85 % inhibition. Rosemary exhibited the weakest antifungal effect among the tested EOs. The growth of test microorganisms was promoted as the

TABLE 3: *Contact and fumigation bioassays to determine inhibitory effects of essential oil type and level on in vitro mycelial growth of P. expansum at different incubation times (n = 9).*

	Mycelial growth diameter (mm)											
	Contact bioassay					Fumigation bioassay					
	EO level	EO type Oregano	Fennel	Sage	Rosemary	Eucalyptus	EO level	EO type Oregano	Fennel	Sage	Rosemary	Eucalyptus
3rd day	Control	18.3 ± 1.0^{a,A}	14.5 ± 0.9^{b,A}	14.0 ± 1.0^{bc,A}	13.3 ± 0.4^{c,A}	14.7 ± 1.5^{b,A}	Control	15.1 ± 1.2^{b,A}	16.9 ± 0.5^{a,A}	14.0 ± 1.0^{c,A}	16.1 ± 0.9^{a,A}	15.1 ± 0.9^{b,A}
	100 µL/L	0.0 ± 0.0^{d,B}	11.9 ± 0.6^{b,B}	11.4 ± 1.3^{b,B}	13.7 ± 1.0^{a,A}	9.4 ± 0.9^{c,C}	1 µl/Petri	0.0 ± 0.0^{c,B}	14.7 ± 1.7^{a,B}	11.1 ± 1.0^{b,B}	15.0 ± 0.7^{a,B}	11.1 ± 0.8^{b,B}
	200 µL/L	0.0 ± 0.0^{c,B}	11.0 ± 0.5^{ab,BC}	10.4 ± 1.2^{b,B}	11.5 ± 0.9^{a,B}	10.4 ± 1.1^{b,B}	3 µl/Petri	0.0 ± 0.0^{d,B}	13.0 ± 0.9^{a,C}	10.4 ± 1.2^{b,B}	12.8 ± 0.6^{a,C}	8.9 ± 1.2^{c,C}
	300 µL/L	0.0 ± 0.0^{d,B}	10.7 ± 0.8^{a,BC}	9.9 ± 0.6^{a,B}	10.4 ± 1.2^{ab,C}	9.0 ± 0.7^{c,C}	5 µl/Petri	0.0 ± 0.0^{d,B}	10.2 ± 0.5^{b,D}	9.9 ± 0.5^{b,B}	10.7 ± 0.5^{a,D}	8.2 ± 0.8^{c,C}
	400 µL/L	0.0 ± 0.0^{b,B}	9.8 ± 0.6^{a,C}	9.5 ± 1.4^{a,B}	9.7 ± 0.5^{a,C}	0.0 ± 0.0^{b}	7 µl/Petri	0.0 ± 0.0^{d,B}	8.4 ± 0.3^{c,E}	9.5 ± 1.4^{b,B}	10.3 ± 0.3^{a,D}	0.0 ± 0.0^{d,D}
	500 µL/L	0.0 ± 0.0^{d,B}	7.7 ± 3.0^{b,D}	3.5 ± 4.2^{c,C}	10.5 ± 0.8^{a,C}	0.0 ± 0.0^{d,D}	9 µl/Petri	0.0 ± 0.0^{c,B}	7.7 ± 0.2^{a,E}	3.5 ± 4.2^{b,B}	9.1 ± 0.7^{a,E}	0.0 ± 0.0^{c,D}
4th day	Control	21.0 ± 0,9^{a,A}	17.2 ± 0.8^{b,A}	16.3 ± 1.0^{b,A}	16.1 ± 0.5^{b,A}	20.3 ± 2.3^{a,A}	Control	18.4 ± 1.3^{b,A}	20.0 ± 1.2^{a,A}	16.3 ± 1.0^{d,A}	17.6 ± 0.5b^{c,A}	17.0 ± 0.7^{cd,A}
	100 µL/L	0.0 ± 0.0^{d,B}	14.5 ± 0.9^{b,B}	13.6 ± 1.5^{bbc,B}	16.3 ± 1.0^{a,A}	12.8 ± 1.5^{c,B}	1 µl/Petri	1.2 ± 3.3^{c,B}	17.2 ± 1.8^{a,B}	13.6 ± 1.5^{b,B}	16.5 ± 0.8^{a,B}	13.1 ± 0.6^{b,B}
	200 µL/L	0.0 ± 0.0^{d,B}	13.4 ± 0.7^{b,C}	12.5 ± 1.3^{c,BC}	14.4 ± 0.9^{a,B}	12.0 ± 1.0^{c,BC}	3 µl/Petri	0.0 ± 0.0^{e,B}	15.2 ± 1.2^{a,C}	12.5 ± 1.3^{c,BC}	14.0 ± 0.8^{b,C}	11.2 ± 0.8^{d,C}
	300 µL/L	0.0 ± 0.0^{c,B}	12.9 ± 1.2^{a,C}	11.3 ± 0.7^{b,C}	13.4 ± 1.6^{a,C}	10.8 ± 1.3^{b,C}	5 µl/Petri	0.0 ± 0.0^{e,B}	12.6 ± 0.5^{a,D}	11.3 ± 0.7^{c,C}	11.9 ± 0.5^{b,D}	10.0 ± 1.0^{d,D}
	400 µL/L	0.0 ± 0.0^{c,B}	12.0 ± 0.7^{a,D}	11.3 ± 1.7^{a,C}	12.1 ± 0.9^{a,D}	9.9 ± 0.9^{b,C}	7 µl/Petri	0.0 ± 0.0^{c,B}	10.9 ± 0.5^{a,E}	11.3 ± 1.7^{a,C}	11.4 ± 0.3^{a,D}	7.9 ± 0.5^{b,E}
	500 µL/L	0.0 ± 0.0^{c,B}	10.4 ± 1.0^{a,E}	5.9 ± 4.6^{b,D}	12.8 ± 0.9^{a,CD}	6.1 ± 4.8^{b,D}	9 µl/Petri	0.0 ± 0.0^{c,B}	9.7 ± 0.2^{a,F}	5.9 ± 4.6^{b,D}	10.2 ± 0.8^{a,E}	7.2 ± 0.7^{b,E}
5th day	Control	23.5 ± 0.8^{a,A}	18.7 ± 0.8^{b,A}	17.2 ± 1.2^{b,A}	18.3 ± 1.4^{a,A}	22.8 ± 2.3^{b,A}	Control	20.5 ± 1.0^{b,A}	21.9 ± 1.0^{a,A}	17.4 ± 1.2^{d,A}	18.9 ± 0.4^{c,A}	18.8 ± 1.1^{c,A}
	100 µL/L	0.0 ± 0.0^{c,B}	15.7 ± 1.8^{a,B}	15.2 ± 2.0^{a,B}	19.3 ± 0.7a	15.2 ± 1.5^{b,B}	1 µl/Petri	1.2 ± 3.5^{c,B}	19.1 ± 1.9^{a,B}	15.2 ± 2.0^{b,B}	18.2 ± 1.0^{a,A}	14.3 ± 0.9^{b,B}
	200 µL/L	0.0 ± 0.0^{d,B}	15.0 ± 0.6^{a,BC}	13.8 ± 1.4^{b,BC}	16.8 ± 1.0^{b}	14.5 ± 0.8^{c,B}	3 µl/Petri	0.0 ± 0.0^{e,B}	17.1 ± 1.1^{a,C}	13.8 ± 1.4^{c,BC}	15.5 ± 1.0^{a,B}	12.3 ± 0.8^{d,C}
	300 µL/L	0.0 ± 0.0^{c,B}	14.0 ± 1.1^{a,CD}	12.5 ± 0.8^{a,CD}	15.9 ± 1.9^{a,BC}	12.6 ± 1.9^{b,C}	5 µl/Petri	0.0 ± 0.0^{d,B}	14.2 ± 0.7^{a,D}	12.5 ± 0.9^{b,C}	13.1 ± 0.5^{b,C}	10.2 ± 1.0^{c,D}
	400 µL/L	0.0 ± 0.0^{d,B}	13.3 ± 0.8^{a,D}	11.5 ± 1.4^{c,D}	14.4 ± 1.2^{b,CD}	12.1 ± 0.8^{d,C}	7 µl/Petri	0.0 ± 0.0^{c,B}	12.4 ± 0.7^{a,E}	12.2 ± 1.9^{a,C}	12.4 ± 0.6^{a,C}	9.1 ± 0.7^{b,E}
	500 µL/L	0.0 ± 0.0^{d,B}	11.3 ± 1.4^{a,E}	8.3 ± 3.3^{c,E}	15.0 ± 0.9^{b,D}	9.6 ± 2.0^{d,D}	9 µl/Petri	0.0 ± 0.0^{c,B}	11.4 ± 0.4^{a,E}	8.3 ± 3.3^{b,D}	11.5 ± 0.9^{a,D}	8.5 ± 0.8^{b,E}
6th day	Control	26.2 ± 1.0^{a,A}	21.9 ± 0.6^{a,A}	20.0 ± 1.7^{a,A}	20.8 ± 1.3^{a,A}	25.2 ± 2.5^{a,A}	Control	24.3 ± 1.2^{a,A}	23.3 ± 1.2^{a,A}	20.0 ± 1.7^{b,}	20.6 ± 1.0^{b,A}	20.9 ± 1.7^{b,A}
	100 µL/L	0.0 ± 0.0^{d,B}	19.4 ± 0.7^{b,B}	17.8 ± 2.2^{a,B}	21.5 ± 0.6^{a,A}	17.8 ± 1.5^{c,B}	1 µl/Petri	1.2 ± 3.7^{c,B}	20.5 ± 1.5^{a,B}	17.8 ± 2.2^{b,B}	20.3 ± 1.3^{a,A}	16.1 ± 0.8^{b,B}
	200 µL/L	0.0 ± 0.0^{d,B}	17.7 ± 1.5^{b,C}	16.6 ± 1.5^{b,B}	16.6 ± 1.0^{a,B}	16.8 ± 1.2^{c,B}	3 µl/Petri	0.0 ± 0.0^{d,B}	18.1 ± 1.2^{a,C}	16.6 ± 1.5^{b,B}	17.0 ± 1.3^{b,B}	13.9 ± 0.8^{c,C}
	300 µL/L	0.0 ± 0.0^{d,B}	17.4 ± 1.3^{b,C}	14.9 ± 0.8^{a,C}	18.3 ± 2.0^{a,B}	14.5 ± 1.7^{c,C}	5 µl/Petri	0.0 ± 0.0^{d,B}	15.4 ± 0.5^{a,D}	14.9 ± 0.8^{ab,C}	14.3 ± 0.4^{b,C}	13.0 ± 1.3^{c,C}
	400 µL/L	0.0 ± 0.0^{d,B}	16.2 ± 0.8^{a,D}	14.2 ± 1.6^{c,C}	16.3 ± 1.7^{b,C}	14.3 ± 0.8^{d,CD}	7 µl/Petri	0.0 ± 0.0^{c,B}	13.5 ± 0.6^{a,E}	14.2 ± 1.6^{a,C}	13.5 ± 0.9^{a,CD}	10.2 ± 0.8^{b,D}
	500 µL/L	0.0 ± 0.0^{d,B}	13.5 ± 2.0^{a,E}	10.4 ± 1.7^{c,D}	16.8 ± 0.9^{b,C}	12.6 ± 3.1^{d,D}	9 µl/Petri	0.0 ± 0.0^{c,B}	12.7 ± 0.7^{a,E}	10.4 ± 1.7^{b,D}	12.6 ± 1.1^{a,D}	9.8 ± 0.5^{b,D}

$^{a-e}$: Within each row, different superscript lowercase letters show differences between the essential oil types within each concentration ($p < 0.05$).
$^{A-F}$: Within each column, different superscript uppercase letters show differences between the essential oil concentrations within each storage period ($p < 0.05$).

TABLE 4: *Contact and fumigation bioassays to determine inhibitory effects of essential oil type and level on in vitro mycelial growth of C. gloeosporioides at different incubation times (n = 9).*

	Mycelial growth diameter (mm)											
	Contact bioassay						Fumigation bioassay					
	EO	EO type					EO	EO type				
	level	Oregano	Fennel	Sage	Rosemary	Eucalyptus	level	Oregano	Fennel	Sage	Rosemary	Eucalyptus
3rd day	Control	34.5 ± 1.3^{ab,A}	34.7 ± 1.7^{a,A}	33.4 ± 0.6^{b,A}	33.4 ± 1.0^{b,A}	33.8 ± 1.3^{ab,A}	Control	37.2 ± 2.1^{a,A}	33.9 ± 0.9^{b,A}	30.4 ± 3.1^{c,A}	36.3 ± 1.5^{a,A}	37.4 ± 1.8^{a,A}
	100 µL/L	0.0 ± 0.0^{e,B}	22.8 ± 1.7^{d,B}	26.7 ± 1.6^{b,B}	25.1 ± 1.6^{c,B}	28.4 ± 1.2^{a,B}	1 µl/Petri	0.0 ± 0.0^{c,B}	29.6 ± 0.8^{a,B}	27.1 ± 1.9^{ab,B}	29.2 ± 5.1^{a,B}	26.3 ± 3.0^{b,B}
	200 µL/L	0.0 ± 0.0^{c,B}	22.0 ± 0.9^{b,B}	25.3 ± 2.2^{a,B}	25.8 ± 1.3^{a,B}	25.6 ± 1.4^{a,C}	3 µl/Petri	0.0 ± 0.0^{e,B}	25.0 ± 0.9^{b,C}	21.0 ± 2.5^{d,C}	28.7 ± 1.5^{a,B}	23.5 ± 0.9^{c,C}
	300 µL/L	0.0 ± 0.0^{d,B}	19.7 ± 2.1^{c,C}	21.3 ± 1.5^{b,CD}	23.0 ± 1.3^{a,C}	23.9 ± 1.2^{a,D}	5 µl/Petri	0.0 ± 0.0^{e,B}	23.0 ± 1.3^{b,D}	16.2 ± 3.1^{d,D}	26.3 ± 1.6^{a,C}	20.1 ± 1.9^{c,D}
	400 µL/L	0.0 ± 0.0^{c,B}	19.6 ± 1.5^{b,C}	22.7 ± 1.2^{a,C}	23.4 ± 1.2^{a,C}	23.5 ± 1.0^{a,D}	7 µl/Petri	0.0 ± 0.0^{d,B}	21.3 ± 0.8^{b,E}	11.6 ± 2.8^{c,E}	23.0 ± 1.5^{a,D}	12.4 ± 1.5^{c,E}
	500 µL/L	0.0 ± 0.0^{d,B}	16.6 ± 1.2^{bc,D}	20.4 ± 1.6^{b,D}	22.6 ± 1.4^{a,C}	21.3 ± 1.4^{b,E}	9 µl/Petri	0.0 ± 0.0^{e,B}	19.9 ± 1.0^{a,F}	4.8 ± 4.5^{d,F}	16.4 ± 1.4^{b,E}	11.0 ± 2.2^{c,E}
4th day	Control	44.5 ± 1.2^{ab,A}	43.7 ± 1.8^{b,A}	45.2 ± 1.3^{a,A}	45.6 ± 1.4^{a,A}	45.1 ± 0.9^{a,A}	Control	50.7 ± 2.4^{a,A}	47.8 ± 1.5^{b,A}	38.0 ± 4.7^{c,A}	49.3 ± 2.3^{ab,A}	49.3 ± 2.8a^{b,A}
	100 µL/L	0.0 ± 0.0^{c,B}	30.9 ± 3.4^{b,B}	37.3 ± 1.9^{a,B}	35.5 ± 2.0^{a,B}	36.8 ± 1.5^{a,B}	1 µl/Petri	0.0 ± 0.0^{c,B}	41.6 ± 1.1^{a,B}	36.9 ± 2.8^{b,A}	42.1 ± 2.1^{a,B}	36.0 ± 3.2^{b,B}
	200 µL/L	0.0 ± 0.0^{d,B}	30.3 ± 1.4^{c,B}	34.7 ± 1.4^{ab,C}	35.7 ± 1.5^{a,B}	34.2 ± 2.2^{b,C}	3 µl/Petri	0.0 ± 0.0^{e,B}	37.0 ± 1.2^{b,C}	28.3 ± 2.2^{d,B}	40.5 ± 1.6^{a,B}	32.8 ± 1.1^{c,C}
	300 µL/L	0.0 ± 0.0^{d,B}	26.7 ± 2.9^{c,C}	30.2 ± 2.4^{b,E}	32.4 ± 1.9^{a,C}	32.1 ± 1.8^{ab,D}	5 µl/Petri	0.0 ± 0.0^{e,B}	34.5 ± 2.0^{b,D}	24.9 ± 4.1^{d,C}	37.1 ± 1.9^{a,C}	27.5 ± 2.0^{c,D}
	400 µL/L	0.0 ± 0.0^{c,b,A}	26.8 ± 2.2^{b,C}	32.3 ± 1.5^{a,D}	32.4 ± 2.5^{a,C}	31.4 ± 1.1^{a,D}	7 µl/Petri	0.0 ± 0.0^{c,B}	32.0 ± 1.3^{a,E}	18.1 ± 3.3^{b,D}	33.5 ± 1.8^{a,D}	19.1 ± 2.0^{b,E}
	500 µL/L	0.0 ± 0.0^{d,B}	22.8 ± 1.4^{c,D}	29.2 ± 2.3^{b,E}	31.4 ± 1.6^{a,C}	28.9 ± 1.8^{b,E}	9 µl/Petri	0.0 ± 0.0^{e,B}	30.5 ± 1.5^{a,F}	11.7 ± 2.1^{d,E}	25.7 ± 2.6^{b,E}	16.8 ± 2.6^{c,F}
5th day	Control	56.2 ± 2.5^{a,A}	56.4 ± 1.6^{a,A}	54.8 ± 2.2^{a,A}	55.1 ± 2.0^{a,A}	55.0 ± 1.8^{a,A}	Control	63.0 ± 3.0^{a,A}	61.5 ± 2.1^{a,A}	46.0 ± 6.3^{b,A}	59.4 ± 3.5^{a,A}	59.3 ± 4.0^{a,A}
	100 µL/L	0.0 ± 0.0^{b,B}	43.2 ± 3.9^{a,B}	45.4 ± 2.2^{a,B}	43.5 ± 2.8^{a,B}	45.2 ± 3.5^{a,B}	1 µl/Petri	0.0 ± 0.0^{d,B}	53.1 ± 1.9^{a,B}	47.2 ± 3.3^{b,A}	51.4 ± 2.3^{a,B}	43.6 ± 2.5^{c,B}
	200 µL/L	0.0 ± 0.0^{c,B}	42.4 ± 2.1^{ab,B}	43.1 ± 1.6^{ab,C}	42.1 ± 2.0^{b,BC}	44.1 ± 2.2^{a,B}	3 µl/Petri	0.0 ± 0.0^{d,B}	48.6 ± 1.6^{a,C}	37.5 ± 2.9^{c,B}	49.3 ± 1.9^{a,B}	41.7 ± 1.4^{b,B}
	300 µL/L	0.0 ± 0.0^{c,B}	34.7 ± 4.0^{b,C}	37.9 ± 2.8^{a,E}	39.9 ± 2.2^{a,CD}	39.6 ± 2.0^{a,C}	5 µl/Petri	0.0 ± 0.0^{c,B}	45.5 ± 2.4^{a,D}	34.7 ± 4.8^{b,B}	46.0 ± 2.0^{a,C}	36.5 ± 3.3^{b,C}
	400 µL/L	0.0 ± 0.0^{c,B}	34.4 ± 2.3^{b,C}	40.3 ± 2.5^{a,D}	39.8 ± 4.3^{a,CD}	37.7 ± 3.2^{a,C}	7 µl/Petri	0.0 ± 0.0^{c,B}	41.5 ± 2.2^{a,E}	25.8 ± 2.2^{b,C}	42.2 ± 2.0^{a,D}	26.4 ± 2.5^{b,D}
	500 µL/L	0.0 ± 0.0^{d,B}	32.4 ± 2.3^{c,C}	36.5 ± 3.0^{ab,E}	38.4 ± 1.6^{a,D}	36.3 ± 2.0^{b,E}	9 µl/Petri	0.0 ± 0.0^{e,B}	40.4 ± 1.1^{a,E}	17.8 ± 3.2^{d,D}	34.3 ± 2.6^{b,E}	23.6 ± 3.6^{c,D}
6th day	Control	66.7 ± 1.9^{ab,A}	67.9 ± 1.5^{a,A}	66.4 ± 1.6^{ab,A}	65.7 ± 2.0^{b,A}	65.8 ± 2.0^{b,A}	Control	74.1 ± 3.0^{a,A}	71.4 ± 2.7^{a,A}	51.6 ± 7.1^{b,A}	72.6 ± 4.8^{a,A}	72.7 ± 5.9^{a,A}
	100 µL/L	0.0 ± 0.0^{c,B}	52.5 ± 8.0^{ab,BC}	56.0 ± 2.3^{a,B}	53.5 ± 2.9^{ab,B}	50.9 ± 8.0^{b,B}	1 µl/Petri	0.0 ± 0.0^{d,B}	61.4 ± 2.7^{a,B}	45.8 ± 5.1^{c,B}	61.2 ± 2.3^{a,B}	54.8 ± 3.6^{b,B}
	200 µL/L	0.0 ± 0.0^{c,B}	53.8 ± 2.4^{a,B}	52.7 ± 1.4^{ab,C}	50.2 ± 4.8^{b,C}	54.8 ± 2.9^{a,B}	3 µl/Petri	0.0 ± 0.0^{d,B}	58.1 ± 4.7^{a,C}	46.1 ± 5.0^{c,B}	58.8 ± 2.6^{a,B}	52.8 ± 2.1^{b,B}
	300 µL/L	0.0 ± 0.0^{c,B}	48.4 ± 3.7^{a,CD}	47.2 ± 2.9^{a,DE}	48.9 ± 1.7^{a,C}	43.5 ± 5.4^{b,C}	5 µl/Petri	0.0 ± 0.0^{d,B}	53.2 ± 3.4^{a,D}	43.1 ± 5.2^{c,B}	54.2 ± 1.1^{a,C}	47.0 ± 4.1^{b,C}
	400 µL/L	0.0 ± 0.0^{d,B}	46.2 ± 4.2^{ab,DE}	49.1 ± 3.5^{a,D}	43.3 ± 4.0^{bc,D}	41.6 ± 5.0^{c,CD}	7 µl/Petri	0.0 ± 0.0^{e,B}	48.0 ± 3.4^{b,E}	33.0 ± 4.0^{d,C}	52.2 ± 3.1^{a,C}	38.2 ± 3.2^{c,D}
	500 µL/L	0.0 ± 0.0^{d,B}	42.2 ± 3.4^{ab,E}	45.0 ± 5.1^{a,E}	41.6 ± 2.6^{bc,D}	38.8 ± 3.4^{c,D}	9 µl/Petri	0.0 ± 0.0^{e,B}	48.1 ± 2.1^{a,E}	22.6 ± 4.5^{d,D}	45.0 ± 2.8^{b,D}	34.0 ± 4.2^{c,E}

$^{a-e}$: Within each row, different superscript lowercase letters show differences between the essential oil types within each concentration ($p < 0.05$).
$^{A-F}$: Within each column, different superscript uppercase letters show differences between the essential oil concentrations within each storage period ($p < 0.05$).

incubation time increased. From the data presented in Tables 2, 3 and 4, it can be stated that *B. cinerea* could grow well on Petries and hence showed stronger resistance to EOs than *C. gloesporioides* and *C. gloesporioides* showed stronger resistance than *P. expansum.*

In fumigation bioassay some of EOs exhibited stronger antifungal activities against the test fungus. This result might be due to the high voltality of phenolic compounds that are found in selected plant EOs in high amounts. Complete inhibition of mycellial growth was observed at 100 µL/L concentration of oregano oil in contact bioassay and 1 µL oregano/petri in fumigation bioassay for all the test fungi studied while 5–7 µL eucalyptus/petri was needed for 100 % inactivation of *B. cinerea* in fumigation bioassay. EOs other than oregano did not show complete inhibition of *P. expansum* and *C. gloesporioides* in both bioassays applied. The test fungi grew rapidly as expected in control plates and significantly less rapidly, except for rosemary, sage and fennel, in those containing oregano EO and to some extent, in the plates containing eucalyptus EO (Figures 1, 2 and 3). Application of rosemary, sage and fennel EOs resulted in slight inhibition of mycellial growth of *B. cinerea, C. gloesporioides* and *P. expansum* in fumigation bioassay and in contact bioassay at specified concentrations. Beside this, in fumigation bioassay tests, the size of the colonies were smaller when compared to that in contact bioassay tests at the same concentrations of EOs used. This demonstrated that the volatility of phenolic compounds acted more effectively on inhibition of the growth of fungi.

Some researchers studied the antifungal effects of thyme oil against *Alternaria alternata* (Wu et al., 2011), *C. gloeosporioides* (Lee et al, 2007) and *B. cinerea* (Reddy et al., 1998). Camele et al (2012) studied the antifungal effects of different plant essential oil components such as carvacrol, cymene and γ-terpinene against selected fungus including *B. cinerea.* They have found that carvacrol at a concentration of 250 ppm had the strongest inhibition against *B. cinerea.* Their finding is in agreement with the results of this study since GC/MS analysis showed that oregano, which showed strongest antifungal activity in this study, had around 80 % carvacrol. According to Camele et al (2012) o-cymene and γ-terpinene showed moderate inhibitory action on mycellial growth against *B. cinerea* and other fungi species. This finding also agrees with the results obtained in this study since eucalyptus, which showed weaker antifungal action, had around 25 % cymene. Some researchers assayed γ-terpinene *in vitro* in gaseous state and they reported that γ-terpinene exhibited strong inhibitory activity against *B. cinerea* (Espinosa-Garcia and Langenheim, 1991). Another EO component, camphene was reported for its antifungal activity against fungi species causing desease in fruits (Pitarokili et al, 2008). In another study it was revealed that carvacrol and thymol at 100 ppm, completely inhibited mycelial growth of several phytopathogenic fungi (Kordali et al, 2008). The strong inhibition effect of carvacrol and thymol against *B. cinerea* was also reported (Bouchra et al, 2003; Tsao and Zhou, 2000). Znini et al (2013) have demonstrated the antifungal effects of *W. saharae* essential oil against *Alternaria* sp. and *P. expansum.* They reported that both fungi species are susceptible to the *W. saharae* essential oil with the EC_{50} (concentration causing 50 % inhibition of mycelial growth on control media) 0.22 and 0.27 µL/mL, respectively. At 2 µL/mL concentration, the % inhibition was found to be 84.44 and 82.00 % for *Alternaria* sp. and *P. expansum,* respectively.

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The concentration of EOs needed to inhibit the growth in fumigation bioassay were found to be much lower than that of EOs in contact bioassay. This means that volatile phases of the essential oils are more toxic than the contact phase to the test fungi. It has been reported that the antifungal activity of EOs are resulted from a direct effect of EO in vapour form on fungal mycelium (Soylu et al, 2010). They also stated that the lipophilic compounds of vaporized EOs were absorbed by fungal mycelia better than the ones diluted in semi-solid agar due to the high lipophilic nature of the fungal mycelia and the high water content of the agar media.

3.3 *In vivo* experimentation

The results obtained from *in vivo* experiments indicated that oregano and eucalyptus EOs had noticable inhibitory effects on mycelial growth of *B. cinerea, C. gloesporioides* and *P. expansum* when tested on apple samples (Tab. 5). Figure 4 shows that oregano EO exhibited the strongest antifungal activity against test microorganisms as it almost inhibited the mycelial growth of *B. cinerea* and *P. expansum* at concentrations of 3 to 5 % after 6 days of incubation. Even though the growth of *C. gloeosporioides* has not been fully inhibited by oregano EO at concentration of up to 5 %, approximately 50 % inhibition of micellial growth was

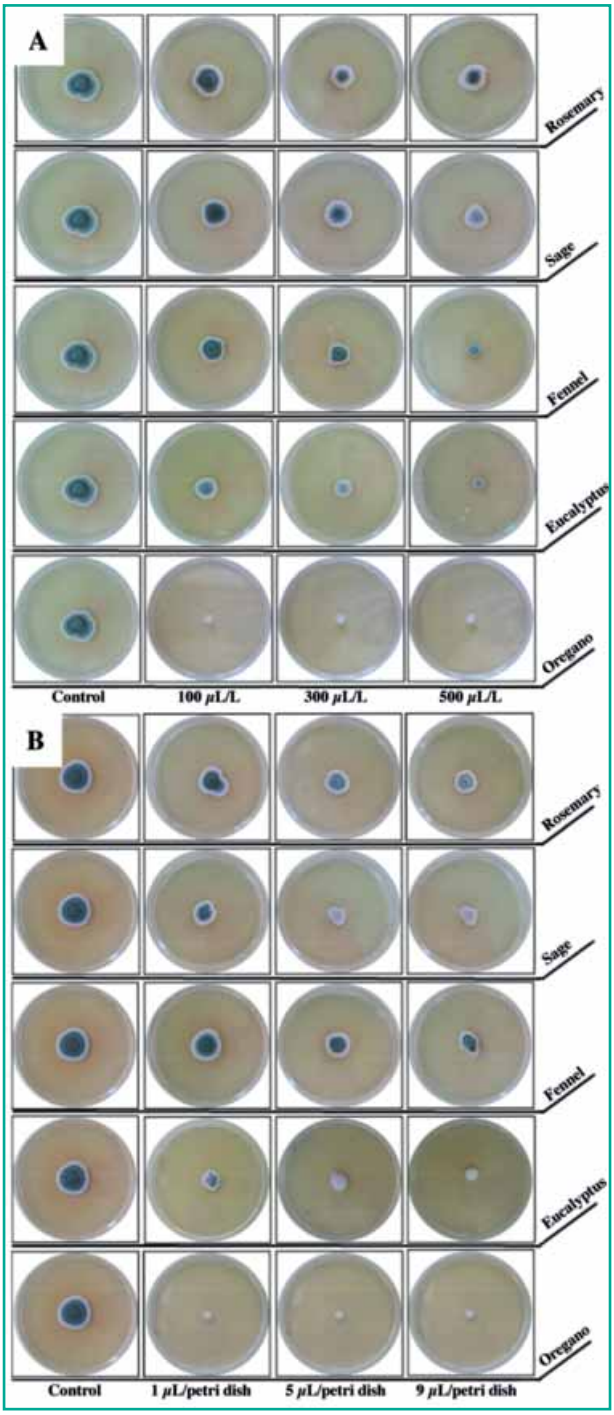

FIGURE 1: *Inhibitory effect of different essential oils against P. expansum. A-contact bioassay, B-fumigation bioassay.*

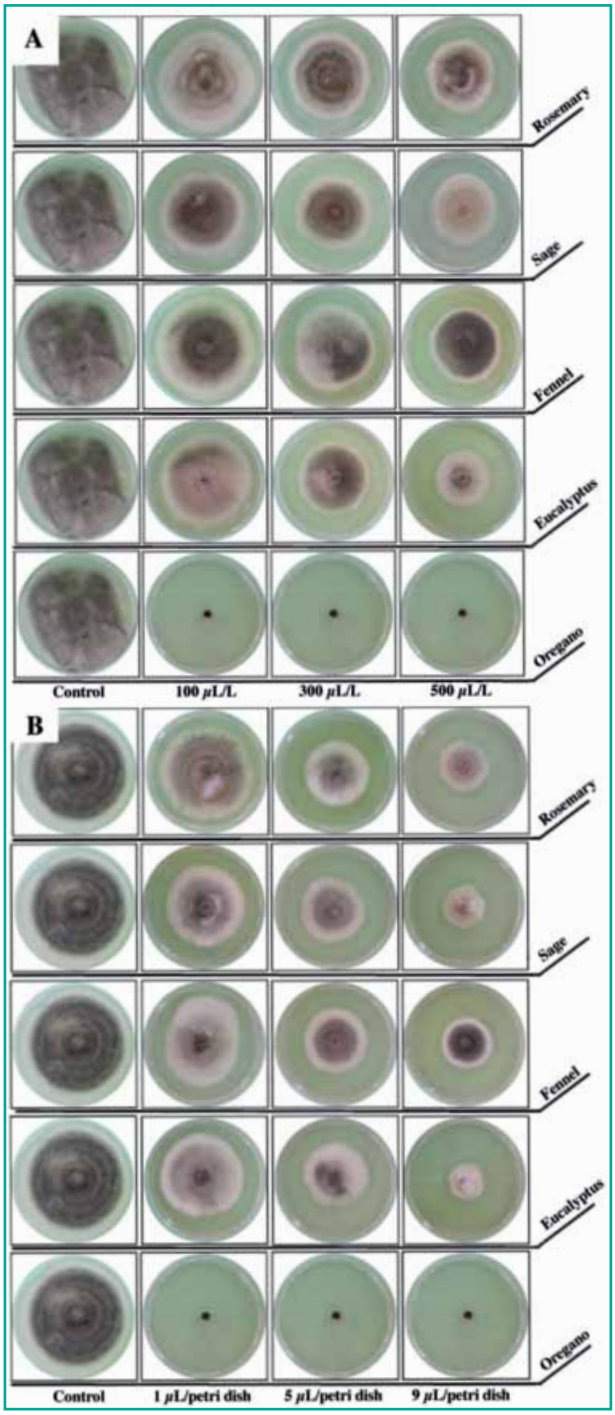

FIGURE 2: *Inhibitory effect of different essential oils against C. gloeosporioides. A-contact bioassay, B-fumigation bioassay.*

TABLE 5: *Inhibitory effects of essential oils on lesion development by B. cinerea, C. gloesporioides and P. expansum on apples (n = 160 for essential oil type and n = 80 for essential oil treatments).*

	Lesion diameter (mm)					
	B. cinerea		*P. expansum*		*C. gloeosporioides*	
EO level	Eucaliptus	Oregano	Eucaliptus	Oregano	Eucaliptus	Oregano
Control	48.2 ± 1.6	28.6 ± 2.6	39.1 ± 3.6	23.8 ± 2.5	34.7 ± 2.2	20.2 ± 1.7
1 %	21.5 ± 1.2	13.8 ± 2.1	34.6 ± 1.5	18.9 ± 1.4	26.4 ± 1.8	16.8 ± 1.9
3 %	6.8 ± 0.6	4.3 ± 1.7	25.1 ± 0.6	14.6 ± 1.6	18.1 ± 1.3	10.5 ± 0.8
5 %	5.2 ± 0.8	3.1 ± 0.7	18.7 ± 0.5	11.3 ±0.8	8.9 ± 1.0	5.4 ± 0.9

observed. Eucalyptus EO showed weaker inhibitory effect on fungal growth comparing to oregano EO (Fig. 4) after 6 days of incubation. At concentration of 5 %, it showed the inhibition of micellial growth for *B. cinerea, P. expansum* and *C. gloeosporioides* as approximately 50 %, 13 % and 23 % respectively.

Gebel and Magurno (2014) reported that thyme oil (consisted 20–25 % p-cymene) delayed the growth of *B. cinerea* in strawberry fruits. According to their findings, the infection by *B. cinerea* started to grow 8 days after inoculation at 500 µl/mL (50 %) concentration. They also noted that 13 days after the inoculation, the strawberries were still in good condition. In this study, oregano EO could almost stop the growth of *B. cinerea* at 1 % concentration and eucalyptus EO showed the same effect at 5 % concentration after 6 days of incubation (Fig. 4). Gebel and Magurno (2014) also added that 200 l/mL (20%) concentration was not effective against *B. cinerea.* Based on these data, oregano and eucalyptus EOs are more effective against *B. cinerea* than thyme EO. This statement agrees with the findings of Soylu et al (2010). They found that oregano EO has stronger antifungal effects against *B. cinerea* than lavender and rosemary EOs. Ibrahim and Al-Ebady (2014) tested antifungal activity of oregano essential oil against test fungi on tomatoes and they reported that oregano oil showed a very strong inhibitory effect on mycellial growth. They found out that the species-depen-

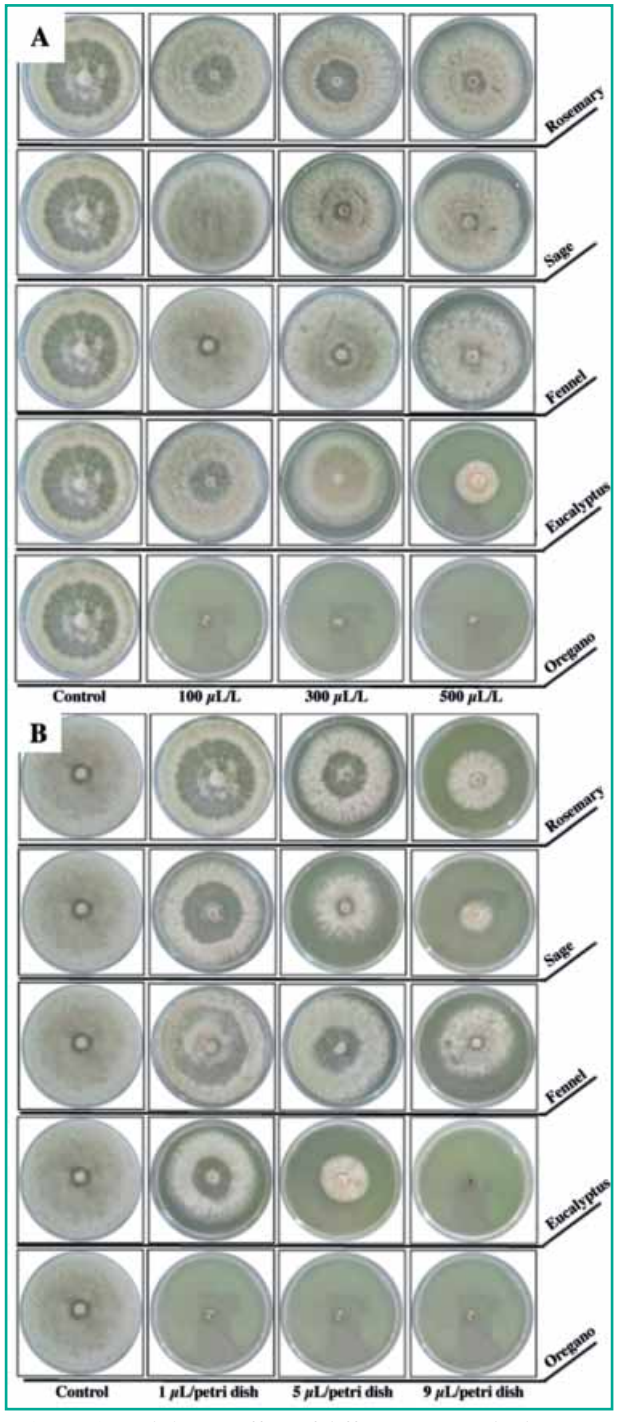

FIGURE 3: *Inhibitory effect of different essential oils against B. cinerea. A-contact bioassay, B-fumigation bioassay.*

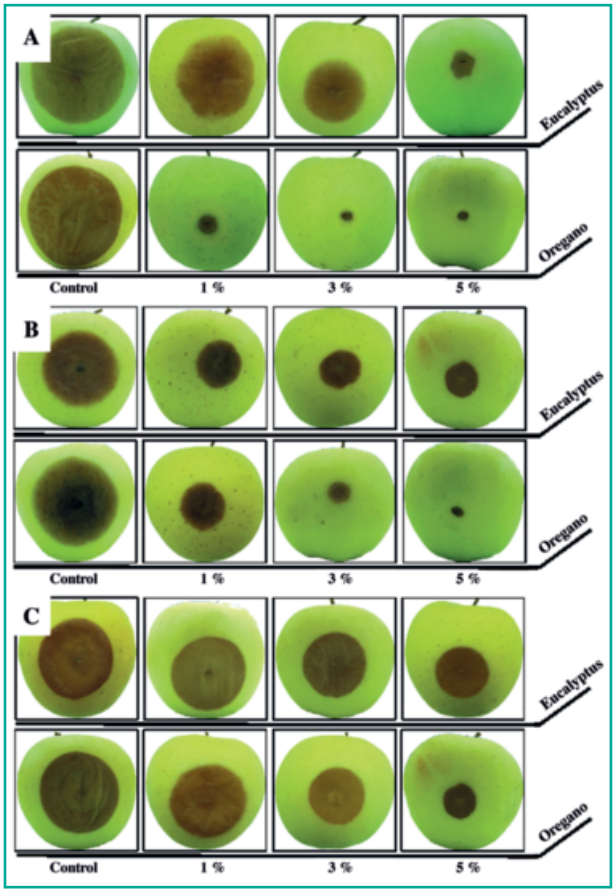

FIGURE 4: *Antifungal effects of oregano and eucalyptus EOs on golden delicious apples against B. cinerea (A), P. expansum (B) and C. gloeosporioides (C) stored at 23 °C for 6 days*

dent minimum inhibitory concentration for *P.* spp. was 4.5 mg/mL (0,45%). In this study, the concentration of oregano EO required to inhibit the growth of *P. expansum* was found to be from 3 to 5 % and the difference in required concentrations might be attributed to the variations in the chemical composition of oregano EOs used and also using different substrates and due to the resisting mode of the fungi against various substances present in EOs (Soylu et al, 2010).

While oregano EO could inhibit the test fungi from 100 µL/L (0,01%) concentration in *in vitro* experiments (Fig. 1, 2 and 3), the minimum concentration to inhibit the growt of *B. cinerea, P. expansum* and *C. gloeosporioides* in *in vivo* experiments were approximately 1 %, 3 % and 5 % respectively (Fig. 4). The differences in concentrations needed to inhibit the test fungi occured in *in vivo* and *in vitro* treatments can be related to apple tissue features such as pH and the amounts of nutrients (vitamins, nitrogen-containing compounds and minerals) and natural phenolic compounds (Rupasinghe et al., 2006). Therefore, it can be stated that *in vivo* antifungal activity of EOs depends on the nature and type of the fungi as well as physical and biochemical characteristics of substrate used (Salas et al., 2011).

4 Conclusions

In this study, the aim was to investigate the antifungal activity of essential oil (EO) of medicinal plants such as eucalyptus, sage, rosemary, oregano and fennel to better understand their inhibition powers against selected postharvest desease causing fungi in apples. The EO of oregano has been shown to reduce growth of test fungi in both *in vitro* (on solid media) and *in vivo* (to control infection of apple). Eucalyptus EO was proved to inhibit the growth of fungi as well but it did not show as strong inhibition as oregano EO did at the same concentrations. According to data obtained from this study and from previous research works, there is a potential to use EOs as natural food protection agents as alternative to synthetic fungicides in postharvest operations since EOs have low level of toxicity, non-persistent in the environment, biodegradable, multifunctional, and are cheap to produce. However, considering the intense smell of the EOs' components, cost and efficacy of these EOs on wide range of diseases may limit their use. Therefore more research and technological studies need to be done to solve these issues.

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Conflict of interest

The authors declare that no conflicts of interest exist.

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