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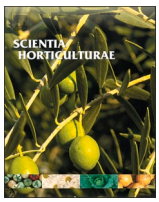

Apple pathogens: Organic essential oils as an alternative solution

A. Di Francesco [a,d,*], E. Aprea [b,c], F. Gasperi [b,c], A. Parenti [d], N. Placì [d], F. Rigosi [d], E. Baraldi [d]

[a] Department of Agriculture, Food, Environmental and Animal Sciences, University of Udine, Via delle Scienze, 206, Udine 33100, Italy
[b] Center Agriculture Food Environment, University of Trento/Fondazione Edmund Mach, San Michele all'Adige, TN 38010, Italy
[c] Research and Innovation Centre, Fondazione Edmund Mach, via Mach 1, San Michele all' Adige, Trento 38010, Italy
[d] Department of Agricultural and Food Sciences, University of Bologna, Viale Fanin, 42, Bologna 40127, Italy

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ABSTRACT

Fusarium avenaceum, Botrytis cinerea, Penicillium expansum, and *Neofabraea vagabunda*, represent postharvest diseases which cause significant apple losses. The aim of this study was therefore to evaluate the effects of organic essential oils (EOs) (*Thymus vulgaris, Lavandula angustifolia, Rosmarinus officinalis*) against apple pathogens both *in vitro* and *in vivo,* as an integrated management tool. By GC–MS analysis a total of 101 compounds principally belonging to the groups of terpenes and terpenoids were detected in the extracted EOs. *In vitro* results showed *T. vulgaris* as the most active EO, both as agar infusion or biofumigant. Through agar infusion, starting from the lowest concentration (0.2 mL L^{-1}), *T. vulgaris* reduced by 74.9%, 86.1%, 66.9%, and 45.7% *F. avenaceum, B. cinerea, P. expansum,* and *N. vagabunda* mycelial growth, respectively; as biofumigant, it completely inhibited the growth of all the tested mycelial pathogens. Application of EOs on apples through dipping treatment displayed some potential to inhibit the above-mentioned pathogens, especially by *T. vulgaris* and L. *angustifolia.* The efficacy of these organic EOs is probably strictly correlated to the chemical composition.

1. Introduction

Apples are cultivated worldwide and can be affected by more than 90 pathogens fungal species (Jones and Aldwinckle, 1990) able to cover different diseases, from the field to the fruit storage, like the apple scab and powdery mildew, roots and collar rots, cankers, black and white rots (Turechek, 2004). Postharvest diseases are caused by wound pathogens such as *Penicillium expansum* (blue mold), *Botrytis cinerea* (gray mold), that are the major postharvest diseases on pome fruit (Konstantinou et al., 2011). These losses may reach as much as 50% during the shelf life of the fruit (Eckert and Ogawa, 1988). *Fusarium avenaceum* together with *Alternaria* spp., *Monilinia* spp., *Mucor* spp. can cause from 5 to 25% of losses in apple during postharvest storage and commercialization (Konstantinou et al., 2011). In addition, bull's eye rot, caused by different fungal species belonging to the genus *Neofabraea,* can cause severe losses with an incidence of 10–20%, exceeding 40% in years favorable to pathogen (Soto-Alvear et al., 2013; Di Francesco et al., 2019). Chemical control in plant protection, both in field and during storage, was the most common method to control fungal diseases that can cause environmental imbalances, pests' resistance, and health risks (Damos et al., 2015). However, its intense use in the past, also during fruit storage determined resistant isolates developing and their widespread within packinghouses (Di Francesco et al., 2015).

In recent years, between the alternative methods and strategies to control fruit fungal pathogens, the application of plant essential oils (EOs) was considered a natural alternative to synthetic fungicides (Lopez-Reyes et al., 2010; Ali et al., 2015; Vilaplana et al., 2018).

EOs are highly complex mixtures of aromatic compounds synthesized by plants, each one with an ecological function (Caputi and Aprea, 2011), including mechanisms against some microorganism's growth (Hosseini et al., 2020). The antifungal activity of EOs can be attributed to the properties of some chemical components able to disrupt cells membrane, causing cell death or inhibiting the sporulation and germination of food spoilage fungi (Nazzaro et al., 2017).

The main EOs chemical compounds of plants are monoterpenes, aldehydes, allyl phenols, alcohols, acids, and esters (Prakash et al., 2012; De Almeida et al., 2018), however the chemical composition differs among plant species and agronomical grown systems. Also, geographical location, environment, plant stage of maturity and method of extraction can be considered as important variable factors. Several studies have been conducted to evaluate the effect of EOs on the control of phytopathogenic fungi (Peighami-Ashnaei et al., 2009), and they are

* Corresponding author.
E-mail address: alessandra.difrancesco@uniud.it (A. Di Francesco).

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considered as potential biocontrol products. The Food and Drug Administration (FDA) considered EOs as safe for use in food and for treating stored fruits and vegetables (Gonzalez-Aguilar et al., 2008; Feng et al., 2008).

However, fruit treatment with EOs needs to be carefully considered, as these might affect their sensory characteristics (Endrizzi et al., 2019) and sometimes compromise consumer's acceptability (Guillén et al., 2007; Servili et al., 2017). EOs application can be conducted through spray or dipping liquid solutions (Fontana et al., 2021), as fruit coating or as biofumigation (Romanazzi et al., 2017; Mehra et al., 2013), depending on the fungal pathogen to control.

Therefore, the objective of the present work was to evaluate the efficacy of organic EOs of *Thymus* (*T.*) *vulgaris, Lavandula* (L.) *angustifolia* and *Rosmarinus* (*R.*) *officinalis* in the control of fungal diseases of apple fruits: *F. avenaceum, B. cinerea, P. expansum,* and *N. vagabunda.*

The study was developed through different strategies: i) by GC–MS analysis to characterize the EOs chemical composition; ii) by *in vitro* assays, testing the efficacy of the EOs at different concentrations and mechanisms of action, through agar infusion or through volatile biofumigation, on mycelial fungal growth; iii) by *in vivo* assays testing the EOs efficacy against apples pathogens through dipping.

2. Materials and methods

2.1. Fruit

'Golden Delicious' apple fruits (*Malus domestica* L. Borkh), homogeneous in size and with the right quality parameters (SSC - soluble solids content, 11; amid content, 3.5), were harvested from an experimental orchard located in Altedo (Bologna, Italy) managed according to the organic regulation, stored at 0 °C and used for the experiments within 5 d from the harvest.

2.2. Pathogens

Fusarium avenaceum, B. cinerea, P. expansum, and *N. vagabunda* isolates, molecularly identified, belonged to Department of Agricultural Sciences of Bologna University Mycological collection. All isolates were grown on PDA (Oxoid, Basingstoke, UK: 39 g in 1 L of distilled) at 20 °C, except for *N. vagabunda* on technical agar (Oxoid, 12 g L^{-1}) amended with tomato juice (250 mL L^{-1}) at 15 °C. Pathogen conidia suspensions were prepared from 10 to 15 days old colonies by scraping and suspending conidia in sterile distilled water added with 0.05% (v/v) Tween 80, and adjusted to the final required concentration (10^4 conidia mL^{-1}) with a hemocytometer.

2.3. Chemical and natural substances

Chemical fungicide Scholar® (fludioxonil 23%) (Syngenta, Basil, Switzerland) was tested at different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4-mL L^{-1}) by *in vitro* (amended medium) and *in vivo* (0.2 mL L^{-1}) by dipping, as positive control. Plant materials (*T. vulgaris,* L. *angustifolia, R. officinalis*) at the BBCH stage 65 were obtained from an organic farm located in Bologna (Italy) (44.4441782, 11.334475, altitude 100 m). According to the evaluation of the plant's behavior in response to seasonal factors, plants were collected during the full flowering (spring 2021) and were representative to the same pedoclimatic and collection conditions. EOs were extracted from wet leaves without any pre-processing by the steam distillation apparatus composed by a 4.000 mL glass boiler heated by an electric resistance, a 3.000 mL glass extraction chamber, and a modified Clevenger trap with 5.00 mL graduated tube. In the boiler a saturated steam at T = 366.65 K and P = 1.01 bar with a steam flow = 3.4 mL/min is generated (Cassel et al., 2009).

2.4. Essential oils: analysis of chemical composition

Gas chromatography coupled with mass spectrometry (GC–MS) was applied to analyze chemical composition of the tested EOs. The EOs were diluted 1:100 in hexane (GC grade; Sigma-Aldrich®, Merk Life Science S.r.l., Milan, Italy). Chromatographic separations were performed injecting 1.0 μL of diluted EO in the injector port (splitting ratio 50:1) of a GC Clarus 500 (PerkinElmer, Norwalk, CT) equipped with an HP-Innowax fused-silica capillary column (30 m, 0.32 mm inner diameter, 0.5 μm film thickness; Agilent Technologies, Palo Alto, CA). The temperature program was set progressively as follows: 50 °C for 2 min, then reached 230 °C with a ramp of 3 °C•min^{-1} and held for 5 min. The total run was 67 min. Helium was used as the carrier gas at a flow rate of 2 ml•min^{-1}. The transfer line temperature was kept at 220 °C. After a solvent delay of 150 s, mass spectra were acquired in the scan range from m/z 33 to 300. Linear retention indices (LRI) were calculated under the same chromatographic conditions injecting C7-C30 n-alkane series (Supelco, Bellefonte, PA). Compounds identification was achieved matching the acquired mass spectra with those present in the NIST-2014/Wiley 7.0 libraries and comparing the calculated LRI with those available from the literature.

2.5. Essential oils efficacy against mycelial pathogens growth

Two kinds of experiments were carried out to evaluate the effectiveness of three EOs for *P. expansum, B. cinerea, F. avenaceum,* and *N. vagabunda* control efficacy: by agar infusion (Fontana et al., 2021) and by volatile compounds (Di Francesco et al., 2015). For the first assay seven doses of each oil and a chemical fungicide 'Scholar®' (fludioxonil), infused on PDA, were tested against pathogen diametral mycelial growth (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 mL L^{-1} of PDA). For this purpose, amended agar plates were inoculated with 6 mm pathogens mycelium plug.

For the volatiles effect, different aliquots of pure EOs (15, 30, and 60 μL) were placed with a microsyringe on a filter paper (Whatmann No. 1, 90 mm diameter) positioned inside a Petri dish (90 mm, Ø) and placed in contact with a PDA plate previously inoculated with 6 mm pathogen mycelial plug. EOs aliquots (15, 30, and 60 μL) corresponded respectively to 1.35, 0.67 and 0.33 μL mL^{-1} headspace, as described by Rouissi et al. (2013). In both experiments, dishes were quickly closed, sealed with Parafilm and incubated at 20 °C, apart from *N. vagabunda* (15 °C). The activity of each EOs concentration against mycelial growth was evaluated after 6 days of incubation, except for *N. vagabunda* (12 days). For the control, no amended PDA was used for agar infusion assay and sterile water for the volatiles assay. There were five replicates per each EOs concentration and fungal isolate. For both assays, the inhibition rate of mycelial growth was calculated using the equation (Chen and Dai, 2012):

$$\%\text{inhibition} = \frac{d1 - d2}{d1} \times 100$$

where (%) is the percent of inhibition of mycelial growth (mm of colony diameter); d1 is the control value; d2 is treated value.

Percent of inhibition values were used to calculate EC_{50} values as the EOs and fludioxonil concentrations (mL L^{-1}) that inhibited fungal mycelial growth by 50% compared with the control. Each experiment was performed twice.

2.6. In vivo assay

After preliminary tests, the lowest tested EOs concentration (0.2 mL L^{-1}) was tested on apple fruits by a dipping treatment. The experiments were conducted reproducing storage warehouse conditions, where fruits can be injured and sanitized by dipping. In fact, fruits were disinfected by immersion for 1 min in 1% sodium hypochlorite solution and washed

twice in sterile water. Apple fruits were wounded with a sterile needle at the equatorial region (3 mm deep and 3 mm wide; one wound/fruit) and inoculated with pathogens suspensions (10^4 conidia mL^{-1}, 20 μL). For dipping, apples were kept at room temperature for 3 h and subsequently dipped in EOs emulsion and in fludioxonil, used a chemical control, both concentrated 0.2 mL L^{-1}. The control samples were represented by apples inoculated with pathogen conidia suspension and dipped in distilled water. Each treatment was represented by 20 fruits, and there were three replicates per treatment (Di Francesco et al., 2019). Treated fruits were stored for a week (*P. expansum, B. cinerea*) (Di Francesco et al., 2015), 14 (*F. avenaceum*) or 21 (*N. vagabunda*) days (Buhulmann et al., 2021) at 20 °C; after that, pathogen's disease severity (mm) was measured with a ruler. The experiment was performed twice.

2.7. Statistical analysis

Data were statistically handled by two-way analysis of variance (ANOVA) performed using the software R. Due to the heteroscedasticity of the residuals a linear mixed model was used to determine the covariance matrix structure, then the Tukey's HSD Test ($\alpha = 0.05$) was used for means separation.

The EC50 of each tested EOs and the chemical compound infused in the growth media were calculated using the probit analysis applied to the percentage of inhibition of mycelial growth (Lesaffre and Molenberghs, 1991).

3. Results

3.1. Essential oils: analysis of chemical composition

The tested EOs were characterized by different chemical composition. Fifty-eight, forty-five, and forty-three chemical compounds were detected respectively for *T. vulgaris,* L. *angustifolia, R. officinalis.* Also, each EO presented some volatile compounds as major component: p-cymene (34.3%), thymol (31.1%), carvacrol (9.28%) for *T. vulgaris*; linalyl acetate (30.32%), trans-β-ocimene (6.75%), linalool isobutyrate (5.10%) for L. *angustifolia;* α-pinene (23.17%), eucalyptol (10.63%), camphene (8.26%) for *R. officinalis* (Table 1).

3.2. Fungal mycelial growth: EOs efficacy modes of application

In order to assess the antifungal effect on mycelial diameter due to EOs, two different assays were set up. In the agar infusion assay the chemical compound fludioxonil was used as positive control. All the tested EOs showed an inhibition against pathogens mycelial growth from the lowest concentration (Fig 1a, b, c, d) except for L. *angustifolia* against *B. cinerea* (Fig. 1b). *T. vulgaris* was the most active EO reducing by 74.9%, 86.1%, 66.9%, and 45.7% *F. avenaceum, B. cinerea, P. expansum,* and *N. vagabunda*, respectively, at 0.2 mL L^{-1}. *T. vulgaris* at 0.8 mL L^{-1} and 1.4 mL L^{-1} displayed the total inhibition of *B. cinerea, P. expansum,* and *N. vagabunda,* comparable to the fungicide activity. Conversely, L. *angustifolia* EO only at 1.4 mL L^{-1} showed a reduction of 52.8%, 15.2%, 51.4%, 61.4% respectively for *F. avenaceum, B. cinerea, P. expansum,* and *N. vagabunda. T. vulgaris* (1.4 mL L-1) displayed the highest efficacy against *N. vagabunda*, At the same condition, the synthetic compound determined an inhibition only by 29.6% of the fungal pathogen (Fig. 1d). Volatile organic compounds (VOCs) produced by EOs significantly inhibited the fungal mycelial growth, with some differences between the pathogen species (Fig. 2a, b, c, d). Starting from the lowest EOs concentration (15 μL) effective results were obtained against fungal mycelial growth. The highest reductions were registered against *F. avenaceum* of 84.7%, 73%, 81.7% respectively by *T. vulgaris, R. officinalis,* and L. *angustifolia*, on average. Also, *R. officinalis* and L. *angustifolia* VOCs reduced by 55% (on average) *B. cinerea* and *P. expansum* at the lowest tested concentration. Conversely, the lowest percentage of reduction was recorded for *N. vagabunda* (21.4% of

Table 1
Chemical composition (%) of *Thymus vulgaris, Lavandula angustifolia,* and *Rosmarinus officinalis* essential oils. The major fractions per each EO are indicated in bold.

Chemical component(%)	Essential oils		
	T. vulgaris	L. *angustifolia*	*R. officinalis*
Tricyclene	0.02	-	0.34
Methyl 2-methylbutanoate	0.05	-	-
α-Pinene	1.00	0.30	23.17
α-Thujene	0.10	0.14	-
α-Fenchene	-	-	0.05
Camphene	0.67	0.12	8.26
Butyl acetate	-	0.04	-
β-Pinene	0.09	0.09	2.22
Sabinene	-	0.07	0.02
Dehydrosabinene	-	-	0.79
δ−3-carene	0.02	0.07	-
3-Carene	-	-	0.05
β-Myrcene	0.33	0.74	4.36
α-Terpinene	0.48	0.07	0.21
Limonene	0.44	0.33	5.25
1,8-Cineole (eucalyptol)	1.11	1.29	10.63
(E)−2-Hexenal	0.01	-	-
Butyl butanoate	-	0.20	-
α-Ocimene	-	5.70	0.01
γ-Terpinene	1.99	0.30	0.29
3-Methyl-5-heptanone	0.06	-	3.49
P-Cymene	34.3	-	1.86
A-Terpinolene	0.02	0.12	0.47
(E)-β-Ocimene	-	6.75	-
n-Hexanol	0.03	0.03	-
3-Methyl-3-buten-1-yl 3-methylbutanoate	0.01	-	-
α-Pinene oxide	-	-	0.02
Hexyl acetate	-	0.72	-
(Z)−3-Hexenyl propanoate	0.01	-	-
Fenchone	-	-	0.02
n-Octan-3-ol	0.12	0.13	0.16
3-Octanyl acetate	-	0.12	-
α-Thujone	-	-	0.88
p-Cymenene	0.09	-	-
(E)-Linalool oxide (furanoid)	0.04	0.06	-
β-thujone		-	0.50
Sabinene hydrate	0.17	0.04	-
(Z.E)−1.3.5-Undecatriene		0.04	-
(Z)-Linaloloxide	0.02	0.05	-
(E)-Sabinene hydrate	-	-	0.04
Butyl hexanoate	-	-	-
α-Cubebene	0.03	-	-
chrysanthenone	-	-	1.10
n-Hexyl butanoate	-	0.56	-
α-Copaene	0.09	-	-
α-Campholenal	-	-	0.04
Hexyl 2-methylbutyrate	-	0.05	-
D-(+)-Camphor ((+)−2-Bornanone	0.70	0.71	8.18
α-Bourbonene	-	-	-
β-Linalool	2.81	27.00	2.48
(Z)−3-Pinanone (cis-Pinocamphone)	-	-	1.1
(E)-β-Terpineol	0.06	-	-
Bornyl acetate	0.30	0.08	-
β-Caryophyllene	1.64	4.70	4.10
Methylthymol	2.13	-	-
α-Pinocarvone	-	-	0.25
4-Terpineol	0.85	6.00	0.82
Linalyl acetate	-	30.32	-
Isobornyl acetate	-	-	3.70
Methyl carvacrol	1.64	-	-
Menthol	0.03	-	-
α-Bergamotene	-	0.08	-
Humulene	-	-	0.63
Pinocarveol (2(10)-Pinen-3-ol)	0.02	-	-
δ-Terpineol	-	-	0.07
Humulene	0.06	0.17	-
(E)-Verbenol	-	-	0.28
(E)-Borneol	2.06	1.77	7.94
Linalool isobutyrate	-	5.10	-

(*continued on next page*)

Table 1 (*continued*)

Chemical component(%)	Essential oils		
	T. vulgaris	L. *angustifolia*	*R. officinalis*
Verbenone	-	-	4.38
α-Muurolene	0.06	-	-
Hexyl tiglate	-	0.04	-
β-Bisabolene	0.07	-	-
(-)-(E)-Myrtanyl acetate	-	-	0.06
D-Carvone	0.05	-	-
(E)-β-Famesene	-	1.26	-
Neodihydrocarveol	-	-	0.12
δ-Cadinene	0.68	-	-
Lavandulol	-	0.67	-
γ-CAMPHOLENOL	-	-	0.05
1-Decanol	0.05	-	-
Campholaldehyde	-	-	0.14
Cumaldehyde	0.02	-	-
Neryl acetate	-	0.26	-
Myrtenol	0.02	-	-
(Z)-Calamenene	0.01	-	-
Nerol	-	0.08	-
p-Cymen-8-ol	0.30	–	0.11
Geraniol	-	0.26	-
Piperitenone	-	-	0.08
Geranyl isovalerate	0.03	-	-
Caryophyllene oxide	1.97	0.21	0.60
α-Calacorene	0.03	-	-
α-epi-Cadinol (tau.-Cadinol)	-	0.08	-
10-epi-γ-Eudesmol	0.25	-	-
Cuminyl alcohol (p-Cymen-7-ol)	0.03	-	-
Spathulenol	0.02	-	-
Hexadecan-2-one	0.04	-	-
10-epi-α-Cadinol	0.26	-	-
2-Acetyl-4-methylphenol	0.31	-	-
Thymol	31.1	-	-
Carvacrol (Isothymol)	9.28	-	-

reduction, on average).

Botrytis cinerea, P. expansum, N. vagabunda were completely (100%) inhibited by VOCs produced by *T. vulgaris* EO at each tested concentration. In addition, VOCs produced by the two other oils at 60 μL were able to stop *Botrytis* and *Fusarium* molds development.

3.3. EC50 values

EOs efficacy by agar infusion was tested *in vitro* experiments against the mycelial growth of the target pathogens: *F. avenaceum*, B. cinerea, P. expansum, and N. vagabunda (Table 2) in order to determine the EC_{50} values. As amended agar, the values ranged between 0.01 mL L^{-1} for *T. vulgaris* against all target pathogens, except for *B. cinerea* (0.67 mL L^{-1}). L. *angustifolia* was the less active EO against all tested pathogens, with the higher EC_{50} values against *F. avenaceum, B. cinerea,* and *P. expansum* with respect to the other oils. In addition, B. cinerea appeared the most resistant pathogen to the tested EOs. F. avenaceum and N. vagabunda were the most sensitive pathogens with EC_{50} values below 1.19 mL L^{-1}. *T. vulgaris* together with fludioxonil resulted the most active treatments displaying the lowest EC50 values against the target pathogens.

3.4. In vivo assay: essential oils efficacy

The antifungal effect of the target EOs by dipping treatment on fungal pathogens was evaluated by the measurements of their severity on wound inoculated apple fruits. *Thyumus* and *Lavandul*a resulted the most active oils showing a comparable reduction of each fungal severity, on average by 61.8%, 69.0%, 35.6% and 18.6% respectively against *F. avenaceum, B. cinerea, P. expansum,* and *N. vagabunda* (Fig. 3). *R. officinalis* displayed a less pronounced, but significant, effectiveness (reduction by 45%, 42.5%, 21.7, and 9.1% respectively against *F. avenaceum, B. cinerea, P. expansum,* and *N. vagabunda*). The chemical compound was the most efficient by totally inhibiting (100%) each tested fungal pathogen.

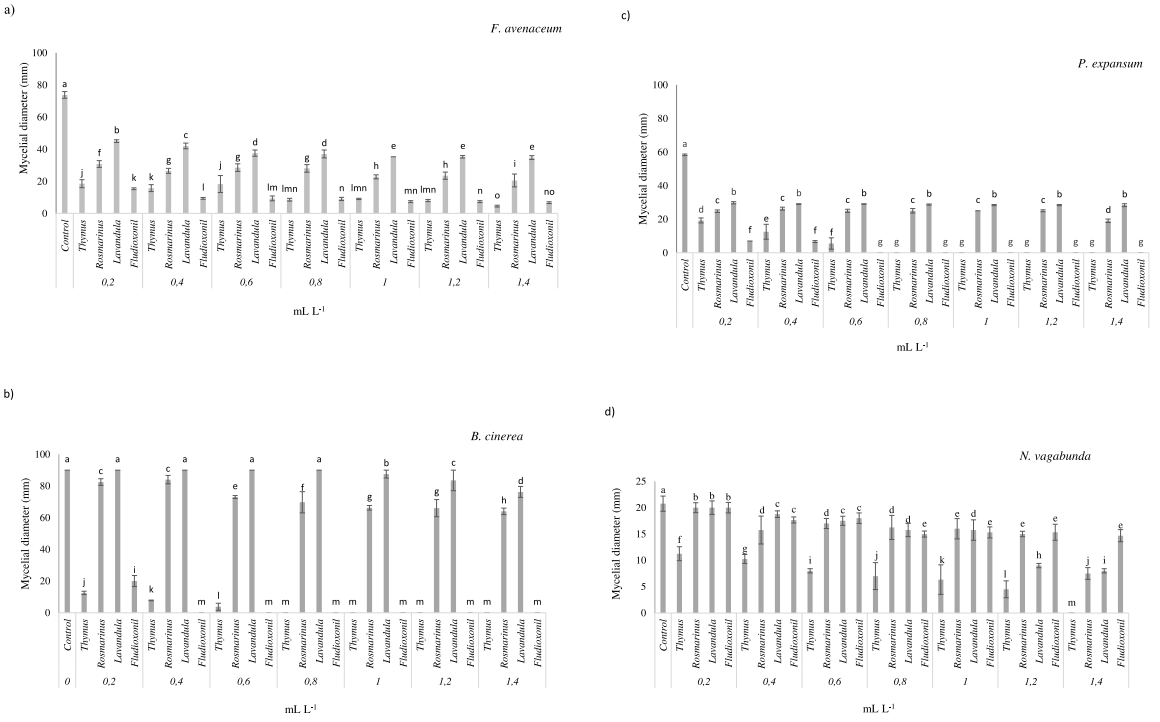

Fig. 1. Effect of essential oils (EOs) (*Thymus vulgaris, Rosmarinus officinalis, Lavandula angustifolia*) and fludioxonil infused on PDA plate (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 mL L^{-1}) on the mycelial growth of a) *Fusarium avenaceum,* b) *Botrytis cinerea*, c) *Penicillium expansum*, d) *Neofabraea vagabunda.* For the control, no amended PDA plates were used. Different letters represent significant differences among EOs and concentration for each pathogen according to Tukey's HSD Test (α = 0.05).

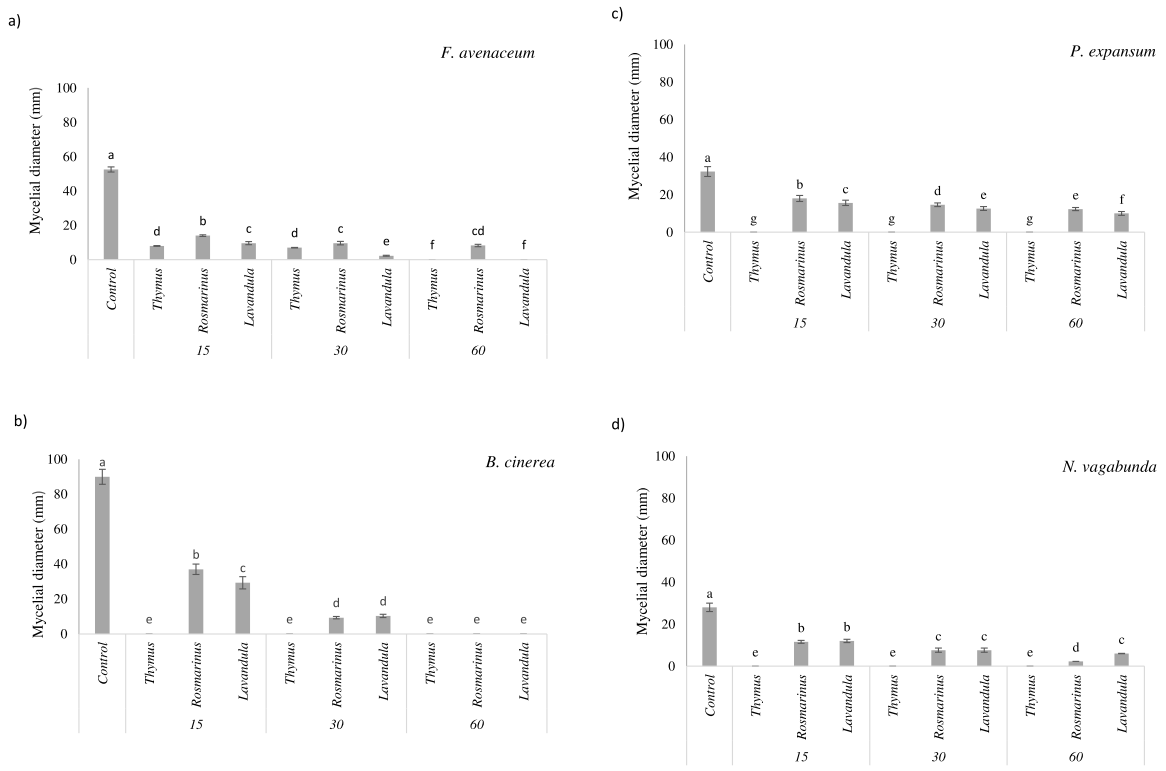

Fig. 2. Effect of essential oils (EOs) (*Thymus vulgaris, Rosmarinus officinalis, Lavandula angustifolia*) volatile compounds produced by different concentrations (control, 15, 30, 60 μL) on the mycelial growth of a) *Fusarium avenaceum,* b) *Botrytis cinerea*, c) *Penicillium expansum,* d) *Neofabraea vagabunda.* Colony diameters were measured after 6 and 12 days, depending on the pathogen. Different letters represent significant differences among the effect of VOCs produced by EOs at different concentrations for each pathogen according to Tukey's HSD Test (α = 0.05).

Table 2
Effect of three essential oils (*Thymus vulgaris, Rosmarinus officinalis, Lavandula angustifolia*) and fludioxonil on four fruit postharvest fungal pathogens. EC50 values (mL L^{-1}).

EC50 (mL L^{-1})	*Thymus vulgaris*	*Rosmarinusofficinalis*	*Lavandulaangustifolia*	Fludioxonil
Fusarium avenaceum	0.01	0.28	1.19	0.01
Botrytis cinerea	0.67	4.29	4.71	<0.01
Penicillium expansum	0.01	1.49	1.52	<0.01
Neofabraea vagabunda	0.01	0.53	0.33	0.02

4. Discussion

Postharvest decay in apples can depend to different conditions such as the cultivar, the harvest maturity, the ripening stage, and the storage. The pathogens reported in the present study could infect by wound infections or when the fruit is still attached to the plant in the field (Sivakumar and Bautista-Baños, 2014) and this can represent one of the major causes for the loss of fruit during the supply chain. Therefore, in the last years, the development of eco-friendly strategies involved also the EOs derived from medicinal plants to substitute synthetic chemical fungicides for the management of postharvest pathogens (Hosseini et al., 2020). EOs efficacy strictly depends on the composition, concentration, microbial species, host on which are applied, time and application form (Droby et al., 2008; Wang et al., 2019). Mechanisms of action of EOs are connected to the plant from which they derive and to the secondary metabolites array composition. These products can inhibit cellular respiration, alter cell morphology growth (Tao et al., 2014) and affect membrane permeability (Viuda Martos et al., 2007). In the last years, EOs gained a great popularity in the postharvest sector probably due to their eco-friendly, safety and effective characteristics, as reported by Hassani et al. (2012); Sellamuthu et al. (2013); Tao et al. (2014) respectively against *Monilinia fructicola, Colletotrichum gloesporioides*, and *Penicillium* spp.

Penicillium expansum, B. cinerea, and *N. vagabunda* are considered to be among the most dangerous pathogens of apples during the postharvest storage. In the present study, we included also *F. avenaceum* as an emerging pathogen on apples (Kou et al., 2014), against which the possibility of using EOs as fungicides for its control is poorly studied. Conversely, different studies were conducted by using EOs against the other above-mentioned pathogens (Amiri et al., 2008; Lopez-Reyes et al., 2010; Znini et al., 2011). Nevertheless, the agronomical and extractive characteristics of the used EOs stimulated our interest in testing their effectiveness against these pathogens. In fact, the susceptibility of the pathogen to EOs depends strictly to its chemical composition, concentration, and solubility (Marino et al., 2020). About the tested EOs known for their active compounds such as thymol, carvacrol, and p-cymene for *T. vulgaris*, α-pinene, eucalyptol for *R. officinalis*, and linalyl acetate for L. *angustifolia*, a good percentage was detected by GC–MS analysis. Abdolahi et al. (2010) reported that thyme EO used in their experiments contained a percentage of thymol and carvacrol respectively of 11% and 7%. Instead, other studies showed an amount of these active compounds respectively of 52% and 3%, or 67.39% and 2.09% (Servili et al., 2017; Lopez-Reyes et al., 2010). Thyme EO used in the present study, if compared to other EOs, presented a slightly lower

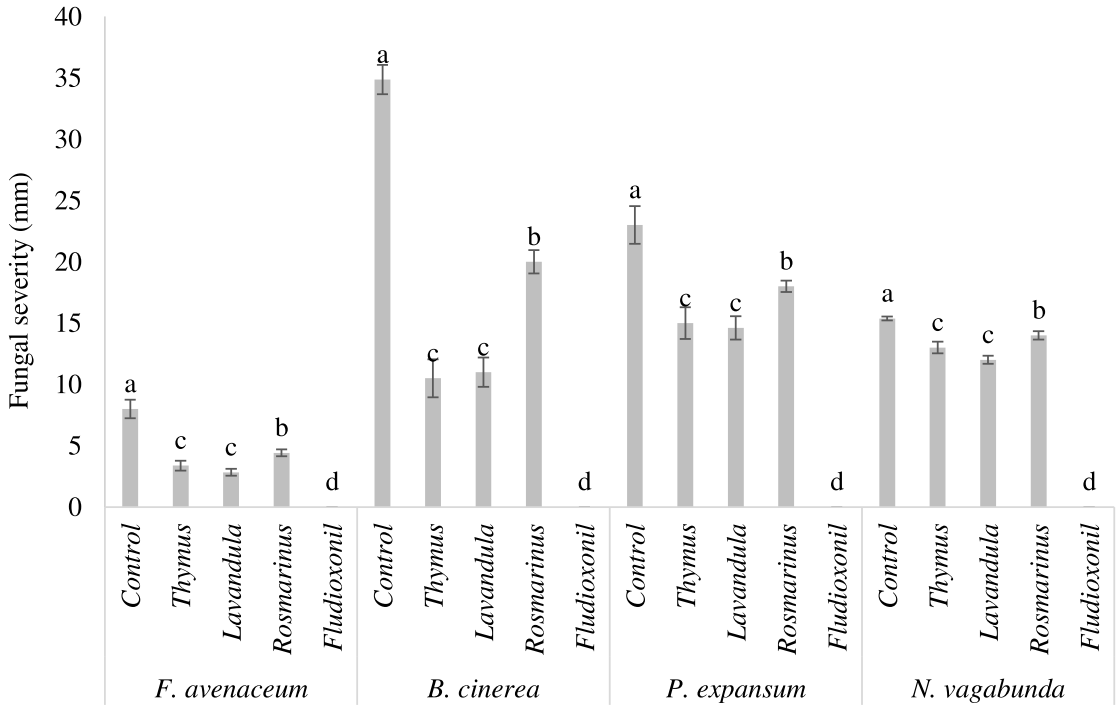

Fig. 3. Efficacy of essential oils (EOs) (*Thymus vulgaris, Rosmarinus officinalis, Lavandula angustifolia*) and fludioxonil (0.2 mL L^{-1}) on the fungal severity on apple of a) *Fusarium avenaceum,* b) *Botrytis cinerea*, c) *Penicillium expansum*, d) *Neofabraea vagabunda.* Fruit wounds were inoculated with 20 μL of each tested pathogen conidial suspension (10^4 conidia mL^{-1}). Fruits were kept at 20 °C for 7, 14 and 21 days depending on the pathogen. Different letters represent significant differences among EOs for each single pathogen according to Tukey's HSD Test (α = 0.05).

percentage of thymol (31.1%) and a higher amount of carvacrol (9.28%), aspect probably correlated to the plant's agronomical practices Martinez Romero et al. (2007). showed how carvacrol was effective on inhibiting spore germination of B. cinerea. In addition, Markovic et al., al. (2011) demonstrated that thymol and carvacrol have a remarkably antifungal potential, with the highest efficacy displayed by carvacrol against Aspergillus spp. and Penicillium spp.. In the case of L. *angustifolia* and *R. officinalis* EOs, the chemical analysis displayed a higher content of trans β-ocimene (6.75%) and α-pinene (23.17%) compounds, respectively, with respect to other EOs (Servili et al., 2017; Lopez-Reyes et al., 2010). Both compounds are known to have an important antifungal activity against *Candida* spp. and *Aspergillus* spp. (Cavaleiro et al., 2015). These reports support the good activity of the tested *Lavandul*a and *Rosmarinus* EOs detected during *in vitro* and *in vivo* assays.

However, the use of *T. vulgaris,* L. *angustifolia,* and *R. officinalis* was already studied and good results were reported (Servili et al., 2017; Lopez-Reyes et al., 2010; Cisarova et al., 2016); in our study, positive results were obtained both *in vitro* and *in vivo* especially by using *T. vulgaris* EO, starting from the lower concentration (0.2 mL L^{-1}), in particular against *B. cinerea.* Indeed, *in vitro* assay conducted by EOs agar infusion, *B. cinerea* mycelial diameter was reduced by 86.1% by the lowest tested concentration (0.2 ml L^{-1}) reaching the total inhibition at 0.8 mL L^{-1}. Other positive results have already been found in the control of *B. cinerea* by using other essential oils (Aguilar-Gonzalez et al., 2015; Combrinck et al., 2011; Lorenzetti et al., 2011).

Against *N. vagabunda* mycelial growth, *T. vulgaris* was the most effective in inhibiting the bull'eye rot pathogen mycelial growth at 1.4 mL L^{-1} with respect to the chemical compound that reduced the fungal growth only by 29.6% at the same concentration. In fact, *in vivo* assay the lowest concentration inhibited only by 15.4% the pathogen severity.

In the case of *F. avenaceum,* the pathogen showed a high resistance to each EOs concentrations and to fludioxonil.

The different efficacy between the treatments depends on the fungitoxic properties of each EO and their synergy (Lopez-Reyes et al., 2010). In fact, by the *in vitro* biofumigation assay, results displayed a higher EOs efficacy with respect to the infused agar assay. *T. vulgaris* VOCs totally inhibited *B. cinerea, P. expansum,* and *N. vagabunda* mycelial growth at 15 μL of concentration. *Fusarium avenaceum* was confirmed to be the most resistant pathogen to EOs volatiles. In fact, that pathogen, only by the VOCs derived from the highest concentration of *T. vulgaris* and L. *angustifolia* EOs, was totally inhibited.

Nevertheless, the use of EOs should be always correlated to the possible phytotoxic effects of treatments on fruits. About this consideration, *in vivo* treatments were performed with the previous lowest concentrations of all essential oils to avoid a phytotoxic reaction on the apple carposphere. In fact, it is known that the concentration with a higher efficacy on the pathogen growth could be that one produces a higher damage on apples carposphere (Lopez-Reyes et al., 2010).

On fruits, visible symptoms such as severe chlorosis and scalds (Palazzolo et al., 2013) are connected to the concentration of the major EOs compounds and their synergy (Lopez-Reyes et al., 2013). Stone and pome fruit are susceptible to this side-effect. About apples, phytotoxicity is strictly connect to the cultivar; in fact, sensitivity of apple to EOs is cultivar dependent, whereby the most sensitive appear to be Golden Delicious and Granny Smith, while Royal Gala and Red Chief are less susceptible (Tarlanovic et al., 2017). In our study, a low percentage of phytotoxicity was detected only for *T. vulgaris* EO as tissue scalds (data not shown). In fact, fruits were artificially wounded and inoculated by pathogens, and suddenly soaked in water and EOs emulsion. EOs efficacy partially confirmed the *in vitro* results. Probably connected to the phytotoxicity effect, *T. vulgaris* not totally inhibits the pathogens infection. An interesting result was obtained by *in vivo* with L. *angustifolia* EO that showed a good activity in reducing fungal pathogens by using the lowest concentration. Rosemary EO has been shown to be less effective with respect to the other EOs, conversely to other studies where it displayed a good efficacy in reducing postharvest decay of stone and pome fruit caused by fungal pathogens (Lopez-Reyes et al., 2010; 2013). However, the interaction between food matrix components and EOs need to be investigated before their application and suddenly proposed for commercial practice. Several variables, such as oils composition,

concentration, timing and form of application, should be taken into account to not affect food sensory properties and safety.

Future perspectives could involve the combination of the organic EOs with other postharvest treatments, preferably under controlled storage conditions.

CRediT authorship contribution statement

A. Di Francesco: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing. **E. Aprea:** Methodology, Validation, Writing – original draft, Resources. **F. Gasperi:** Methodology, Validation, Writing – original draft, Resources. **A. Parenti:** Software, Formal analysis. **N. Placì:** Investigation. **F. Rigosi:** Investigation. **E. Baraldi:** Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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