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ORIGINAL ARTICLE

Assessment of the efficiency of *Mentha pulegium* essential oil to suppress contamination of stored fruits by *Botrytis cinerea*

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Abstract

Essential oils are a real ore rich in bioactive compounds characterized by a wide spectrum of various biological activities. In this context, our research focused on the study of the chemical composition of *Mentha pulegium* and *Myrtus communis* essential oils growing in Northeast Algeria as well as the exploration of their antifungal activities in vitro and in vivo against *Botrytis cinerea* responsible for moulding on strawberries. GC–MS analysis indicated that *M. pulegium* essential oil was an isomenthone chemotype (55.59%) while *M. communis* essential oil was characterized as a eucalyptol chemotype (36.82%). *M. pulegium* essential oil expressed the best antifungal activity either with poisonous medium method (MIC = MFC:2.66 µl/ml) or with volatile activity method (MIC:30 µl) compared to *M. communis* essential oil, (MIC:5.33 µl/ml, MFC:10.66 µl/ml) which expressed no volatile activity. Both crude oils completely inhibited the germination of *B. cinerea* spores and resulted in up to 88% morphological changes in conidia. In vivo tests have revealed the effectiveness of *M. pulegium* essential oil in completely suppressing grey mould from strawberries previously inoculated with conidia of *B. cinerea* by direct contact or exposure to vapours. *M. pulegium* essential oil display weak phytotoxicity towards fumigated strawberries at low temperatures ($T < 16$ °C). This low phytotoxicity was confirmed by the preservation of some physical parameters of strawberries stored at 7 °C such as colour and weight loss.

Keywords Antifungal activity · *Botrytis cinerea* · Conidia · *Mentha pulegium* · Phytotoxicity · Strawberry

Introduction

Agricultural products are often subject to biotic and abiotic damage during production, conservation and processing. Post-harvest losses are mainly due to oxidative processes or attacks by destructive agents mainly insects and moulds. In developing countries, pre- and post-harvest fungal diseases cause 12% crop loss (El Reza et al. 2010) and up to 50% fruit loss during storage and transport (De Cicco et al. 2008). On the other hand, about 35% of crops are lost annually due to pests (Bounechada and Arab. 2011). In addition, fungal and insect contamination decreases the post-harvest storage life and declines the marketability of the fruits (Tripathi et al. 2007). *Botrytis cinerea,* the causa agent of grey mould disease, is multidrug resistant to anti-botrytis fungicides. It is a polyphagous pathogen attacking more than 200 plant species (Jarvis 1980) including greenhouse grown crops (Rosslenbroich and Stuebler 2000), as well as cold-stored fruits (Williamson et al. 2007). In particular, strawberries

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are very sensitive to the grey rot agent during cultivation, storage and marketing.

To control postharvest fungi, a variety of synthetic chemicals have been applied. However, overreliance on synthetic pesticides is disheartened because of their harmful effects on health and the environment, in addition to the development of resistance among strains of pests and pathogens. Moreover, pesticides are characterized by significant drawbacks including toxicity to non-target organisms (Soylu et al. 2010), carcinogenic and teratogenic potential, and persistence in the food chain (Lingk 1991). Furthermore, increasing public awareness of human health, food safety and environment preservation has led consumers to ban products treated with chemical agents (Teixeira et al. 2012). Against this background, the scientific community was compelled to seek new sustainable and healthy alternatives by exploring new natural molecules from plants to create a new generation of botanical pesticides for pests and disease management. Since ancient times, essential oils have been used in natural pharmaceutical preparations and in food conservation (Prabuseenivasan et al. 2006). The use of essential oils has recently become an attractive technique applied in postharvest disease control (Znini et al. 2013); it is associated with the fungicidal and insecticidal activities of their vapour phase allowing them to be used as bio-fumigants for the management of rot in stored products (Tripathi et al. 2007) and as a relevant alternative to extend the shelf life and preservation of the overall quality of the fruit after harvest (Aloui et al. 2014). Due to their very rich and complex composition, resistance of organisms to essential oils is rarely established (Siroli et al. 2015). It is difficult to relate the biological activity of essential oils to a single component, but it seems to be the result of additive and synergistic action of their components (Bagamboula et al. 2004).

The interest of the present work was to characterize the chemical composition of *Mentha pulegium* L (Lamiaceae) and *Myrtus communis* L (Myrtaceae) essential oils from oriental Numidia (Algeria), then to assess their antifungal potential in vitro and in vivo against *Botrytis cinerea* on strawberries.

Material and methods

Plant material

Aerial parts of each species were sampled from two different sites in the Annaba region located in the Northeast of Algeria (Oriental Numidia) where they grow spontaneously in the wild state. For *M. pulegium*, samples were harvested during the flowering period (July, 2018) from Zâamcha locality (12 km south of Annaba: 36° 47′ N, 7° 45′ E), whereas *M. communis* samples were collected during the setting period (October, 2017) from the Edough Massif (13 km west of Annaba: 36° 55′ N, 7° 36′ E). The plant material was air-dried in the shade at room temperature (20–25 °C) for 7 days and then kept in glass boxes and stored in a dry place.

Essential oils extraction and GC/MS analysis

Dried leaves of each plant (100 g) were submitted to hydrodistillation for 90 min using a Clevenger-type apparatus. The obtained essential oils were stored in opaque airtight flasks and kept at 3–4 °C. Essential oil yields of each plant species were calculated according to Carrée (1953). The identification of the volatile compounds of essential oils was carried out using an Agilent 7890A gas chromatograph combined to an Agilent 5972C mass spectrometer with electron impact ionization (70 eV). The mass spectrometer was equipped with an HP-5 MS capillary column (19091S-433), length 30 m, diameter 250 µm and film thicknesses 2.5 µm (5% phenyl methyl silicone, 95% dimethylpolysiloxane; Hewlett-Packard, CA, USA). Column temperature was automated to rise from 50 to 250 °C at a rate of 7 °C/ min. The essential oils components were identified by comparing their retention indices (RI) relative to n-alkanes with those of authentic compounds published in the literature or available in our laboratory. Additionally, the identification was confirmed by matching their mass spectra with those recorded in the Wiley Registry 9th Edition/NIST 2011 Edition mass spectral library. The composition of the essential oils was given as a relative percentage of the total peak area.

Antifungal bioassay

Fungal isolation and molecular identification

A virulent strain of *B. cinerea* was isolated from a symptomatic strawberry showing grey mould symptoms. It was purified and identified on the basis of morphological criteria according to Jarvis (1980). The purified strain was maintained on potato dextrose agar (PDA) medium at 25 ± 2 °C for routine use and stored at − 80 °C on 30% glycerol for long term storage.

Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA were amplified using primer pair (White et al 1990) ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′) according to Kalai et al. (2010). PCR amplification products were then sequenced and the resulting sequence was checked and processed for sequence similarities in DNA databases by The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Poisoned food technique

The toxicity of *M. pulegium* and *M. communis* essential oils against *B. cinerea* was studied using the poisoned food technique (Mohana and Raveesha 2007). Eight increasing concentrations (0.16, 0.33, 0.66, 1.33, 2.66, 5.33, 10.66 and 21.33 µl/ml) of each essential oil were tested by being incorporated into 15 ml of sterile PDA medium cooled to 40 °C and mixed well before solidification. Subsequently, an 8 mm diameter agar plug was aseptically removed from the edge of seven-day-old *B. cinerea* cultures and placed in the centre of each Petri dish. The control was conducted under the same conditions but without any supplementation. Petri dishes were incubated for 5 days in dark at 25 ± 2 °C. Mycelial growth (cm) was measured daily. Three replicates were performed for each essential oil and concentration. Growth inhibition was calculated according to the formula of Cakir et al. (2005), in percentage inhibition of radial growth of the treated samples compared to the control.

$$\% \text{ inhibition} = \left[(C - T)/C\right] \times 100$$

where C Mean radial mycelial growth of pathogen alone (control), T Mean radial mycelial growth of treated samples.

The lowest concentration of essential oils without fungal growth was considered a minimum inhibitory concentration (MIC).

Effect of essential oil vapour exposure

The toxicity of *M. pulegium* and *M. communis* essential oils against *B. cinerea* was also performed using the volatile activity technique as described by Neri et al. (2006) slightly modified. For this assay, 8 mm of mycelial disc was inoculated in PDA dishes and exposed to volatiles compounds. Sterile squares of Whatman filter paper N °1 were glued with sterile adhesive tape to the inner surface of the lids of the petri dishes. Subsequently, the sterile squares were supplemented by 0, 25, 30 or 35 µl of pure essential oils. Petri dishes were sealed with Parafilm, inverted and incubated for 5 days in the dark at 25 ± 2 °C. Three replicates were carried out for each concentration and each oil. Mycelial growth diameters were noted daily and data were expressed as per cent inhibition of radial mycelial growth according to Plaza et al. (2004). Minimum inhibitory concentration (MIC) was assigned to the lowest concentration able to inhibit totally fungal growth.

Minimum fungitoxic concentration (MFC)

To determine the fungitoxic concentration of *M. pulegium* and *M. communis* essential oils against *B. cinerea*, fungal discs showing no visual fungal growth when exposed or contacted with a volatile compound were transferred and reinoculated into fresh PDA medium. Fungal growth was observed after 7 days of incubation at 25 ± 2 °C.

Spore germination assay

A conidial suspension of *B. cinerea* was prepared by scraping off a ten-day-old culture and resuspending the resulting mycelium in sterile glucose solution 5%. Conidial suspension was aseptically filtrated and adjusted to 10^5 spores/ml by hemocytometer (Malassez). In vitro assays were performed using concave micro-culture slides by mixing 40 µl of either crude or MFC (diluted in 5% DMSO) of essential oil with 40 µl of conidial suspension (10^5 cells/ml). Control was prepared by mixing 40 µl of sterile glucose solution 5% with 40 µl of conidial suspension (10^5 cells/ml). Slides were incubated in a wet and dark chamber at 25 ± 2 °C for 48 h then observed with an optical microscope (Leica) at 1000 magnification. Each treatment was conducted in quadruplicate. The percentage of conidial germination was evaluated using four regions per slide and corresponding to at least 300 conidia.

Microscopic observation

Mycelium was obtained by mixing on a slide 40 µl of sterile glucose solution 5% with 40 µl of conidial suspension (10^5 cells/ml). Four slides were incubated in a wet chamber at 25 °C in the dark and allowed to grow till a well-developed mycelium was obtained. The effect of essential oils on the mycelium structure was evaluated by adding 40 µl of crude essential oils to the mycelium (two replicates). Incubation was performed under the same conditions as previously described and observation of the mycelia morphology was monitored daily using an optical microscope. Control was run by mixing sterile glucose solution 5% instead of essential oil with the mycelium. Conidia were obtained from the previous spore germination test.

In vivo *application*

Selection of the most suitable essential oil to undertake in vivo testing was based on the higher yield and lower MFC. Therefore, firm and freshly harvested strawberries (cultivar camaroza) were first superficially sterilized by soaking in 2% sodium hyperchlorite solution for 5 min, rinsed in sterile distilled water and then dried on sterile Whatman filter paper before starting the tests. Fruits were then inoculated on the surface with 8 mm mycelium discs diameter obtained from 10-day-old culture of *B. cinerea* at the rate of one disc per strawberry. Treatment of inoculated fruits with essential oil was assessed either by direct

contact or by fumigation immediately after inoculation assessed in plastic boxes covered with moistened sterile filter paper. Thus, 30 µl of pure essential oil was applied directly to the fruits for the first test, whereas in the second test, 400 µl/l air (in vitro MIC) was deposited in sterile Whatman filter paper glued inside the lids of the box. All boxes were sealed with parafilm to ensure airtightness and were incubated at 7 °C for 13 days. Treatments and controls were replicated three times at the rate of three strawberries by box and one box per repeat. The incidence of contamination was assessed using a rot index. The severity of strawberry disease in each replicate was assessed according to the empirical scales of Zhao et al. (2011) slightly modified: 0: healthy fruit, 1: rotten area less than 12% of the total fruit surface; 2: rot surface between 12 and 25% of the fruit surface, 3: rot surface between 25 and 40% of the fruit surface, 4: symptoms extend over an area ranging from 50 to 75% of the fruit surface; 5: rotten area greater than 75% of the fruit surface. The disease index was calculated by the following formula:

$$1 \times N1 + 2 \times N2 + 3 \times N3 + 4 \times N4 + 5 \times 5/5 \times \mathrm{NT}$$

where $N1$ to $N5$ is the respective number of fruits on each scale and NT is the total number of fruits examined. The evaluation of decay inhibition (DI) was assessed according to De Corato et al. (2010) as follows:

$$\mathrm{DI} = \left[\left(N_c - N_o\right) / N_c\right] \times 100$$

where N_c is the average of the number of infected fruits in the untreated sets and N_0 is the average of the number of infected fruits in the oil-treated sets.

Phytotoxicity assay of M. pulegium essential oil vapour

Healthy, firm and uniform strawberries were selected to assess the optimal conditions for safe application of essential oils vapour to the fruit by testing different storage temperatures (7, 12, 16, 20, 25 °C). Symptoms of phytotoxicity were evaluated by storing strawberries treated with the MIC of *M. pulegium* essential oil vapour at different temperatures according to a phytotoxicity index: 0: no change, 1: colour change**,** 2: superficial softening, 3: important softening**,** 4: modification of the pulp. The injury index was calculated by the following formula:

$$1 \times N1 + 2 \times N2 + 3 \times N3 + 4 \times N4 + 5 \times N5/5 \times \mathrm{NT}$$

where N1 to N5 is the respective number of fruits of each index and NT is the total number of fruit examined. Fruits were stored in airtight boxes containing four strawberries and each box was considered as a replicate. In this experience, three replicates were assessed.

Effect of M. pulegium essential oil vapour on physical parameters of stored strawberries

a. Surface colour change measurement

Fruit surface colour was measured on three batches of strawberries considering 10 fruits/batch. Measurements were performed on M. *pulegium* essential oil vapour treated fruits and untreated control fruits before and after 6 days of storage at 7 °C. The CIE L*, *a**, and *b** parameters were provided by a chromameter (CR 400, Minolta) at the two opposite sides of the fruit. *a** values indicate green or red colour from negative to positive values. *b** values designate yellow colour: the higher this parameter, the more the colour becomes yellow. H or hue degree was also calculated according to the formula: h° = arctangent [*b**/*a**], considering 0° = red purple, 90° = yellow, 180° = bluish green and 270° = blue. Chroma or intensity or colour saturation is finally computed according to the formula $C^* = [a^*2 + b^*2]1/2)$.

b. Weight loss measurement

Weight loss was evaluated by measuring the weight of three batches of each strawberry treated and untreated with *M. pulegium* essential oil considering 10 fruits/batch. Weight loss was calculated before and after 6 days of storage at 7 °C by the formula:

$$((\mathrm{NSW} - \mathrm{SW})/\mathrm{NSW}) \times 100$$

With NSW: weight of not stored fruits; SW: weight of stored fruits.

Statistical analysis

Results were expressed as mean ± SD. Data were compared by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. All analyses were performed using SPSS22.0. A P value < 0.05 was considered significant.

Results

Essential oils chemical composition

The oil yield of *M. pulegium* and *M. communis* was 2.14 and 0.63%, respectively (Table 1). Statistical analysis showed a significant difference in oil yield between the two species ($F_{1,4} = 950.47$, $P < 0.001$). The identified components, the percentage (%) and retention index (RI) of two essential oils are listed in Table 1. A total of 17 volatile constituents

Table 1 Chemical composition of *Mentha pulegium* and *Myrtus communis* essential oils collected from Bône (North eastern Algeria)

Volatile components	RI	*M. pulegium*	*M. communis*
α-pinene	939	0.86	**29.08**
Camphene	954	0.48	1.91
Sabinene	976	**3.29**	–
β-pinene	980	0.36	0.77
3-Octanone	985	0.48	–
β-myrcene	991	0.31	–
Δ-3-carene	1011	–	0.64
p-cimene	1023	–	1.52
D-Limonene	1028	**8.61**	–
Eucalyptol	1033	0.29	**36.82**
γ-terpinene	1053	–	0.53
α-terpinolene	1089	–	0.47
β-linalool	1098	–	**4.04**
p-menthone	1126	**17.66**	–
Trans-Pinocarveol	1138	–	1.04
2.8-p-Menthadien-1-ol	1140	1.37	–
Camphor	1143	0.54	0.46
Isomenthone	1159	**55.59**	–
Neomenthol	1167	1.82	–
Terpinen-4-ol	1178	0.76	1.09
α-terpineol	1189	–	**6.42**
Isopiperitenol	1210	**2.11**	–
trans-carveol	1230	–	0.33
Pulegone	1237	0.3	–
Piperitone	1245	**5.15**	–
Geraniol	1255	–	1.15
Linalylacetate	1257	–	0.51
Geranylacetate	1383	–	**4.38**
Methyleugenol	1401	–	**2.59**
Caryophyllene	1420	–	0.42
Caryophylleneoxide	1581	–	0.96
Grouped components (%)			
Total monoterpenehydrocarbons		**14.39**	**35.25**
Total oxygenatedmonoterpenes		**85.59**	**58.92**
Total sesquiterpenehydrocarbons		–	**0.42**
Total oxygenatedsesquiterpenes		–	**0.96**
Total identified		**99.98**	**95.13**
Yield (%) (w/w)		2.14	0.64

–Compound not detected; RI: Retention Index calculated on a HP-5MS capillary column (30 m × 0.25 mm × 0.25 mm)

Major compounds (> 2%) and chemical classes are marked in bold form

amounting 99.98% of the total oil of *M. pulegium* have been detected. The chemical composition of *M. pulegium* oil was strongly dominated by oxygenated monoterpenes (85.59%) represented especially by isomenthone (55.59%) followed by p-menthone (17.66%), piperitone (5.15%) and isopiperitenol (2.11%). The hydrocarbon monoterpenes class was weakly represented (14.39%), while the sesquiterpenes class was totally absent in the essential oil. GC–MS analysis of *M. communis* essential oil revealed the presence of 20 main components representing 95.13% of the total composition of the oil. The essential oil of *M. communis* exhibited a high percentage of oxygenated monoterpenes (58.92%) and hydrocarbon monoterpenes (35.25%), unlike to a very weak presence of the sesquiterpenoids class (1.38%). Eucalyptol and α-pinene were the main active compounds accounting for 36.82 and 29.08%, respectively, in common myrtle oil.

Molecular identification of Botrytis cinerea isolate

The sequence obtained (Accession number: OM530236) matched 100% with *Botrytis cinerea* ATCC 11,542 sequence deposited in Genbank (Accession number KU729081).

Antifungal bioassay

Poisoned food technique

The minimum inhibitory concentration of *M. pulegium* and *M. communis* essential oils not allowing the growth of *B. cinerea* was determined at 2.66 µl/ml and 5.33 µl/ml, respectively, in comparison with a negative control showing 100% growth (Fig. 1). At 1.33 µl/ml, the inhibition of mycelial growth reached 87.66%, for *M. pulegium* and 19.26% for *M. communis.* Below this concentration, no significant antifungal effect was registered for the two essential oils, showing that inhibition percentage increased with increasing concentrations of the oils with a dose–response way.

Re-incubation of fully inhibited mycelium discs at doses equal to or greater than the MIC on fresh culture medium (PDA) determined that the minimum fungitoxic concentration (MFC) corresponded to the MIC for *M. pulegium* and 10.66 µl/ml for *M. communis*.

Effect of essential oil vapour exposure

Statistical analysis allowed to classify *M. pulegium* essential oil as the most effective against *B. cinerea* when it is applied by volatile activity method. In fact, *M. communis* oil showed a very weak volatile effect in inhibiting *B. cinerea* mycelial growth even at the highest concentration of 35 µl not exceeding 3.67% (Fig. 2). Whereas *M. pulegium* allows fungal inhibition varying from 77.48 to 100% at 25 µl and 30 µl, respectively (Fig. 2), knowing that, 30 µl of *M. pulegium* essential oil is the minimum inhibitory concentration towards *B. cinerea*. Mycelial discs completely inhibited by *M. pulegium* essential oil (30 and 35 µl) displayed normal

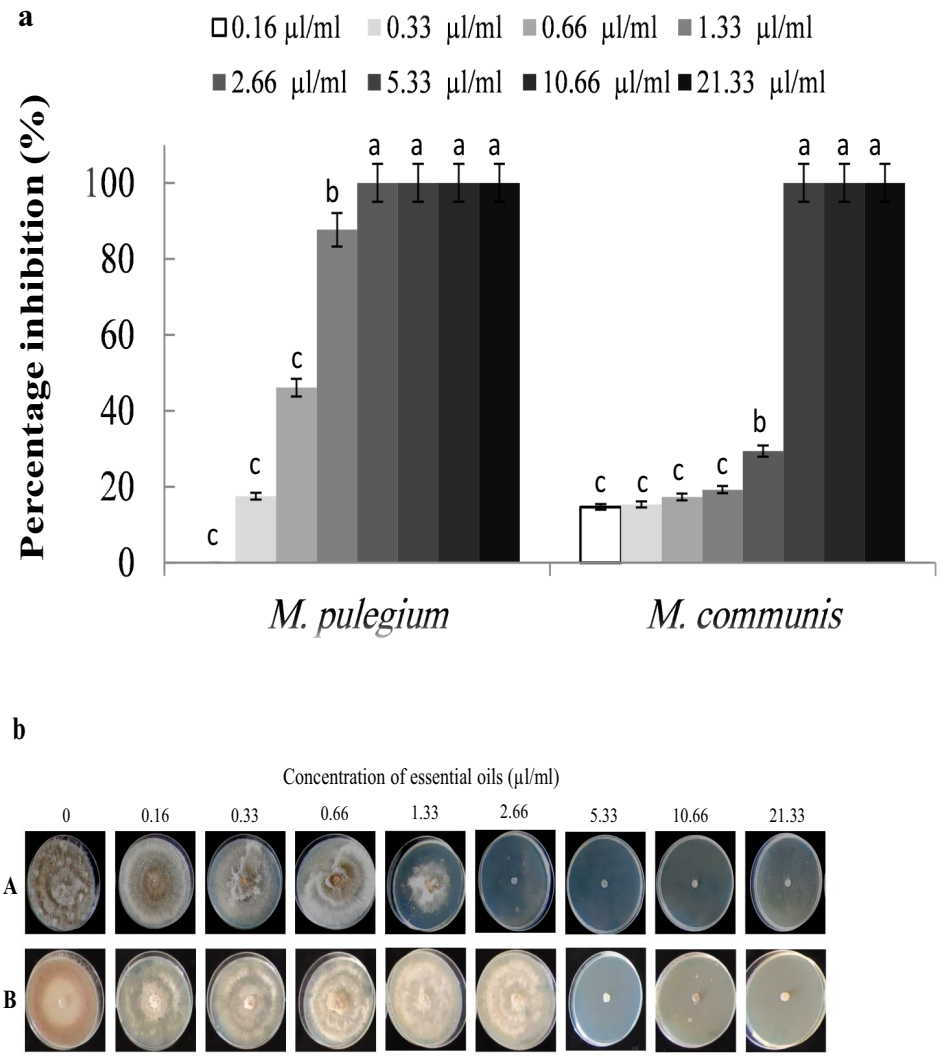

Fig. 1 Inhibition of *Botrytis cinerea* mycelial growth by *Mentha pulegium* and *Myrtus communis* essential oils by poisonous medium method at 7 days incubation. **a** inhibition index; **b** Mycelial growth inhibition; a: *Mentha pulegium*; b: *Myrtus communis.* Different letters are significantly different according to Duncan test at $P \leq 0.01$)

fungal growth when deposited in culture medium not treated with essential oil. This experiment demonstrated that the essential oil vapours of *M. pulegium* exhibit a fungistatic effect against *B. cinerea*.

Spore germination assay

The observation of the effect of *M. pulegium* and *M. communis* essential oils on *B. cinerea* conidia showed that the two essential oils totally inhibited fungal germination compared to untreated control (Fig. 3and Table 2). Crude essential oil treated conidia also exhibited 88.68% and 87.28% of morphological modifications (Macrovacuolization, swelling and crumbling) related, respectively, to *M. pulegium* and *M. communis* assay (Fig. 3 and Table 2). MFC treated conidia showed a low percentage of germination reaching, respectively, 13.78% and 8.32% for the essential oils of *M. pulegium* and *M. communis* compared to the control (92.45%) (Table 2). However, ungerminated conidia treated with minimal fungitoxic concentrations of both oils showed no structural modifications.

Microscopic observation of essential oil treated mycelium

Microscopic observation of the mycelium treated with essential oils exhibited serious structural alterations compared to the control (Fig. 3h, i and j). In fact, the treated hyphae appeared thinner and were characterized by an irregular surface, the absence of septation and the presence of macrovacuolization (Fig. 3i and j) when the untreated control hyphae showed a typical septate structure with a regular cell wall and microvacuoles (Fig. 3h).

In vivo *assay*

Symptoms evaluation on inoculated and treated strawberries showed complete inhibition of *B. cinerea* decay by *M. pulegium* essential oil both by direct contact and by vapour

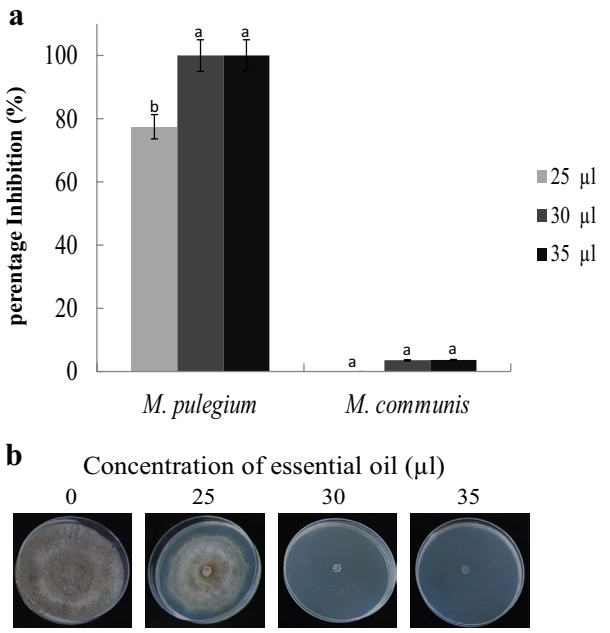

Fig. 2 Inhibition of *Botrytis cinerea* mycelial growth by *Mentha pulegium* and *Myrtus communis* essential oils using volatile activity method. **a** inhibition index; **b** Mycelial growth inhibition. Different letters are significantly different according to Duncan test at $P \leq 0.01$)

exposure (Fig. 4). Untreated control strawberries showed 100% symptom development corresponding to a maximum disease index (Table 3 and Fig. 4).

Phytotoxicity test

Strawberries exposed to oil vapours displayed a very good appearance aspect at low temperatures (7, 12 and 16 °C). Statistical analysis showed a significant effect of storage temperature on the phytotoxicity of essential oils. Indeed, the increase in temperatures induced an increase in damage to the strawberries (Fig. 5) with injury indices varying from 0.08 to 1 for temperatures ranged from7 to 25 °C**.**

Effect of *M. pulegium* essential oil fumigation on strawberries surface colour during storage Evaluation of *M. pulegium* essential oil fumigation on stored strawberries revealed that there was overall no significant change in the surface colour of strawberries compared to untreated control strawberries. However, there was a significant preservative effect on certain fruit colour parameters (Table 4). Indeed, $H°$ and $b*$ decreased in the stored fruits treated or not treated compared to the fruits not preserved (Table 4). $a*$ also showed a significant difference between strawberries stored at 7 °C and

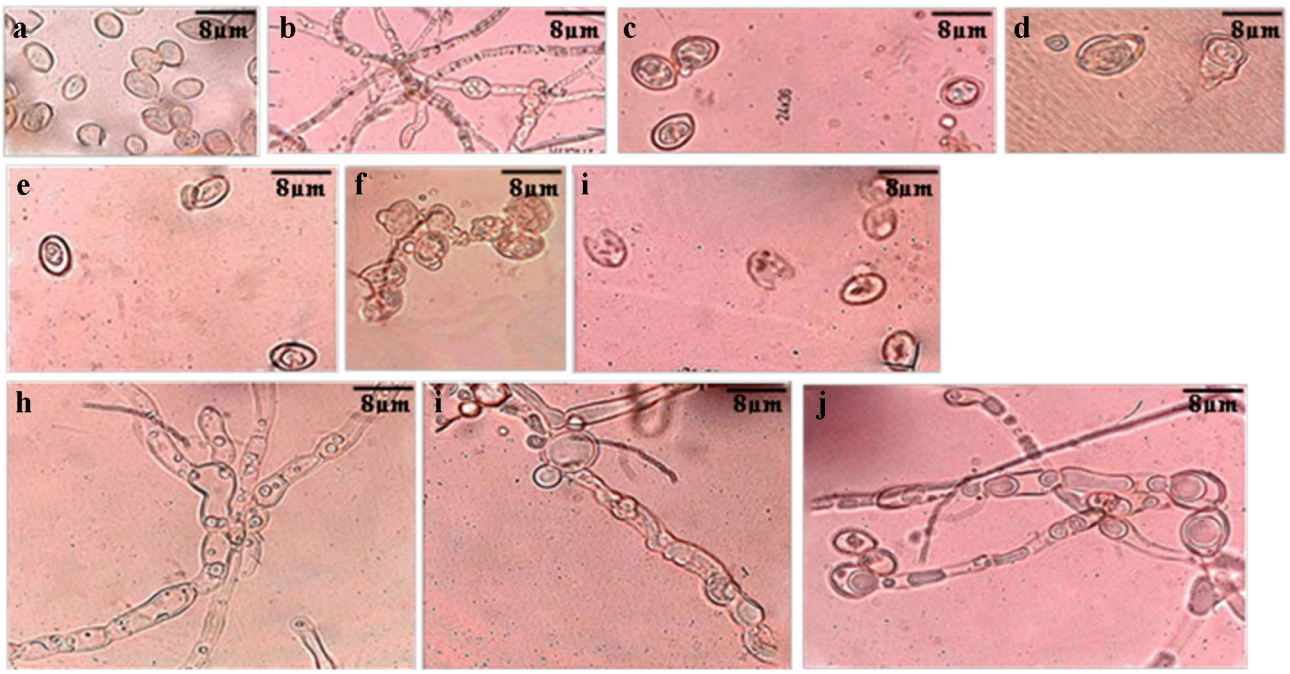

Fig. 3 Micrograph of morphological modifications of *Botrytis cinerea* conidia and mycelium treated with *Mentha pulegium* and *Myrtus communis* essential oils. **a** untreated conidia before germination; **b** untreated positive control of germinated conidia; **c** conidia treated with *M. communis* oil showing macrovacuolization of the cytoplasm and pore formation; **d** conidia treated with *M. communis* oil broken up releasing its intracellular content; **e** conidia treated with *M. pulegium* oil showing macrovacuolization; **f** Conidia treated with *M. pulegium* oil showing cell wall deformations; **g** conidia treated with *M. pulegium* oil broken up; **h** normal untreated mycelium; **i** mycelium treated with *M. communis* showing macrovacuolization and membrane deformation; **j** Mycelium treated with *M. pulegium* oil showing macrovacuolization

Table 2 Percentage of *B. cinerea* spore germination and modifications after treatment with *M. pulegium* and *M. communis* essential oils

Treatment	Crude essential oils		Minimal fungitoxic concentration	
	Germination (%)	Modified spores (%)	Germination (%)	Modified spores (%)
Control	$78.112^{a} \pm 10.174$	0^{b}	$92.455^{a} \pm 2.265$	0^{a}
M. pulegium	0^{b}	$88.687^{a} \pm 6.433$	$13.789^{b} \pm 4.143$	0^{a}
M. communis	0^{b}	$87.285^{a} \pm 10.268$	$8.329^{c} \pm 2.653$	0^{a}

Different letters are significantly different according to Duncan test at $P \leq 0.01$)

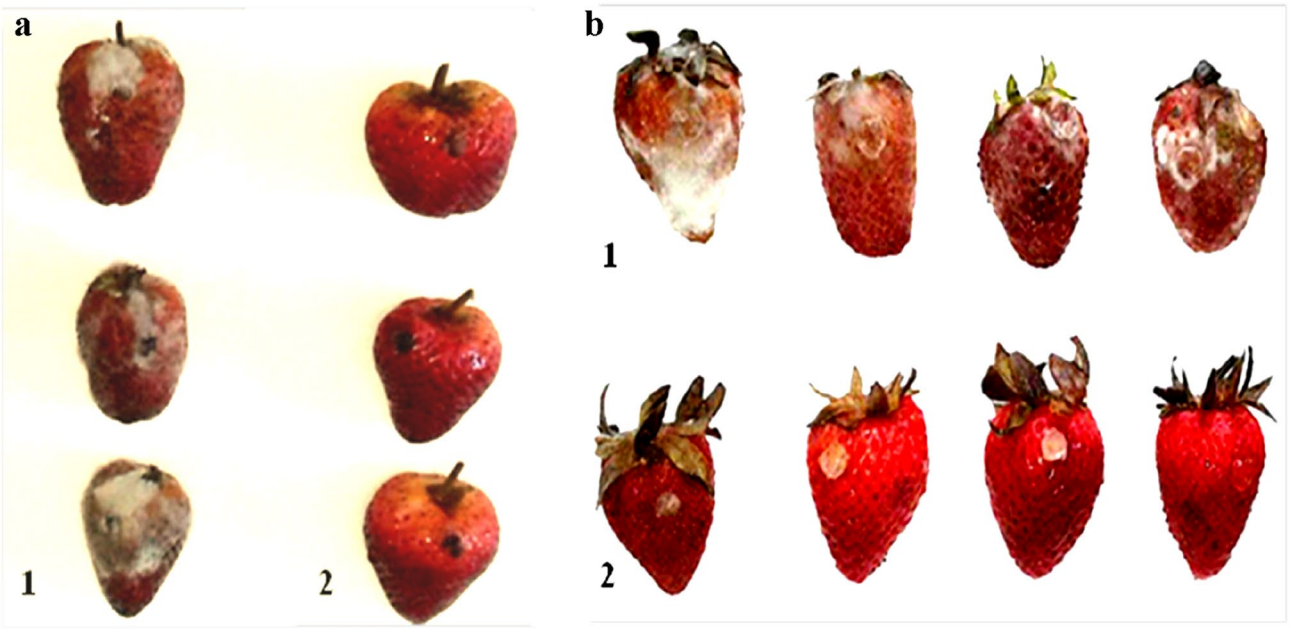

Fig. 4 Protective effect of *Mentha pulegium* essential oil against *Botrytis cinerea* on strawberries by direct contact (**A**) and vapor phase (**B**) treatment after 13 days of inoculation.**1**: Control strawberries non treated with essential oil and inoculated with *Botrytis cinerea*; **2**: strawberries treated with essential oil and inoculated with *Botrytis cinerea*

Table 3 Inhibition of grey mould disease index on *Mentha pulegium* essential oil treated strawberries

Treatment	Disease index	Decay inhibition (%) (DI)
Control	1^{a}	–
Direct contact	0^{b}	100
Exposure to volatile	0^{b}	100

Different letters are significantly different according to Duncan test at $P \leq 0.01$)

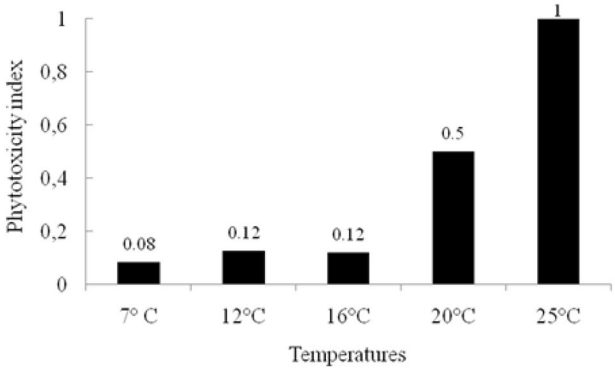

Fig. 5 The effect of storage temperatures on *Mentha pulegium* essential oil vapor phase phytotoxic effect on strawberries

pre-stored fruits highlighting a storage effect. Otherwise, chroma (c) was not affected by storage but fruits treated with essential oils showed a slight decrease in colour intensity (− 8%) compared to untreated strawberries before and after storage. Knowing that the treatment with essential oils did not affect any colour parameter before storage.

Effect of *M. pulegium* essential oil fumigation on strawberries weight during storage Statistical analysis revealed a

Table 4 Colour parameters measurement of not treated and treated strawberries with *M. pulegium* essential oil before and after 6 days of storage

Storage period (Days)	Treatment	H	C	*a**	*b**
6	Control	$0.84^{b} \pm 0.061$	$51.24^{a} \pm 4.691$	$33.74^{a} \pm 2.16$	$38.44^{ab} \pm 5.22$
6	*M. pulegium* EO fumigation	$0.88^{ab} \pm 0.0526$	$47.16^{b} \pm 3.19$	$29.82^{c} \pm 2.11$	$36.45^{b} \pm 3.44$
0	Control	$0.89^{a} \pm 0.086$	$50.99^{a} \pm 3.779$	$31.70^{b} \pm 2.68$	$39.69^{a} \pm 5.22$
0	*M. pulegium* EO fumigation	$0.9^{a} \pm 0.091$	$52.33^{a} \pm 4.407$	$32.24^{b} \pm 3.59$	$40.95^{a} \pm 5.46$

Different letters are significantly different according to Duncan test at $P \leq 0.01$)

Table 5 Weight loss of not treated and treated strawberries with *M. pulegium* essential oil after 6 days long storage at 7 °C

Treatment	Weight loss%
M. pulegium EO fumigation	$1.92^{b} \pm 0.60$
Control	$22.907^{a} \pm 2.04$

Different letters are significantly different according to Duncan test at $P \leq 0.01$)

significant difference between the weight loss of strawberries treated or not with *M. pulegium* essential oils. Indeed less than 2% of weight loss was recorded in fruits treated with essential oils and preserved, i.e. 1420 times less than untreated and preserved fruits showing a weight loss of 22.90% (Table 5).

Discussion

In this study, the antifungal activities of essential oils (EO) of *Mentha pulegium* and *Myrtus communis* were evaluated on the mycelial growth and the sporulation rate of *B. cinerea* on synthetic medium and on the development of grey mould in strawberries. The results showed that the two oils completely stopped the mycelial growth of the fungus in the solid medium. In particular, essential oil of *M. pulegium.* was the most effective against *B. cinerea* with a lower MIC (2.66 µl/ml) and allowing a fungitoxic effect (by Poisoned food technique and exposure to essential oil vapours)). However, *M. communis* essential oil expressed higher MIC (5.33 µl/ml) and MFC (10.66 µl/ml) by the Poisoned food technique and showing a very weak volatile antagonist effect against mycelial growth of *B. cinerea*. This difference in the antifungal potential of the essential oils of the two plants can be attributed to their chemical compositions. This is because *M. pulegium* oil is dominated by a ketone while *M. communis* oil is mainly constituted of terpene oxide. Previous works (El Arch et al. 2003; Saeed et al. 2012) have shown that ketones are more active against microbial agents than terpene oxides. Although, according to Koba et al. (2004), the inhibitory power of essential oils towards a microbial strain is classified as excellent inhibitory power for: MIC < 50 µl/ml. Thus, on the basis of their MIC, the oils of *M. pulegium* and *M. communis* originating from Algeria are considered to be natural antifungal compounds potent against *B. cinerea*. These results corroborate previous works which demonstrated the good antifungal effect of these two essential oils against dermatophyte strains (*M. furfur*, *M. sympodialis*, *Malassezia* sp (Barac et al. 2017)) and non-dermatophyte strains (*Alternaria alternata*, *Penicillium expansum*, *Botrytis cinerea*, *Fusarium culmorum*, *Fusarium oxysporum*, *Aspergillus flavus*, *Aspergillus niger* and *Trichoderma* sp (Uwineza et al. 2018); *Rhizopus* sp, *Aspergillus* sp. and *Penicillium* sp (Amalich et al. 2016)). This antifungal potency of essential oils is certainly attributed to their chemical composition rich in oxygenated compounds (Soltani and Kellouche. 2004; Bourkhiss et al. 2007; Amarti et al. 2010) representing 85.59% in *M. pulegium* and 58.92% in *M. communis.*

In addition to the quantitative dissimilarities, there are mainly qualitative differences, between the essential oils of *M. pulegium* and *M. communis*. In fact, *M. communis* essential oil mainly consists of eucalyptol (36.82%) and α-pinene (29.08%) whereas in *M. pulegium* essential oil the major compound is isomenthone (55.59%) followed by p-menthone (17.66%).

These results corroborate previous works on the chemical composition of *M. communis* essential oil (Aouadi et al. 2020), whereas they contradict the results of Beghidja et al. (2007), Zekri et al. (2013), Ouakouak et al. (2015); Abdelli et al. (2016) stipulating that Algerian pennyroyal oils are characterized by the predominance of pulegone. Likewise, Tunisian (Snoussi et al. 2008; Hajlaoui et al. 2009), Portuguese (Rodrigues et al. 2013), Moroccan (Farah et al. 2001; Chebli, et al. 2003; Ouraini et al. 2005; Ouraini et al. 2007), Brazilian (Silva et al. 2015), Uruguayan (Lorenzo et al. 2002), Egyptian (El Ghorab 2006) and Yugoslavian (Teixeira et al. 2012) *M. pulegium* essential oils are reported to be predominantly pulegone. Nevertheless, Spanish (De Gavina and Ochoa. 1974), Uruguayan (Grosso and Moyna. 1985), Cuban (Pino et al. 1996) and Greek (Kokkini et al. 2004) pennyroyal oils are characterized by the prevalence of isomenthone which

is in concordance with our findings. Although, another chemotype rich in pipéritone/pipéritenone has also been reported in Morocco (Derwich et al. 2010) and in Greece (Kokkini et al. 2004).

Indeed, the study of essential oils from different populations of *M. pulegium* allowed to classify three chemotypes: pulegone-type, piperitenone/piperitone-type and isomenthone neoisomenthol-type (Lawrence 1978).

According to Rodrigues et al. (2013), the chemical composition is closely related to the phenological stage; thus, the relative amount of pulegone in *M. pulegium* essential oil increases until the vegetative phase then decreases while anticipating the pre-flowering phase. Towards full flowering, the relative amount of pulegone increased further. These changes are followed by changes in the relative amounts of isomenthone and menthone, as pulegone decreased isomenthone and menthone tended to increase.

Chemical composition also depends on seasonal variations, geographic areas, climatic and edaphic conditions (Müller-Riebau et al. 1997) generating variable bioactivity profiles within the same species (Gon Çalves et al. 2007).

However, most essential oils rich in alcohols and/or ketones have a stronger antimicrobial activity than those which have high hydrocarbon contents (Charai et al. 1996; Koroch et al. 2007). Indeed, pulegone have been widely reported to be responsible for the antifungal activity of essential oil (Muller-Riebau et al. 1995; Samber et al. 2014; Boni et al. 2016) but is also known to be hepatotoxic and is not suitable for aromatherapy or post-harvest or stored food processing. Moreover, 1,8-cineole, piperitenone (Dorman and Deans. 2000; El Arch et al. 2003; Satrani 2010), menthone (Hmiri et al. 2011) and isomenthone (Gon Çalves et al. 2007) have also been reported to exhibit an antifungal effect.

Biological activity could also be the result of the synergistic effect of minor compounds (Bouzouita et al. 2008; Saban et al. 2008). The work of Chebli et al. (2003) and Vilela et al. (2009) has shown that major compounds inhibit mycelial growth, but at higher concentrations when the essential oils are in their entirety; thus, the activity of essential oil is the result of its major compounds and also of the synergistic effect of minor compounds (Chebli et al. 2003; Ouraini et al. 2007).

The mechanism of action of terpenes is not fully understood, but it is likely that these lipophilic compounds soluble in aqueous media cause significant damage to the cell walls of microorganisms (Griffin et al. 1999) through loss of membrane integrity (Cowan 1999; Hajlaoui et al. 2009) and inducing deleterious effects on mitochondrial membranes leading to inhibition of mitochondrial energy metabolism, resulting in disturbances in a wide range of physiological and biochemical processes in the cell (Yoshimura et al. 2010). It is also suggested that the essential oil compound can act as an H^+ carrier or a depleting adenosine triphosphate pool (Farag et al. 1989; Adams et al. 1996; Ultee et al. 2002).

In this context, microscopic observations have corroborated the loss of membrane integrity of *B. cinerea* conidia treated with crude essential oil exhibiting nearly 90% morphological modifications (macrovacuolization, swelling and crumbling). The two crude essential oils studied abolished the germination process probably resulting from disturbances of physiological and biochemical processes induced by essential oils. Whereas, treated conidia with the minimum fungitoxic concentration (MFC) exhibited a germination inhibition of nearly 80% and no membrane deformation which corroborating the dose-response activity of the two oils. In fact, the activity of essential oils has been investigated by many authors who implied that antimicrobial activity results from the interaction of the active compound at low concentrations, with the enzymatic systems of microbial cells (Omidbeygi et al. 2007; Russo et al. 2013), whereas at higher concentrations, they cause protein denaturation (Russo et al. 2013).

The ability of *M. pulegium* essential oil to inhibit the growth of *B. cinerea* was also confirmed by direct application of the oil to the fruit surface or by fumigation. Accordingly, both methods of application were efficient when in vitro MIC was tested. Indeed no rot symptoms were observed on the fruits treated and inoculated with essential oil compared to the untreated and inoculated control fruits showing severe symptoms of grey rot. The in vivo tests were carried out under storage condition (7 °C) and indicated that at low temperature, *M. pulegium* essential oil did not affect the quality of the fruits. Nevertheless, knowing that several essential oils could be phytotoxic (Kalai-Grami et al. 2019; Tsao and Zhou. 2000), the optimal conditions for a safe application of the essential oil of *M. pulegium* were evaluated. Results revealed a significant effect of storage temperature on the phytotoxicity of essential oils. Indeed, when the temperature exceeds 16 °C, the quality of the strawberries is drastically affected. Similarly, Tsao and Zhou (2000) found that the phytotoxicity of monoterpenoids is also related to temperature and becomes negligible at 2 °C against post-harvest pathogens.

Therefore, phytotoxicity testing is necessary before considering an essential oil as an alternative disease control method. However, these drawbacks can be overcome by the formulation (Plotto et al. 2003), the association with other compounds and the application temperature (Tsao and Zhou. 2000). Besides, the level of phytotoxicity depends on the species, variety and phonological stage treated by the essential oil (Vidal et al. 2018). Therefore, *M. pulegium* oil could be non-phytotoxic when applied to other fruits or other stored products even at higher temperature.

The low phytotoxicity of this essential oil at 7 °C was also confirmed by studying the effect of *M. pulegium* essential oil vapour on the physical parameters of stored strawberries. Indeed, no significant colour change was recorded in the stored treated strawberries compared to the untreated and preserved strawberries although a small decrease in the chroma value was observed. These results are in accordance with Ulukanli and Tulin (2015) and Aitboulahsen et al. (2018) who noted a slight decrease in the chroma value in treated stored fruits.

Similarly, no significant weight loss was registered in the treated and stored strawberries compared to the stored control. In addition, the *M. pulegium* essential oil even had a protective effect on the treated fruits by reducing the weight loss by 22.90% compared to the control being up to the strawberry acceptance limit (6%) (Aitboulahsen et al. 2018). The reduction in weight loss by treatment with essential oils has also been reported by many authors (Martinez-Romero et al. 2005; Serrano 2005) which implies that it could probably be attributed to the creation of a protective layer on the surfaces of fruit openings preventing water loss by minimizing metabolic activity, respiration and transpiration during post-harvest fruit storage (Shafiee et al. 2010).

Conclusion

Overall, it can be concluded that the essential oil of *M. pulegium* displays interesting contact and fumigant toxicity against *B. cinerea*. Accordingly, *M. pulegium* essential oil could be applied as a potential botanical pesticide and can be considered as a safer and cleaner alternative to preserve the fruits from fungal attacks.

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Declarations

Conflict of interest All authors have no conflict of interest of any authority or persons in the field of our work in national and international levels.

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