



Article

The Use of Essential Oils from Thyme, Sage and Peppermint against *Colletotrichum acutatum*

Armina Morkeliūnė * , Neringa Rasiukevičiūtė , Lina Šernaitė  and Alma Valiuškaitė 

Laboratory of Plant Protection, Lithuanian Research Centre for Agriculture and Forestry, Institute of Horticulture, LT-54333 Babtai, Lithuania; neringa.rasiukeviciute@lammc.lt (N.R.); lina.sernaite@lammc.lt (L.Š.); alma.valiuskaite@lammc.lt (A.V.)

* Correspondence: armina.morkeliune@lammc.lt

Abstract: The *Colletotrichum* spp. is a significant strawberry pathogen causing yield losses of up to 50%. The most common method to control plant diseases is through the use of chemical fungicides. The findings of plants antimicrobial activities, low toxicity, and biodegradability of essential oils (EO), make them suitable for biological protection against fungal pathogens. The aim is to evaluate the inhibition of *Colletotrichum acutatum* by thyme, sage, and peppermint EO in vitro on detached strawberry leaves and determine EO chemical composition. Our results revealed that the dominant compound of thyme was thymol 41.35%, peppermint: menthone 44.56%, sage: α,β -thujone 34.45%, and camphor: 20.46%. Thyme EO inhibited *C. acutatum* completely above 200 $\mu\text{L L}^{-1}$ concentration in vitro. Peppermint and sage EO reduced mycelial growth of *C. acutatum*. In addition, in vitro, results are promising for biological control. The detached strawberry leaves experiments showed that disease reduction 4 days after inoculation was 15.8% at 1000 $\mu\text{L L}^{-1}$ of peppermint EO and 5.3% at 800 $\mu\text{L L}^{-1}$ of thyme compared with control. Our findings could potentially help to manage *C. acutatum*; however, the detached strawberry leaves assay showed that EO efficacy was relatively low on tested concentrations and should be increased.

Keywords: biocontrol; chemical composition; inhibition; *Mentha piperita*; *Salvia officinalis*; *Thymus vulgaris*



Citation: Morkeliūnė, A.; Rasiukevičiūtė, N.; Šernaitė, L.; Valiuškaitė, A. The Use of Essential Oils from Thyme, Sage and Peppermint against *Colletotrichum acutatum*. *Plants* **2021**, *10*, 114. <https://doi.org/10.3390/plants10010114>

Received: 11 December 2020

Accepted: 3 January 2021

Published: 8 January 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The strawberry anthracnose can be considered as one of the most important diseases, caused by several species complex of *Colletotrichum* spp.: *Colletotrichum acutatum* J. H. Simmonds, Brooks and *C. gloeosporioides* (Penz.) Penz. and Sacc. [1,2]. It also infects and causes diseases in many economically important crops. Strawberry anthracnose causes yield losses of up to 50% and plant death up to 80% and was considered to be a warmer climate zone pathogen, where the optimal temperature is from 15 to 30 °C, with optimal 25 °C temperature [3–5]. Strawberry diseases are controlled by several fungicide applications [6,7]. The growing resistance to pesticides and their adverse environmental effects leads to a new environmentally-safe disease control strategy [8–10].

Essential oils (EO) demonstrate a distinct level of antimicrobial activity to various ranges of strawberry pathogens [6,9,11,12]. The EO includes terpenes, terpenoids, aromatic, and aliphatic constituents, and most importantly, they contain antioxidants and biologically active compounds.

The EO is commonly described as secondary metabolites with high defence plant effect as they have antimicrobial properties and are non-toxic and biodegradable [13–17]. Harvest dates, storage period, plant extraction method, and climate may affect plant essential oils' chemical compositions. Several factors that could influence the composition of the EO: climate, geographical location, harvest dates, storage period, and extraction method [18,19].

Plant essential oils are developed commercially on a large scale, most of which are the Lamiaceae family members, including *Thymus vulgaris*, *Salvia officinalis*, *Mentha piperita* [18,20]. Thyme (*T. vulgaris* L.), sage (*S. officinalis* L.), and peppermint (*M. piperita* L.) EO, as products from plants, have a wide application in pharmacy, fragrance, food industries, however recent studies of essential oils revealed their potential antimicrobial activity [21–23]. *S. officinalis* EO affects *Fusarium* spp. growth [15]. *M. piperita* EO inhibits the spread of *Alternaria* spp. and *Fusarium* spp. pathogens [24]. *T. vulgaris* EO has antifungal activity against plant pathogens such as *Monilinia fructicola*, *Botrytis cinerea*, *Aspergillus flavus* [16,25,26]. *T. vulgaris*, EO can be used as a natural food preservative against casual agents of food-borne diseases like *E. coli*, *Pseudomonas* spp. and others [27].

Plant protection products against plant diseases are necessary to avoid yield and crop losses. However, pesticides have an adverse effect on plants and humans, as they leave residues. The European Green Deal provides a plan to increase environmentally friendly technologies by supporting strategies to reduce pesticides and make agriculture more sustainable. Growing pathogens resistance occurs because of the extensive use of chemical pesticides for plant protection. The new sources of natural active ingredients for plant protection may solve pesticide resistance problems and reduce environmental and food contamination [7,11,12,28]. This study aims to evaluate the inhibition of *C. acutatum* by thyme, sage, and peppermint EO in vitro also on the detached strawberry leaves, and determine EO chemical composition.

2. Results

2.1. Essential Oils Chemical Composition

The chemical composition of EO is presented in Table 1. In total, 99.33% of thyme and 99.94% of peppermint EO components were identified. Three dominant compounds were determined of thyme: thymol 41.35%, *p*-cymene 16.95%, γ -terpinene 10.81%. Peppermint: menthone 44.56%, isomenthone 12.81%, pulegone 10.74%. 99.94% of the total identified compounds in sage EO the highest quantities were set of α , β -thujone 34.45%, camphor 20.46%, and eucalyptol 10.33%.

Table 1. Composition of the essential oil compounds of thyme (*T. vulgaris*), sage (*S. officinalis*), and peppermint (*M. piperita*).

| Essential Oils Component | <i>Thymus vulgaris</i> | | <i>Salvia officinalis</i> | | <i>Mentha piperita</i> | |
|-----------------------------|------------------------|-----------------|---------------------------|--------|------------------------|--------|
| | PA ¹ (%) | RT ² | PA (%) | RT | PA (%) | RT |
| Tricyclene | | | 0.16 | 6.388 | | |
| α -thujene | 1.06 | 6.488 | 0.17 | 6.503 | | |
| α -pinene | 1.09 | 6.667 | 3.23 | 6.689 | 0.73 | 6.682 |
| Camphene | 0.38 | 7.079 | 5.5 | 7.079 | | |
| Sabinene | | | 0.12 | 7.702 | 0.55 | 7.701 |
| β -pinene | 0.43 | 7.779 | 2.65 | 7.798 | 0.87 | 7.795 |
| 1-octen-3-ol | 0.94 | 7.888 | | | | |
| Myrcene | 2.42 | 8.139 | 1.18 | 8.147 | 0.48 | 8.147 |
| 3-octanol | | | | | 0.21 | 8.344 |
| α -phellandrene | 0.32 | 8.517 | | | | |
| δ -3-carene | 0.15 | 8.673 | | | | |
| α -terpinene | 2.52 | 8.863 | 0.15 | 8.871 | 0.47 | 8.869 |
| <i>p</i> -cymene | 16.95 | 9.157 | 0.18 | 9.107 | 0.47 | 9.1 |
| Limonene | 0.81 | 9.228 | 1.21 | 9.237 | 0.92 | 9.219 |
| Eucalyptol | 1.88 | 9.285 | 10.33 | 9.3 | 3.35 | 9.283 |
| cis- β -ocimene | 0.11 | 9.432 | 0.23 | 9.444 | 0.16 | 9.442 |
| γ -terpinene | 10.81 | 10.092 | 0.32 | 10.053 | 0.73 | 10.053 |
| 4-pentenyl butyrate | 0.15 | 10.214 | | | | |
| cis-sabinene hydrate | 1.04 | 10.328 | 0.16 | 10.351 | 2.51 | 10.352 |
| Terpinolene | 0.17 | 10.876 | 0.32 | 10.894 | 0.2 | 10.895 |
| trans-sabinene hydrate | | | | | 0.21 | 11.257 |
| Linalool | 3.47 | 11.255 | 0.47 | 11.339 | 0.33 | 11.291 |
| α -thujone | 0.6 | 11.39 | 25.8 | 11.507 | | |
| β -thujone | 0.21 | 11.708 | 8.65 | 11.775 | | |

Table 1. Cont.

| Essential Oils Component | <i>Thymus vulgaris</i> | | <i>Salvia officinalis</i> | | <i>Mentha piperita</i> | |
|---|------------------------|-----------------|---------------------------|--------|------------------------|--------|
| | PA ¹ (%) | RT ² | PA (%) | RT | PA (%) | RT |
| Isothujol | | | 0.17 | 12.371 | | |
| cis-p-menth-2-en-1-ol | | | | | 0.19 | 11.939 |
| trans-p-menth-2-en-1-ol + trans-Sabinol | | | | | 0.22 | 12.548 |
| Menthone | | | | | 44.56 | 12.964 |
| Isomenthone | | | | | 12.81 | 13.2 |
| Camphor | 0.74 | 12.619 | 20.46 | 12.616 | | |
| trans-pinocamphone | | | 0.14 | 12.98 | | |
| Borneol | 0.75 | 13.208 | 4.37 | 13.223 | | |
| δ-terpineol + borneol | | | | | 0.24 | 13.24 |
| cis-pinocamphone | | | 0.2 | 13.385 | | |
| Menthol | | | | | 7.95 | 13.473 |
| Terpinen-4-ol | 1.05 | 13.483 | 0.28 | 13.494 | 2.58 | 13.549 |
| α-terpineol | 0.25 | 13.999 | 0.22 | 13.879 | 0.32 | 13.916 |
| Thymol methyl ether | 0.61 | 15.01 | | | | |
| Carvacrol methyl ether | 0.63 | 15.272 | | | | |
| Carvone Z, dihydro | | | | | 0.31 | 14.039 |
| Myrtenol | | | 0.28 | 14.056 | | |
| cis-3-hexenyl-isovalerate | | | | | 0.11 | 15.072 |
| Pulegone | | | | | 10.74 | 15.282 |
| Bornyl acetate | | | 1.39 | 16.431 | | |
| Thymol | 41.35 | 16.984 | | | | |
| Carvone | | | | | 0.45 | 15.346 |
| Carvacrol | 2.57 | 17.123 | | | | |
| trans-sabinyol acetate + thujyl acetate | | | 0.1 | 16.597 | | |
| Piperitone | | | | | 1.65 | 15.636 |
| Caryophyllene E | 1.86 | 20.018 | 2.84 | 20.013 | 0.75 | 20.004 |
| Menthyl acetate | | | | | 1.41 | 16.639 |
| α-humulene | 0.17 | 20.859 | 3.25 | 20.872 | | |
| Geranyl propanoate | 0.12 | 21.212 | | | | |
| γ-cadinene | 0.14 | 22.324 | | | | |
| δ-cadinene | 0.22 | 22.517 | | | | |
| Piperitenone | | | | | 0.36 | 17.97 |
| β-elemene | | | | | 0.34 | 19.258 |
| Germacrene D | | | | | 0.79 | 21.536 |
| Bicyclogermacren | | | | | 0.2 | 21.908 |
| Caryophyllene oxide | 0.31 | 24.066 | 0.19 | 24.059 | | |
| Viridiflorol | 0.11 | 24.337 | 3.03 | 24.363 | | |
| Humulene epoxide II | | | 0.26 | 24.796 | | |
| Manool | | | 0.74 | 32.227 | | |
| α-muurolol | | | | | | |
| Unknown | 0.49 | | | | | |
| Squalene | 1.19 | 36.912 | | | | |
| Di-n-octyl phthalate | 0.13 | 37.496 | | | 0.2 | 37.486 |
| Other ³ | 1.64 | | 1.41 | | 1.68 | |
| Total Identified | 99.33 | | 99.94 | | 99.94 | |

¹ PA—peak area. ² RT—retention time. ³ The compounds that were less than 0.1% of the quantity of the essential oil.

2.2. Essential Oils Antifungal Activity In Vitro

EO antifungal activity was assayed at different concentrations on potato dextrose agar (PDA). The inhibition of *C. acutatum* by thyme EO is shown in Figure 1. Thyme EO showed 100% mycelial growth inhibition at 4 and 7 days after inoculation (4 and 7 days after inoculation (DAI)) at 100 µL L⁻¹ but did not demonstrate the high antifungal effect at 150 µL L⁻¹ at 4 (87.96%) and 7 DAI (89.9%). However, mycelial pathogen growth was inhibited above 200 µL L⁻¹.

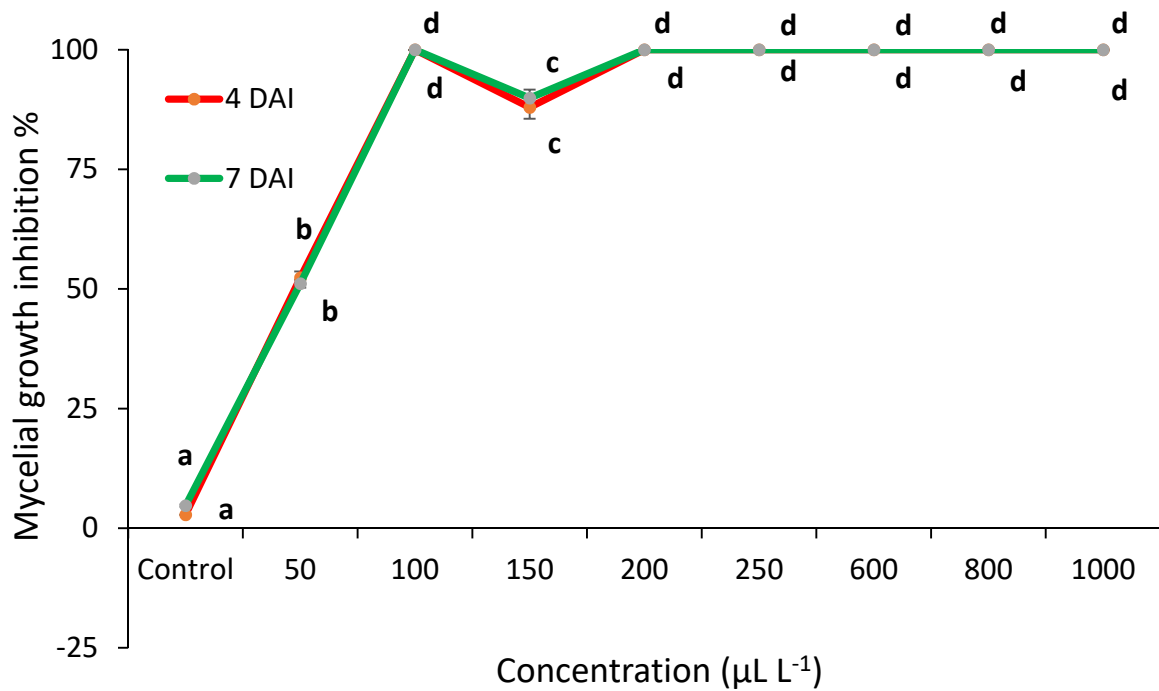


Figure 1. *C. acutatum* mycelial growth inhibition (%) by thyme (*T. vulgaris*) EO at 4 and 7 days after inoculation (4 DAI and 7 DAI). The results presented as means ($n = 4$). The same letter indicates no significant differences between treatments ($p < 0.05$).

The mycelial growth inhibition of *C. acutatum* by sage EO is presented in Figure 2. Data indicate that this EO was less effective than thyme. Sage EO showed antifungal activity up to 1000 μL L⁻¹ at 4 DAI and achieved the highest effect of 88.14% at 1800 μL L⁻¹. However, this EO's effectiveness at 7 DAI was lower in 1800 μL L⁻¹ and reached 62.54%.

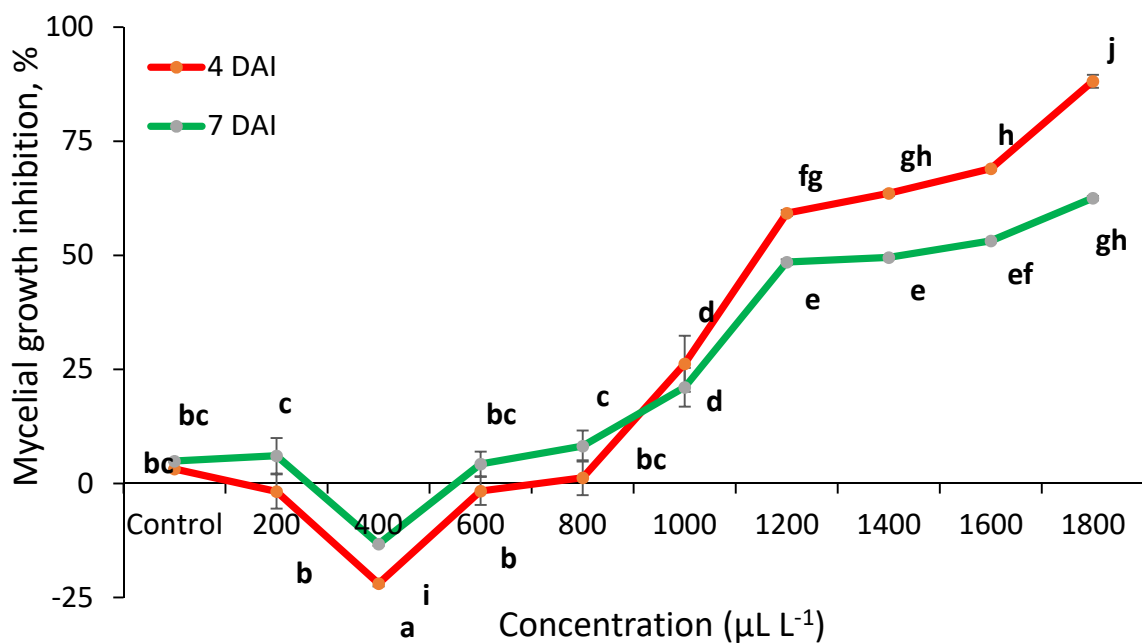


Figure 2. *C. acutatum* mycelial growth inhibition (%) by sage (*S. officinalis*) EO at 4 and 7 days after inoculation (4 DAI and 7 DAI). The results are presented as means ($n = 4$). The same letter indicates no significant differences between treatments ($p < 0.05$).

The fungicidal activity of peppermint EO against *C. acutatum* is shown in Figure 3. This EO had a similar effect on *C. acutatum* comparing with sage. Meanwhile, peppermint EO reduced the mycelial growth at 600–1800 $\mu\text{L L}^{-1}$ from 20% to 88%. However, the highest antifungal activity was reached at 1600 $\mu\text{L L}^{-1}$ at 4 DAI. 1800 $\mu\text{L L}^{-1}$ EO efficiency decreased to 62.54%.

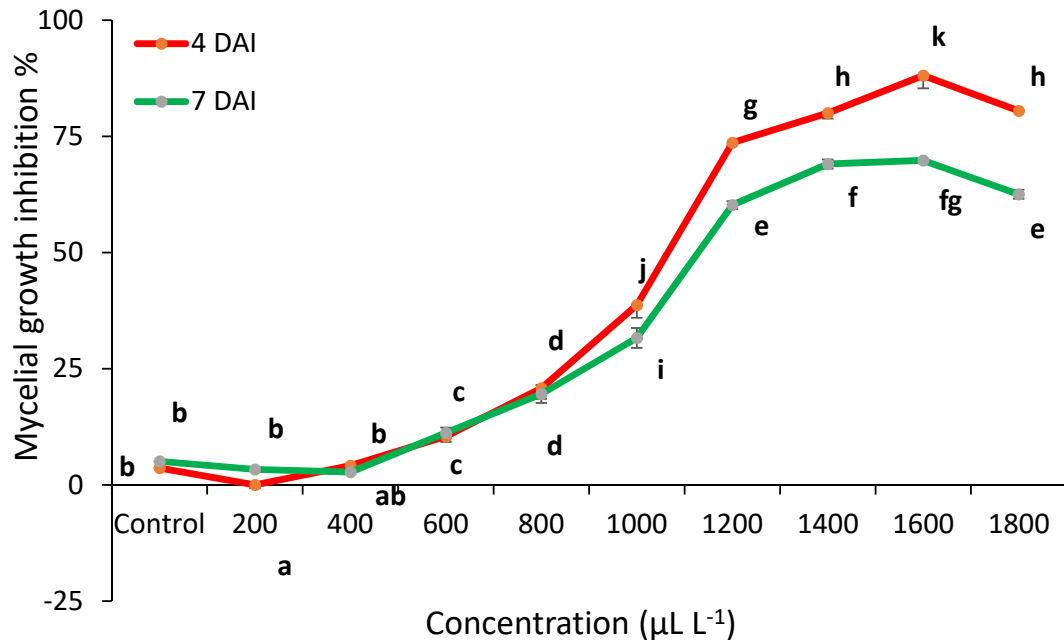


Figure 3. *C. acutatum* mycelial growth inhibition (%) by peppermint (*M. piperita*) EO at 4 and 7 days after inoculation (4 DAI and 7 DAI). The results are presented as means ($n = 4$). The same letter indicates no significant differences between treatments ($p < 0.05$).

T. vulgaris EO totally inhibited the mycelial growth at 200–1000 $\mu\text{L L}^{-1}$. The MIC was determined (minimal inhibitory concentration) an equal to 200 $\mu\text{L L}^{-1}$. *S. officinalis* and *M. piperita* EO reduced mycelial growth compared to control. However, this EO was insufficient to inhibit the spread of anthracnose infection (Figure 4) (Detailed information can be found in supplementary materials Tables S1 and S2, Figures S1 and S2).

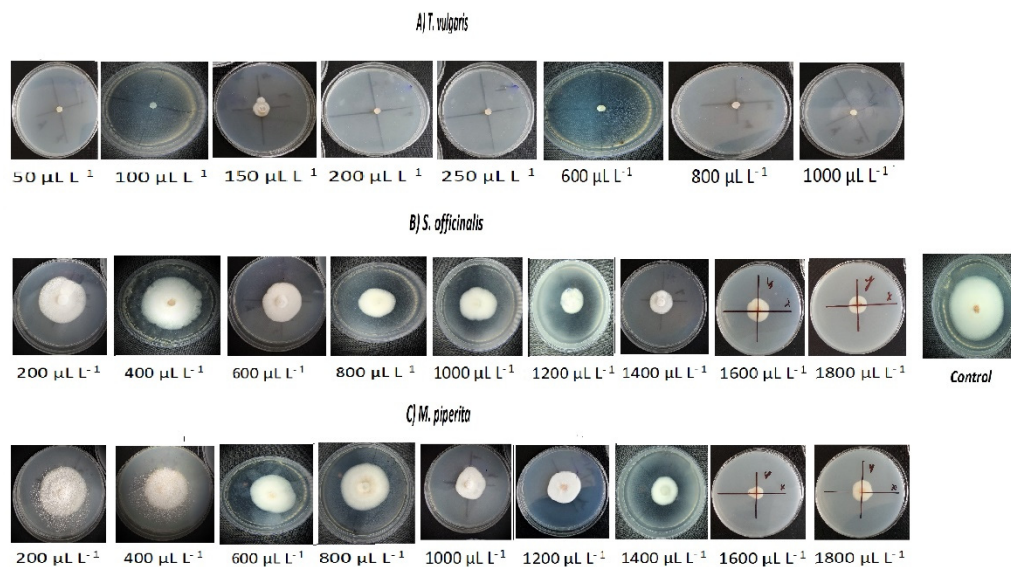


Figure 4. *C. acutatum* mycelial growth inhibition by EO at various concentrations. (A) thyme EO; (B) sage EO; (C) peppermint EO.

2.3. Antifungal Activity on Detached Strawberry Leaves

The detached strawberry leaf assay was developed to determine the efficiency of essential oils against *C. acutatum* (Figure 5). The results revealed that among all the investigation treatments, only 1000 $\mu\text{L L}^{-1}$ concentration of peppermint EO (5.3%) and 800 $\mu\text{L L}^{-1}$ concentration of thyme (15.8%) to decrease the infection on detached strawberry leaves compared to inoculated control 4 DAI (Table 2). Sage EO had no positive influence on infection spread.

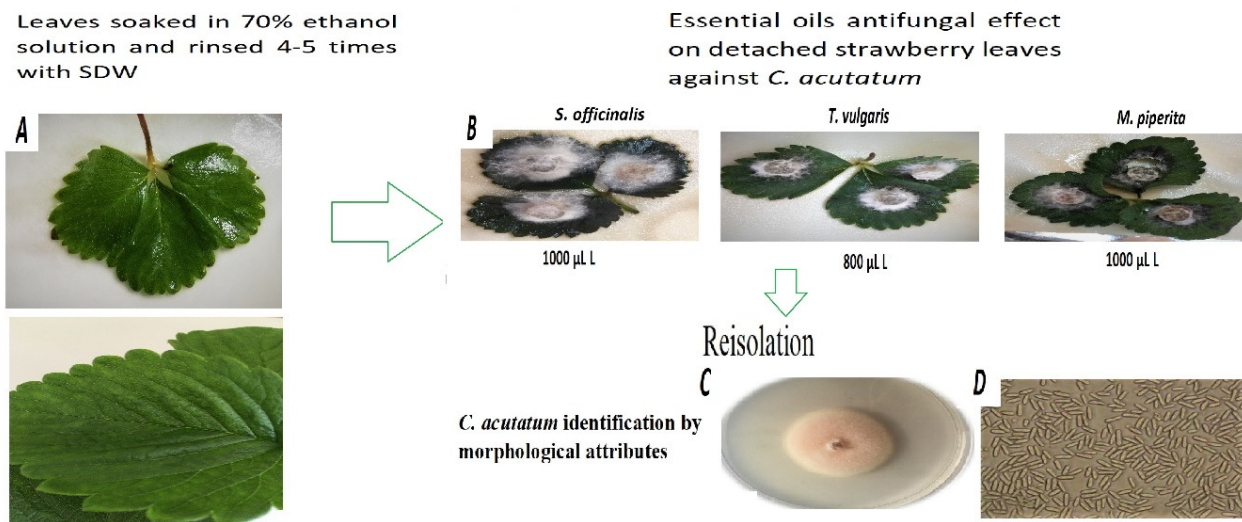


Figure 5. Infection of *C. acutatum* on detached strawberry leaves after EO application. (A) Control—not inoculated leaves; (B) Leaves treated with EO thyme 800 $\mu\text{L/L}$, sage and peppermint 1000 $\mu\text{L/L}$ concentrations; (C) after reisolation, morphologically confirmed *C. acutatum*; (D) after reisolation confirmed *C. acutatum* spores.

Table 2. The disease severity and reduction in anthracnose regarding strawberry cultivar ‘Deluxe’ by different essential oils concentrations at 4 days after inoculation. Means $n = 4 \pm \text{SE}$.

| Treatments | Disease Severity (%) | Disease Reduction (%) |
|--|----------------------|-----------------------|
| Inoculated control | 79.2 \pm 0.2 | n.a.* |
| <i>Thymus vulgaris</i> 800 $\mu\text{L/L}$ | 75 \pm 0.3 | 5.3 |
| <i>Salvia officinalis</i> 1000 $\mu\text{L/L}$ | 80.6 \pm 0.2 | 0 |
| <i>Mentha piperita</i> 1000 $\mu\text{L/L}$ | 66.7 \pm 0.2 | 15.8 |

* n.a.—not applicable.

3. Discussion

There is a growing interest in EO, and their components due to their volatility, relative safety, and wide acceptance by consumers, as well as their ecological and biodegradable properties. For our study, we selected *T. vulgaris*, *S. officinalis*, and *M. piperita* EO, and analyzed the chemical composition, antifungal activities and EO effect on detached strawberry leaves, to assess the feasibility of using EO as biocontrol agents in disease control. EO from thyme, sage, peppermint presented noticeable antifungal activity against *C. acutatum* in vitro.

Our research data confirmed that the chemical composition of tested EO is in line with what is already have been described in the literature [16,17,19,20,26,29–34]. Oliveira et al. [16] reported that thymol and *p*-cymene were abundant components in identified 28 compounds of thyme EO. Kim et al. [19] stated that the most common compound differed according to the area. These results were equivalent to our study. In an investigation conducted by Duduk et al. [31], thyme EO showed good antifungal efficacy against *C. acutatum* on strawberry fruit. In our study, thyme EO inhibited *C. acutatum* mycelial growth in vitro above 200 $\mu\text{L L}^{-1}$. This suggests that the antifungal effect presence of the dominant components of

EO, as main activity carriers. Palfi et al. [17] reported that thyme, sage, and peppermint EO totally inhibited the mycelial growth of *F. oxysporum* in vitro; however, sage EO had a low inhibitory effect against *B. cinerea*. Oliveira et al. [32] observed that 5 $\mu\text{L}/\text{mL}$ peppermint EO showed 100% MGI on all tested *Colletotrichum* stains. In our research peppermint EO highest antifungal activity reached 1600 $\mu\text{L L}^{-1}$ (88%). In comparison, Oliveira et al. [32] identified 26 different constituents of peppermint EO, and the dominant compounds were: menthol (41.34%), isomenthone (23.47%), cis-menthone (23.47%), while in our studies 7.95, 12.81, 44.56%, respectively. Hong et al. [6] evaluated plant EO component menthone antimicrobial activity during conidial germination and mycelial growth of *C. gloeosporioides*. Menthone demonstrated relatively low antifungal activity on conidia germination and pepper fruits. After reviewing the previous literature [6,24,32], the assumption could be that the higher content of menthol in the essential oil has a higher inhibitory effect against *Colletotrichum* spp. The antifungal properties of sage EO primarily affect the main abundant constituents α,β -thujone, camphor, and eucalyptol [20,29,33,34]. In our research, the antifungal activity of sage EO against *C. acutatum* achieved the highest effect of 88.14% at 1800 $\mu\text{L L}^{-1}$. In comparison, Yilmaz et al. [34] studied, that the application of sage EO resulted in slight inhibition on mycelial growth of *C. gloeosporioides* in fumigation bioassay and contact bioassay in vitro (solid media) and in vivo (apple) conditions. These results supported our findings, where the predominant compounds of sage EO, showed antifungal effect against *C. acutatum* but did not suppress it. Chemical compounds found in lower amounts in EO may also influence its antifungal properties.

However, to the best of our knowledge, no investigations have been previously performed on the antifungal effect of thyme, sage, and peppermint EO on detached strawberry leaves against *C. acutatum*. The present research, investigated EO result on the detached strawberry leaves assay indicated a less positive effect of reducing the spread of anthracnose infection. 1000 $\mu\text{L L}^{-1}$ concentration of peppermint EO (15.8%) and 800 $\mu\text{L L}^{-1}$ concentration of thyme decreased (5.3%) the infection on strawberry leaves. A higher concentration of this EO's should be investigated, to obtain greater efficiency. Plants effect as volatile compounds on EO may induce a stressful environment on the surface of strawberry leaves [11,35].

In summary, examining various EO and their concentrations in vitro exhibited promising prospects against strawberry anthracnose; however, the antifungal effect on detached strawberry leaves was low.

4. Materials and Methods

4.1. Essential Oil Extraction

The essential oils (EO) were extracted by Clevenger-type hydro-distillation. EO obtained from common thyme (*Thymus vulgaris* L.), common sage (*Salvia officinalis* L.), peppermint (*Mentha piperita* L.) was chosen for the determination of antifungal activity against strawberry pathogen *C. acutatum* at different concentrations. Plants for essential oils extraction were obtained from Lithuanian Research Centre for Agriculture and Forestry (LAMMC) Institute of Horticulture (IH) experimental fields (55.081052, 23.806630).

4.2. Identification of the Essential Oils Chemical Composition

Volatile compounds of essential oils were established by gas chromatography - mass spectrometry (GC-MS). The analysis was executed on GC-2010Plus/GCMS-QP2010 Ultra system (Shimadzu, Kyoto, Japan) equipped with Rxi-5MS capillary column (33 m \times 0.25 mm; 0.25 μm) (Restek, Bellefonte, PA, USA), as defined in previous studies [11,12].

4.3. *C. acutatum* Isolates

The research carried out at the Laboratory of Plant Protection, LAMMC IH in 2017–2020. To obtain pure single-spore culture isolation from *C. acutatum* (infected strawberry 'Deluxe' fruits) performed. The selected isolates cultured on potato dextrose agar (PDA)

at 25 °C for 7 days. The isolates initially identified by morphological traits typical of the colonies [36] and confirmed by PCR as *C. acutatum* by Moreira et al. [37].

4.4. Essential Oils Antifungal Activity In Vitro

To evaluate antifungal activity against *C. acutatum* 50–1000 $\mu\text{L L}^{-1}$ concentrations of thyme EO and 200–1800 $\mu\text{L L}^{-1}$ sage and peppermint EO were used. Different concentrations of pure essential oil were added to cool at 45 °C PDA. Four repetitions with four replications were carried out. Petri plates inoculation was with 5 mm *C. acutatum* mycelial plugs of 7-day old fungus. The mycelium was put upside down (mycelia side) in Petri's center containing PDA with different EO concentrations. The Petri plates were incubated at 25 ± 2 °C in the dark. The control treatments were oil-free.

The diameter (mm) of *C. acutatum* colony growth measured in two directions after 2, 4, and 7 days after inoculation (DAI). The mean of colony growth diameter used for mycelial growth inhibition calculations. The mycelial growth inhibition (MGI) (%) was determined using the formula:

$$\text{Mycelial growth inhibition (\%)} = (C - T)/C \times 100,$$

C is the mycelium diameter of the pathogen colony in control Petri dish, mm; T —mycelium diameter of the pathogen colony in the essential oil-treated Petri dish, mm [11,38,39]. Minimal inhibitory concentration (MIC) was determined as EO concentration with 100% MGI [11].

4.5. Essential Oils Antifungal Activity on Detached Strawberry Leaves

The essential oils inhibitory effect evaluated on detached strawberry cultivar 'Deluxe' leaves. Healthy strawberry leaves, consisting of three petiole leaflets, without any visible symptoms of the disease were soaked in 70% ethanol solution for 3 min and rinsed 4–5 times with sterile distilled water (SDW). Each leaf was placed in a Petri dish with 5 mL of SDW. Detached strawberry leaves were sprayed with essential oils (800 and 1000 $\mu\text{L L}^{-1}$), then were wounded with sterile needle and a 9-mm plug of 7-day-old *C. acutatum* was placed on the wound. Incubation was carried out at 25 ± 2 °C in the dark for 7 days. The 16 leaves used in treatment; the experiment repeated three times with four replicates. The control treatments were not sprayed with EO but inoculated with *C. acutatum* (Inoculated control). The treatments antifungal activity is assessed by the disease severity (DS) and disease reduction (DR) in the leaves.

Disease severity (DS) of each inoculated plant leaf assessed at 4 and 7 DAI by calculating the percentage of leaf area affected: (1) 0%—no visible infection, (2) 5%, (3) 10%, (4) 20% and (5) 50% or more area of leaf infected [40–42].

$$DS (\%) = (((0 \times P_0) + (1 \times P_1) + (2 \times P_2) + (3 \times P_3) + (4 \times P_4) + (5 \times P_5))/N \times G) \times 100$$

where P_0 to P_5 is the total number of the evaluated leaves in each corresponding scale, N —total number of leaves, G —number of maximum grades observed in scale [42].

$$DR (\%) = (X_c - X_t)/X_c \times 100$$

where X_t is the mean of DS per treatment, and X_c is the mean of DS in the inoculated control [43].

4.6. Statistical Analysis

The SAS Enterprise Guide 7.1 program (SAS Institute Inc., Cary, NC, USA) used for the analysis of experimental data. The analysis of variance (ANOVA) processed, and Duncan's multiple range test ($p < 0.05$) used for the comparison of obtained means. The standard error (SE) in the figures marked as an error bar estimated for isolates growth rates.

5. Conclusions

The essential oil of thyme, containing dominant compound thymol, showed total inhibition against *C. acutatum* in vitro. Peppermint and sage EO containing predominant components menthone, isomenthone, and thujone, camphor, respectively, showed significant antifungal activity at the highest concentrations. *C. acutatum* mycelial growth on detached strawberry leaves was slightly reduced by applying thyme EO and more suppressed by peppermint EO at tested concentration. Sage EO did not influence the spread of *C. acutatum* on detached strawberry leaves. The detached strawberry leaves assay revealed that the investigated essential oils were not equally effective and needed further investigations with higher concentrations.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2223-7747/10/1/114/s1>.

Author Contributions: Conceptualization: A.M., N.R.; methodology: A.M., N.R. and A.V.; software: A.M., L.Š.; validation: N.R.; formal analysis: A.M.; investigation: A.M., N.R.; resources: N.R.; data curation: A.M.; writing—original draft preparation: A.M.; writing—review and editing: A.M., N.R., L.Š., and A.V.; visualization: A.M., N.R., L.Š.; supervision: A.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cannon, P.F.; Damm, U.; Johnston, P.R.; Weir, B.S. *Colletotrichum*—Current status and future directions. *Stud. Mycol.* **2012**, *73*, 181–213. [[CrossRef](#)] [[PubMed](#)]
2. Udayanga, D.; Manamgoda, D.S.; Liu, X.; Chukeatirote, E.; Hyde, K.D. What are the common anthracnose pathogens of tropical fruits? *Fungal Divers.* **2013**, *61*, 165–179. [[CrossRef](#)]
3. Jayawardena, R.S.; Huang, J.K.; Jin, B.C.; Yan, J.Y.; Li, X.H.; Hyde, K.D.; Bahkali, A.H.; Yin, S.L.; Zhang, G.Z. An account of *Colletotrichum* species associated with strawberry anthracnose in china based on morphology and molecular data. *Mycosphere* **2016**, *7*, 1147–1163. [[CrossRef](#)]
4. He, L.; Li, X.; Gao, Y.; Li, B.; Mu, W.; Liu, F. Characterisation and fungicide sensitivity of *Colletotrichum* spp. from different hosts in Shandong, China. *Plant Dis.* **2019**, *103*, 34–43. [[CrossRef](#)] [[PubMed](#)]
5. Forcelini, B.B.; Seijo, T.E.; Amiri, A.; Peres, N.A. Resistance in strawberry isolates of *Colletotrichum acutatum* from Florida to quinone-outside inhibitor fungicides. *Plant Dis.* **2016**, *100*, 2050–2056. [[CrossRef](#)]
6. Hong, J.K.; Yang, H.I.; Jung, H.; Yoon, D.J.; Sang, M.K.; Jeun, Y.C. Application of volatile antifungal plant essential oils for controlling pepper fruit anthracnose by *Colletotrichum gloeosporioides*. *Plant Pathol.* **2015**, *31*, 269–277. [[CrossRef](#)]
7. Rasiukevičiūtė, N.; Uselis, N.; Valiuškaitė, A. The use of forecasting model IMETOS[®] for strawberry grey mould management. *Zemdirb. Agric.* **2019**, *106*, 143–150. [[CrossRef](#)]
8. Zhang, L.; Song, L.; Xu, X.; Zou, X.; Duan, K.; Gao, Q. Characterisation and fungicide sensitivity of *Colletotrichum* species causing strawberry anthracnose in Eastern China. *Plant Dis.* **2020**, *7*, 104.
9. Aguado, A.; Pastrana, A.M.; Santos, B.; Romero, F.; Sánchez, M.C.; Capote, N. The efficiency of natural products for the control of *Colletotrichum acutatum* monitored by real-time PCR. *Acta Hort.* **2014**, *1049*, 329–334. [[CrossRef](#)]
10. Mahilrajana, S.; Nandakumar, J.; Kailayalingam, R.; Manoharan, N.A.; Srivijeindran, S. Screening the antifungal activity of essential oils against decay fungi from palmyrah leaf handicrafts. *Biol. Res.* **2014**, *47*, 35. [[CrossRef](#)]
11. Šernaitė, L.; Rasiukevičiūtė, N.; Valiuškaitė, A. The extracts of cinnamon and clove as potential biofungicides against strawberry grey mould. *Plants* **2020**, *9*, 613. [[CrossRef](#)] [[PubMed](#)]
12. Šernaitė, L.; Rasiukevičiūtė, N.; Valiuškaitė, A. Application of plant extracts to control postharvest gray mold and susceptibility of apple fruits to *B. cinerea* from different plant hosts. *Foods* **2020**, *9*, 1430. [[CrossRef](#)] [[PubMed](#)]
13. Lopez, N.L.; Gutiérrez-Grijalva, E.P.; Vazquez-Oliva, G.; Heredia, J.B. Essential oils of Oregano: Biological activity beyond their antimicrobial properties. *Molecules* **2017**, *22*, 989. [[CrossRef](#)] [[PubMed](#)]
14. Naeini, A.; Ziglari, T.; Shokri, H.; Khosravi, A.R. Assessment of growth inhibiting effect of some plant essential oils on different *Fusarium* isolates. *J. Mycol. Med.* **2010**, *20*, 174–178. [[CrossRef](#)]
15. Foltinová, D.; Tančinová, D.; Císarová, M. Influence of essential oils on the growth of *Aspergillus flavus*. *Potravinárstvo* **2017**, *11*, 322–331. [[CrossRef](#)]

16. Oliveira, R.C.; Moreno, M.C.; Correa, B.; Callejas, F.J. Cellular, physiological and molecular approaches to investigate the antifungal and anti-aflatoxigenic effects of thyme essential oil on *Aspergillus flavus*. *Food Chem.* **2020**, *315*, 126096. [[CrossRef](#)]
17. Palfi, M.; Konjevoda, P.; Vrandečić, K. Antifungal activity of essential oils on mycelial growth of *Fusarium oxysporum* and *Botrytis cinerea*. *Emir. J. Food Agric.* **2019**, *31*, 544–554. [[CrossRef](#)]
18. Sil, A.; Pramanik, K.; Samantaray, P.; Firoz, M.; Yadav, V. Essential oils: A boon towards eco-friendly management of phytopathogenic fungi. *J. Entomol. Zool. Stud.* **2020**, *8*, 1884–1891.
19. Kim, J.E.; Lee, J.E.; Huh, M.J.; Lee, S.C.; Seo, S.M.; Kwon, J.H.; Park, I.K. Fumigant Antifungal Activity via Reactive Oxygen Species of *Thymus vulgaris* and *Satureja hortensis* Essential Oils and Constituents against *Raffaelea quercus-mongolicae* and *Rhizoctonia solani*. *Biomolecules* **2019**, *9*, 561. [[CrossRef](#)]
20. Moumni, S.; Elaissi, A.; Trabelsi, A.; Merghni, A.; Chraief, I.; Jelassi, B.; Chemli, R.; Ferchichi, S. Correlation between chemical composition and antibacterial activity of some Lamiaceae species essential oils from Tunisia. *BMC Complement. Med. Ther.* **2020**, *20*, 103. [[CrossRef](#)]
21. Kumar, A.; Kudachikar, V.B. Antifungal properties of essential oils against anthracnose disease: A critical appraisal. *J. Plant Dis. Prot.* **2018**, *125*, 133–144. [[CrossRef](#)]
22. Starović, M.; Ristic, D.; Pavlović, S.; Ristic, M.; Stevanović, M.; Aljuhaimi, F.; Svetlana, N.; Özcan, M.M. Antifungal activities of different essential oils against anise seeds mycopopulations. *J. Food Saf. Food Qual.* **2016**, *67*, 72–78.
23. Micucci, M.; Protti, M.; Aldini, R.; Frosini, M.; Corazza, I.; Marzetti, C.; Mattioli, L.B.; Tocci, G.; Chiarini, A.; Mercolini, L.; et al. *Thymus vulgaris* L. Essential Oil Solid Formulation: Chemical Profile and Spasmolytic and Antimicrobial Effects. *Biomolecules* **2020**, *10*, 860. [[CrossRef](#)] [[PubMed](#)]
24. Desam, N.R.; Al-Rajab, A.J.; Sharma, M.; Mylabathula, M.M.; Gowkanapalli, R.R.; Albratty, M. Chemical constituents, in vitro antibacterial and antifungal activity of *Mentha × piperita* L. (peppermint) essential oils. *J. King Saud. Univ. Sci.* **2017**, *31*, 528–533. [[CrossRef](#)]
25. Santoro, K.; Maghenzani, M.; Chiabrande, V.; Bosio, P.; Gullino, M.L.; Spadaro, D.; Giacalone, G. Thyme and savory essential oil vapor treatments control brown rot and improve the storage quality of peaches and nectarines, but could favor gray mold. *Foods* **2018**, *7*, 7. [[CrossRef](#)]
26. Taghavi, T.; Kim, C.; Rahemi, A. Role of Natural Volatiles and Essential Oils in Extending Shelf Life and Controlling Postharvest Microorganisms of Small Fruits. *Microorganisms* **2018**, *6*, 104. [[CrossRef](#)]
27. Çetin, B.; Çakmakçi, S.; Çakmakçi, R. The investigation of antimicrobial activity of thyme and oregano essential oils. *Turk. J. Agric. For.* **2011**, *35*, 145–154.
28. Park, J.Y.; Kim, S.H.; Kim, N.H.; Lee, S.W.; Jeun, Y.C.; Hong, J.K. Differential Inhibitory Activities of Four Plant Essential Oils on In Vitro Growth of *Fusarium oxysporum* f. sp. *fragariae* Causing Fusarium Wilt in Strawberry Plants. *Plant Pathol. J.* **2017**, *33*, 582–588.
29. Tančinová, D.; Mašková, Z.; Foltinová, D.; Štefániková, J.; Árvay, J. Effect of essential oils of Lamiaceae plants on the *Rhizopus* spp. *Potravinárstvo* **2018**, *12*, 491–498. [[CrossRef](#)]
30. Wan, J.; Zhong, S.; Schwarz, P.; Chen, B.; Rao, J. Physical properties, antifungal and mycotoxin inhibitory activities of five essential oil nanoemulsions: Impact of oil compositions and processing parameters. *Food Chem.* **2019**, *291*, 199–206. [[CrossRef](#)]
31. Duduk, N.; Markovic, T.; Vasic, M.; Duduk, B.; Vico, I.; Obradovic, A. Antifungal activity of three essential oils against *Colletotrichum acutatum*, the casual agent of strawberry anthracnose. *Essent. Oil-Bear. Plants* **2015**, *18*, 529–537. [[CrossRef](#)]
32. Oliveira, K.Á.R.; Berger, L.R.R.; Araújo, S.A.; Câmara, M.P.S.; Souza, E.L. Synergistic mixtures of chitosan and *Mentha piperita* L. essential oil to inhibit *Colletotrichum* species and anthracnose development in mango cultivar Tommy Atkins. *Food Microbiol.* **2017**, *66*, 96–103. [[CrossRef](#)] [[PubMed](#)]
33. Tančinová, D.; Medo, J.; Mašková, Z.; Foltinová, D.; Árvay, J. Effect of essential oils of Lamiaceae plants on the *Penicillium commune*. *J. Microbiol. Biotech. Food Sci.* **2019**, *8*, 1111–1117. [[CrossRef](#)]
34. Yilmaz, A.; Ermis, E.; Boyraz, N. 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*. *J. Food Saf. Food Qual.* **2016**, *67*, 113–148.
35. Idris, F.M.; Ibrahim, A.M.; Forsido, S.V. Essential oils to control *Colletotrichum musae* in vitro and in vivo on banana fruits. *AEJAES* **2015**, *15*, 291–302.
36. Živković, S.; Stojanović, S.; Ivanović, Ž.; Trkulja, N.; Dolovac, N.; Aleksić, G.; Balaž, J. Morphological and Molecular Identification of *Colletotrichum acutatum* from Tomato Fruit. *Pestic. Phytomed.* **2010**, *25*, 231–239. [[CrossRef](#)]
37. Moreira, R.R.; Silva, G.A.; Mio, L.L.M. *Colletotrichum acutatum* complex causing anthracnose on peach 457 in Brazil. *Australas. Plant Pathol.* **2020**, *49*, 179–189. [[CrossRef](#)]
38. Šernaitė, L.; Rasiukevičiūtė, N.; Dambrauskienė, E.; Viškėlis, P.; Valiuškaitė, A. Efficacy of plant extracts and essential oils for biocontrol of strawberry pathogen *Botrytis cinerea*. *Zemdirb. Agric.* **2020**, *107*, 147–152. [[CrossRef](#)]
39. Oliveira, J.; Gloria, E.M.; Parisi, M.C.M.; Baggio, J.S.; Silva, P.P.M.; Ambrosio, C.M.S.; Spoto, M.H.F. Antifungal activity of essential oils associated with carboxymethylcellulose against *Colletotrichum acutatum* in strawberries. *Sci. Hortic.* **2019**, *243*, 261–267. [[CrossRef](#)]
40. EPPO. *Efficacy Evaluation of Fungicides: Leafspots of Vegetables*; European and Mediterranean Plant Protection Organization: Paris, France, 1996.

41. Bajpai, S.; Shukla, P.S.; Asiedu, S.; Pruski, K.; Prithiviraj, B. A biostimulant preparation of brown seaweed *Ascophyllum nodosum* suppresses powdery mildew of strawberry. *Plant Pathol. J.* **2019**, *35*, 406–416.
42. Kone, N.; Asare-Bediako, E.; Silue, S.; Kone, D.; Koita, O.; Menzel, W.; Winter, S. Influence of planting date on incidence and severity of viral disease on cucurbits under field condition. *Ann. Agric. Sci.* **2017**, *62*, 99–104. [[CrossRef](#)]
43. Hosseini, S.; Amini, J.; Saba, M.K.; Karimi, K.; Pertot, I. Preharvest and postharvest application of garlic and rosemary essential oils for controlling anthracnose and quality assessment of strawberry fruit during cold storage. *Front. Microbiol.* **2020**, *11*, 1855. [[CrossRef](#)] [[PubMed](#)]