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Mandarin Essential Oils as an Alternative Method of Controlling the Fungus *Alternaria alternata* (Fr.: Fr.) Keissler

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Abstract: Alternaria brown spot (ABS) is a disease caused by the fungus *A. alternata* f. sp. *citri*, which results in lesions on the fruits, leaves, and branches of several mandarin varieties and their hybrids. Due to the high cost of fungicide application, alternative methods for controlling ABS need to be studied. Therefore, this study aimed to evaluate the use of essential oils (EOs) from different mandarin varieties to mitigate the effects of ABS. The inhibitory effect of different concentrations (1, 2, 4, 8, and 16 $\mu L \cdot mL^{-1}$) of the EOs of Fremont IAC 543 mandarin, IAC 2019Maria mandarin, Murcott IAC 221 tangor, and Late IAC 855 willowleaf on the in vitro mycelial growth of the fungus *A. alternata* was evaluated. Additionally, the curative and preventive effects of these EOs on the ABS symptoms in detached leaves of Murcott IAC 221 tangor were also assessed. The EO of IAC 2019 Maria mandarin induced less mycelial growth, and consequently, a greater inhibition of the growth of the fungus *A. alternata* at a concentration of 16 $\mu L \cdot mL^{-1}$. This EO was more effective for control than the other oils tested. In the detached leaf experiment, both the curative and preventive treatments at a concentration of 16 $\mu L \cdot mL^{-1}$ showed lower values of disease severity.

Keywords: alternaria brown spot; mandarin; *Citrus reticulata*; alternative control

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1. Introduction

Brazil is the largest world producer of oranges (17.1 million tons); however, it ranks seventh as a world producer of tangerines, having produced around 850 thousand tons in 2021, a much lower production than China, the first producer, with approximately 20 million tons. Ahead of Brazil are Spain, Turkey, Morocco, Egypt, and the United States, which stand out as the largest producers [1].

In the state of São Paulo, the main commercially grown mandarin varieties are the Ponkan mandarin (*Citrus reticulata* Blanco) and the Murcott tangor (*C. reticulata* × *C. sinensis* (L.) Osbeck), which represent about 80% of orchards. The Rio mandarin (*C. deliciosa* Tenore) and Cravo mandarin (*C. reticulata*) are also grown, as well as smaller plantings of Fremont IAC 543 mandarin (*C. clementina* Hort. ex Tan. × *C. reticulata*), IAC 2019Maria mandarin (*C. reticulata* × *C. sinensis* (L.) Osbeck) × Pera IAC orange (*C. sinensis* (L.) Osbeck), and Late IAC 855 willowleaf [2].

Mandarin production in Brazil is limited to a few varieties with low genetic variability, making it vulnerable to phytosanitary problems. Among the diseases that cause significant damage to mandarins are Alternaria brown spot (ABS) (*Alternaria alternata* (Fr.:Fr.) Keissler) and huanglongbing (HLB) (*Candidatus* Liberibacter spp.), which have compromised orchards and hindered the management and production of the crop [3,4]. Consequently, the planted area and production of mandarins in Brazil have declined in recent years [5].

As most mandarin varieties are susceptible to ABS, producers need to apply numerous fungicides for their control, around 12 to 18 times a year, increasing their production

costs [4]. However, the growing concern about the resistance of the fungus *A. alternata* to registered fungicides and their toxicity necessitates alternative means to managing the disease, besides genetic improvement. Cases of resistance of the fungus *A. alternata* to strobilurin group fungicides (Quinone Outside Inhibitor Resistance) have been observed since 2012 in Florida, USA [6], and were recently detected in 2019 in mandarin orchards in the state of São Paulo, Brazil [7].

Given the above, studies related to the antifungal activity of plant products have become a target of research. Essential oils (EOs) extracted from several plant species are an alternative to traditional chemical treatments, which rely on synthetic fungicides and may select resistant phytopathogenic fungi [8]. Tropical plants are a reservoir of secondary metabolites and a significant source of chemical components with different biological properties. The use of EOs extracted from these plants can act as a natural fungicide, inhibiting the activity of a range of fungi and leaving no toxic residues on humans or treated food [9]. They act both by direct fungitoxic action, inhibiting mycelial growth and spore germination, and by the action of phytoalexins [10,11]. Certain terpenes present in citrus EOs, such as limonene, can make the cell membrane of the fungus permeable, causing the leakage of its content [12].

The objective of this study was to evaluate the inhibitory effect of essential oils from mandarin varieties on the fungus *Alternaria alternata*, which causes ABS.

2. Materials and Methods

2.1. Plant Material and Extraction of Essential Oils

The EOs were extracted from the ripe and unripe fruit peels of varieties showing differential responses to ABS: Murcott IAC 221 tangor (susceptible), Fremont IAC 543 mandarin, IAC 2019 Maria mandarin, and Late IAC 855 willowleaf (tolerant/resistant) grafted on Rangpur lime. The plants were located in an experimental area in Cordeirópolis, São Paulo State, Brazil, at a latitude of 22°27′35′′ south and a longitude of 47°24′27′′ west, with an average altitude of 712 m above sea level. The climate at the site is classified as subtropical Cwa, with dry winters (temperatures below 18 °C) and hot summers (temperatures above 22 °C), according to the Köppen–Geiger climate classification system [13].

The EO extraction process was carried out in the Laboratory of Improvement and Analysis of Fruit Quality (LMQF) of the Sylvio Moreira Citrus Center (IAC). The EOs were extracted using the hydrodistillation method, employing the modified Clevenger apparatus [14]. In total, 400 g of chopped peel (1 cm^2) of each variety was used for the oil extraction. The material was placed in 6 L flasks and kept boiling at a constant temperature of 180 °C for three hours. After this time, the oil was collected, quantified, and stored in light-protected bottles at 4 °C.

2.2. Isolation of Alternaria alternata Fungus and Preparation of Inoculum

The *Alternaria alternata* fungus was isolated from typical lesions on highly susceptible Murcott tangor fruits collected in the field from a plantation located in Cordeirópolis-SP at the Sylvio Moreira Citrus Center (CCSM) of the Agronomic Institute of Campinas (IAC), where the fungus is endemic. To obtain the isolate, the methodology described by Canihos et al. [15] was used, with modifications [4]. The lesions were removed from the fruits and disinfected with 70% ethanol, 3% hypochlorite, and distilled water. They were then incubated on Petri plates containing potato dextrose agar (PDA) medium (200 g potato, 20 g dextrose, and 14 $g{\cdot}L^{-1}$ agar), with the addition of the fungicide carbendazim (0.64 $g{\cdot}L^{-1}$), which does not affect the pathogen, but other opportunistic fungi. The plates were kept in the Biological Oxygen Demand (BOD), with a photoperiod of 12 h at approximately 27 °C for 48 h.

After 48 h, the characteristic hyphae and conidia (Figure S1) of the pathogen were identified on the plates using a Leica DM750 light microscope (LM) (Leica Microsystems, Co., Wetzlar, Germany). Serial dilution was then performed with distilled water until a concentration of 10^5 conidia mL^{-1} was obtained to obtain a single-spore culture. The

solution of the single-spore culture was transferred to other Petri plates containing PDA. The fungus was repotted in PDA every three months using an autoclaved loop to transfer the spores to the new plate. All the procedures were performed in the Biotechnology Laboratory of the Citrus, in Cordeirópolis, SP.

To prepare the inoculum, inverted discs of the fungus's mycelium (8 mm diameter) were transferred to Petri plates containing the same condition of the isolate and kept in the BOD for seven days, with a photoperiod of 12 h at approximately 27 °C, following the methodology of Canihos et al. [15]. After mycelial growth, 10 mL of distilled water was added to the surface of the plate and the conidia were removed from the plate's surface using a sterile Drigalski spatula. The suspension was filtered on a layer of sterile gauze to remove mycelial fragments from the plate, then the concentration was adjusted to 10^5 conidia mL^{-1} with the aid of the Neubauer chamber.

2.3. Analysis of Essential Oils by GC-FID and GC-MS

The chemical compositions of the EOs were analyzed using gas chromatography coupled with mass spectrometry (GC-MS) and conventional gas chromatography (GC-FID), following the procedure described by Frizzo et al. [16]. A Shimadzu GC-14B gas chromatograph (Tokyo, Japan) equipped with flame ionization (FID), data-processing software (EZ-Chrom, Shimadzu Corp., Kyoto, Japan), and a GC-MS QP 5050A (Shimadzu Europe, Duisburg, Germany) were used. The chemical identification of the components was performed by comparing the mass spectra with commercial libraries and calculating the linear retention rates (LRR) on two capillary columns of different polarities: weakly polar (SE-52, Mega, Legnano, Italy) and polar (CW-20M, Mega, Legnano, Italy). The quantification of each component was performed according to Frizzo et al. [16], using tetradecane (Sigma Aldrich, USA) as an internal standard. All the chromatographic analyses were performed on a single sample and the results of the components were expressed as a relative percentage.

2.4. Inhibition of Alternaria alternata Fungus In Vitro

In 2019 and 2020, the antimicrobial activity of the EOs from the different varieties was tested against *Alternaria alternata* fungus using the agar diffusion method [17]. The effects of five concentrations (1, 2, 4, 8, and 16 $\mu L \cdot mL^{-1}$) on the growth and mycelial inhibition of the fungal cultures were evaluated in triplicate.

Approximately 20 mL of the potato dextrose agar (PDA) culture medium was added to 9 cm diameter Petri plates in an aseptic laminar flow hood. The concentrations of 0 (control), 1, 2, 4, 8, and 16 $\mu L \cdot mL^{-1}$ of the EOs, along with Tween 80 in a 1:1 ratio, were added to the media with a micropipette, and the mixture was agitated using a vortex to make it homogeneous. Tween® 80 was used as a surfactant in this study due to its low irritation, low toxicity, and high stability, and also because it does not have a fungitoxic effect against *Alternaria* spp. [18] and others when used at concentrations of 1% to 3% ($v \cdot v^{-1}$) [19,20].

After the solidification of the culture media, an 8 mm diameter inverted disc containing *A. alternata* mycelium (taken from a twelve-day-old colony in PDA) was deposited in the center of each plate. The assay was performed in a 2 × 4 × 6 × 7 fully randomized design, with EOs extracted from unripe and ripe fruits of four varieties: Fremont IAC 543, Murcott IAC 221, IAC 2019Maria, and Late IAC 855, five concentrations, and the control being evaluated at seven different times (24, 48, 72, 96, 120, 144, and 168 h). The plates were sealed with plastic film, identified, and incubated in a germination chamber under a 12 h photoperiod at a temperature of 25 °C. The experiment was performed twice over two years (2019 and 2020), and because there were no statistical differences between these years, the mean values between them were used.

2.5. Preventive and Curative Control on Detached Leaves

During 2019 and 2020, new leaves of the tangor Murcott IAC 221, a variety susceptible to Alternaria brown spot (ABS) with approximately 2–3 cm, were collected from the upper third of 12-month-old plants after two weeks of pruning. The leaves were grafted onto Rangpur lime and maintained in a greenhouse. In vitro mycelial growth inhibition tests were conducted to stipulate the concentrations of the EOs from the four varieties of mandarins under study for the preventive and curative control of the *Alternaria alternata* fungus. The concentrations used were 2, 4, 8, and 16 $\mu L \cdot mL^{-1}$ of EOs, together with Tween 80 in a 1:1 ratio, and a control with only distilled water.

In sterile test tubes, a solution of water and Tween 80 was added, and an aliquot of the EOs was added to obtain the desired concentration. The mixture was then stirred until it was completely homogenized. A suspension of the spores from the single-spore culture at a concentration of 10^5 was prepared by adding 20 mL of sterile distilled water into the Petri dishes, according to the literature [15].

The trial was conducted in 2021 in an entirely randomized design, with a 2×3 factorial scheme, two control methods (preventive and curative), and three replicates with three leaves each per concentration. The experiment was set up according to the in vitro severity assessment model [4], using the diagrammatic assessment scale [21].

The preventive control test was installed by applying approximately 1 mL of the solution (EO + Tween 80) per leaf, in different concentrations, onto the abaxial part and spreading it with a brush. About two hours after this application, when the solution had dried on the leaf surface, the pathogen was sprayed with approximately 1 mL of the conidia suspension on the leaves and then maintained in a BOD at approximately 27 ± 2 °C with a photoperiod of 12 h. For the curative control test, the leaves were inoculated with the conidia solution and kept for 24 h in the BOD, along with the other control test. After 24 h, the solution with oil was applied in the same way as in the preventive test.

2.6. Assessments and Data Analysis

The evaluations of mycelial growth (assay 1) were performed seven days after the experiment was set up by taking diametrically opposite measurements (average of two measurements) of the pathogen's mycelial growth. The percentage of the mycelial growth inhibition was calculated for each concentration (treatment) and compared to the control, using Equation (1) as follows:

$$Pi = \frac{(dc - dt) \times 100}{dc} \quad (1)$$

where, Pi = percentage of growth inhibition

dc = the mean diameter of the colony of the fungus in the control

dt = the mean diameter of the fungus colony in the treatment

The assessment of the lesions on the detached leaves caused by the fungus was performed seven days after inoculation by observing the typical symptoms of the disease and subsequently determining the injured area (% of leaf taken by the disease), as described by Martelli et al. [21]. This represented the levels of symptoms in ten illustrated grades, where "0" represented a leaf without symptoms and the severity grades ranged from 0.3 to 97% of the leaf area affected by *A. alternata* symptoms.

All the data underwent variance analyses, and when there was significance for a concentration, a regression analysis was performed. The models were selected based on the determination coefficient, using the software SISVAR 4.5 [22].

3. Results and Discussion

3.1. Chemical Composition of Essential Oils

The chemical compositions of the EOs were determined using GC-FID and GC-MS chromatographic analyses, which identified 48 volatile compounds (Table 1), grouped as follows: 2 acids, 12 alcohols, 6 aldehydes, 1 ketone, 20 terpenes, and 7 sesquiterpenes.

Table 1. Chemical composition (%) and relative percentage of essential oils of unripe (U) and ripe (R) fruit peel of mandarins grafted on Rangpur lime.

Volatile Compounds	Relative Percentage (%) *							
	Fremont IAC 543 Mandarin		Late Mandarin IAC 855		Mandarin IAC 2019Maria		Tangor Murcott IAC 221	
	U	R	U	R	U	R	U	R
Acids								
benzoic acid	-	-	-	1.03	-	-	-	-
formic acid	-	-	-	-	-	0.06	-	-
Alcohols								
3,7-Dimethyloct-7-en^{-1}-ol	-	-	-	-	-	0.41	-	-
cis-homomenthol	-	-	-	-	0.02	0.02	0.05	-
citronellol	-	0.16	0.34	0.2	-	-	0.32	0.14
isocarveol	-	-	-	-	0.6	0.87	0.04	-
Linalool	3.04	3.39	1.05	0.41	13.13	9.7	2.89	1.37
octanol	0.15	0.22	-	-	0.49	-	-	-
p-menth-2-en^{-1}-ol	-	-	0.04	-	-	-	-	-
trans-isocarveol	-	-	0.32	-	1.3	1.5	0.49	-
terpinen-4-ol	0.05	0.23	2.74	0.63	0.92	0.44	0.09	0.06
terpineol	-	-	2.65	-	0.09	0.04	-	-
trans-p-mentha-dien-2-ol	-	0.05	-	-	0.66	-	0.28	-
α-Terpineol	0.25	0.45	-	1.06	1.03	0.71	0.61	0.21
Aldehydes								
citronellal	0.19	0.24	0.08	0.07	0.37	0.45	0.02	0.3
decanal	0.32	0.72	0.07	0.18	0.45	0.82	0.03	0.44
neral	-	-	0.11	0.1	0.08	-	-	-
octanal	1.45	0.98	0.22	-	-	-	1.01	-
perillaldehyde	0.16	0.16	0.29	0.15	0.15	0.18	0.18	-
Ketones								
carvone	-	0.02	-	-	-	-	0.02	0.3
Monoterpenes								
Δ-carene	0.02	-	0.75	0.55	0.15		0.02	0.14
1,3,6-Heptatriene, 2,5,5-trimethyl-	0.01	-	-	-	-	-	0.02	0.4
1,3,8-p-Menthatriene	-	-	-	-	-	0.02	-	0.03
bornanone	-	-	0.04	-	-	-	-	-
carvacrol	-	0.03	2.71	2.06	-	-	-	-
citronellol	-	-	0.34					
felandrene	-	0.01	0.13	0.11	-	0.01	-	0.11
isolimonene	-	0.05	0.39	0.25	-	-	-	-
Isoterpinolene	-	-	1.3	1.12	-	0.04	0.07	0.03
limonene	90.37	88.86	58.89	66.19	77.18	80.62	88.77	90.89
ocimene	-	-	-	-	0.09	0.04	-	2.79
p-Cymeno	-	0.03	0.29	0.12	-	-	-	0.44
sabinene	0.5	0.71	0.27	-	-	0.87	1.01	0.54
Terpinene	-	0.02	-	-	-	-	0.02	-
tujeno	-	0.01	0.79	0.74	0.01	-	-	-
α-Pinene	0.75	0.73	1.98	1.95	0.6	0.63	0.68	0.92
α-terpinene	-	0.06	-	-	0.02	0.12	0.02	-
β-myrcene	2.47	2.52	1.89	2.03	2.13	2.02	2.5	0.08
β-ocimene	-	0,02	0.02	-	0.09	-	-	-
β-pinene	0,06	0.08	1.75	1.47	0.07	0.07	0.04	-
γ-terpinene	0,06	0.17	19.88	18.93	0.22	0.13	0.06	0.08
Sesquiterpenes								
caryophyllene	-	-	0.39	0.38	-	-	-	-
farneceno	-	-	0.11	0.12	-	-	-	0.15
germacrene	0.07	0.02	-	-	-	-	-	0.03
selinene	-	-	0.11	0.1	-	-	-	-
valencene	-	0.01	-	-	-	0.2	0.11	0.23
α-copaene	0.04	0.02	0.02	0.02	0.02	-	0.56	-
β-cadinene	0.04	0.03	0.04	0.03	0.04	0.03	0.09	0.21
β-Copaene	-	-	-	-	-	-	-	0.02
TOTAL	100	100	100	100	100	100	100	100

* Relative area of the chromatogram.

The number of compounds identified in the EOs was consistent with the literature, which reported 29 to 116 compounds in the EOs of different mandarin varieties, with

limonene being the predominant compound [23,24]. The amount of limonene found in this study was similar to that reported in the literature for mandarins, which ranged from 65 to 75% [25], except for the oil of Late IAC 855 mandarin (58.9%). Myrcene or linalool are generally the second most abundant compounds in citrus essential oils, with terpinene also being prominent [26]. A high percentage of linalool was observed in the IAC 2019Maria mandarin, distinguishing it from the other varieties for this component.

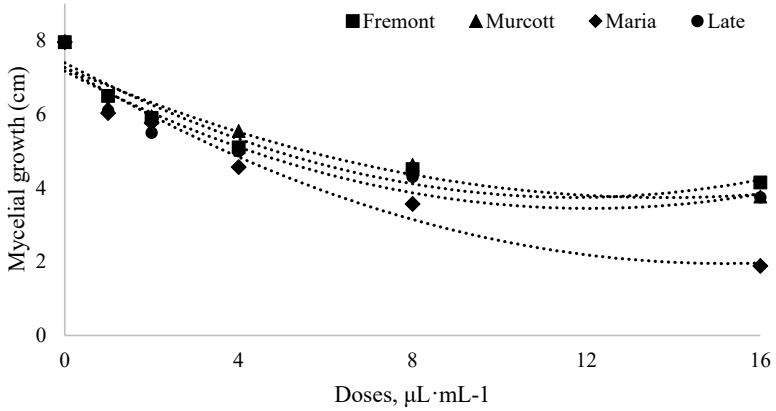
This characterization also highlights the presence of myrcene and linalool (0.08 to 2.47%) in mandarins and terpinene (>18%) in the oil of Late IAC 855 mandarin. Although the commercialized volumes of mandarin and mandarin oils are low, they have a high added value, mainly in the cosmetics and perfumery industries. Furthermore, their antimicrobial potential is also known [14].

It is worth noting that the inhibitory activity (antimicrobial) of an EO is explained by a complex interaction between its constituents, leading to additive, synergistic, or antagonistic effects, especially considering those present in low concentrations [27]. Therefore, it is important to study different varieties of mandarins, which present significant differences in the chemical constitutions of their essential oils.

3.2. In Vitro Inhibition of Alternaria alternata Fungus

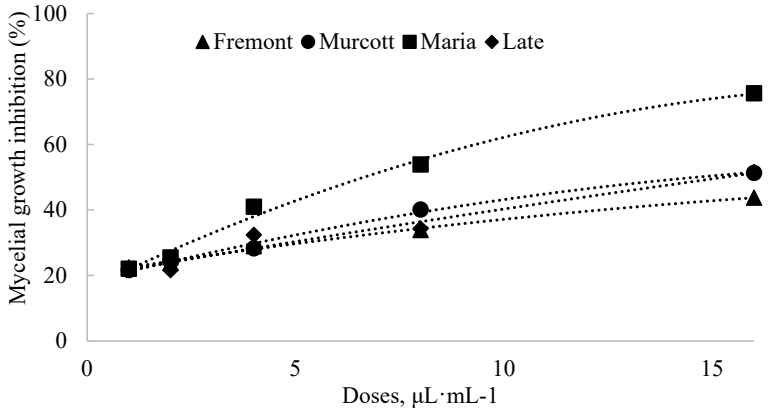
No interactions were observed between the mandarin fruit oils in the fruits at different stages of maturity (unripe and ripe) and between the years of evaluation (2010 and 2020). On the other hand, significant differences were observed when comparing concentrations within the same variety and among the varieties studied (Table S1).

The growths (Figure 1) and percentages of the mycelial growth inhibition (Figure 2) of the fungus *Alternaria alternata* in vitro, with different concentrations (1, 2, 4, 8, and 16 μL·mL^{-1}) of EOs from mandarins, after a regression analysis, fitted linear models with a high degree of determination. As the concentration increased, the mycelial growth decreased after seven days (Figure 1). The oil extracted from the IAC 2019Maria mandarin provided the lowest mycelial growth to the pathogen (1.10 cm) when using the highest concentration (16 μL·mL^{-1}), followed by Late IAC 855 willowleaf (3.20 cm), Murcott IAC 221 tangor (4.53 cm), and Fremont IAC 543 mandarin (4.87 cm), while the control had a mycelial growth of 7.96 cm.



Essential Oils	Equation	R^2
Fremont IAC 543 mandarin	$y = 0.0266x^2 - 0.6228x + 7.3953$	0.92
Murcott IAC 221 tangor	$y = 0.0185x^2 - 0.5102x + 7.263$	0.91
IAC 2019Maria mandarin	$y = 0.0230x^2 - 0.6997x + 7.2699$	0.95
Late IAC 855 willowleaf	$y = 0.0256x^2 - 0.6175x + 7.1715$	0.90

Figure 1. In vitro mycelial growth of *Alternaria alternata*, in PDA culture media, added with different concentrations (1, 2, 4, 8, and 16 μL·mL^{-1}) of essential oils from fruit peel (unripe and ripe) of mandarin fruits, grafted on Rangpur lime (Cordeirópolis/SP, 2019 and 2020). n = 12.



Essential Oils	Equation	R^2
Fremont IAC 543 mandarin	$y = 1.4101x^2 - 21.835x + 2.437$	0.98
Murcott IAC 221 tangor	$y = 2.0034x^2 - 20.740x + 1.876$	0.97
IAC 2019Maria mandarin	$y = 3.5394x^2 - 21.682x + 3.132$	0.96
Late IAC 855 willowleaf	$y = 1.9582x^2 - 20.202x + 2.954$	0.95

Figure 2. Inhibition of mycelial growth (%) in vitro of *Alternaria alternata*, in PDA culture media with different concentrations (1, 2, 4, 8, and 16 $\mu L \cdot mL^{-1}$) of essential oils from the peel of unripe and ripe mandarin fruits, grafted on Rangpur lime (Cordeirópolis/SP, media trials of 2019 and 2020). $n = 12$.

The results of the mycelial growth inhibition showed that all the EOs had an inhibitory effect on the fungus and indicated that the higher the concentration used in the culture media, the greater the direct fungitoxic effect on the pathogen, inhibiting the mycelial growth. Among the tested EOs, the one from the IAC 2019Maria mandarin presented the best mycelial growth inhibition result (75.67%), followed by Late IAC 855 willowleaf (51.55%), Murcott IAC 221 tangor (51.34%), and Fremont IAC 543 mandarin (43.80%) (Figures 2 and S2).

Studies corroborate these results and have described that the effect of EOs on citrus varieties (lemon, orange, grapefruit, and mandarin) is associated with a decrease in fungal growth with the antimicrobial potential of the tested oils [28,29]. This occurs due to the high chemical complexity of these EOs, attributing this antimicrobial effect to the synergism or antagonism among their constituents [30].

Better results were obtained with the IAC 2019Maria mandarin, which may be related to the higher concentration of isocarveol (perillyl alcohol), along with the presence of limonene and linalool in the composition of its essential oil. Studies have shown the antifungal capacity of perillyl alcohol against *Candida* spp. strains, demonstrating fungicidal activity at concentrations ranging from strong to moderate due to the presence of bulky lipophilic groups attached to the aromatic ring, which contribute to potentiating bioactivity [31]. The fungicidal effect of EOs may be related to the presence of limonene in the constitution of these oils. In several studies, the fungitoxic effect of citrus EO (*C. sinensis*) in the control of Asiatic Rust (*Phakopsora pachyrhizi*) in soybean has been observed, with limonene being a major compound with elicitor characteristics [10]. Additionally, it has been found that orange EO has a high concentration of monoterpenes and phenolic compounds, where these components inhibit the mitochondrial respiration of the fungus membrane [32], and the same concentration was found in the EOs of this study.

The fungitoxic activity of plant EOs is attributed to small terpenoids and phenolic compounds such as thymol, carvone, menthol, carvacrol, and limonene, as was the case with the majority of compounds present in the varieties studied in this work. The effects of limonene, linalool, and myrcene on the inhibition of mycelial growth and the germination of the spores of the *Colletotrichum acutatum* species isolated from Valencia orange plants have been reported [33].

The fungicidal activity caused by the application of EOs has also been described against a wide range of postharvest fungi, including *Alternaria alternata*, *Colletotrichum*

gloeosporioides, *Rhizopus stolonifer*, *Aspergillus* spp., and several species of *Penicillium spp*., among others, which can be effectively controlled with the use of EOs [34,35]. It has already been found in the literature that the fungitoxic effect of the essential oil of orange, which was observed for *Phakopsora pachyrhizi*, was due to the presence of limonene, a majority compound with elicitor characteristics [10,11].

The components citral, linalool, and β-pinene found in the tested mandarin oils have effects against different phytopathogenic fungal species, and the antimicrobial efficacy of this combination of chemical compounds has been observed [32]. The fungitoxic effects of citrus EOs were observed in samples with a high concentration of monoterpenes and phenolic compounds, where these components inhibited the mitochondrial membrane respiration of fungi [36]. Although the characterization of the action mechanisms of EOs is not known for sure, the accumulation of compounds of a lipophilic character in the membrane causes a loss of energy with the microbial cells [37].

Limonene has antifungal activity attributed to the inhibition of pectinmethylesterase (PME), which modifies the degree of the methylesterification of pectins, the main components of the cell walls of fungi [38]. Citral (a mixture of the neral and geranial isomers present in EOs) reduced the mycelial growth of *Fusarium oxysporum cubense*, *C. gloeosporioides*, *Bipolaris* spp., and *Alternaria alternata* [39]. Mandarin EOs with 46.7% of limonene can inhibit the growth of *A. alternata*, *Rhizoctonia solani*, and *Curvularia lunata* [40].

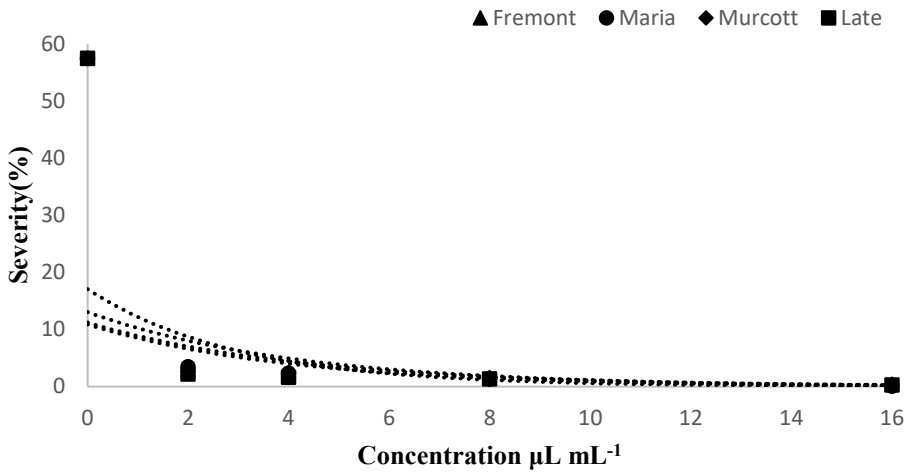
According to Lopes et al. [41], the mode of action of phytoalexins on fungi includes cytoplasmic granulation, the disorganization of cell contents, the rupture of the plasma membrane, and the inhibition of fungal enzymes. These effects are reflected in the inhibition of germination and germ tube elongation and the reduction or inhibition of the mycelial growth of fungi. Sharma et al. [42] showed the inhibition of the mycelial growth of the fungus *Aspergillus niger* when the EO of orange peels was used. The morphology of this fungus was evaluated using scanning electron microscopy, and after the test with the EO, they reported that the hyphae were damaged, and in some cases, their death occurred. The same authors also evaluated the fungitoxic effects of orange EO on ten pathogens, observing a broad spectrum of action on microorganisms, with a minimum inhibitory concentration of 400 to 500 $\mu g \cdot mL^{-1}$. Hani et al. [43] observed that the chemical components present in the oils of *C. sinensis* and *C. reticulata* inhibited intercellular and extracellular enzymes, acting as regulators of cellular metabolism and affecting the enzyme synthesis in the nucleus and/or ribosome. They also interacted with the nutrient uptake from the environment, affecting mycelial growth.

Similarly, in research conducted to verify the effect of 22 EOs on eukaryotic cells, it was revealed that they acted as pro-oxidants, affecting mainly the cell membranes and interior of organelles such as mitochondria, as exposed in an important review on the subject [44]. According to these authors, the cytotoxic effects of EOs on cells may be associated with changes in the intracellular oxireduction potential resulting from the activity of this exposure to EOs.

In this way, essential oils can exert antimicrobial activity on a wide diversity of microorganisms. However, to obtain an expressive effect, different concentrations are necessary according to the pathogen being inhibited. Moreover, the antifungal effect depends directly on the chemical components present in the oil, acting directly or indirectly on the biological activity and sensitivity of the microorganisms [45].

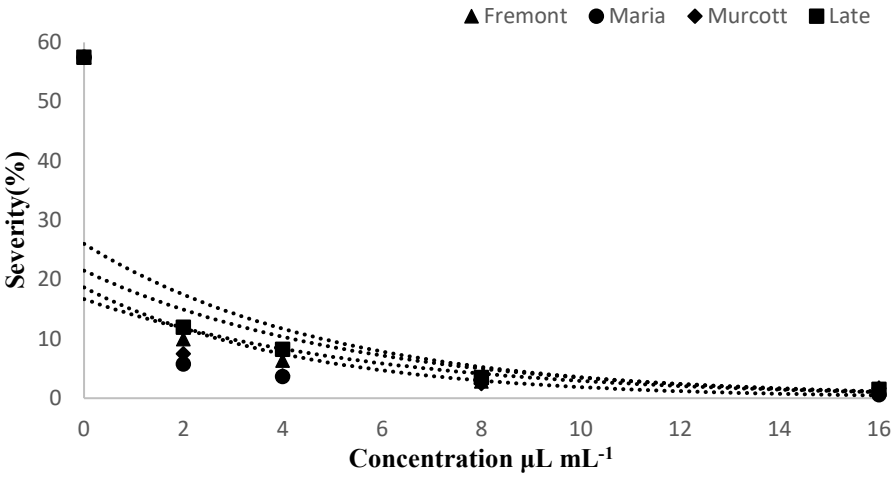
3.3. Preventive and Curative Control on Detached Leaves

There was no interaction between the EOs extracted from the ripe and unripe fruits; therefore, the results of these tests were grouped (Table S2). When analyzing the effect of the oils in the preventive and curative treatments, all the essential oils tested at the concentration of 16 $\mu L \cdot mL^{-1}$ provided lower values for disease severity within each treatment (preventive and curative) when compared to other concentrations and the control, which showed an average severity of 57.5% of the injured leaf area (Figures 3,4 and S3), demonstrating the aggressiveness of ABS in susceptible varieties such as Murcott tangor [46].



Essential Oils	Equation	R^2
IAC 2019Maria mandarin	$y = 17.064^{-0.335x}$	0.87
Fremont IAC 543 mandarin	$y = 13.060^{-0.243x}$	0.71
Murcott IAC 221 tangor	$y = 11.223^{-0.234x}$	0.65
Late IAC 855 willowleaf	$y = 10.896^{-0.249x}$	0.67

Figure 3. Severity (%) in preventive treatment, in vitro, of *Alternaria alternata*, on detached leaf with different concentrations of essential oil (2, 4, 8, and 16 µL·mL^{-1}) of unripe and ripe fruits of mandarins, grafted on Rangpur lime. *n* = 12.



Essential Oils	Equation	R^2
IAC 2019Maria mandarin	$y = 18.688^{-0.23x}$	0.79
Fremont IAC 543 mandarin	$y = 21.512^{-0.183x}$	0.74
Murcott IAC 221 tangor	$y = 16.710^{-0.174x}$	0.63
Late IAC 855 willowleaf	$y = 26.032^{-0.2x}$	0.84

Figure 4. Severity (%) in the curative treatment, in vitro, of *Alternaria alternata*, in leaf detached with different concentrations of essential oil (2, 4, 8, and 16 µL·mL^{-1}) of unripe and ripe fruits of mandarins, grafted on Rangpur lime. *n* = 12.

In the preventive control test (Figure 3), it was observed that a greater control of the disease was obtained at concentrations of 2, 4, and 8 µL·mL^{-1} when compared to the same concentrations in the curative control test (Figure 4). At a concentration of 16 µL·mL^{-1}, a lower leaf severity was observed when using the EO of the IAC 2019Maria mandarin, with

an average of 0.1% of the injured leaf. All the oils, when used at a dose of 16 $\mu L \cdot mL^{-1}$, had severities below 0.5.

The curative effect of citronella EO on rice brusone (*Pyricularia grisea*) was observed to reduce disease incidence by up to 50% in the replicates [47]. Better results were also observed for the preventive effect of noni EO application when compared to the curative test for the control of anthracnose (*C. gloeosporioides*) in mango plants [48].

The use of EOs as antimicrobial agents is considered to be low risk because it is believed to be difficult for a pathogen to develop resistance to the complex mixture of active components that comprise these oils [49].

The IAC 2019Maria and Late IAC 855 mandarin EOs showed greater effectiveness in the curative control of ABS (Figure 4 and Table S3), leading to 0.6% and 1.3% of the infected area on the leaf being affected, respectively, at a concentration of 16 $\mu L \cdot mL^{-1}$, 168 h after inoculation. However, the application of the Murcott IAC 221 tangor oil at lower concentrations kept the disease severity level stable, preventing it from evolving in the plants and causing more damage. In the curative test, the concentration of 16 $\mu L \cdot mL^{-1}$ reduced the severity of the disease by half when compared to the lowest concentration used.

Díaz Dellavalle et al. [50] performed an experiment using extracts of *R. officinalis* on the growth of *Alternaria* spp., concluding that the antimicrobial action may be due to the presence of substances such as EOs in the form of alpha- and beta-pinene, limonene, camphene, myrcene, terpenoids such as carnosol, and oleanic acid [51], compounds similar to the monoterpenes found in the mandarin EOs extracted in this work.

The antifungal activity of EOs is related to their hydrophobicity, which allows them to interact with lipids of the cell wall, cell membrane, and mitochondria, changing the permeability and causing disturbances in these structures [12].

This study shows that EOs are a rich source of research, as many of them have shown promise and may become another option for the control of ABS. However, for the definitive and safe insertion of EOs in recommendations for producers, studies on their concentrations, time of application, residual period, mechanisms of action, phytotoxicity, real safety to mammals, other vertebrates, and the environment, the availability of their products, and their costs deserve more attention [52].

4. Conclusions

After seven days, the Mandarin IAC 219Maria essential oil, at a concentration of 16 $\mu L \cdot mL^{-1}$, showed promising results for controlling the *A. alternata* fungus in vitro. Furthermore, in detached leaves, the essential oils of all the varieties tested, at the highest concentration (16 $\mu L \cdot mL^{-1}$), provided the lowest values for the disease severity in the leaves, both curatively and preventively.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9060613/s1, Figure S1: Conidia of Alternaria alternata observed in Leica DM750 light microscope (LM) (Leica Microsystems, Co., Wetzlar, Germany); Figure S2: In vitro mycelial growth of the fungus Alternaria alternata after 168 h in culture medium BDA with different concentrations (1, 2, 4, 8 and 16 µL mL−1) of mandarin essential oils; Figure S3: Alternaria brown spot symptoms on detached leaves after 168 h, where 0 = control and different concentrations 2, 4, 8 and 16 µL mL−1 of mandarin essential oils, in curative and preventive control. Table S1: In vitro mycelial growth of Alternaria alternata, in PDA culture media, added with different concentrations (1, 2, 4, 8 and 16 µL mL−1) of essential oil from fruit peel (means of unripe and ripe) of mandarin fruits, grafted on Rangpur lime (Cordeirópolis, SP, 2019 and 2020). (*) means followed by different lowercase letters, in the column, indicate significant differences between the doses within each variety and different uppercase letters, in the row, indicate significant differences between the varieties at the same dose (Tukey, 5%). n = 12; Table S2: Severity (%) in preventive treatment, in vitro, of Alternaria alternata, on detached leaf with different concentrations of essential oil (2, 4, 8 and 16 µL mL−1) means of unripe and ripe fruits of mandarins, grafted on Rangpur lime. (*) means followed by different lowercase letters, in the column, indicate significant differences between the doses within each variety and different uppercase letters, in the row, indicate significant differences between the

varieties at the same dose (Tukey, 5%). n = 12; Table S3: Severity (%) in the curative treatment, in vitro, of Alternaria alternata, in leaf detached with different concentrations of essential oil (2, 4, 8 and 16 µL mL−1) means of unripe and ripe fruits of mandarins, grafted on Rangpur lime. (*) means followed by different lowercase letters, in the column, indicate significant differences between the doses within each variety and different uppercase letters, in the row, indicate significant differences between the varieties at the same dose (Tukey, 5%). n = 12.

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