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Essential Oils as Natural Fungicides to Control *Rhizopus stolonifer*-Induced Spoiled of Strawberries

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Abstract: Strawberry is a highly desirable fruit with a unique taste and a good source of bioactive compounds beneficial to human health. However, it has a short post-harvest shelf life, mainly due to the soft rot caused by *Rhizopus stolonifer*. This study aimed to evaluate the antimicrobial properties of essential oils (EOs) of *Mentha piperita*, *Cymbopogon martinii*, *Cinnamomum camphora*, and *Mentha spicata* using spore germination and micro-well dilution assays, and to test the effects of the vapor-phase application of *M. spicata* and *C. martini* on the incidence and severity of soft rot in strawberry artificially inoculated with *R. stolonifer*. In *in vitro* tests, *C. martinii* and *M. spicata* EOs were the most effective, inhibiting more than 95% of the spore germination. Additionally, in the microwell dilution test, these EOs had the lowest minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) (5 and 10 µg/mL, respectively), and for the microwell dilution test, the lowest MIC (5 µg/mL and 10 µg/mL, respectively) and MFC (10 µg/mL for both). High *in vivo* inhibitory effects of *M. spicata* and *C. martinii* EOs were observed at 10% concentration, with 100 and 78% reduction, respectively, in the *R. stolonifer*-induced spoilage. Our results suggest that *C. martinii* and *M. spicata* EOs can be used as efficient natural fungicides and can be an alternative to synthetic fungicides for preserving fresh strawberries from soft rot.

Keywords: plant essential oils; minimal inhibitory concentration; natural antifungal agent; soft rot.

1. Introduction

Strawberry (*Fragaria* × *ananassa*) is a fruit cultivated and appreciated worldwide, mainly for direct consumption and processing value-added products [1]. It is rich in beneficial bioactive compounds, such as vitamins, β-carotene, and phenolic compounds [2]. However, because of their high respiration rate and soft texture, ripe fruits have a short post-harvest life, mainly due to infection of phytopathogenic fungi, which changes their physical-chemical characteristics and reduces shelf life [3].

Rhizopus stolonifer is one of strawberry's main storage pathogens that contributes to the ripe fruits' high spoilage during post-harvest life. It causes soft rot that, under high humidity conditions, covers the fruit with mycelium, interspersed with pathogens' structures [4]. Although this infection can be controlled efficiently using synthetic fungicides, these additives can be harmful to human and plant health. In addition, fungicides can form long-term residues

in the environment and promote resistance to their active principles in the fungal populations [5,6]. A better alternative to synthetic fungicides must reduce these indirect economic costs [6].

Among the new natural alternatives, essential oils (EOs) show great potential for controlling phytopathogenic fungi because of their high antifungal activity, low toxicity, and low environmental impact [7]. EOs are secondary metabolites produced by plants in response to adverse conditions. They have high volatility, intense aroma, and good solubility in organic solvents [8]. *In vitro* studies have indicated their high antimicrobial activity in direct contact tests (liquid phase); however, relatively higher concentrations are necessary to see these effects *in vivo*. At high concentrations, their strong aroma can modify the flavor of the food [9]. To minimize the sensory impact of EOs and make their application feasible for the products that are sensitive to immersion treatments, the use of EOs in the volatile vapor phase is an option. This can reduce the amount needed to guarantee the optimum antimicrobial effect and the least impact on the target food's sensory properties [10]. Applying EOs using the vapor phase is an interesting, new alternative that allows its useful bioactivity as an antimicrobial agent to preserve perishable food products [10-12]. El Ouadi *et al.* [13] demonstrated that the EO of *Melissa officinalis* in the vapor phase could control *Botrytis cinerea, Penicillium expansum,* and *Rhizopus stolonifer* in apples. Thus, this study aimed to evaluate the antimicrobial activity of *Cymbopogon martinii*, *Cinnamomum camphora*, *Mentha spicata,* and *Mentha piperita* OEs using spore germination and micro-well dilution methods as well as the incidence and severity of soft rot in strawberry artificially inoculated with *R. stolonifer* after vapor-phase application of *M. spicata* and *C. martini*.

2. Materials and Methods

2.1. Materials.

Essential oils of pippermint (*Mentha piperita*) lot 1123, palmarosa (*Cymbopogon martinii*) lot 2489, and ho wood (*Cinnamomum camphora*) lot 1369 were purchased from Laszlo Aromaterapia (Belo Horizonte, Minas Gerais, Brazil), and mint (*Mentha spicata*) lot 197 EO was purchased from Ferquima Ind. e Com. Ltda. (Vargem Grande Paulista, São Paulo, Brazil). All EOs used in this study were extracted by hydrodistillation, as per the companies' information on the product labels. The fungal strain *R. stolonifer* CCT 0276 was obtained from Andre Tosello Foundation (Campinas, São Paulo, Brazil). The chemical profiles of EOs were determined using gas chromatography and mass spectrometry (GC-MS, QP-5000, Shimadzu, Columbia, MD, USA) previously and published by our research group [14]. The major volatile contents (i.e., ≥ 1%) of *M. piperita* EO were menthol (45.37%), menthone (20.13%), isomenthone (16.94%), menthyl acetate (3.81%), pulegone (1.89%), α-terpinene (1.88%), isopulegol (1.83%), neoisomenthol (1.19%), and α-terpineol (1.08%), while that of *C. camphora* EO was oxygenated monoterpene linalool (98.39%). The EO of *C. martinii* had geraniol (83.82%), geranyl acetate (7.49%), linalool (2.48%), and caryophyllene (1.33%) as the major volatile compounds, whereas that of *M. spicata* showed carvone (61.71%), limonene (20.22%), 1,8-cineole (5%), sabinene (2.28%), cis-dihydrocarvone (1.63%), and α-thujene (1.4%) as the major volatile compounds.

2.2. Determination of the in vitro antifungal activity of essential oils.

2.2.1. Spore germination assay.

Germination tests were conducted as described by Pimentel *et al.* [15], with some modifications. Briefly, 100 µL of *R. stolonifer* or *B. cinerea* conidia suspension (2×10^5 conidia per mL) was mixed with 100 µL of each EO. Tween 80 (0.05% v/v) was used as a negative control. Microscope cavity slides containing the mixtures were incubated at 25°C for 24 h in a Petri dish with wet filter paper, and the germination was observed under an Optical Systems Nova 107 light microscope. A total of 200 spores were counted, and the percentage of germinated conidia was calculated using the number of conidia germinated/total number of conidia.

2.2.2. Micro-well dilution method.

Fungal growth inhibition was tested as described by Broekaert *et al.* [16]. Briefly, a mixture of 10 µL of conidial suspension and 90 µL of yeast potato dextrose broth were incubated in a 96-well microtiter plate for 16 h at 26 ± 2°C. An aliquot (100 µL) of each 2-fold-diluted sample was added to a 96-well plate at 0.156–20 µg/mL and incubated again at 26 ± 2°C. Tween 80 (0.05% v/v) served as a negative control. After incubation, 10 µL of cell viability indicator (2,3-5-triphenyl tetrazolium chloride 1%) was added to each well and incubated at 37°C for 1 h. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of essential oil that shows no color change, while minimal fungicidal concentration (MFC) was defined as the lowest concentration that completely inhibits subculture growth; for this, 5 µL of cultures were subcultured subsequently on PDA plates at 26 ± 1°C for 5 days.

2.3. In vivo tests: determination of the antifungal activity of essential oil vapors against R. stolonifer on strawberries.

For this, the EOs of *M. spicata* and *C.* martini, which were more effective in the *in vitro* experiments, were emulsified at 2.5%, 5%, and 10% (v/v) concentrations distilled water using Tween 80 (0.05% v/v) as a surfactant. Emulsions were prepared by mixing the EO and the aqueous phase in a high-speed mixer (UltraTurrax T25, IKA Werke GmbH & Co, Staufen, Alemanha) for 5 min at 5,000 rpm.

Strawberries (*Fragaria x ananassa*) were purchased from a local market (São Carlos, SP, Brazil). The specimens were selected based on their appearance and freshness and then sanitized using 2.5% sodium hypochlorite. Batches of 3 fruits were inoculated with *R. stolonifer* spores using 30 µL of a spore suspension (10^5 spores mL^{-1}) in 3 mm deep wounds and were placed in transparent plastic containers (157×130×40mm, Galvanotec, GA 92, Carlos Barbosa, Rio Grande do Sul, Brazil). These were treated with 1 mL of each EO emulsion soaked in cotton balls attached to the containers' edge and sealed with Parafilm® to prevent vapors' leakage. The fruits were stored at 25± 2°C/ at 80 ± 5% relative humidity for 7 days, with a photoperiod of 12 h. The treatments' efficacy was evaluated based on the incidence and severity of the infection in the fruits. Seven days after the inoculation, disease incidence was calculated from the number of symptomatic fruits in relation to the total number of fruits used for each treatment. The results were expressed as a percentage (%) [17]. Severity of infection was calculated on a scale of 6 (0 = no symptoms; 1 = infection in 1%–20% of the wounded

area; 2 = in 21%–40% area; 3 = in 41%–60% area; 4 = in 61%–80% area and 5 = infection in more than 81% of the wounded area) [18].

2.4. Statistical analysis.

The standard deviation of the means was calculated, and the statistical difference of the means at a 5% significance level ($p < 0.05$) was determined by the Tukey test. In the *in vivo* tests, the experimental design was randomized in a factorial scheme (7×3), with seven treatments and three concentrations (Control; 2.5%, 5%, and 10% EO of *M. spicata*; 2.5%, 5%, and 10% EO of *C. martinii*). The relative frequency was calculated, and the statistical difference at 5% significance level ($p < 0.05$) was calculated using the Kruskal–Wallis test.

3. Results and Discussion

3.1. Spore germination assay.

Essential oils of *M. spicata* and *C. martinii* showed respectively 95.5% and 98.6% inhibition of germination of *R. stolonifer* spores, the highest among the EOs tested, while that of *C. camphora* had the lowest inhibition (69.3%) (Figure 1). Under the experimental conditions, control (0.5% Tween 80) was completely unable to inhibit spore germination, presenting a 100% score. Present results match those reported previously [19-21]. Although the capacity of EOs to inhibit spore germination is reported in the literature, the possible mechanisms of action of these oils and their major constituents are not known.

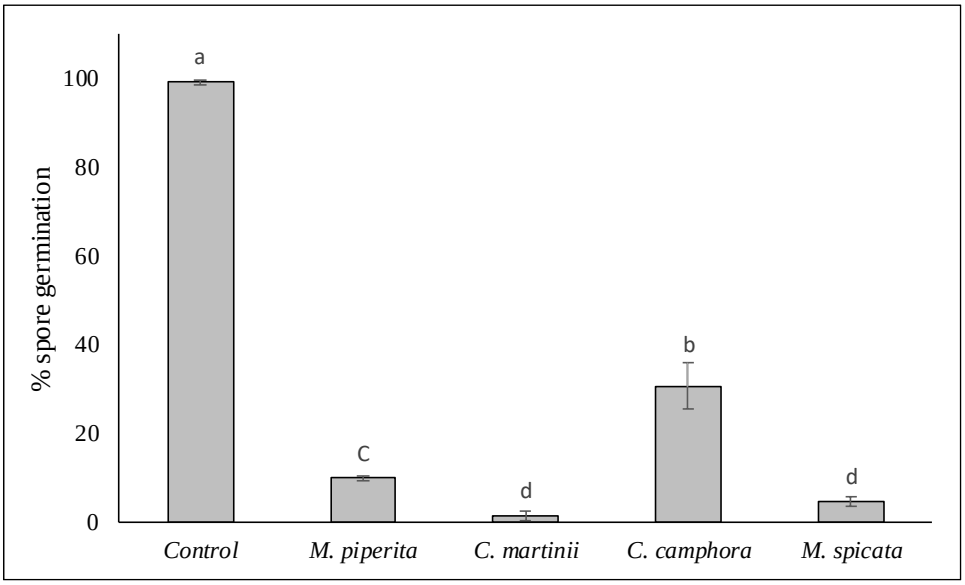

Figure 1. Inhibition of *R. stolonifer* spore germination by essential oils of *Mentha piperita*, *Cymbopogon martinii*, *Cinnamomum camphora*, and *Mentha spicata* at 0.1% concentration. Different letters represent a significant difference between the essential oils by the Tukey test ($p < 0.05$).

3.2. Micro-well dilution assay.

Among the EOs tested, those of *C. martinii* and *M. spicata* showed the highest antifungal activity, with MIC values of 5 µg/mL and 10 µg/mL and both with MFC of 10 µg/mL, respectively. In comparison, EOs of *M. piperita* and *C. camphora* had the lowest antifungal activity with MIC and MFC values of 20 µg/mL, against *R. stolonifer* (Table 1).

Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of essential oils against *R. stolonifer* by micro-well dilution method.

Essential oils	MIC (µg/mL)	MCF (µg/mL)
M. piperita	20	20
C. martini	5	10
C. camphora	20	20
M. spicata	10	10

The high antifungal activity of EOs of *M. spicata* and *C. martinii* appears to be related to their chemical composition. *M. spicata* EO is rich in carvone, which has high antifungal activity [22], while that of *C. martinii* EO may be related to the presence and interactions between its major ingredients, geraniol, geranyl acetate, linalool, and karyophylene [23].

Relatively lower antifungal activity of *M. piperita* EO compared to those of *M. spicata* and *C. martini* (Table 1) may be due to the presence of poorly-soluble menthol acetate [22] in it composition. This result corroborates the weak antifungal activity of *M. piperita* EO compared to that of *M. spicata* and *C. martinii* EOs, reported by others [22,24]. C. camphora EO's low efficacy here is probably due to the high concentration of linalool (98%), which is reported to have poorer antifungal effects than compounds found in other EOs [25,26].

3.3. In vivo tests: determination of the antifungal activity of essential oil vapors against B. cinerea on strawberries.

Since EOs of *M. spicata* and *C. martinii* showed the best results in *in vitro* tests, their *in vivo* antifungal activity in the vapor phase was investigated. Figures 2 and 3 show the effectiveness of *M. spicata* and *C. martinii* EOs in controlling *R. stolonifer* in strawberries. *M. spicata* and *C. martinii* EOs were able to reduce the incidence and severity of rot caused by *R. stolonifer* in strawberries, stored at 25°C for 3 days. The inhibitory effect of the EOs was directly dependent on their dose (Figure 3). At the highest concentration tested (10%), *M. spicata* and *C. martinii* EOs showed 100 and 78% inhibition, respectively. These results are consistent with the report of Hosseini, Amini, Saba, Karimi, and Pertot [11], using EOs of *Allium sativum* (garlic) and *Rosmarinus officinalis* (rosemary) in the vapor phase to control *Colletotrichum gloeosporioides* in strawberries. In the present study, a similarly created controlled atmosphere to evaluate the EOs against *R. stolonifer* in strawberries resulted in inhibition comparable to that described by Hosseini, Amini, Saba, Karimi, and Pertot [11].

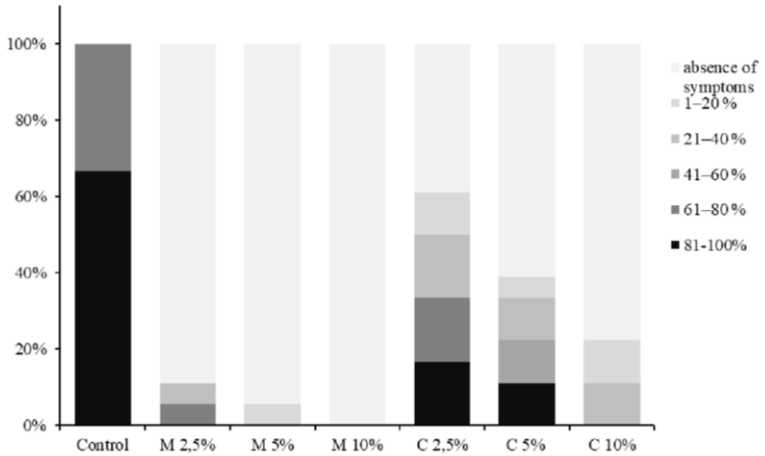

Figure 2. Severity of *B. cinerea* in post-harvest strawberries treated with *M. spicata* and *C. martinii* essential oils in vapor-phase and stored at 25± 2°C/80 ± 5% RH for 7 days (n = 18). Different letters represent a significant difference between the treatments using the Kruskal-Wallis ($p < 0.05$). Control: distilled water containing Tween 80; M2.5, M5, and M10: *M. spicata* essential oil at 2.5, 5, and 10%, respectively; C2.5, C5, and C10: *C. martinii* essential oil at 2.5, 5, and 10%, respectively.

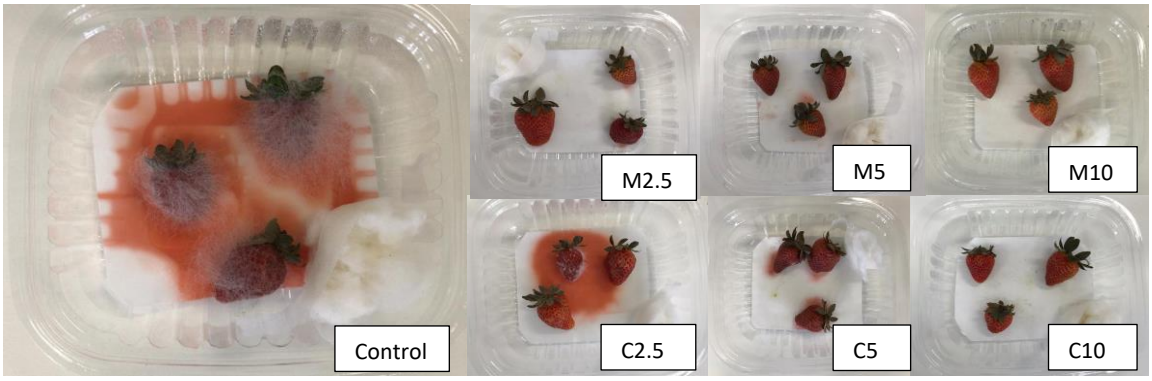

Figure 3. Strawberries inoculated with *R. stolonifera* and treated with *M. spicata* and *C. martinii* essential oils in vapor-phase before being stored at 25± 2°C/80± 5 % RH, for 7 days. Control: distilled water containing Tween 80; M2.5, M5, and M10: *M. spicata* essential oil at 2.5, 5, and 10%, respectively; C2.5, C5, and C10: *C. martinii* essential oil at 2.5, 5, and 10%, respectively

4. Conclusions

Our results show that essential oils of *C. martinii* and *M. spicata* had the greatest antifungal activity according to spore germination and micro-well dilution assays. The application of essential oils in the vapor phase effectively reduced the severity and incidence of *R. stolonifer* infection in artificially inoculated strawberries. Therefore, *C. martinii* and *M. spicata* essential oils can be used as potential natural fungicides, which can be better alternatives to synthetic fungicides to control the soft rot in fresh strawberries.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the study's design; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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