Antifungal activity of essential oils and their combinations against postharvest fruit pathogen

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Abstract

***Rhizopus stolonifer* is considered the most devastating storage fungus of horticultural commodities such as strawberries and peaches. The antifungal activity of the essential oils (EOs) from *Mentha piperita, Cymbopogon martinii, Mentha spicata* and *Cinnamomum camphora*, as well as effect of their possible double combinations M1 (*M. piperita* and *C. martinii*), M2 (*M. piperita/C. camphora*), M3 (*M. piperita/M. spicata*), M4 (*C. martinii/C. camphora),* M5 (*C. martini/M. spicata*), and M6 (*C. camphora/M. spicata*), were investigated in vitro by direct contact and by vapor contact against *R. stolonifer*. The highest antifungal activity by the contact method was promoted by the EOs of *M. piperita, M. spicata* and *C. martinii*, individually, and by the combinations M2, M3, M4, M5 and M6 with total inhibition of mycelial growth between concentrations of 500 and 750 µL L^{-1}. *C. camphora* EO and M1 mixture presented lower antifungal activity with total inhibition of mycelial growth between 750 and 1000 µL L^{-1}. On the other hand, the exposure of fungi to volatiles showed that *C. martinii* EO had the highest activity, with total inhibition of mycelial growth of *R. stolonifer* at a volume of 5µL per closed Petri dish. Although the EOs of *C. martinii, M. spicata* and *M. piperita* presented the highest antifungal potentials when evaluated individually, their combination did not result in better antifungal development by direct contact or volatile exposure. Among all the oils and mixtures evaluated in vitro, the EO of *C. martinii, M. spicata* and *M. piperita* showed the greatest inhibitory capacity against *R. stolonifer*. Therefore, these oils can be a potential alternative to the synthetic fungicides for disease postharvest control.**

Keywords: *Mentha piperita*, *Cymbopogon martinii*, *Cinnamomum camphora*, *Rhizopus stolonifer*

INTRODUCTION

Filamentous fungi are widely dispersed in nature and can cause deterioration in food and agricultural crops. Most fresh vegetables are susceptible to phytopathogenic fungi infection during postharvest. Fungal contamination is the main cause of economic losses in the global fresh vegetable industry, as it affects their quality and decreases shelf life (Leyva Salas et al., 2017; Dukare et al., 2018).

Rhizopus stolonifer is one of the most common fungi and is considered as one of the most devastating pathogenic agents (Bautista-Banos et al., 2014). *Rhizopus* rot appears particularly on mature fruits, when temperatures are above 5°C, and spread rapidly infecting healthy fruits (Ogawa et al., 1995). Due to their wide array of hosts, rapid penetration and colonization, *R. stolonifer* has become an important control target for synthetic fungicides. However, alternatives are needed to handle this pathogen and others because of the growing concerns on environmental impacts, human health, the generation of resistant strains of phytopathogens, and the widespread and sustained use of these substances (Palou et al.,

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2016).

In this sense, plant essential oils (EOs) and their constituent molecules have received special attention and are generally recognized as safe for human consumption by the FDA (Burt, 2004). EOs have been shown to possess a broad spectrum of activities against postharvest pathogens and, therefore, have been considered as natural, safe and biodegradable alternatives in the last decade (Oliveira et al., 2019; Karimi et al., 2016).

Considering that, in some cases, applications in high concentrations are required for the EO to have an in vivo effect, their use may have a negative impact on the sensory properties of the food. To avoid this adverse effect, several EOs can be mixed to increase their antimicrobial action and reduce the individual concentrations required for in vivo application (Rentsenkhand and Vágvölgyi, 2010). The possible synergistic effect produced by the combination of plant essential oils was reported as an efficient strategy to combat microbial development (Nikkhah et al., 2017). Only a few studies about the synergistic effects of the combination of EOs have been reported.

Thus, the objective of this study was to evaluate the effect of *Cymbopogon martinii*, *Cinnamomum camphora*, *Mentha spicata* and *Mentha piperita* EOs and their binary mixtures against the growth of *R. stolonifer* by the methods of direct contact and vapor contact, in order to contribute to the development of a new efficient and safe strategy for the control of *R. stolonifer* on postharvest fruit.

MATERIALS AND METHODS

Materials

Pippermint (*Mentha piperita*), palmarosa (*Cymbopogon martinii*) and ho wood (*Cinnamomum camphora*) essential oils (EOs) were purchased from Laszlo Aromaterapia (Belo Horizonte, Brazil). Mint (*Mentha spicata*) EO was purchased from Ferquima Ind. e Com. Ltd.a (Vargem Grande Paulista, São Paulo, Brazil). Fungal strains *R. stolonifer* CCT 0276 was purchased from Andre Tosello Foundation (Campinas, SP, Brazil).

Determination of the in vitro antifungal activity of EOs

The in vitro antifungal activity of EOs *M. piperita, C. martinii, C. camphora* and *M. spicata*, individually or in combinations M1 (*M. piperita* and *C. martinii*), M2 (*M. piperita* and *C. camphora*), M3 (*M. piperita* and *M. spicata*), M4 (*C. martinii* and *C. camphora*), M5 (*C. martinii* and *M. spicata*), M6 (*C. camphora* and *M. spicata*) were tested to evaluate the combination effect of EOs on the control of *R. stolonifer* by direct contact and vapor contact methodology.

Method by direct contact

The antifungal activity of EOs was evaluated by measuring *R. stolonifer* growth inhibition by the direct contact of the fungus with the potato dextrose agar (PDA) culture medium containing the EO either individually and with their binary mixtures (50% each), at concentrations 31; 62.5; 125; 250, 500, 750 and 1000 μL L^{-1} (Plaza et al., 2004). For the homogenization of the EOs and the mixtures to the PDA medium, the emulsifier Tween 80 (0.05% v/v) was used. A control treatment, containing only the emulsifier and the culture medium, was also employed.

After solidification of the PDA medium, *R. stolonifer* was transferred to the center of the plate, from a disk (5 mm diameter) containing mycelium (inoculum). Plates were maintained in 12 h photoperiod at 25 °C (Baggio et al., 2016), with measurements of the mycelial growth of each colony performed every 8 h, in two perpendicular directions (diameter in cm). Fungal growth inhibition at the different concentrations of individual EOs and mixtures were measured by the equation PI (%) = (Control Growth – Treatment Growth / Control Growth) × 100 (Plaza et al., 2004). The Minimum Inhibitory Concentration (MIC), when present, was considered as the lowest concentration of the treatment, among the concentrations evaluated, capable of completely inhibiting *R. stolonifer* development.

Method by vapor contact

The antifungal activity of EOs was also evaluated by the method of exposure to volatiles, according to Yun et al. (2013). Fungal growth inhibition was checked for different amounts of individual EOs and their mixtures (0, 5, 10, 20, and 30 µL), which were applied on a circle of filter paper (20 mm^2), fixed in the center of the internal part of the Petri dish lid, which contained solidified PDA. Pathogen inoculation procedure, incubation, mycelial growth measurement and MIC determination were the same as described for the contact method.

Statistical analysis

In the in vitro methods, the experimental design was the randomized in factorial scheme (10×7), with ten treatments (Control; *M. piperita, M. spicata, C. martinii, C. camphora* and the binary mixtures), and seven concentrations, for the contact method, and in a 10×6 factorial scheme, with ten treatments (Control; *M. piperita, M. spicata, C. martinii, C. camphora* and the binary mixtures), and six concentrations, for the method of exposure to volatiles. Both experiments contained five repetitions per treatment and were repeated three times. 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.

RESULTS AND DISCUSSION

Table 1 shows the percentage of mycelial growth inhibition of *R. stolonifer* and the minimum inhibitory concentration by the direct contact method. All EOs and their binary mixtures inhibited *R. stolonifer*'s mycelial growth in a dose-dependent behavior. The results indicated significant differences in the mycelial growth inhibition of *R. stolonifer* according to the increase of the concentrations of oils used.

The highest antifungal activity was provided by the EOs of *M. piperita*, *C. martinii* and *M. spicata*, and the mixtures M2, M3, M4, M5 and M6 with total mycelial growth inhibition between concentrations of 500 and 750 µL L^{-1}. *C. camphora* EO and mixture M1 presented lower antifungal activity when compared to other treatments, with total mycelial growth inhibition between 750 and 1000 µL L^{-1}.

Although the EOs of *M. piperita* and *C. martinii* presented the highest antifungal potentials when assessed individually by direct contact, their combination did not result in a better antifungal development. The mixture M1 (*M. piperita* and *C. martinii*) showed the highest MIC range among all the mixtures evaluated (Table 1). Similar behavior was observed by Ji et al. (2019) for the *Allium sativum* EO, which showed the lowest MIC against *Penicillium corylophilum*, but when combined with the EOs of *Cinnamomum zeylanicum*, *Cymbopogon nardus*, *M. spicata*, and *Thymus zygis* did not exhibit synergistic antifungal activity. Indeed, interactions between EO components may sometimes lead to antagonistic effects (Bassolé and Juliani, 2012; Tserennadmid et al., 2010). The causes of antagonism are basically unknown, but some hypotheses have been presented, such as the antimicrobials having the same site of action (competing to each other) or interacting with each other in an undesirable way (Hyldgaard et al., 2012).

Another important property of EOs is that they also show antimicrobial activity in the vapor phase, which makes them suitable as potential fumigants for the preservation of stored fresh products that are sensitive to immersion treatments (Tzortzakis, 2009). Table 2 shows the percentage of mycelial growth inhibition of *R. stolonifer* and the minimal inhibitory concentration of EOs in vapor phase.

Table 1. Percentage of mycelial growth inhibition (PI) of *Rhizopus stolonifer* after exposure by contact at different concentrations (µL L^{-1}) of essential oils incorporated to PDA medium (mean values ± SD, *n*=4).

Concentrations (µL L^{-1})	EOs PI (%)									
	M. piperita	*C. martinii*	*C. camphora*	*M. spicata*	M1	M2	M3	M4	M5	M6
0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
31	4.6±0.0^{b}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
62.5	5.6±5.4^{b}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
125	19.2±3.2^{c}	6.1±1.6^{b}	0.0±0.0^{a}	18.6±3.1^{b}	9.1±1.2^{b}	12.2±1.7^{b}	18.9±1.2^{b}	5.5±1.9^{b}	0.0±0.0^{a}	0.0±0.0^{a}
250	30.0±4.2^{d}	31.5±6.4^{c}	16.1±0.8^{b}	37.6±4.1^{c}	21.9±4.2^{c}	29.9±3.2^{c}	73.3±8.4^{c}	29.3±2.2^{c}	32.6±1.9^{b}	53.3±2.8^{b}
500	68.0±1.2^{e}	83.4±3.6^{d}	48.4±11.0^{c}	89.0±1.4^{d}	63.8±7.3^{d}	73.6±10.6^{d}	93.4±4.5^{d}	94.1±6.8^{d}	84.3±0.9^{c}	82.3±3.4^{c}
750	100.0±0.0^{f}	100.0±0.0^{e}	87.4±3.6^{d}	100.0±0.0^{e}	92.0±0.6^{e}	100.0±0.0^{e}	100.0±0.0	100.0±0.0^{e}	100.0±0.0^{d}	100.0±0.0^{d}
1000	100.0±0.0^{g}	100.0±0.0^{e}	100.0±0.0^{e}	100.0±0.0^{e}	100.0±0.0^{f}	100.0±0.0^{e}	100.0±0.0	100.0±0.0^{e}	100.0±0.0^{d}	100.0±0.0^{d}
MIC	500<MIC ≤ 750	500<MIC ≤ 750	750<MIC ≤ 1000	500<MIC ≤ 750	750<MIC ≤ 1000	500<MIC ≤ 750	500<MIC ≤ 750	500<MIC ≤ 750	500<MIC ≤ 750	500<MIC ≤ 750

PI=percentage of mycelial growth inhibition in relation to the control treatment. MIC=Interval between concentrations in which values of 100% of mycelial growth inhibition can be found. M1=binary mixture of from *M. piperita* and *C. martinii* EOs, M2=binary mixture of *M. piperita* and *C. camphora* EOs, M3=binary mixture of *M. piperita* and *M. spicata* EOs, M4=binary mixture of *C. martini* and *C. camphora* Eos, M5=binary mixture of *C. martinii* and *M. spicata* EOs, M6=binary mixture of *C. camphora* and *M. spicata* EOs. SD=Standard deviation, n=number of repetitions used in the experiment.

Values in the same column not sharing a common letter are significantly different ($p<0.05$).

Table 2. Percentage of mycelial growth inhibition (PI) of *Rhizopus stolonifer* after exposure by exposure to the volatiles at different volumes (µL) of essential oils (mean values ± SD, *n*=4).

Volume (µL)	*M. piperita*	*C. martinii*	*C. camphora*	*M. spicata*	M1	M2	M3	M4	M5	M6
0	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
2,5	0.0±0.0^{a}	36.01±3.0^{b}	0.0±0.0^{a}	23.3±1.9^{b}	34.1±6.8 ^{b}	34.1±6.8 ^{b}	37.1±9.6^{b}	25.4±1.8^{b}	35.1±7.1^{b}	0.0±0.0^{a}
5	54.8±0.9^{b}	100.0±0.0^{c}	0.0±0.0^{a}	93.3±4.8^{c}	76.9±8.4^{c}	76.9±8.4^{c}	78.7±9.4^{c}	64.0±1.1^{c}	61.9±1.7^{c}	28.7±2.2^{b}
10	100.0±0.0^{c}	100.0±0.0^{c}	43.6±4.8^{b}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{c}
20	100.0±0.0^{c}	100.0±0.0^{c}	100.0±0.0^{c}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{c}
30	100.0±0.0^{c}	100.0±0.0^{c}	100.0±0.0^{c}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{d}	100.0±0.0^{c}
MIC	5<MIC ≤ 10	2,5<MIC ≤ 5	10<MIC ≤20	5<MIC ≤ 10	5<MIC ≤ 10	5<MIC ≤ 10	5<MIC ≤ 10	5<MIC ≤ 10	5<MIC ≤ 10	5<MIC ≤ 10

PI=percentage of mycelial growth inhibition in relation to the control treatment. MIC=Interval between concentrations in which values of 100% of mycelial growth inhibition can be found. M1=binary mixture of the EO from *M. piperita* and *C. martinii*, M2=binary mixture of the EO from *M. piperita* and *C. camphora*, M3=binary mixture of the EO from *M. piperita* and *M. spicata*, M4=binary mixture of the EO from *C. martini* and *C. camphora,* M5=binary mixture of the EO from *C. martinii* and *M. spicata,* M6=binary mixture of the EO from *C. camphora* and *M. spicata*. SD=Standard deviation, n=number of repetitions used in the experiment.

Distinct letters represent a significant difference between concentrations of treatments by the Tukey test ($p<0.05$).

All EOs and their binary mixtures were able to inhibit the mycelial growth of *R. stolonifer* in a dose-dependent behavior, as observed in the direct contact method. The highest antifungal activity was observed for *C. martinii* EO, with a total mycelial growth inhibition in the volume of 5 µL. The EO of *C. camphora* presented the lowest antifungal activity in the vapor phase when compared to the other treatments, with total capacity of mycelial growth inhibition in the volume of 20 µL, corroborating with what was observed from direct contact. Similar results were reported by da Rocha Neto et al. (2019), which demonstrated that the EO of palmarosa (*C. martinii*) was more effective when evaluated in the vapor phase in vitro against *P. expansum* than EOs of *Elettaria cardamomum*, *Eugenia caryophyllus*, *Copaifera officinalis*, *Cupressus sempervirens*, *Eucalyptus globulus*, *Zingiber officinale*, *M. piperita*, *Pogostemon cablin*, *Citrus aurantium*, *Rosmarinus officinalis*, *Salvia sclarea* and *Vetiveria zizanoides*.

In this study, the combination of EOs did not result in an increased antifungal activity and all the mixtures evaluated showed a total mycelial growth inhibition at the volume of 10 µL. Similar behavior was reported by Hossain et al. (2016), in a vapor phase assay using mandarin and eucalyptus EOs, which when combined with thyme and oregano EOs, respectively, did not produce interaction and did not result in an improvement in antifungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium chrysogenum*.

The evaluation of the interaction of EOs against the *R. stolonifer* strain when applied in combination explores the possibility of reducing the required doses of EOs to reduce the total growth of the target microorganism, when applied in combination. However, none of the combinations showed a better antifungal development, that is, the total inhibition of fungus growth in a lower concentration when compared to the individual EOs, in the two methods tested, indicating the absense of synergy.

The evaluation by exposure to volatiles also showed that the antifungal activity of the EOs of *M. spicata* and *M. piperita* was reduced, compared to the activity observed in the contact evaluation, since the MIC by this method was 10 µL, superior than the one observed for *C. martinii* (5 µL). In the contact method, the three EOs presented the same value of MIC (between 500 and 750 µL L^{-1}) (Table 1). Similar behavior was observed by Oliveira et al. (2019) who noticed a higher percentage of mycelial growth inhibition when in direct contact than when exposed to the volatile fraction of *Lippia sidoides* EO against *R. Stolonifer*. In another study, Karimi et al. (2016) observed a higher percentage of mycelial growth inhibition when in direct contact than when exposed to the volatile fraction of *Anethum graveolens* EO against *Colletotrichum nymphaeae*.

In this study, the lower EO efficiency in the vapor phase method can be explained due to a possible lower concentration of the effective compounds in the volatile fraction compared to the present in the direct contact test. Another possible explanation is the faster accumulation of inhibitory compounds in the pathogen structure during incubation time, which could be more efficient in the contact assay, since there is direct contact of EO with the fungal structure (Karimi et al., 2016).

Among all oils and mixtures evaluated, *C. camphora* EO had the lowest antifungal potential, since higher concentrations were required for total mycelial growth inhibition of the pathogen. Although the activity of this oil has been already reported previously (Pragadheesh et al., 2013), it has not yet been evaluated in *R. stolonifer*. Thus, the results showed the importance of evaluating the antifungal capacity for this species and highlighted the greater potential of the EOs of *M. piperita*, *C. martinii* and *M. spicata* for use in the control of this pathogen.

The findings of this study are consistent with those reported by Znini et al. (2013), who observed that the EO of *Pulicaria mauritanica* presented a fungicidal effect against *Alternaria alternata* and *Penicillium expansum in vitro*, depending on the method, the synergism between the EO's compounds and the doses used. Thus, it is suggested that the antifungal activity of the EOs evaluated in this study depends on several factors, such as the chemical nature of their components, their doses, the target fungus, and the methods used to evaluate the antifungal activity.

CONCLUSIONS

The in vitro antifungal effects of four essential oils isolated and in combination against *R. stolonifer* were studied. Based on MIC values, *M. piperita*, *C. martinii* and *M. spicata* exhibited the highest antifungal activity by the direct contact method and *C. martinii* by the vapor phase assay. The combinations of the essential oils did not show significant synergy in inhibiting the growth of *R. stolonifer*. Therefore, these oils may be a potentially efficient and safe alternative to synthetic fungicides for control of *R. stolonifer* in the postharvest of fruits.

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