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Control of *Rhizopus stolonifer* in strawberries by the combination of essential oil with carboxymethylcellulose

J. Oliveira[a,*], M.C.M. Parisi[b], J.S. Baggio[c], P.P.M. Silva[a], B. Paviani[a], M.H.F. Spoto[a], E.M. Gloria[a]

[a] Department of Agroindustry, Food and Nutrition, "Luiz de Queiroz" College of Agriculture, University of São Paulo, Pádua Dias Av., 11, 13418-900 Piracicaba, São Paulo, Brazil

[b] São Paulo's Agency for Agribusiness Technology, SP 127 Highway, km 30, 13400-970 Piracicaba, São Paulo, Brazil

[c] Department of Plant Pathology and Nematology, "Luiz de Queiroz" College of Agriculture, University of São Paulo, Pádua Dias Av., 11, 13418-900 Piracicaba, São Paulo, Brazil

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ABSTRACT

Strawberry has a limiting postharvest shelf life, especially because of soft rot. The antifungal activity of the essential oils (EOs) of *Eucalyptus staigeriana*, *Lippia sidoides* and *Pimenta pseudocaryophyllus* was tested *in vitro* against plant pathogen *Rhizopus stolonifer*. The chemical composition of the EO with the highest activity and its effects on pathogen morphology were verified. The *in vivo* antifungal activity of this EO associated with carboxymethylcellulose (CMC) coating, in preventive and curative applications, was also evaluated. *L. sidoides* EO presented the highest *in vitro* antifungal activity. The analysis of the chemical composition of this EO showed a prevalence of the compound thymol and the scanning and transmission electron microscopy showed that L. *sidoides* EO was able to cause damage to the cell wall and the intracellular components of the pathogen. Strawberries treated with L. *sidoides* EO associated with CMC presented a reduction in disease severity, especially when treated in a curative way.

1. Introduction

Strawberry has a short shelf life, especially due to microbial deterioration, which results in alterations that reduce its quality and its commercial value at postharvest (Basu et al., 2014). Management of this crop is a challenge, since several fungal diseases occur, from ground to postharvest (Zamani-Zadeh et al., 2014). Soft rot, caused by species of *Rhizopus* and *Mucor*, is one of the main postharvest diseases in strawberries. The disease is usually controlled with synthetic chemical products, which are commonly employed in inadequate and excessive ways. Pesticide monitoring in *in natura* foods has shown that, of nine crops studied, strawberry was the one which presented the highest percentages of samples with unsatisfactory results, with the presence of active pesticide ingredients above the Maximum Residue Limit (MRL) allowed, or residues of active ingredients that are not authorized for this culture (Sanitária, 2009). Besides this issue, fungicides can promote the selection of resistant mutants, risking disease management (Mari et al., 2014). Efficient and low cost products are necessary for the control of pests in plants (Aguilar-González et al., 2015).

Essential oils (EOs), are classified as "generally recognized as safe" (GRAS) food additives and, are an alternative for fungal disease control, because of their high antimicrobial potential (Basak and Guha, 2017; Burt, 2004; Rehman et al., 2016). Given the multicomponent nature of EOs, the development of pathogen resistance to these products would be more difficult to occur (Alikhani and Daraei Garmakhany, 2012). Studies have evaluated the antifungal efficacy of different EOs on *R. stolonifer* (Castaño et al., 2017; Shao et al., 2013). Nonetheless, studies with the EOs extracted from *Eucalyptus staigeriana*, *Lippia sidoides* and *Pimenta pseudocaryophyllus* on fruit-deteriorating fungi are scarce, despite the proven antimicrobial action of these EOs (de Menezes Cruz et al., 2012; Yokomizo and Nakaoka-Sakita, 2014). Moreover, a synergism between the compounds may happen with the combination of EOs in mixtures, causing the mixtures, sometimes, to be more efficient than the EO by itself (Nikkhah et al., 2017).

Nevertheless, the use of EOs in fruits poses a great challenge, since their efficacy can be reduced by interactions of EO components with fruit constituents and loss of the active compounds by fast volatilization or the action of other factors, such as light (Turek and Stintzing, 2013). Thus, an alternative is the incorporation of EOs in formulations of edible coatings, preserving fruit quality, extending their shelf life and

* Corresponding author at: University of São Paulo, "Luiz de Queiroz" College of Agriculture - USP/ESALQ, Pádua Dias Av., 11, Piracicaba 13418-900, SP, Brazil.
E-mail addresses: jacqueline.oliveira@usp.br (J. Oliveira), pporrelli@usp.br (P.P.M. Silva), bruna.paviani@usp.br (B. Paviani), martaspoto@usp.br (M.H.F. Spoto), emgloria@usp.br (E.M. Gloria).

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reducing microbial growth (Guerreiro et al., 2015; Peretto et al., 2014).

Carboxymethylcellulose (CMC), is a promising polysaccharide polymer which consists of β-D-glucose and β-D-gluco-pyranosyl-2-O-(carboxymethyl)-monosodium salt connected *via* β-(1,4-glycosidic) bonds and has been used as edible coating. It is not toxic, presents good solubility, low viscosity, and provides uniform coating, being of stable matrix and high structural integrity during storage (Arnon et al., 2014; Gregorová et al., 2015). CMC can be originated from abundant and low-cost sources, such as sugarcane bagasse, one of the major by-products of sugar cane industry. The addition of EOs to the coatings for application in foods can minimize the intense aroma of EOs, besides enabling the release of effective agents for a larger period (Vieira et al., 2016). Previous studies have demonstrated that CMC has the potential to extend storage period and to control deterioration of fruits by microrganisms when associated with EOs, as in papaya (Zillo et al., 2018), avocados (Tesfay and Magwaza, 2017) and strawberries (Badawy et al., 2017).

Therefore, the main objective was to verify the possibility to use the combination of an edible coating based on CMC with an EO as an alternative to synthetic fungicides to minimize postharvest loss of *R. stolonifer* on strawberries.

2. Material and methods

2.1. Plant material, extraction of the essential oils

The EOs were extracted from leaves of *E. staigeriana* (Itatinga - SP, Brazil), *L. sidoides* (Campinas - SP, Brazil) and *P. pseudocaryophyllus* (Cananéia - SP, Brazil), by hydrodistillation, for 4 h, in a Clevenger equipment at up to 100 °C until ebullition point. Subsequently, the EO was dehydrated in anhydrous sodium sulfate and stored at −5 °C. The EOs presented a translucent appearance with yellowish coloration.

2.2. Isolation and molecular identification of Rhizopus stolonifer

The pathogen *R. stolonifer* was obtained by direct isolation of fungal structures present in strawberries harvested in a commercial conventional farm, located in Jarinu (SP, Brazil), with typical symptoms of soft rot. Genomic DNA was extracted following the CTAB method described by Doyle and Doyle (1987). The region of the 28S ribosomal gene was amplified by PCR using the primers LR0R (5′ – ACCCGCTGAACTTAAGC – 3′) and LR5 (5′ – TCCTGAGGGAAACTTCG – 3′) (Vilgalys and Hester, 1990). The purified product was sequenced and compared with DNA sequences of *R. stolonifer* deposited at GenBank: EU6222 *Rhizopus stolonifer* isolate NW643, AF117935 *Rhizopus stolonifer* ATCC 14037 and AF117936 *Rhizopus stolonifer* ATCC 6227A.

2.3. Determination of the in vitro antifungal activity of EOs

The *in vitro* antifungal activity of EOs, individually or in combination, on *R. stolonifer* was verified first by direct contact. The highest antifungal treatment was also verified for its antifungal activity by the exposure to volatiles methodology.

2.3.1. Method by contact

The antifungal activity of EOs was initially 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 or with its binary (50% each) and ternary mixtures (33,33% each), at concentrations 31; 62.5; 125; 250 and 500 μL/ L (Plaza et al., 2004). The mixtures M1 (*L. sidoides* + *E. staigeriana*); M2 (*P. pseudocaryophyllus* + *E. staigeriana*); M3 (*L. sidoides* + *P. pseudocaryophyllus*) and M4 (*L. sidoides* + *P. pseudocaryophyllus* + *E. staigeriana*) were tested to evaluate if the combination of EOs could present a higher activity on the control of the pathogen than when evaluated individually. For the homogenization of the EOs and the mixtures to the PDA medium, the emulsifier soy lecithin (0.2% w/v in ethanol) 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 an inoculum suspension containing 10^5 spores/mL. Plates were maintained in 12 h photoperiod at 25 ° (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 formula $PI\ (\%) = (Control\ \ Growth - Treatment\ \ Growth / Control\ \ Growth) \times 100$ (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.

2.3.2. Method by exposure to the volatiles

The treatment that presented the highest *in vitro* antifungal activity by the contact method was also evaluated by the method of exposure to volatiles, according to Yun et al. (2013). Fungal growth inhibition was observed in EO concentrations and their mixtures (0; 31; 62.5; 125; 250 and 500 μL/L), which were emulsified in tween-80 (at the proportion 2:1 v/v) and 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 (item 2.3.1).

In the *in vitro* methods, the experimental design was the randomized in factorial scheme (8 × 5), with eight treatments (Control; *L. sidoides*; *E. staigeriana*; *P. pseudocaryophyllus* and the binary and ternary mixtures), and five concentrations, for the contact method, and in a 2 × 5 factorial scheme, with two treatments (Control; *L. sidoides*), and five concentrations, for the method of exposure to volatiles. Both experiments contained five repetitions per treatment and were repeated three times.

2.3.3. Effects of the essential oil on Rhizopus stolonifer morphology

The effect of the essential oil selected as presenting the highest antifungal activity on the pathogen morphology was evaluated by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The samples were prepared according to Yu et al. (2015), with modifications. For this, a suspension composed of 150 mL of potato-dextrose (PD) broth with the addition of 1 mL of spore suspension (10^6 spores/mL), obtained from colonies grown for 5 d in PDA, was incubated for 2 d at 25 °C, and 12 h photoperiod (Baggio et al., 2016). After this period, the concentration corresponding to the MIC determined in the *in vitro* experiment by the method of dilution in agar was firstly emulsified with soy lecithin (0.2% w/v in ethanol) and added to the broth. Subsequently, the sample was incubated for further 6 h at the same conditions already described. The broth without the addition of the treatment was used as control. The samples were processed according to Escanferla et al. (2009) for SEM analyses and the observations were performed in a Scanning Electron Microscope LEO 435 (Zeiss, England). On the other hand, for the TEM analyses the samples were processed according to Ramos-González et al. (2017) and the observations were performed in a transmission electron microscope JEOL JEM 1011 (JEOL, Akishima, Japan). These analyses were repeated twice, with three repetitions.

2.4. Evaluation of the essential oil chemical composition

The chemical composition was determined only for the EO that better controlled pathogen growth in the *in vitro* test. The characterization was performed by gas chromatography coupled to mass spectrometry, using the equipment GCMS 2010 (SHIMADZU) and capillary-column gas chromatography diphenyl dimethylpolysiloxane (5%

diphenyl and 95% dimethylpolysiloxane). Reactions were performed at 50 °C for 1.5 min and then elevated at 4 °C/min until 200 °C, followed by 10 °C/min until 240 °C, remaining at 240 °C for 7 min. The injector temperatures were set at 240 and 220 °C for the ions and interface sources, respectively. Injection was done on "Split" mode: 1 μL of EO was injected and the "Split" ratio was 1:20. Helium gas was used as the drag gas at 1.2 mL/min. The mass detector was run on scan mode with scanning range of 40 to 500 *m/z*. The volatile compounds were identified by the comparison between their Linear Retention Indexes (LRI) and the calculated and observed mass spectra, with data published in the literature (Adams, 2017), and with the existing mass spectrum libraries (NIST, WebBook, NIST 07 and WILEY 8). Only the peaks with presence higher than 0.5% of the total chromatogram area were considered to be identified.

2.5. Determination of the in vivo antifungal activity

The treatment with the highest *in vitro* antifungal activity was evaluated *in vivo* in a preventive and curative way, regarding fungal infection, and associated or not to CMC coating, resulting in the treatments: "C" – fruit without CMC and EO application; "COP" – fruit treated preventively with CMC + EO; "CP" – fruit treated preventively with only CMC; "COC" – fruit treated curatively with CMC + EO; "CC" – fruit treated curatively with only CMC.

For emulsion preparation, CMC was used, with 99.8% purity, 7.6% moisture, pH of 7.0, viscosity of 340 cP, determined in a 1% solution at 25 °C, and degree of substitution of 0.86. The emulsion was prepared at 1% (p/v) in distilled water at 60 °C under continuous mechanical stirring at 2.000 rpm for 20 min, in a 3-bladed propeller stirrer. Subsequently, 0.5 mL (50% w/w dry weight of CMC) of glycerol was added as plasticizer and stirring followed for further 15 min. In the treatments involving EO, as the emulsion reached 25 °C, the EO previously emulsified with Tween-80 was added (proportion 2:1 v/v). In this test, the EO concentration used was ten times superior to the MIC obtained *in vitro*. According to Hyldgaard et al. (2012), to maintain the efficacy observed in the *in vitro* test, it is necessary to extrapolate the concentration *in vivo*.

'Oso Grande' strawberries from an organic farm in Cambuí (MG, Brazil), were visually selected regarding appearance and health, and were subsequently sanitized in 2.5% sodium hypochlorite. For the evaluation in the preventive mode of action, the strawberries were immersed for 2 min in the emulsion of CMC associated to the EO and after natural drying, the fungus was inoculated with 30 μL of a spore suspension (10^5 spores/mL) placed on a 3 mm deep wound. The fruits were maintained in a growth chamber with 95% relative humidity, at 25 °C and photoperiod of 12 h for 24 h, ideal conditions for pathogen development. To evaluate the curative mode of action, the inoculation of the fungus was performed 24 h before treatment application. The further procedures were the same described for the preventive mode of action. The control treatment (C) was composed of only the fruit immersed in sterilized distilled water. Thus, the experimental design in this step was in a 5 × 7 factorial scheme, involving five treatments ("C"; "COP"; "CP"; "COC" and "CC") and 7 d of evaluation. Six repetitions of each treatment were used, each of them composed of 12 strawberries. This experiment was conducted twice.

The antifungal activity of the treatments was evaluated by the incidence and severity of the disease in the fruit. Disease incidence was evaluated after seven days of storage, and was calculated from the number of symptomatic fruit in relation to the total fruit number in each treatment, with the results expressed in percentage (Ali et al., 2015). Severity was evaluated daily by a scale of scores composed of six degrees (0 = absence of symptoms; 1 = 1 to 20% of injured area; 2 = 21 to 40%; 3 = 41 to 60%; 4 = 61 to 80% and 5 = > 81% of the area with injury), with the results expressed in Disease Index (DI), according to Cia et al. (2010): $DI(\%) = [(1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4) + (5 \times n5)] \times 100 / 5 \times N$, in which ni is the number of infected fruit in the corresponding scale of scores and N is the total number of fruit. Based on the DI values with time, in each repetition, the area under the disease progress curve (AUDPC) was calculated for severity according to Campbell and Madden (1990): $AUDPC = \Sigma [(y_i + y_{i+1}) / 2 \times (t_{i+1} - t_i)]$, with y_i referring to the DI at time t_i; y_{i+1}; DI at time, t_i the initial time of reading and t_{i+1} the time in days of each evaluation.

2.6. Statistical analysis

In the determination of the *in vitro* antifungal activity of EOs by contact and by exposure to the volatiles, the data referring to MIC and PI were evaluated with the program Statistical Analysis System model 9.3 (Institute, 2010) and subjected to the analysis of variance (ANOVA) for the F test. 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.

The data referring to AUDPC determined in the *in vivo* antifungal activity of OEs were evaluated with the program Statistical Analysis System model 9.3 (Institute, 2010) and subjected to the analysis of variance (ANOVA) for the F test in randomized blocks, each block corresponded to each of the two repetitions of the experiment *in vivo*. 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.

3. Results and discussion

3.1. In vitro antifungal activity of EOs

All individual EOs and their mixtures presented some ability of inhibiting *R. stolonifer* mycelial growth, in a dose-dependent behavior. *E. staigeriana* EO presented the lowest potential for antifungal activity, when compared to the other treatments, with a maximum inhibition of 28,48% in the highest concentration (500 μL/L). Conversely, *L. sidoides* EO was the treatment that presented the highest antifungal activity, in other words, the lowest MIC, with a total inhibition of *R. stolonifer* mycelial growth occurring between the concentrations 62.5 and 125 μL/L. Besides presenting the highest antifungal potential when individually evaluated by direct contact, *L. sidoides* EO also influenced a better antifungal development of the mixtures when it was present. Mixtures M1, M3 and M4, which presented this oil in their composition, had the lowest MIC intervals among all mixtures evaluated (Table 1).

The antifungal activity of L. *sidoides* EO, which presented the lowest inhibitory concentration among the oils evaluated in the tests *in vitro*, has been observed in other studies. According to Aquino et al. (2012), mycelial growth and conidia germination of *C. gloeosporioides*, isolated from passion fruit trees, were affected by the EO of this species. De Menezes Cruz et al. (2012) also verified the antifungal effect in fungi that caused postharvest diseases in mangoes. Other filamentous fungi, such as *Aspergillus niger*, *Penicillium* sp., *Fusarium* sp. and *Fusarium oxysporum* also had their inhibition proven by the use of *Lippia* EO (de Oliveira et al., 2008). The antifungal activity of this oil may be related to the elevated presence of thymol and carvacrol in its composition, since these compounds act on the fungal cell wall, causing distortions and altering its permeability, thus affecting its growth and shape (Burt, 2004; Guimarães et al., 2014). Nonetheless, although there are already reports on antifungal activity for the major compounds detected in the oil studied here, the antifungal activity observed for this oil may be a result of the combination of the major and minor compounds (Alitonou et al., 2012; Sharifi et al., 2008).

E. staigeriana EO presented the lowest antifungal activity in relation to the other oils, since none of the concentrations evaluated led to the total inhibition of the pathogen mycelial growth. *P. pseudocaryophyllus* EO also presented a lower potential on *R. stolonifer* (250 < MIC ≤ 500)

Table 1
Mycelial growth (MG) and percentage of mycelial growth inhibition (PI) of *Rhizopus stolonifer* from strawberry, and minimum inhibitory concentration (MIC) at two days of incubation, after exposure by contact at different concentrations (μL/L) of essential oils incorporated to PDA medium (mean values ± SD, n = 5).

Treatments	Concentrations (μL/L)	MG (cm)	PI[1] (%)	MIC[2]
	0.00	8.70 ± 0.00^{a}	0.00 ± 0.0^{c}	
Eucalyptus staigeriana	31.0	7.95 ± 0.42^{ab}	8.66 ± 4.8^{bc}	MIC > 500
	62.5	7.86 ± 0.47^{b}	9.66 ± 5.3^{b}	
	125.0	7.42 ± 0.28^{b}	14.73 ± 3.2^{b}	
	250.0	7.62 ± 0.58^{b}	12.43 ± 6.6^{b}	
	500.0	6.22 ± 0.62^{c}	28.48 ± 7.1^{a}	
	0.00	8.70 ± 0.00^{a}	0.00 ± 0.00^{d}	
Lippia sidoides	31.0	7.22 ± 0.90^{b}	16.99 ± 10.3^{c}	62.5 < MIC ≤ 125
	62.5	5.46 ± 0.45^{c}	37.21 ± 5.2^{b}	
	125.0	0.00 ± 0.00^{d}	100 ± 0.0^{a}	
	250.0	0.00 ± 0.00^{d}	100 ± 0.0^{a}	
	500.0	0.00 ± 0.00^{d}	100 ± 0.0^{a}	
	0.00	8.70 ± 0.00^{a}	0.00 ± 0.0^{e}	
P. pesoudocaryo-phyllus	31.0	8.07 ± 0.46^{ab}	7.30 ± 5.3^{de}	250 < MIC ≤ 500
	62.5	7.86 ± 0.47^{ab}	17.93 ± 11.9^{cd}	
	125.0	7.42 ± 0.28^{c}	21.41 ± 6.4^{c}	
	250.0	4.20 ± 0.24^{d}	51.76 ± 2.7^{b}	
	500.0	0.00 ± 0.00^{e}	100 ± 0.0^{a}	
	0.00	8.70 ± 0.00^{a}	0.00 ± 0.0^{c}	
M1	31.0	7.11 ± 0.79^{ab}	18.26 ± 11.6^{bc}	125 < MIC ≤ 250
	62.5	6.94 ± 0.92^{ab}	20.29 ± 10.6^{bc}	
	125.0	5.43 ± 0.48^{b}	37.54 ± 5.5^{b}	
	250.0	0.97 ± 1.76^{c}	88.84 ± 20.2^{a}	
	500.0	0.00 ± 0.00^{c}	100 ± 0.0^{a}	
	0.00	8.70 ± 0.00^{a}	0.00 ± 0.0^{d}	
M2	31.0	8.31 ± 0.13^{a}	4.44 ± 1.2^{d}	MIC > 500
	62.5	7.86 ± 0.47^{b}	18.38 ± 14.0^{c}	
	125.0	7.42 ± 0.28^{c}	23.52 ± 2.9^{bc}	
	250.0	5.99 ± 0.38^{c}	31.11 ± 4.3^{b}	
	500.0	1.34 ± 0.67^{d}	84.56 ± 7.6^{a}	
	0.00	8.70 ± 0.00^{a}	0.00 ± 0.0^{d}	
M3	31.0	7.09 ± 0.56^{b}	17.84 ± 4.6^{c}	125 < MIC ≤ 250
	62.5	6.48 ± 0.47^{b}	25.27 ± 2.8^{c}	
	125.0	3.61 ± 0.28^{c}	58.45 ± 10.6^{b}	
	250.0	0.00 ± 0.00^{d}	100 ± 0.0^{a}	
	500.0	0.00 ± 0.00^{d}	100 ± 0.0^{a}	
	0.00	8.70 ± 0.00^{a}	0.00 ± 0.0^{c}	
M4	31.0	8.42 ± 0.18^{a}	3.18 ± 2.0^{c}	125 < MIC ≤ 250
	62.5	7.86 ± 0.47^{ab}	16.85 ± 8.0^{bc}	
	125.0	7.42 ± 0.28^{b}	32.67 ± 12.0^{b}	
	250.0	1.33 ± 1.90^{c}	84.68 ± 21.8^{a}	
	500.0	0.00 ± 0.00^{c}	100 ± 0.0^{a}	

M1 = mixture of *L. sidoides* and *E. staigeriana* EO, M2 = mixture of *E. staigeriana* and *P. pseudocaryophyllus* EO, M3 = mixture of *L. sidoides* and *P. pseudocaryophyllus* EO, M4 = mixture of L. *sidoides*, *E. staigeriana* and *P. pseudocaryophyllus* EO, 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 ($p < 0.05$).

[1] PI = percentage of mycelial growth inhibition in relation to the control treatment.

[2] MIC = Interval between concentrations in which values of 100% of mycelial growth inhibition can be registered.

in comparison with L. *sidoides* EO (62.5 < MIC ≤ 125). The antifungal activity of these oils, although already reported for other filamentous fungi (Ribeiro et al., 2013; Tyagi and Malik, 2011), still had not been evaluated for the fungal species under study. Therefore, the results observed in this work show the importance of evaluating the antifungal activity of L. *sidoides* EO on the species *R. stolonifer* and highlight the higher potential of this EO among those studied as a means to control this pathogen.

Another important property of EOs is their antimicrobial activity also in the vapor phase, a characteristic that makes them appropriate as potential fumigants for the conservation of stored fresh products (Tzortzakis, 2009). Thus, as L. *sidoides* EO presented the lowest MIC interval among all treatments evaluated by contact, this treatment was selected to optimize the following steps. Thus, a new MIC was determined by the methodology of exposure to volatiles (Table 2).

The evaluation by exposure to volatiles demonstrated that L. *sidoides* EO antifungal activity was reduced, in comparison to its activity observed in the evaluation by contact, since MIC by this method was higher than 500 μL/L, with that observed in the contact method staying

Table 2
Mycelial growth (MG) and percentage of mycelial growth inhibition (PI) of *Rhizopus stolonifer* from strawberry, and minimum inhibitory concentration (MIC) at two days of incubation, after exposure to volatiles at different concentrations (μL/L) of *Lippia sidoides* essential oil (mean values ± SD, n = 5).

Treatments (μL/L)	MG (cm)	PI[1] (%)
0.0	8.54 ± 0.00^{a}	0.00 ± 0.00^{e}
31.0	7.45 ± 0.70^{ab}	12.76 ± 8.14^{de}
62.5	6.72 ± 0.59^{b}	21.31 ± 6.91^{d}
125.0	5.18 ± 0.58^{c}	39.34 ± 6.74^{c}
250.0	3.07 ± 0.37^{d}	64.05 ± 4.39^{b}
500.0	1.74 ± 0.87^{e}	79.62 ± 10.24^{a}
MIC[2]	MIC > 500	

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

[1] PI = percentage of mycelial growth inhibition in relation to the control treatment.

[2] MIC = Interval between concentrations in which values of 100% of mycelial growth inhibition can be registered.

between 62.5 and 125 μL/L.

In the vapor phase, *L. sidoides* EO presented a lower efficacy against *R. stolonifer*, with an inhibition rate of 39.34% at 125 μL/L against 100%, when in direct contact with this same EO concentration. The same was observed by Karimi et al. (2016) who 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*. Many studies evidence the efficacy of EO vapor phase against postharvest fruit pathogens. The exposure to the volatiles of the EO of *Melaleuca alternifolia* L. significantly reduced spore germination and mycelial growth of *R. stolonifer* isolated from strawberries (Shao et al., 2013). The vapor phase of *Lippia scaberrima* EO controlled *Colletotrichum gloesporioides* and *Botryosphaeria parma*, pathogens of postharvest deterioration in mango (Regnier et al., 2008), and oregano volatiles inhibited *Botrytis cinerea* growth in tomato (Soylu et al., 2010). However, in this study, the lower efficiency of the volatiles can be explained due to a possible lower concentration of the effective compounds in the volatile fraction in comparison with that present in the direct contact assay. Another possible explanation is the faster accumulation of inhibiting compounds in the pathogen structure with incubation time, which could be more efficient in the assay by contact, since there is a direct contact of the EO with the fungal structure (Karimi et al., 2016).

3.2. Effects of the essential oil on R. stolonifer morphology

The structural alterations resulting from the exposure to L. *sidoides* EO can be observed by SEM in *R. stolonifer* mycelia, presenting superficial wrinkles, distortions and destruction of the hyphae (Fig. 1C and D). In the absence of EO, *R. stolonifer* mycelia exhibited homogeneous and regular tubular hyphae, as well as a smooth and long external surface (Fig. 1A and B). By TEM, the sections of *R. stolonifer* control revealed a typical fungal ultrastructure, that is, normal cell wall thickness, regular and intact plasma membrane, mitochondrion in a regular form and uniform cell cytoplasm in the mycelium (Fig. 2A). When the pathogen was subjected to the EO, the general cell ultrastructure was modified and lost its regularity when compared to the control. It was possible to observe that the plasma membrane separated from the cell wall and the intracellular components were seriously damaged, presenting indistinct intracellular organelles and cytoplasm loss.

These alterations suggest that the EO antifungal activity may include an attack to the hypha plasma membrane, resulting in mycelial death, since it has a vital role in the maintenance of a homeostatic environment for the cell, exchanging materials and transferring energy and information (Shao et al., 2013). *L. sidoides* EO is rich in thymol, that can alter fungal cell wall and plasma membrane structure and facilitate ion exchange, increasing its permeability and hampering cell survival (Moreira et al., 2010; Rao et al., 2010). The application of chitosan associated to *Origanum vulgare* essential oil caused morphological alterations in *R. stolonifer* hyphae, such as wrinkling and loss of the cytoplasmic material (dos Santos et al., 2012). The same alterations were observed in *Aspergillus niger* hyphae when subjected to *Matricaria chamomilla* EO (Tolouee et al., 2010).

3.3. Evaluation of the essential oil chemical composition

The chemical composition of the L. *sidoides* essential oil was determined, since it presented the best antifungal activity among the EOs evaluated. In this oil, 20 compounds had an area of chromatographic peaks higher than or equal to 0.38% of the total area of the peaks present in the chromatogram. The major compound present was thymol (49,46%), with cymene and Iso-caryophyllene being the second and third most abundant compounds, with 11 and 8% of area, respectively (Table 3).

The identification of the chemical components of L. *sidoides* EO revealed thymol as the major compound (49.46%), followed by cymene (11.40%). Many studies have been demonstrating thymol as the major component of the EO of L. *sidoides* cultivated in several places of Northeast Brazil, with concentrations varying between 30.24 and 84.09% (Aquino et al., 2012; Marco et al., 2012). Carvacrol, which is an isomer of thymol, has also been frequently observed as a major

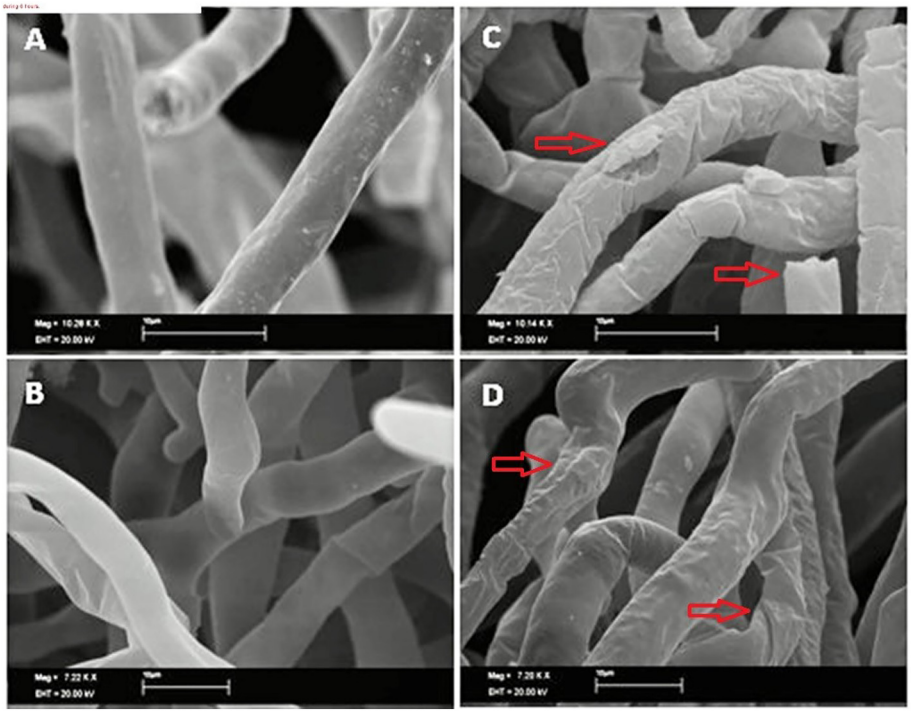

Fig. 1. Scanning electron microscopy (SEM) of the hyphae of *Rhizopus stolonifer* from strawberry, cultivated in potato broth for two days and subjected (C and D) or not (A and B) to *Lippia sidoides*. EO at 125 μL/L after 6 h. Arrows show the main points of destruction caused in the pathogen hyphae by the EO.

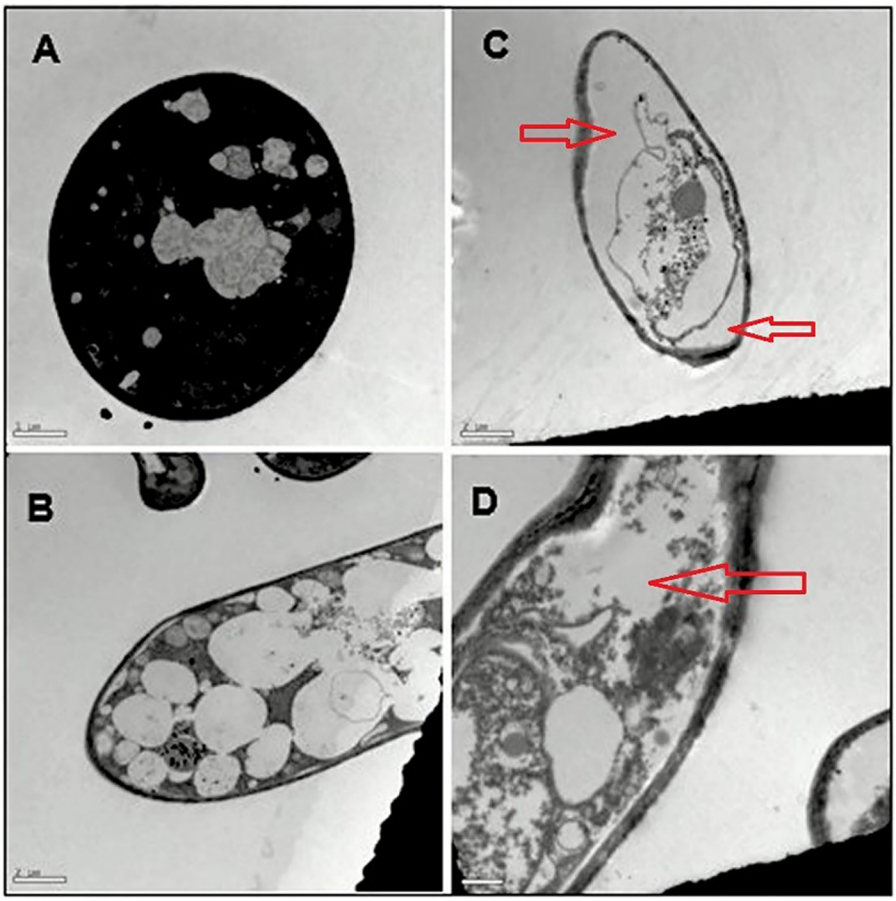

Fig. 2. Transmission electron microscopy (TEM) of the hyphae of *Rhizopus stolonifer* from strawberry, cultivated in potato broth for two days and subjected (C and D) or not (A and B) to *Lippia sidoides* EO at 125 μL/L after 6 h. Arrows show the main points of destruction caused in the pathogen hyphae by the EO.

Table 3
Chemical composition of the EO extracted from *Lippia sidoides* leaves.

Compounds[1]	*L. sidoides*[2] (%)[2]	LRI[3]
α-Felandrene	1.51	930
α-Pinene	0.54	937
Myrcene	2.37	995
α- Ferpinene	2.50	1021
Cymene	11.40	1029
Limonene	1.08	1033
Cineol	2.31	1036
γ –Terpinene	8.06	1063
Cis-Hydrate Sabinene	0.38	1072
2-Methyl-6-methylene-2,7-octadien-4-ol	1.07	1151
4-Terpineol	1.31	1183
α-Terpenol	0.38	1198
Anisole	4.08	1240
Thymol	49.46	1299
Copaene	1.00	1384
Iso-caryophyllene	8.80	1430
Aromadendrene	0.69	1449
Humulene	0.55	1464
Lepidozene	1.71	1507
δ –Cadinene	0.68	1533
Total	99.86	

[1] Identified by GC/MS.
[2] Relative amounts of the compounds identified based on the area of each peak in the total chromatogram area.
[3] Linear Retention Indices calculated.

compound in the composition of the EO of this species (Guimarães et al., 2014); however, it was not observed in the EO studied in this work. It has been reported in previous studies the existence of different genotypes of L. *sidoides* that can confer different production levels of carvacrol and thymol (dos Santos et al., 2015). Nevertheless, despite the presence of the major compounds here identified, 18 other compounds were present in this oil in sufficient amounts to produce individually peak areas higher than 0.38% of the total chromatogram area and thus were identified and considered as minor components.

The EO fungicide activity cannot be only attributed to its major component; minor compounds or synergism effect among compounds present in the EO can also provide the antifungal activity (Hyldgaard et al., 2012). The chemical composition of the L. *sidoides* EO indicates other monoterpenos with > 5%, such as cyneme, y-terpinene, and cineol. These compounds have been shown to inhibit ergosterol biosynthesis, affecting the integrity and stability of fungi membrane, which are vital for most phytopathogenic fungi (Wang et al., 2018). According to Tian et al. (2018), monoterpenos can also cause significant losses on pigmentation of *Aspergillus flavus* colonies, suggesting a blockage on the biosynthesis of fungi melanin, contributing to a decrease in pathogenicity and increase on sensibility to several biotic and abiotic stresses (Hamada et al., 2014).

Although chemical composition analyses of the other EOs were not performed in this study, it is known that the major components of the essential oils of the genus Pimenta mainly consisted of phenylpropanoids (eugenol, methyleugenol, transmethyl isoeugenol, timol), monoterpene (1,8-cineol), monoterpenic aldehydes (neral, geranial), and alcohol derivatives (geraniol, linalol, α-terpineol) (Paula et al., 2012). According to Barata et al. (2011), seven compounds were identified, two phenylpropanoids (chavibetol: 70,9% and methyl eugenol: 20,7%)

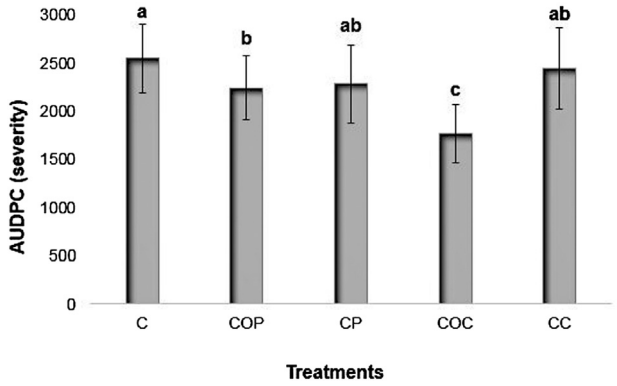

Fig. 3. Area under the disease progress curve (AUDPC) for *Rhizopus* rot severity, caused by *Rhizopus stolonifer*, in "Oso Grande" strawberries.

and five monoterpenes (tricyclene, *o*-cymene, 1,8-cineole, *p*-mentha-2,4(8)-diene and terpinen-4-ol). The *Eucalyptus staigeriana* EO has as main components 1,8-cineol (34,8%), neral (10,8%), geranial (10,8%) e α-felandreno (8,8%) (Gilles et al., 2010).

3.4. Determination of the in vivo antifungal activity

The *in vivo* evaluation of the antifungal activity of the application of the emulsion of *Lippia sidoides* oil with CMC resulted in a lower severity of Rhizopus rot, in comparison to the fruit without the application. This reduction was observed in both preventive and curative applications (COP; COC) (Fig. 3). On the other hand, the application of only CMC (CP; CC), in either the preventive or curative ways, produced numerically lower severity values than those detected in the fruit without the application (C), but this difference was not sufficient to produce statistical difference ($P < 0.05$). Thus, it was possible to observe that the application of only the CMC emulsion in a preventive or curative way did not produce a significant protective effect aiming the control of Rhizopus rot in strawberries. Nevertheless, when this emulsion was combined to L. *sidoides* oil, its application in strawberries provided an efficient control of this disease, especially when the emulsion combined with oil was applied in a curative way. Regarding disease incidence, there was a similarity among the treatments, which reached 100% in the second day. *R. stolonifer* is a very aggressive pathogen with fast growth rates. In the *in vitro* test, the pathogen had completely grown on 10 cm plates after two days of incubation. This could also be observed in the *in vivo* test in fruit treated with CMC and in the non-treated control. This was also observed for peaches inoculated with *R. stolonifer* (Baggio et al., 2017). Moreover, the area under the disease progress curve (AUDPC) analysis takes into consideration the disease progress over time, and differences among the treatments would have been observed. The experiments were performed under conditions of elevated inoculum pressure for the ripe fruit. Therefore, the present assay provided ideal conditions for the development of the disease, especially without the use of low temperatures.

Distinct letters represent a significant difference among the treatments by the Tukey test ($p < 0.05$). Vertical bars indicate the standard error of the mean ($n = 12$). C: fruit without the application of CMC and EO; COP: fruit treated preventively with CMC and EO; CP: fruit treated preventively with only CMC; COC: fruit treated curatively with CMC and EO; CC: fruit treated curatively with only CMC.

The antifungal activity of EO association to other types of edible coatings when applied in fruits has already been proven in other works. In strawberries, a slow disease progress caused by *R. stolonifer* and *Botrytis cinerea* was also verified in the fruits treated with chitosan associated to different EOs (Khalifa et al., 2016; Mohammadi et al., 2015). In other fruits, as papaya and grape, a reduction in disease severity caused by *Colletotrichum gloesporioides*, *R. stolonifer*, and *Aspergillus niger* was also observed, after application of coatings associated with EOs (Ali et al., 2016; Bosquez-Molina et al., 2010). Works associating EO incorporation to CMC coatings aiming at the control of pathogenic fungi in fruits are absent, but the antibacterial potential of this mixture has already been verified by Dashipour et al. (2015). Although, CMC is known to induce the production of cellulases by fungi (Abdel-Fatah et al., 2012; Zhang et al., 2017), and the production of esterases, a type of celullases, has been reported in *R. stolonifer* enabling its direct penetration into unwounded stone fruit (Baggio et al., 2016), the fungus is only able to produce these enzymes when there is spores germination. However, spore germination is usually favored when an external source of nutrients is provided (Baggio et al., 2016). That was the case of our experiments, in which strawberry fruit were wounded, contributing for the release of nutrients essential for spore germination and, as consequence, the production of enzymes.

Although disease severity was lower in the fruit treated with CMC incorporated with EO, disease incidence was high in all treatments. This is justifiable, since the experiment was performed under conditions of elevated inoculum pressure for the ripe fruit. Therefore, the present assay provided ideal conditions for the development of the disease, especially without the use of low temperatures. Furthermore, higher concentrations of essential oil are usually necessary in *in vivo* experiments, since there might be interactions among the compounds of EO and the food matrix (Feng and Zheng, 2007). Food matrix complexity, for being an environment rich in nutrients, can provide an excellent growth medium for fungal development, as well as an adequate medium for the repair and regeneration of the cell components (Espitia et al., 2012). Consequently, it can be expected that *R. stolonifer* exhibits a lower sensibility to L. *sidoides* EO when it grows on the surface of strawberries than in agar. The results here observed can be considered important aiming at optimizing the technique of using the combination of this essential oil with CMC in the control of *R. stolonifer* in strawberries at postharvest. The effects of the EOs volatilization on the nutritional and organoleptic aspects on the fruits and on the consumer health were not addressed in our study. However, it is known that essential oils are classified as GRAS (Generally Regarded As Safe), by United States Food and Drug Administration (FDA), and do not present toxic effects to consumers, even when they are employed in relatively high concentrations (Burt, 2004).

4. Conclusion

The *in vitro* evaluation of the EO(s) allowed the observation that, among all oils and mixtures evaluated, the one from L. *sidoides* presented the highest capacity of *R. stolonifer* inhibition, being more effective when applied in direct contact to the pathogen than when by exposure to the volatiles. Furthermore, this EO causes a morphological degeneration in the pathogen hyphae, suggesting its action on the fungal cell wall and the intracellular components. The incorporation of L. *sidoides* EO to a CMC emulsion was efficient in disease severity reduction in the *in vivo* evaluation, being more effective as a curative control method. Therefore, the association of L. *sidoides* EO to CMC coating can be a potential alternative to the synthetic fungicides for the control of the postharvest disease caused by *R. stolonifer* in strawberries.

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