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Antifungal activity of essential oils associated with edible coatings for the postharvest control of spoilage fungi in strawberry (*Fragaria* spp.)

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Thesis presented to obtain the degree of Doctor in Science. Area: Food Science and Technology

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DEDICATION

To my sons Enzo and Cauã, the reason for all my effort

To my husband Oscar, for the love, care, incentives and for believing

To my parents José Carlos and Maria de Fátima, for the life, dedication and every example

To my siblings, Amanda and Raphael, for the love and friendship

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SUMMARY

RESUMO

Atividade antifúngica de óleos essenciais associados a recobrimentos comestíveis para o controle pós-colheita de fungos deterioradores do morango (*Fragaria* spp.)

A cultura do morangueiro tem como problemática limitante o manejo fitossanitário, o que associado aos resíduos dos fungicidas tóxicos, à resistência dos patógenos e à fragilidade dos frutos durante o transporte e armazenamento, tornam a comercialização deste produto um desafio. A identificação e o desenvolvimento de novos produtos com ação antifúngica, como óleos essenciais (OEs), é uma alternativa promissora. Assim, o presente estudo discute: a atividade antifúngica *in vitro* de três óleos essenciais, *Eucalyptus staigeriana*, *Lippia sidoides* e *Pimenta pseudocaryophyllus;* a composição química e o modo de ação do OE de maior potencial e sua atividade *in vivo* quando associado com carboximetilcelulose (CMC), assim como seus efeitos na qualidade sensorial e pós-colheita dos morangos. Nos capítulos dois, três e quatro os estudos foram direcionados para *Colletotrichum acutatum*, *Rhizopus stolonifer e Botrytis cinerea,* respectivamente. No último capítulo avaliou-se a atividade antifúngica de uma formulação comercial, alternativa e de baixa toxidez contra os mesmos três patógenos. Dentre os OEs avaliados, o de *L. sidoides* apresentou melhor atividade antifúngica, com a predominância do timol em sua composição, causando alterações morfológicas nos patógenos. *In vivo*, os morangos tratados com CMC associado ao OE apresentaram redução na severidade das doenças e melhorias nas propriedades físico-químicas com pouca alteração na qualidade sensorial da fruta. A formulação comercial também apresentou potencial de controle, *in vitro* e *in vivo*, dos principais fungos deteriorantes pós-coheita do morango.

Palavras-chave: *Coletotrichum acutatum*; *Botrytis cinerea*; *Rhizopus stolonifer*; *Lippia sidoides;* Carboximetilcelulose

ABSTRACT

Antifungal activity of essential oils associated with edible coatings for the postharvest control of fungi that deteriorate strawberry (*Fragaria* spp.)

Strawberry cultivation presents as limiting problem the phytosanitary management, which, associated with the residues of the toxic fungicides, the resistance of pathogens and the fragility of the fruit during transport and storage, make the commercialization of this product a challenge. The identification and development of new products with antifungal action, such as essential oils (EOs), is a promising alternative. Therefore, the present study addresses: the *in vitro* antifungal activity of three essential oils, *Eucalyptus staigeriana*, *Lippia sidoides* and *Pimenta pseudocaryophyllus;* the chemical composition and the mode of action of the EO with the highest potential and its *in vivo* activity when associated with carboxymethylcellulose (CMC), as well as its effects on the sensory quality and postharvest of strawberries. In chapters two, three and four, the studies were directed to *Colletotrichum acutatum*, *Rhizopus stolonifer* and *Botrytis cinerea,* respectively*.* In the last chapter, the antifungal activity of a commercial, alternative formulation with low toxicity was evaluated against the same three pathogens. Among the EOs evaluated, the one from *L. sidoides* presented the best antifungal activity, with the predominance of thymol in its composition, causing morphological alterations in the pathogens. *In vivo*, the strawberries treated with CMC associated to the EO presented a reduction in the severity of the diseases and improvements in the physicochemical properties with little alteration in the sensory quality of the fruit. The commercial formulation also presented a potential for the control, *in vitro* and *in vivo*, of the main postharvest strawberry-deteriorating fungi.

Keywords: *Coletotrichum acutatum*; *Botrytis cinerea; Rhizopus stolonifer; Lippia sidoides*; Carboxymethylcellulose

1. INTRODUCTION

Strawberry (*Fragaria* spp.) is a pseudofruit with importance in several countries. According to the last survey performed by the Food and Agriculture Organization in 2016, the five major producers of this fruit are China, the United States, Spain, Turkey and Mexico (FAO, 2018). In South America, the Brazilian production is the highest, with 155 thousand tons of the fruit in 4,300 hectares, with Minas Gerais, Rio Grande do Sul and São Paulo comprising the main producing States (FAGHERAZZI et al., 2016). The production and consumption are increasing, since the fruit offers a variety of nutrients, such as potassium, manganese, vitamins C and E, folic acid and carotenoids, besides being rich in phenolic compounds. It is very appreciated because of its excellent sensory properties; however, it has a short shelf life, caused by the high metabolism and microbial spoilage, which result in alterations that reduce its commercial value in the postharvest (BASU et al., 2014; UGOLINI et al., 2014; MOHAMMADI et al., 2015).

The culture presents a major problem in relation to the presence of fungal diseases during cultivation and, also, in the postharvest, given the fact that there is a great rate of resistance among the pathogens to many active ingredients used for the control of the diseases. Furthermore, the number of products registered for the control of fungi in the culture is reduced or absent, as is the case of strawberry postharvest, a fact confirmed in the system AGROFIT of the Ministry of Agriculture, Livestock, and Supply (BRASIL, 2015).

Currently, the study of new sources of food conservation is in wide development. The consumers are increasingly giving credits to the foods which did not receive chemical products along their production, and which, in the postharvest, have sensory and nutritional characteristics close to those *in natura* (ROMANAZZI et al., 2016). Therefore, there is a need for products that are differentiated, efficient, of low cost and that control the growth of pathogens in vegetables (AGUILAR-GONZALEZ et al., 2015). Additionally, such products must not damage the environment and human health, maintaining the sensory characteristics of the product and expanding its shelf life.

Natural bioactive substances extracted from plants, such as extracts or essential oils, appear as a promising option for the development of phytosanitary products intended for the management of disease in several cultures (FARZANEH et

al., 2015; PALOU et al., 2016). These substances present several advantages when compared to the synthetic pesticides, such as a lower aggressiveness to the environment, biodegradation, the use in both organic and conventional cultivations, besides serving as starting point for the synthesis of new compounds (NGUEFACK et al., 2012; ENYIUKWU et al., 2014). On the other hand, the use of essential oils with antimicrobial capacity poses a big challenge, since interactions of the oil components with fruit constituents and losses in the active compounds by fast volatilization or the action of other factors, such as light, can reduce or preclude their application (MOHAMMADI et al., 2015). Thus, an interesting alternative to overcome this challenge and maintain the effectiveness of the essential oil is the incorporation of these substances in the formulation of edible coatings, preserving fruit quality and reducing microbial deterioration (SANGSUWAN et al., 2016; VIEIRA, JORGE M. et al., 2016).

The identification and the development of new products with antifungal action obtained from plants is of major importance, as well as the evaluation of the physicochemical quality and the appearance of the fruits after the application of these products. Thus, it becomes necessary to verify whether the original characteristics of the fruit *in natura* will be maintained and to determine its shelf life period. A formulation which is natural and efficient in controlling postharvest diseases in strawberries might bring highly positive impacts in the economic and environmental sectors, aggregating a better quality and lifespan to the strawberry.

In this work, the general goal was to evaluate the *in vitro* antifungal activity of the EOs from *Eucalyptus staigeriana*, *Lippia sidoides* and *Pimenta pseudocaryophyllus* on the main strawberry postharvest pathogens, determining the one with the highest activity. The chemical composition and the effects on the morphology of the pathogens were also observed for the EO with the highest activity, besides the *in vivo* effect of the association of EO with CMC, considering the preventive and curative applications, as well as their effects on the postharvest quality (physicochemical and sensory) of the fruit.

For this, the work was divided into five chapters, with specific goals:

Chapter I – Literature review.

Chapter II – Antifungal activity of essential oils associated with carboxymethylcellulose against *Colletotrichum acutatum* in strawberries.

Chapter III – Control of *Rhizopus stolonifer* in strawberries by the combination of essential oil with carboxymethylcellulose.

Chapter IV – Impact of carboxymethylcellulose-based edible coatings with essential oil on the development of *Botrytis cinerea* and the quality of strawberries

Chapter V - *In vitro* and *in vivo* antifungal activity of the formulation 'Acquativ Agro' against strawberry-deteriorating pathogens in the postharvest.

1.1. Literature Review

1.1.1. Strawberry

The strawberry, according to the botanical classification, belongs to the family Rosaceae, to the genus *Fragaria* and species *Fragaria x ananassa* Duch (SILVA *et al.*, 2007). It is originated from the regions of temperate climate of Europe and of the Americas, being botanically considered a pseudofruit, since it originates from a single flower with several ovaries. The true fruit are actually small achenes, commonly called "seeds". This fruit has a non-climacteric respiratory pattern, with a short shelf life, because of its elevated metabolism and the microbial deterioration, providing alterations which reduce its commercial value in the postharvest. The strawberry has a great importance in several countries, being very appreciated due its excellent sensory properties, being consumed either fresh or in the form of candies, beverages, pulps and jams (CHITARRA and CHITARRA, 2005; GOL *et al.*, 2013; MOHAMMADI *et al.*, 2015).

In Brazil, the culture of strawberry is present in several States and is usually developed in small properties, presenting an important socio-economic role (OLIVEIRA *et al.*, 2017). Minas Gerais, Rio Grande do Sul and São Paulo are the main producing States, responsible for 66% of the total produced in the country (CEPEA, 2017). The national production in 2017 was around 155 thousand tons. Data from the State of São Paulo indicate that in 2017, the strawberry production of the State was around 9,500 t (IEA, 2018).

Strawberry cultivation has as limiting drawback the phytosanitary management, since the intensive cultivation and the ideal microclimatic conditions favor the onset of fungal diseases. This drawback, associated to the fragility of the

fruit to mechanical damages during transportation and storage, makes the commercialization of this product a challenge (LORENZETTI, 2012; SHUZHEN *et al.*, 2012). Several fungal diseases occur from the newly planted seedling to the ripe pseudofruit, which reduces culture productivity, causing losses in both commercialization and shelf life. The postharvest losses can vary from 5 to 20% or more in developed countries, depending on the type of plant species, with the possibility of increasing to 50% in developing countries (ZAMANI-ZADEH *et al.*, 2014).

Economical damages are evidenced by the strawberry producers, especially when the fruit are attacked by *Botrytis cinerea*, a filamentous fungus that causes the "gray mold" or "gray rot", which might occur in field and postharvest conditions (DE OLIVEIRA *et al.*, 2011). This is an important postharvest pathogen, since the environmental conditions prevailing in transportation and storage favor its infection and development (DROBY and LICHTER, 2007). Other important rots in strawberries are anthracnose and soft rot. Anthracnose is caused by the fungi of species *Colletotrichum acutatum*, *C. gloesporiodes* or *C. fragariae*, which produce wounds and strangulation in strawberry stolons, petiole, peduncle, pseudofruit and crown. In the pseudofruits, the wounds are rounded, deep and firm, being more common in ripe pseudofruit. The soft rot is caused by fungi of the genera *Rhyzopus* and *Mucor*, which produce a watery rot and can infect in any stage of development, being more severe during storage and commercialization (TEMPERADO, 2005; YANG *et al.*, 2015).

To minimize these diseases during strawberry production, synthetic pesticides are used and, since it is an extremely fragile and perishable fruit, there is a tendency of using them inappropriately and excessively (ZAMBOLIN; COSTA, 2006; CANTILLANO, 2006). The postharvest pathogens are also usually controlled by synthetic chemical products, which impact human health and the environment. Furthermore, they are more aggressive in this period, since there is a short time interval between treatment and consumption. Some fungi have been presenting resistance against broad spectrum fungicides, such as the benzimidazoles, imazalil and procloraz, because of the repeated use, reaching a point where some pathogens, such as *Rhizopus stolonifer*, are not sensitive anymore, with special fungicides being required to control them (ADAY *et al.*, 2011; MARI *et al.*, 2014; LIMA et al., 2015).

A study performed by the National Health Surveillance Agency (*Agência Nacional de Vigilância Sanitária*, ANVISA) in 2008, based on monitoring pesticide residues in foods *in natura*, showed that from 2001 to 2007, among nine products studied, strawberry presented the highest percentages of samples with unsatisfactory results (44.2%) and in 2008, they were 36.1%. 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 is considered worrying (ANVISA, 2009). Therefore, researches aiming at proposing natural products to control diseases in the strawberry culture are of extreme importance.

1.1.2. Essential oils

The term essential oil (EO) is defined as chemically complex natural substances of variable composition, volatile oily liquids, with strong odor, liposoluble and soluble in organic solvents. Their synthesis in plants might occur in the shoots, flowers, leaves, stems, branches, seeds, fruits, roots and peels, where they are stored in secretory cells, cavities, channels and epidermal cells. They can be extracted by hydrodistillation, steam distillation, dry distillation or by mechanisms of mechanical pressing (HYLDGAARD *et al.*, 2012; REGNAULT-ROGER *et al.*, 2012; TONGNUANCHAN and BENJAKUL, 2014; REHMAN *et al.*, 2016). The EOs are composed mainly by monoterpenes, sesquiterpenes, phenylpropanoids, esters and other substances with low molecular weight (MOHAMMADI *et al.*, 2015).

These substances have been studied because of their flavor and aroma, in order to aggregate quality to the foods, beverages and other goods, either with their antioxidant properties (TEIXEIRA *et al.*, 2012; ROBY *et al.*, 2013), or to expand the shelf life of these products, given their antimicrobial activity (BAJPAI, 2012; REYES-JURADO *et al.*, 2015). The antimicrobial power of EOs is related to their main constituents. Those presenting phenols, such as thymol, carvacrol and eugenol, exhibit a more pronounced activity on various fungal and bacterial species (KALEMBA *et al.*, 2012). Nevertheless, it is not possible to affirm that the major compounds present in the EOs are the only ones responsible for the biological activity under study, that is, antifungal or antibacterial. Components of the oil that are present in a lower amount can be, either individually or in synergy with other compounds, responsible for this activity.

The mechanism of antimicrobial action present in the EOs is still little known (GONÇALVES *et al.*, 2015). According to Kumar *et al.* (2008), the activities of the EOs and their constituents are related to their hydrophobicities, which enables them to interact with the lipid layer of cell membranes, causing alterations in their structures and making them less selective, causing the leakage of ions and other cell constituents, facilitating cell death. There are different results of the antifungal power of the EOs, which might be related to the type of technical assay, growth medium used, target microorganism, as well as EO composition (IRKIN and KORUKLUOGLU, 2009; DOS SANTOS *et al.*, 2012). The composition of the EOs is variable depending on the plant species and plant tissue used, on the stage of development, on the soil and climate conditions and on the methodology employed for extraction.

The use of EO as antimicrobial agent offers low risk of development of pathogen resistance, because of the different compounds present in the oil and of their different mechanisms of action, making them important in the combat to the development of microbial resistance (DAFERERA *et al.*, 2003). Thus, they can be used for the control of diseases in the vegetation house and in packages, or used as active ingredients in the preparation of new formulated products (ASLAN *et al.*, 2004; CZEPAK, 2008).

Some works have evidenced the efficacy in the use of EO for the control of some fungi. Anaruma *et al.* (2010), evaluating 28 EOs, verified the activity of fifteen of them against *Colletotrichum gloeosporioides*, causal agent of anthracnose in yellow passion fruit. EOs from plants of the family *Lamiaeceae*, such as oregano (*Origanum syriacum*), lavender (*Lavandula stoechas*) and rosemary (*Rosmarinus officinalis*) have demonstrated an effect in the control of *Botrytis cinerea* in tomato (SOYLU *et al.*, 2010), whereas the EO from clove, rich in eugenol, was efficient in controlling several fruit postharvest pathogens, among them *Botrytis cinerea* in grape (COMBRINCK *et al.*, 2011).

The application of EO as food preservative requires a detailed knowledge on their properties, minimum inhibitory concentration (MIC), range of target organisms, mode of action and the effect of the food matrix components on their microbial properties (HYLDGAARD *et al.*, 2012). Therefore, for this research, the selection of *Eucalytus staigeriana, Lippia sidoides* and *Pimenta pseudocaryophyllus* was based on the major compounds present in the EO of these species, their use in the popular

medicine and their antimicrobial potential proven in previous studies. In the literature, although there are works proving the antimicrobial action of the EO of these species, the researches evaluating the action of these oils on the fungi that cause postharvest diseases in strawberries are scarce or absent.

1.1.3. *Eucalyptus staigeriana*

Eucalyptus staigeriana is a medium-sized tree species, belonging to the family Myrtaceae, whose EO has a pleasant aroma, with yield varying from 1.2 to 1.5%, presenting as main constituents 1.8-cineol (34.8%), neral (10.8%), geranial (10.8%) and α-felandren (8.8%) (VITTI and BRITO, 2003; HASEGAWA *et al.*, 2008); GILLES *et al.*, 2010). Eucalyptus EOs have been used for the alternative control of phytopathogenic agents. Among the compounds present in these oils, the antimicrobial activity has been related to limonene and citronellal (MESQUITA, 2012; RIBEIRO *et al.*, 2013). Nevertheless, most of these studies were restricted to evaluating few eucalyptus species, especially *Corymbia citriodora* (former *E. citriodora*), which presents an aroma typical of lemon, that has been demonstrating a broad spectrum of antifungal activity. (GILLES *et al.*, 2010).

The fungistatic action of the extract of *Eucalyptus staigeriana* leaves was observed by Ceschini (2011), who observed a delay in the germination of *Aspergillus flavus* and by Martins *et al.* (2013), who observed a significant antifungal action on *Candida albicans*. For Da Silva *et al.* (2011), the EO of *E. globulus* was efficient in inhibiting the germination of *C. gloeosporioides.*

1.1.4. *Lippia sidoides*

Lippia sidoides, popularly known as rosemary-pepper, is native to the Brazilian Northeast and North of Minas Gerais, belonging to the family Verbenaceae and is widely used as herbal remedy in the popular medicine (DE FARIAS *et al.*, 2012). Its leaves can present up to 6% of EO, which has an elevated commercial value and is rich in thymol and carvacrol, the first being responsible for the characteristic smell of the plant (FONTENELLE *et al.*, 2007; ALMEIDA *et al.*, 2010). Both the extract and EO of this species have antimicrobial action. By

physicochemical and biological data, the antifungal potential of this species has been confirmed, evidencing that it is a natural alternative, which can be used in the development of plant-based medicines or even in food products and cosmetics (DA SILVA *et al.*, 2011; VERAS *et al.*, 2012). Regarding its action on fungi that cause postharvest diseases in fruits, studies are scarce, mainly for the strawberry culture. According to Silva *et al.* (2009), the EO of this species was efficient in inhibiting mycelial growth and the germination of *C. gloeosporioides* isolated from passion fruit. It also presented a fungitoxic effect, inhibiting the mycelial growth of *Rhizoctonia solani*, an important fungal species that causes losses in various agricultural crops (GONÇALVES, 2012; SILVA, 2013). In studies *in vivo*, the EO of this species has controlled rots caused in mangoes by *Lasiodiplodia theobromae* and *Botryosphaeria dothidea* (DE MENEZES CRUZ *et al.*, 2012).

1.1.5. *Pimenta pseudocaryophyllus*

Pimenta pseudocaryophyllus is a tree species of the family Myrtaceae, popularly known as "pau-cravo", "cataia" or "carnation", being found in mountain and coastal regions of the South and Southeast of Brazil, having been registered in Minas Gerais, Espírito Santo, São Paulo, Rio de Janeiro, Paraná, Santa Catarina, Rio Grande do Sul and Distrito Federal (PAULA, 2007; SOBRAL, 2010). The genus *Pimenta* (*Myrtaceae*), broadly used in the popular medicine, has been widely studied because of its biological properties, such as antimicrobial, anti-inflammatory, antinociceptive and hypotensive activities, among others (FERNÁNDEZ et al., 2001; GARCIA et al., 2004).

The antimicrobial potential of the EO from *Pimenta pseudocaryophyllus* leaves was active against strains of *C. albicans*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (LIMA et al., 2006). This EO, which can have eugenol as main constituent (92.8%), presented a good activity on filamentous fungi, such as *Alternaria* sp*.*, *Botryosphaeria ribis*, *Botryosphaeria* sp*.* and *Aspergillus niger* (CUSTODIO, 2007, CUSTÓDIO et al., 2010; LACERDA, 2014). Yokomizo and Nakaoka-Sakita (2014) also observed the activity of this oil against the fungi *Aspergillus niger* and *Penicillium verrucosum*. Paula et al. (2012), in a study performed with extracts, fractions and EO from leaves of this species, collected in São Gonçalo de Abaté (Minas Gerais), verified several levels of antifungal activity

against *Candida* spp. and *Cryptococcus* spp. Given the presence of chemical components with known biological activity in its EO, *P. pseudocaryophyllus* becomes a species with potential for the development of new substances in the pharmaceutical industry and in agriculture (D'ANGELIS and NEGRELLE, 2014; YOKOMIZO and NAKAOKA-SAKITA, 2014).

Thus, given the potential of the antifungal effect of the EOs of these plant species, studies on their effects against fungi that cause postharvest diseases in fruits of economic importance are necessary.

1.1.6. Edible coatings – Carboxymethylcellulose

Although the EOs present a potential for being used in the conventional and organic agriculture as a new option for the control of diseases, their use is many times limited because of their costs of application, their intense aroma and potential toxicity. Thus, an interesting alternative to reduce EO concentration and maintain its efficacy can be the incorporation of these compounds in the formulation of edible coatings, preserving fruit quality and reducing microbial deterioration (PERDONES *et al.*, 2012; GUERREIRO *et al.*, 2015; VIEIRA, JORGE M *et al.*, 2016).

The edible coatings are thin layers of a material made from biodegradable ingredients, which can be consumed as a part of the food, acting as a selective barrier for the transfer of gas and moisture, also preventing the attack of microorganisms (VARGAS *et al.*, 2008). The edible coatings that are most used in fruits are those composed of polysaccharides, such as chitosan, carboxymethylcellulose, gum arabic and pectin. Their major advantages are the good solubility in water and low viscosity in high concentrations (XIAO *et al.*, 2014).

Carboxymethylcellulose (CMC) is one of the most common cellulose derivatives. It is a long-chain anionic polysaccharide, soluble in water, non-toxic and non-allergenic (TONGDEESOONTORN *et al.*, 2011). Bioactive compounds, such as the EOs, can be incorporated to the edible coatings in order to extend the shelf life, prevent the growth of microorganisms and preserve the nutritional and sensory value of the foods (PERETTO *et al.*, 2014). This technique prevents these agents to be added directly to the foods, which can cause damages to the product, since the active substances can be neutralized or partially inactivated, besides sensory deteriorating the food. Furthermore, if these compounds are added to the coatings of

foods, the oil concentrations used can be reduced, maintaining their efficacy, with a resulting minimization of their intense aroma and possible toxicity to the consumer (VARGAS *et al.*, 2008; FALGUERA *et al.*, 2011; PERDONES *et al.*, 2012). Another advantage of the use of edible coatings with the addition of antimicrobial agents is that they can be more efficient, since they allow the release of the effective agents for a longer period (WATTANASATCHA *et al.*, 2012).

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2. ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS ASSOCIATED WITH CARBOXYMETHYLCELLULOSE AGAINST *Colletotrichum acutatum* IN STRAWBERRIES

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Abstract

The antifungal activity of the essential oils (EOs) of *Eucalyptus staigeriana, Lippia sidoides* and *Pimenta pseudocaryophyllus* was evaluated *in vitro*, by direct contact and by exposure to volatiles, against *Colletotrichum acutatum*, an important pathogen of strawberry. The chemical composition of the EO with the highest activity and its effects on the morphology of the pathogen 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 antifungal activity *in vitro*, being more efficient by direct contact than by volatilization. This EO has a predominance of the compound thymol and was able to cause dehydration and rupture of the pathogen hyphae. *In vivo*, strawberries treated with CMC associated with *L. sidoides* EO presented a reduction in disease severity, when treated in a curative way. Thus, the association of *L. sidoides* EO with CMC can be a potential alternative for the control of this disease.

Keywords: *Lippia sidoides; Pimenta pseudocaryophyllus*; *Eucalyptus staigeriana*; Scanning electron microscopy; Edible coating

2.1. Introduction

Strawberry, a fruit appreciated because of its pleasant flavor and aroma, represents a valuable source of bioactive compounds. Nevertheless, these properties can be undermined since this fruit is susceptible to mechanical damages, desiccation, physiological disorders and, especially, the degradation by fungal diseases, limiting its commercialization and consumption (Ugolini, Martini, Lazzeri, D'Avino, & Mari, 2014; Mohammadi, Hashemi, & Hosseini, 2015).

Anthracnose fruit rot, an important disease in strawberries, can be caused by several fungal species of the genus Colletotrichum (Freeman, 2008), with *C. acutatum* being one of the most prejudicial in the economic standpoint (Zhang et al.,

2016). The application of synthetic fungicides, which represents 15 % of the total operational costs, and the use of less susceptible cultivars, are important tools for the control of this disease (Legard, MacKenzie, Mertely, Chandler, & Peres, 2005; Forcelini, Gonçalves, & Peres, 2017). Nevertheless, the use of these fungicides can bring potential risks to consumers and the environment and contribute to the selection of resistant pathogens. Thus, there is a strong need of alternative strategies for the control of this disease and the extension of strawberry postharvest shelf life (Aguado et al., 2012; Romanazzi, Smilanick, Feliziani, & Droby, 2016).

In this sense, the essential oils (EOs) are alternatives to extend fruit shelf life, due to their antimicrobial activity and low risk for the development of pathogen resistance, because of their complex composition and their different mechanisms of action (Rehman, Hanif, Mushtaq, & Al-Sadi, 2016). The use of EOs in fruits represents a challenge, since their components can interact with fruit constituents, besides the occurrence of losses of their active compounds by rapid volatilization or the action of other factors, such as light, which reduces or impairs its application (Turek & Stintzing, 2013).

The incorporation of EOs in edible coating, for the protection of their compounds and controlled release, has been indicated as an efficient strategy. The carboxymethylcellulose (CMC), one of the most common cellulose derivatives (Embuscado & Huber, 2009), can be used in association with the EO, acting as a selective barrier for the transfer of gas and moist, in the reduction of microbial growth and in the decrease of the intense aroma of the EOs, thus preserving its quality with shelf life extension (Vieira et al., 2016). CMC can be originated from abundant and low-cost sources, such as sugarcane bagasse, one of the major by-products of sugar cane industry. Some studies have shown the association of EOs with edible coating, like chitosan, as an alternative for increasing the shelf life of strawberries (Perdones, Escriche, Chiralt, & Vargas, 2016; Badawy, Rabea, AM El-Nouby, Ismail, & Taktak, 2017). However, few studies have been done for this purpose combining EOs with edible coatings less expensive and from abundant and affordable sources, such as CMC is.

Some studies have revealed the antifungal power of EOs on *C. acutatum* (Elshafie, Ghanney, Mang, Ferchichi, & Camele, 2016; Elshafie et al., 2016), However, studies regarding the antifungal activity of the EOs extracted from leaves of the species *Eucalyptus staigeriana*, *Lippia sidoides* and *Pimenta pseudocaryophyllus*

on fruit-deteriorating fungi in the postharvest period are scarce, despite the proven antimicrobial action of these oils (Yokomizo & Nakaoka-Sakita, 2014; Herculano, de Paula, de Figueiredo, Dias, & Pereira, 2015). Therefore, this study evaluated the in vitro antifungal activity of these EOs on *C. acutatum* by different methods (direct contact and exposure to volatiles), determining the EO with the highest activity. The chemical composition and the effects on pathogen morphology were also observed for the EO with the highest activity. Still, the effect of the association of the EO with CMC, considering the preventive and curative application, was evaluated in vivo.

2.2. Materials and Methods

2.2.1. Plant material and 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. The EO was dehydrated in anhydrous sodium sulfate and stored at -5 °C. The yield of each EO was calculated by the formula R (%) = [(EO volume x density) / dry mass of the leaves] x 100 (Girard, Koehler, & Netto, 2017).

2.2.2. Isolation and Molecular identification of *C. acutatum*

C. acutatum was obtained by direct isolation of fungal structures present on strawberries, from a conventional grower in Jarinu (SP, Brazil), with typical symptoms of anthracnose fruit rot. Genomic DNA of the isolate was extracted using the Kit FastDNA®MP Biomedicals, following the procedures recommended by the manufacturer. Polymerase chain reaction (PCR) was used to confirm the identity of the species complex using the primers ITS4-Universal (5'-TCCTCCGCTTATTGATATGC-3') and Cacut-Int2 (5'-GGGGAAGCCTCTCGCGG-3'). Water was used as negative control and a previously identified isolate of the species complex *C. acutatum* was used as positive control.

2.2.3. Determination of EO antifungal activity *in vitro*

2.2.3.1. Method by contact

EO antifungal activity was initially evaluated by measuring *C. acutatum* growth inhibition by the direct contact of the fungus with potato dextrose agar (PDA) culture medium either containing the individual EO or with its binary and ternary mixtures, at concentrations of 31; 62.5; 125; 250 and 500 µl/L (Plaza, Torres, Usall, Lamarca, & Vinas, 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 installed.

After solidification of the PDA medium, *C. acutatum* was transferred to the central point of the Petri dish, from an inoculum suspension containing 10^5 spores mL^{-1}. Plates were maintained in growth chambers at 25 °C with photoperiod of 12 h, and mycelial growth measurements of each colony were taken every two days, in two perpendicular directions (diameter in cm). Mycelial growth inhibition at the different concentrations of the individual EOs and of the mixtures was measured by the formula PI (%) = (Growth of the Control - Growth of the Treatment/ Control Growth) x 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 the development of C. acutatum visible at naked eye.

2.2.3.2. Method by exposure to volatiles

The treatment that presented the highest antifungal activity *in vitro* by the method of contact was also evaluated by the method of exposure to volatiles, according to (Yun, Fan, & Li, 2013). The inhibition of fungal growth was observed at concentrations of the EOs 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 inner part of a Petri dish that contained solidified PDA. Pathogen inoculation, incubation, mycelial growth measurement and determination of the MIC, were the same as described in the method by contact (item 2.2.3.1).

In the methods in vitro, the experimental design was randomized in factorial scheme, 8 x 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 x 5 factorial scheme, with two treatments (Control; *L. sidoides*) and five concentrations, for the method of exposure to the volatiles. Both experiments had five repetitions per treatment and were performed three times. Data from repeated experiments were combined after tests of homogeneity indicated that variances were homogeneous.

2.2.3.3. Effects of the essential oil on *C. acutatum* morphology

The damages caused by the EO with the highest antifungal activity on the pathogen morphology was evaluated by Scanning Electron Microscopy (SEM) according to (Yu, Wang, Shao, Xu, & Wang, 2015) with modifications. For this, 150 mL of potato dextrose broth (PDB) with the addition of 1 mL of a suspension of pathogen spores (10^6 spores mL^{-1}) was incubated for two days at 25 °C and under a photoperiod of 12 h. After this period, the volume of EO corresponding to the MIC determined in the experiment in vitro by the method of direct contact in PDA was emulsified with soy lecithin and added to the broth. Subsequently, the incubation followed for additional 6 h at the same conditions described. A treatment with potato broth without the addition of the EO was used as control. The samples were processed according to Escanferla, Moraes, Salaroli, & Massola Jr (2009) and the observations were performed in a Scanning Electron Microscope LEO 435 (Zeiss, England). This analysis was repeated twice, with three repetitions.

2.2.4. Evaluation of the chemical composition of the essential oil

The chemical composition was determined only for the EO that presented the best result in the in vitro test. The characterization was performed by gas chromatography coupled to mass spectrometry, using the equipment CGMS 2010 (SHIMADZU) and capillary-column gas chromatography diphenyl dimethylpolysiloxane (5% diphenyl and 95% dimethylpolysiloxane). Reactions were performed at 50°C for 1.5 minutes, then elevated at 4°C min-1 until 200°C, followed by 10°C min-1 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-1. 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.

2.2.5. Determination of the antifungal activity *in vivo*

The treatment with the highest antifungal activity *in vitro* was evaluated *in vivo* in preventive and curative ways, either in association or not with CMC coating, resulting in the treatments: "C" – fruit without the application of CMC and EO; "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 purity of 99.8 %, humidity of 7.6 %, 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 % (w / v) in distilled water at 60 °C under continuous mechanical agitation at 2.000 rpm for 20 min, in a 3-bladed propeller stirrer (Fisatom - 713D). Subsequently, 0.5 mL (50 % w/w weight of CMC) of glycerol was added as plasticizer, following stirring for further 15 min. In the treatments involving EO, as the emulsion reached 25 °C, the EO previously

emulsified with Tween-80 (proportion 2:1 v/v) was added. In this test, the EO concentration used was ten times superior to the MIC obtained in the test *in vitro* (Hyldgaard, Mygind, & Meyer, 2012).

'Oso Grande' strawberries, from an organic farm located in Cambuí (MG, Brazil, 22° 36' 43" S 46° 03' 28" W), were visually selected regarding appearance and sanity, and then sanitized in sodium hypochlorite at 2.5 %. For the evaluation of the preventive 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^{-1}) on a 3 mm-deep wound. The fruit were stored for 24 h in a wet chamber with 95 % of relative humidity, in a growth chamber at 25 °C and photoperiod of 12 h. To evaluate the curative mode of action, fungus inoculation was performed 24 h before the application of the treatments. The subsequent procedures were the same described for the preventive mode of action. The control treatment (C) consists only of fruit immersed in sterilized distilled water. Therefore, the experimental design in this step was in a 5 x 7 factorial scheme, involving five treatments ("C"; "COP"; "CP; "COC" and "CC") and seven days of evaluation. Six repetitions of each treatment were used, each of them consisting of 12 strawberries.

The antifungal activity of the treatments was evaluated by the incidence and severity of the disease in the fruit. Disease incidence was calculated from the number of symptomatic fruit in relation to the total number of fruit in each treatment, evaluated after seven days of storage, with the results expressed in percentage (%) (Ali, Wee Pheng, & Mustafa, 2015).

Severity was daily evaluated by a scale of scores composed of six degrees (0 = absence of symptoms; 1 = 1 to 20 % of wounded area; 2 = 21 to 40 %; 3 = 41 to 60 %; 4 = 61 to 80 % and 5 = more than 81 % of wounded area), with the results expressed in Disease Index (DI), according to Cia, Benato, Pascholati, & OLIVEIRA GARCIA (2010): $DI(\%) = [(1 x n1) + (2 x n2) + (3 x n3) + (4 x n4) + (5 x n5)] \times 100/5 x N$, in which ni is the number of fruit infected 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 & Madden (1990): $AUDPC = \sum [(y_i + y_{i+1}) / 2 \times (t_{i+1} - t_i)]$, with y_i corresponding to wound DI at time t_i; y_{i+1}; DI with time, t_i the initial reading time and t_{i+1} the time in days of each read.

This experiment was conducted twice to confirm the results. The data relative to the AUDPC were evaluated by 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, which corresponded to the two experiments *in vivo*. The standard deviation of the means was calculated and the statistical difference of the means, at the significance level of 5 % (p<0.05), was determined by the Tukey test.

2.3. Results

2.3.1. Yield of the EOs

The plant material with the highest yield of EO was the one from *E. staigeriana*, followed by *L. sidoides* and *P. pseudocaryphyllus* (Table 1).

Table 1 – Yield of the essential oils (EOs) extracted from the leaves of *Eucalyptus staigeriana*, *Lippia sidoides* and *Pimenta pseudocaryophyllus* by hydrodistillation.

Plant species	Volume[1]	Density[2]	LM[3]	Y[4]
Eucalyptus staigeriana	8.30	0.8980	422,70	1.76
Lippia sidoides	6.40	0.9495	400	1.52
Pimenta pseudocaryophyllus	5.10	0.9879	550	0.92

Note: [1]Volume = Volume of the average essential oil obtained in the hydrodistillations (mL); [2]Density = density measured from the volume (mL) of oil obtained in the process, divided by sample dry mass (g); [3]LM = Leaf mass (g); [4]Y (%) = Mean yield in percentage.

2.3.2. Molecular identification of C. acutatum

The isolate used in this study was identified as belonging to the species complex *C. acutatum*. PCR product visualized under UV light on 1% agarose gel presented the same band profile as the *C. acutatum* isolate used as positive control.

2.3.3. Determination of the antifungal activity *in vitro*

2.3.3.1. Method of evaluation by contact

The highest antifungal activity on *C. acutatum* was provided by the EO of *Lippia sidoides* when applied individually, which presented a dose-dependent activity, with the capacity of total inhibition of the mycelial growth observed between the concentrations 125 and 250 µl/L. The binary mixtures M1 and M3, which contained *L. sidoides* EO in their composition, also showed high rates of pathogen inhibition. Conversely, the EOs of *E. staigeriana* and *P. pseudocaryophyllus* presented a low potential for the control of *C. acutatum*, since the maximum inhibition observed in the highest concentration was of 3 and 11 %, respectively (Table 2).

Table 2 – Mycelial growth (MG), percentage of mycelial growth inhibition (PI) and minimum inhibitory concentration (MIC) of *Colletotrichum acutatum*, isolated from strawberry, at 12 d of incubation, after exposure by contact to different concentrations (µl/L) of essential oils incorporated to the medium Potato-Dextrose-Agar (PDA). Mean values ± SD, n = 5.

Treatments	Concentrations (µl/L)	MG (cm)	PI[1] (%)	MIC[2]
Eucalyptus staigeriana	0.0	8.70±0.0[a]	0.00±0.0[a]	
	31.0	8.70±0.0[a]	0.00±0.0[a]	
	62.5	8.70±0.0[a]	0.00±0.0[a]	
	125.0	8.70±0.0[a]	0.00±0.0[a]	**MIC > 500**
	250.0	8.62±0.11[a]	0.87±1.9[a]	
	500.0	8.60±0.22 [a]	1.15±2.5[a]	
Lippia sidoides	0.0	8.70±0.0[a]	0.00±0.0[d]	
	31.0	6.34±1.08[b]	27.12±12.4[c]	
	62.5	6.14±0.46[b]	29.42±5.2[c]	
	125.0	3.43±0.79[c]	60.50±9.1[b]	**125 < MIC ≤ 250**
	250.0	0.00±0.00[d]	100±0.0[a]	
	500.0	0.00±0.00[d]	100±0.0[a]	
Pimenta pesoudocaryophyllus	0.0	8.70±0.0[a]	0.00±0.0[b]	
	31.0	8.70±0.00[a]	0.00±0.0[b]	
	62.5	8.70±0.00[a]	0.00±0.0[b]	
	125.0	8.17±0.7[b]	6.00±8.2[ab]	**MIC > 500**
	250.0	7.82±0.63[b]	10.09±7.3[a]	
	500.0	7.65±0.39[b]	12.02±4.5[a]	
M1	0.0	8.70±0.00[a]	0.00±0.0[a]	
	31.0	8.70±0.00[a]	0.00±0.0[a]	
	62.5	8.70±0.00[a]	0.00±0.0[b]	
	125.0	6.33±0.61[b]	27.17±7.1[b]	**MIC > 500**
	250.0	4.86±2.05[b]	44.06±23.5[b]	
	500.0	0.41±0.93[c]	95.19±10.7[a]	
M2	0.0	8.70±0.00[a]	0.00±0.0[c]	
	31.0	8.70±0.00[a]	0.00±0.0[c]	**MIC > 500**

	Concentration			MIC
	62.5	8.70 ± 0.00^a	0.00 ± 0.0^c	
	125.0	8.70 ± 0.00^a	0.00 ± 0.0^c	
	250.0	7.32 ± 0.47^b	15.86 ± 5.8^b	
	500.0	5.95 ± 0.65^c	31.52 ± 7.6^a	
M3	0.0	8.70 ± 0.00^a	0.00 ± 0.0^d	
	31.0	8.70 ± 0.00^a	0.00 ± 0.0^d	
	62.5	6.90 ± 0.77^b	20.62 ± 8.9^c	
	125.0	6.56 ± 0.97^b	24.55 ± 11.2^c	**$250 < MIC \leq 500$**
	250.0	3.74 ± 1.54^c	56.98 ± 17.7^b	
	500.0	0.00 ± 0.00^d	100 ± 0.0^a	
M4	0.0	8.70 ± 0.00^a	0.00 ± 0.0^b	
	31.0	8.70 ± 0.00^a	0.00 ± 0.0^b	
	62.5	8.62 ± 0.17^a	0.92 ± 2.6^b	
	125.0	8.46 ± 0.21^a	2.71 ± 2.5^b	**$MIC > 500$**
	250.0	6.76 ± 0.64^a	22.30 ± 7.4^b	
	500.0	3.12 ± 3.25^b	64.14 ± 37.4^a	

Note: [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 found. M1= binary mixture of the EO from *L. sidoides* and *E. staigeriana*, M2= binary mixture of the EO from *E. staigeriana* and *P. pseudocaryophyllus*, M3= binary mixture of the EO from *L.sidoides* and *P. pseudocaryophyllus*, M4= ternary mixture of the EO from *L.sidoides*, *E. staigeriana* and *P. pseudocaryophyllus*, 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 ($P < 0.05$).

2.3.3.2. Method of evaluation by exposure to the volatiles

The EO from *L. sidoides* presented the lowest MIC interval among all treatments evaluated by contact and, therefore, this EO was selected to compose the subsequent steps of the experiment. Thus, the evaluation of the antifungal activity by exposure to the volatiles of this EO demonstrated that pathogen exposure to the volatiles was less efficient in inhibiting mycelial growth than when in direct contact with the EO, not allowing the detection of a MIC in the concentrations evaluated by this method (Table 3).

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Table 3 – Mycelial growth (cm) and percentage of mycelial growth inhibition of *Colletotrichum acutatum*, isolated from strawberry, at 12 d of incubation, after exposure to the volatiles of different concentrations of *Lippia sidoides* (mean values ± SD, n = 5).

Concentrations (µl/L)	Mycelial growth (cm)	PI[2] (%)
0	8.54±0.00[a]	0.00±0.00[e]
31	4.74±0.89[ab]	46.26±9.71[de]
62.5	4.73±0.47[b]	44.65±5.48[d]
125	3.86±0.27[c]	54.84±3.06[c]
250	2.67±0.32[d]	68.84±3.20[b]
500	0.60±0.58[e]	92.99±6.14[a]
MIC[1]	MIC[1] > 500	

Note: [1]MIC= Minimum Inhibitory Concentration, with interval between concentrations in which values of 100 % of mycelial growth inhibition can be detected. [2]PI= percentage of mycelial growth inhibition in relation to the control treatment. 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).

2.3.3.3. Effects of the essential oil on *C. acutatum* morphology

In the absence of the EO, *C. acutatum* mycelia exhibited tubular hyphae that were homogeneous, regular, with defined septa, besides a smooth and long external surface (Figures 1A and 1B). The opposite occurred in the hyphae exposed to *L. sidoides* EO, which presented structural alterations, such as superficial wrinkles, peeling, distortion and destruction (Figures 1C and 1D).
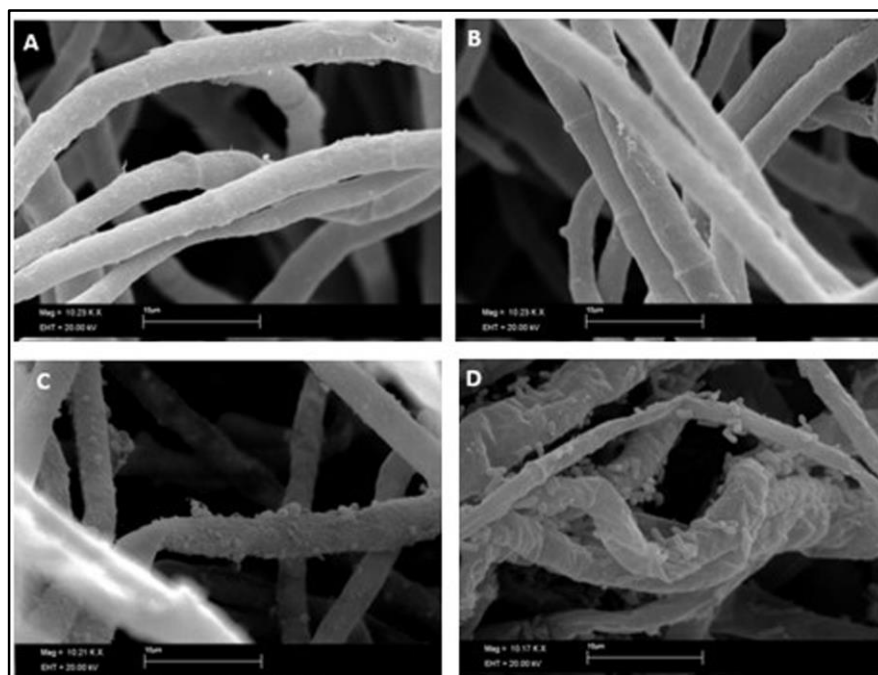
Figure 1 – Scanning electron microscopy (SEM) of the hyphae of *Colletotrichum acutatum* from strawberry, cultivated in potato broth for two days and subjected (C and D) or not (A and B) to *Lippia sidoides* essential oil, at the concentration of 250 µl/L.

2.3.4. Evaluation of the chemical composition of the essential oil

The chemical composition was performed for the oil of L. sidoides, which had the best antifungal activity among the EOs evaluated. In this oil, 20 compounds presented an area with chromatographic peaks higher or equal to 0.5 % of the total area of the peaks present in the chromatogram. The major compound present was thymol (49 %), followed in descending order by cimene and Iso-caryophyllene, with 11 and 8 % of area, respectively (Table 4).

Table 4 – Chemical composition of the EO extracted from the leaves of *Lippia sidoides*.

Compounds[1]	*Lippia sidoides*[2] (%)	LRI[3]
α-Felandrene	1.51	930
α-Pinene	0.54	937
Myrcene	2.37	995
α- Terpinene	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**	

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

2.3.5. Determination of the antifungal activity *in vivo*

Disease severity was lower in the strawberries treated in a curative way with CMC, either associated or not to the EO, in other words, in treatments COC and CC, respectively. Nevertheless, the treatment that involved the EO presented the lowest AUDPC value, besides being the only one that differed statistically from the others.

Thus, this treatment (COC) was the most adequate for the control of anthracnose fruit rot, confirming the antifungal potential of the EO in the test *in vivo* (Figure 2).

Disease incidence reached 100 % at the seventh day of evaluation, with a resemblance among the treatments.
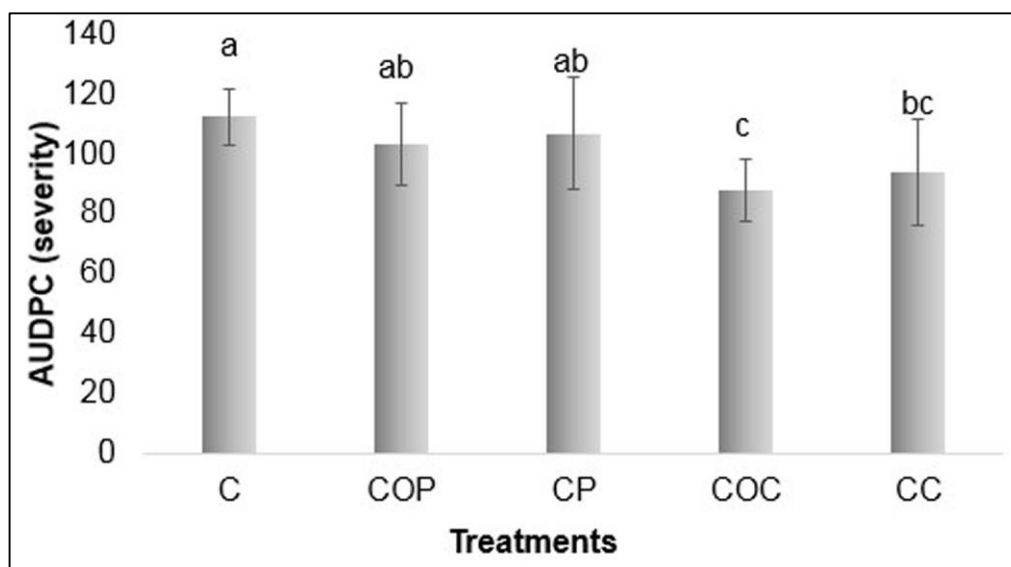
Figure 2 - Area under the disease progress curve (AUDPC) for disease severity, caused by *Colletotrichum acutatum*, in "Oso Grande" strawberries.
Distinct letters represent a significant difference among the treatments by the Tukey test (p<0.05). Vertical bars indicate the standard error of the mean (n=12). C: fruit without the application of carboxymethylcellulose and essential oil; COP: fruit treated preventively with carboxymethylcellulose and essential oil; CP: fruit treated preventively with only carboxymethylcellulose; COC: fruit treated curatively with carboxymethylcellulose and essential oil; CC: fruit treated curatively with only carboxymethylcellulose.

2.4. Discussion

For *E. staigeriana,* the mean yield of the EO was 1.52 %, a value inferior to that obtained by Gilles, Zhao, An, & Agboola (2010), who obtained yields varying between 2.13 and 3.12 %. On the other hand, the yield of *L. sidoides* EO (1.76 %) was superior to that found by Veras et al. (2012) e de Morais et al. (2016) and inferior to that found by Cavalcanti et al. (2010), who obtained yields varying from 5 to 8 %. *P. pseudocaryophyllus* yielded 0.92 % of EO, a value close to those obtained in other works, which presented variations from 1 to 2.3 % (Yokomizo & Nakaoka-Sakita, 2014; Ribeiro et al., 2015). Both the yield and chemical composition of the EO may vary because of intrinsic and extrinsic factors, such as period of harvest, climate

condition, seasonal variation, amount of light, water availability, among others (Dhouioui, Boulila, Chaabane, Zina, & Casabianca, 2016).

L. sidoides EO, which presented the highest antifungal activity in the *in vitro* test, has in its composition an elevated content of thymol, which is a phenolic compound that belongs to a class of natural antioxidants, due to the presence of a hydroxyl group bound to the aromatic ring, and that can act on the fungal cell wall (Chavan & Tupe, 2014; de L, da Silva, Reis, Costa, & Alves, 2015). Nevertheless, EOs are a complex mixture of active chemical compounds, which can present a synergistic effect of its components, being therefore responsible for its antifungal activity (Khoury et al., 2014; Guerreiro, Gago, Faleiro, Miguel, & Antunes, 2015). The mechanisms of action of EOs on the microorganisms have been related to the alteration in permeability and integrity of the cell membranes, leading to leaks of nucleic acids and proteins, affecting their growth and shape (Guo et al., 2017).

The fungitoxic or fungistatic potential of *L. sidoides* EO on pathogens, including those of postharvest, has been proven in other studies (Laranjeira et al., 2013; Aquino, Sales, Soares, Martins, & Costa, 2014). Therefore, this EO has potential for use independently or for the development of new formulations for the control of numerous pathogenic fungi.

The EOs that presented the lowest antifungal activities in relation to the other treatments were those from *E. staigeriana* and *P. pseudocaryophyllus,* with maximum inhibition of 3 and 11 %, respectively. The antifungal activity of these oils, despite being positive in other filamentous fungi (Custódio et al., 2010; Herculano et al., 2015), still had not been evaluated on *C. acutatum.* Thus, the results showed the importance of evaluating the antifungal capacity for this species and highlighted the highest potential of *L. sidoides* EO for use in the control of this pathogen.

The bioactivity of the EOs is also relevant when applied in the vapor phase, a characteristic that makes them appropriate as potential fumigants for the conservation of fresh stored products and that are sensitive to treatments by immersion (Tzortzakis, 2009). However, in the vapor phase, *L. sidoides* EO presented the lowest efficiency against *C. acutatum,* with an inhibition rate of 68 % at 250 µl/L, against 100 % when compared to the application in direct contact. According to Karimi et al. (2016), this fact can be justified due to a possible lower concentration of the active components in the volatile fraction in comparison to the concentration present in the assay by direct contact. Another possible explanation is

the faster accumulation of inhibiting compounds in the pathogen structure throughout incubation time, which could be more efficient in the assay of contact, since there is a direct contact of the EO with the fungal structure. Nonetheless, many studies indicate a promising potential for the volatile phase of the EO in pathogen control (Chu, Liu, & Zhou, 2001; Sellamuthu, Sivakumar, Soundy, & Korsten, 2013).

Alterations in the fungus in contact with the EO, observed by SEM, suggest that the alteration of the EO can include an attack to the cell wall with consequent loss of the ability of pathogen infection, colonization, and disease progress. The EO of *L. sidoides* is rich in thymol, a compound that can alter the structure of the fungal cell wall and facilitate ion exchange, increasing its permeability and hampering cell survival, since it affects essential processes, influencing in morphogenesis and fungal growth (Sharma & Tripathi, 2008; Rao, Zhang, Muend, & Rao, 2010). These alterations were similar to those observed in studies conducted with *Rhizopus stolonifer and Fusarium graminearum*, exposed to EO(s), in which hyphae *compression and grouping* were observed without the original tubular form, presenting the appearance of dry, wrinkled and with loss of the cytoplasmic material *(dos Santos et al., 2012; Kumar et al., 2016).*

The identification of chemical components of *L. sidoides* EO revealed thymol as the major compound (49.46 %), followed by cimene (11.40 %), but the minor compounds of the essential oils, as well as the interaction among them, can also present some antifungal activity (Alitonou et al., 2012). Many studies exhibited thymol as a major compound in the EO of *L. sidoides* cultivated in several places of Northeast Brazil, with concentrations varying between 30.24 and 84.09 % (Marco et al., 2012; Aquino et al., 2014). The thymol has been approved by the U.S. Food and Drug Administration (FDA) as "generally recognized as safe" (GRAS) and can be used as a food additive (Marchese et al., 2016).

Fruit treated in curative way presented a significant reduction in disease severity after seven days of storage at 25 °C. Although there was no statistical difference among treatments "COC" and "CC", it was observed that the gross value of the AUDPC was lower for "COC", which involved *L. sidoides* EO, suggesting a higher safety for disease control, since CMC is a natural and inert polymer and does not present any chemical effect on the pathogen (Carmona-Ribeiro & de Melo Carrasco, 2013). According to Sivakumar & Bautista-Baños (2014), thymol, a major compound present in the *L. solids* EO, has the capacity to increase the levels of

antioxidant enzymes in fruits, which may induce the resistance of the fruit tissues to the pathogens, thus reducing their physiological degradation. Works that associate the incorporation of EOs to CMC coatings aiming at the control of pathogenic fungi in fruits are lacking; nevertheless, the antimicrobial content of this association has already been verified *in vitro* by (Dashipour et al., 2015)*,* who, associating CMC with the EO from *Zataria multiflora Boiss*, observed the capacity of inhibition against some pathogenic bacteria. The efficiency of the combination *of other EOs* with different types of coatings, aiming at the postharvest control of fungal diseases in fruits, has already been proven. In strawberries, chitosan coatings containing EO(s) were described as efficient in the control of fungal decomposition caused by *Rhizopus stolonifer* and *Botrytis cinerea* (Mohammadi et al., 2015*; Perdones et al., 2016).*

Although disease severity was lower in the fruit treated with CMC incorporated with *L. sidoides* EO, the incidence of the disease was high in all treatments, even though the test *in vitro* indicated a capacity of 100 % of *C. acutatum* growth inhibition at 250 µl/L. This is justifiable, since the experiment was performed under conditions of high inoculum pressure for ripe fruit, without the use of low temperatures. Thus, this assay provided ideal conditions for the disease development. Furthermore, higher concentrations of EO are usually required in experiments *in vivo,* since there might be interactions between the compounds of the EO and the food matrix (Hyldgaard et al., 2012). The complexity of the food matrix, for being an environment rich in nutrients, provides an excellent growth medium for fungal development, as well as the repair and regeneration of cell components (Espitia et al., 2012)*.* Consequently, it can be expected that *C. acutatum* exhibits a lower sensitivity to *L. sidoides* EO on the surface of strawberries.

2.5. Conclusions

Among all essential oils and mixtures evaluated *in vitro*, *Lippia sidoides* EO presented the highest capacity of *C. acutatum* inhibition. The EO caused morphological degradation of pathogen hyphae, suggesting its action on the fungal cell wall. Incorporation of *L. sidoides* EO to a CMC emulsion was efficient in the reduction of anthracnose severity in strawberries, being more effective as a curative method of control. Association of *L. sidoides* EO with CMC coating can be a potential

alternative to the synthetic fungicides for the control of the postharvest disease caused by *C. acutatum* in strawberries.

Acknowledgements

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3. CONTROL OF *Rhizopus stolonifer* IN STRAWBERRIES BY THE COMBINATION OF ESSENTIAL OIL WITH CARBOXYMETHYLCELLULOSE

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.

Keywords: Edible coatings; *Fragaria* spp.; *Pimenta pseudocaryophyllus*; *Eucalyptus staigeriana*; *Lippia sidoides; Rhizopus stolonifer*

3.1. Introduction

Strawberry, an important pseudofruit in several countries, is a relevant source of bioactive compounds, beneficial substances for health, being very appreciated by its excellent sensory properties. Despite these characteristics, it 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 of *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 classed 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). Considering 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 reducing microbial growth (Guerreiro et al., 2015; Peretto et al., 2014).

Carboxymethylcellulose (CMC) has been used as edible coating for not being toxic, presenting good solubility and low viscosity (Tongdeesoontorn et al., 2011). 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). Some studies have shown the association of EOs with edible coating, like chitosan, as an alternative for increasing the shelf life of strawberries (Badawy et al., 2017; Perdones et al., 2016). However, few studies have been done for this purpose combining EOs with edible coatings less expensive and from abundant and affordable sources, such as CMC is.

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.

3.2. Material and Methods

3.2.1. 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.

3.2.2. 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.

3.2.3. 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.

3.2.3.1. 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 growth chambers under a 12 h photoperiod at 25 °C, 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*) x 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. 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.

3.2.3.2. 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 3.2.3.1).

In the *in vitro* methods, the experimental design was the randomized in factorial scheme (8 x 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 x 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.

3.2.3.3. 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. 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.

3.2.4. 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 minutes 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.

3.2.5. 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. 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 x 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.

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 = more than 81 % of the area with injury), with the results expressed in Disease Index (DI), according to Cia et al. (2010): DI (%)=[(1x$n1$) +(2x$n2$) +(3x$n3$) +(4x$n4$) +(5x$n5$)] x100 / 5 x 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 = $\sum [(y_i + y_{i+1}) / 2 \times (t_{i+1} - t_i)]$, 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.

This experiment was conducted twice and the data referring to AUDPC 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.

3.3. Results

3.3.1. *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).

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]
Eucalyptus staigeriana	0	8.70±0.00 [a]	0.00±0.0 [c]	
	31.0	7.95±0.42 [ab]	8.66±4.8 [bc]	
	62.5	7.86±0.47 [b]	9.66±5.3 [b]	
	125.0	7.42±0.28 [b]	14.73±3.2 [b]	MIC > 500
	250.0	7.62±0.58 [b]	12.43±6.6 [b]	
	500.0	6.22±0.62 [c]	28.48±7.1 [a]	
Lippia sidoides	0.00	8.70±0.00 [a]	0.00±0.00 [d]	
	31.0	7.22±0.90 [b]	16.99±10.3 [c]	
	62.5	5.46±0.45 [c]	37.21±5.2 [b]	
	125.0	0.00±0.00 [d]	100±0.0 [a]	62.5 < MIC ≤ 125
	250.0	0.00±0.00 [d]	100±0.0 [a]	
	500.0	0.00±0.00 [d]	100±0.0 [a]	
P. pesoudocaryophyllus	0.00	8.70±0.00 [a]	0.00±0.0 [e]	
	31.0	8.07±0.46 [ab]	7.30±5.3 [de]	
	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 < MIC ≤ 500
	250.0	4.20±0.24 [d]	51.76±2.7 [b]	
	500.0	0.00±0.00 [e]	100±0.0 [a]	
M1	0.00	8.70±0.00 [a]	0.00±0.0 [c]	
	31.0	7.11±0.79 [ab]	18.26±11.6 [bc]	
	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 < MIC ≤ 500
	250.0	0.97±1.76 [c]	88.84±20.2 [a]	
	500.0	0.00±0.00 [c]	100±0.0 [a]	
M2	0.00	8.70±0.00 [a]	0.00±0.0 [d]	
	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]	

	Conc.	PI	%	
	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]	
M3	0.00	8.70±0.00 [a]	0.00±0.0 [d]	
	31.0	7.09±0.56 [b]	17.84±4.6 [c]	
	62.5	6.48±0.47 [b]	25.27±2.8 [c]	
	125.0	3.61±0.28 [c]	58.45±10.6 [b]	**125 < MIC ≤ 250**
	250.0	0.00±0.00 [d]	100±0.0 [a]	
	500.0	0.00±0.00 [d]	100±0.0 [a]	
M4	0.00	8.70±0.00 [a]	0.00±0.0 [c]	
	31.0	8.42±0.18 [a]	3.18±2.0 [c]	
	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 < MIC ≤ 500**
	250.0	1.33±1.90 [c]	84.68±21.8 [a]	
	500.0	0.00±0.00 [c]	100±0.0 [a]	

[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. 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).

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).

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	

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

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 between 62.5 and 125 µl/L.

3.3.2. 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 (Figures 1C and 1D). In the absence of EO, *R. stolonifer* mycelia exhibited homogeneous and regular tubular hyphae, as well as a smooth and long external surface (Figures 1A and 1B). 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 (Figure 2 A). 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.
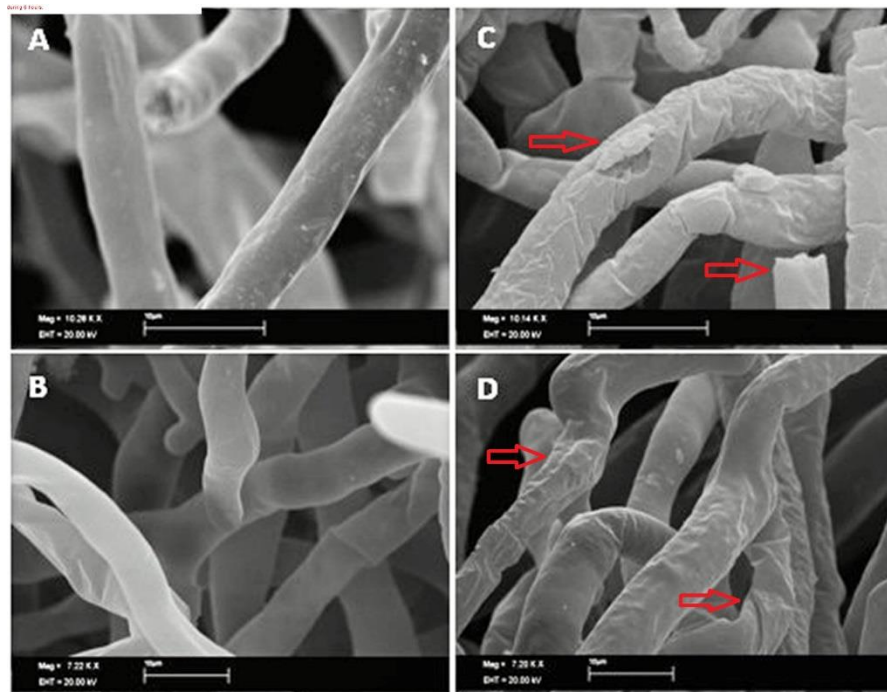
Figure 1 – Scanning electron microscopy (SEM) of the hyphae of *Rhizopus stolonifer* from strawberry, cultivated in potato broth for two days and subjected (C and D) or not (A and B) to *Lippia sidoides.* EO at 125 µl/L after 6 hours. Arrows show the main points of destruction caused in the pathogen hyphae by the EO.
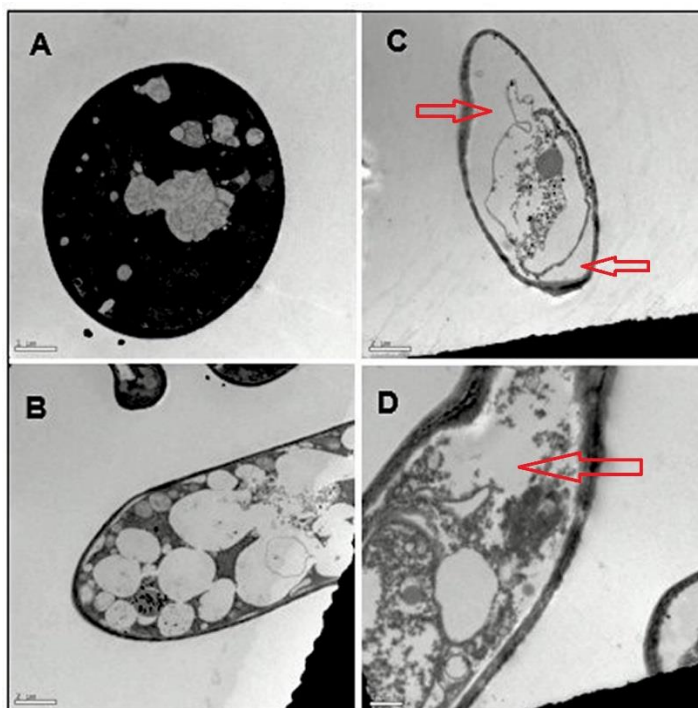
Figure 2 – Transmission electron microscopy (TEM) of the hyphae of *Rhizopus stolonifer* from strawberry, cultivated in potato broth for two days and subjected (C and D) or not (A and B) to *Lippia sidoides* EO at 125 µl/L after 6 hours. Arrows show the main points of destruction caused in the pathogen hyphae by the EO.

3.3.3. 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).

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

Compounds[1]	*L. sidoides*[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.

3.3.4. 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) (Figure 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.
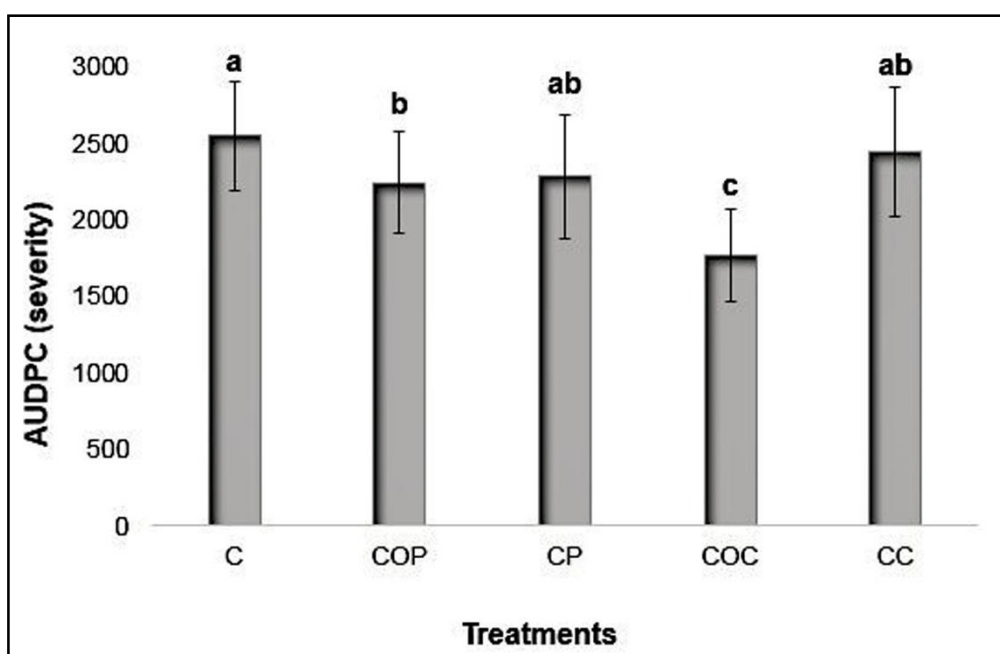
Figure 3 - Area under the disease progress curve (AUDPC) for *Rhizopus* rot severity, caused by *Rhizopus stolonifer*, in "Oso Grande" strawberries.
Distinct letters represent a significant difference among the treatments by the Tukey test (p<0.05). Vertical bars indicate the standard error of the mean (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.

3.4. Discussion

3.4.1. Chemical composition of the essential oil

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 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, probably because the genetic material used for EO extraction was from a variety of *L. sidoides* unable to produce Carvacrol. 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.

3.4.2. Minimal inhibitory concentration (MIC)

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) 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). Nevertheless, 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).

3.4.3. Effects of the essential oil on *R. stolonifer* morphology

The exposure of *R. stolonifer* mycelium to *L. sidoides* EO resulted in significant morphological alterations in the hyphae, such as wilting, rupture, thinning and damages to the intracellular components. 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).

3.4.4. *In vivo* antifungal activity

Disease severity evaluation showed that the fruit treated with the combination of CMC and EO presented lower disease severity, and the curative action was more effective than the preventive one. The coating, although serving as a physical barrier to microbial attacks (Vargas et al., 2008), was not efficient in inhibiting disease progress when applied alone, in other words, without the incorporation of *L. sidoides* EO, as it did not differ from the control treatment. 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 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.

3.5. 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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4. IMPACT OF CARBOXYMETHYLCELLULOSE BASED EDIBLE COATINGS WITH ESSENTIAL OIL ON THE DEVELOPMENT OF *BOTRYTIS CINEREA* AND THE QUALITY OF STRAWBERRIES

Abstract

The antifungal activity of the essential oils (EOs) from *Eucalyptus staigeriana*, *Lippia sidoides* and *Pimenta pseudocaryophyllus* was evaluated *in vitro,* by direct contact on *Botrytis cinerea*, causal agent of gray mold in strawberry*.* The chemical composition of this EO, its effects on pathogen morphology, its activity *in vivo* when associated with carboxymethylcellulose (CMC), and its effects on strawberries postharvest and sensory quality, were also verified. *L. sidoides* EO presented the highest antifungal activity *in vitro*. This EO, rich in thymol, caused dehydration and rupture of the pathogen hyphae. Strawberries treated with CMC associated with *L. sidoides* EO presented a reduction in disease severity, showed improvements in the physicochemical properties during postharvest storage and had the sensory quality little altered.

Keywords: *Lippia sidoides; Pimenta pseudocaryophyllus; Eucalyptus staigeriana;* Antifungal activity; Physicochemical quality; Sensory evaluation

4.1. Introduction

Strawberry is very perishable mainly because it is affected by many fungal diseases. Therefore, delaying microbial deterioration and reducing its metabolic rate can extend its marketable periods, representing a gain for the chain (Feliziani & Romanazzi, 2016; Sangsuwan, Pongsapakworawat, Bangmo, & Sutthasupa, 2016). *Botrytis cinerea* is a necrotrophic pathogen, which secretes phytotoxic metabolites and enzymes, degrading the cell wall, facilitating infection and developing disease in strawberries (Choquer et al., 2007*;* Qin, Xiao, Cheng, Zhou, & Si, 2017). Despite being a classic "high risk" pathogen in the sense of resistance management, its control is mainly performed with synthetic fungicides (Rosslenbroich & Stuebler, 2000). Nevertheless, the decreasing efficacy and the increasing concern with the adverse effects of these products brought the need for the development of alternatives for control and methods for the protection of cultures with or without the reduced use of conventional fungicides (Romanazzi, Smilanick, Feliziani, & Droby, 2016).

EOs become safe and environmentally friendly alternatives, because of their low toxicity and biodegradability (Erland, Bitcon, Lemke, & Mahmoud, 2016). These

compounds are natural antioxidants and rich in monoterpenes, sesquiterpenes, phenylpropanoids, and esters. Nonetheless, their use in fruit can be limited because of their intense aroma, degradation of their volatile compounds under the action of heat, pressure, light and oxygen; and its possible phytotoxic effects depending on the method of application employed, the concentration of the EOs and the perishability of the food (Wuryatmo, Klieber, & Scott, 2003; Mohammadi, Hashemi, & Hosseini, 2015).

To make their application in fruit feasible, they can be associated with edible coatings (Sangsuwan et al., 2016), such as carboxymethylcellulose (CMC), which is derived from cellulose, is soluble in water, non-toxic, and non-allergenic (Tongdeesoontorn, Mauer, Wongruong, Sriburi, & Rachtanapun, 2011), has high permeability to water vapor and does not have antimicrobial properties (Raeisi, Tajik, Aliakbarlu, Mirhosseini, & Hosseini, 2015). These properties can be improved by the incorporation of EO (Dashipour et al., 2015), in other words, a hydrophobic compound that improves the CMC characteristic of selective barrier for the transfer of gas and moisture from the fruit, besides aggregating antimicrobial characteristics to this coating (Dong & Wang, 2017) resulting in quality maintenance and extension of the fruit shelf life (Guerreiro, Gago, Faleiro, Miguel, & Antunes, 2015). Some studies demonstrate the association of the EOs with edible coating as an alternative to increase the shelf life of strawberries (Perdones, Escriche, Chiralt, & Vargas, 2016; Badawy, Rabea, AM El-Nouby, Ismail, & Taktak, 2017). Nevertheless, few studies have been performed with this purpose, combining EOs with edible coatings that are less expensive and derived from abundant and affordable sources, such as CMC, which can be originated from sugarcane bagasse, one of the main by-products of the sugarcane industry.

The antifungal power of different EOs on *B. cinerea* is proven (Li et al., 2016; Liu et al., 2016). Nevertheless, studies on the antifungal activity of the EOs from *E. staigeriana*, *L. sidoides* and *P. pseudocaryophyllus* during postharvest are scarce (Yokomizo & Nakaoka-Sakita, 2014; Herculano, de Paula, de Figueiredo, Dias, & Pereira, 2015). Therefore, this study evaluated the *in vitro* antifungal activity of these EOs on *B. cinerea* by different methods, determining the one with the highest activity. The chemical composition and the effects on pathogen morphology were observed for the EO with the highest activity. The *in vivo* effect of the association of the EO

with CMC, considering the preventive and curative applications and their effects on strawberries postharvest (physicochemical and sensory) quality, were also verified.

4.2. Materials and methods

4.2.1. Acquisition of the EOs

The EOs were extracted from the leaves of *E. staigeriana* (Itatinga - SP, Brazil), of *L. sidoides* (Campinas - SP, Brazil) and of *P. pseudocaryophyllus* (Cananéia - SP, Brazil) by hydrodistillation in a clevenger device, for four hours, at a maximum temperature of 100 °C. Subsequently, the EO was dehydrated in anhydrous sodium sulfate and stored at -5 °C.

4.2.2. Isolation and Molecular identification of *Botrytis cinerea*

B. cinerea was obtained from the direct isolation of fungal structures in strawberries. For the molecular identification, pathogen DNA was extracted according to Doyle and Doyle (1987). The ITS region (Internal Transcribed Spacer region) and part of the gene coding for the translation elongation factor (EF) were amplified by PCRs. For the amplification of the ITS region, the primers ITS1 (5' – TCCGTAGGTGAACCTGCGG – 3') and ITS4 (5' – TCCTCCGCTTATTGATATGC – 3') (White et al., 1990) were used, and for the gene of the elongation factor, primers EF-F (5' – GTYGTYATYGGYCACGTYGAYTC – 3') (De Souza, Pires-Zottarelli, Dos Santos, Costa, & Harakava, 2012) and tef997R (5' – CAGTACCGGCRGCRATRATSAG – 3') (Shoukouhi, 2008) were employed. PCRs were conducted and the sequences of the ITS region were compared to the sequences deposited at the database of GenBank KF859918 *B. cinerea* CBS 131.28 and KU729081 *B. cinerea* ATCC 11542, and for gene EF, to the sequences DQ471045 *B. cinerea* AFTOL-ID 59.

4.2.3. *In vitro* antifungal activity by contact

The EO antifungal activity was initially evaluated by measuring *B. cinerea* mycelial growth inhibition by the direct contact of the fungus with potato dextrose

agar (PDA) culture medium either containing the individual EO or with its binary and ternary mixtures, at concentrations of 31; 62.5; 125; 250 and 500 µl L^{-1} (Plaza, Torres, Usall, Lamarca, & Vinas, 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 PDA, 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 installed. After PDA solidification, *B. cinerea* was transferred to the central point of the Petri dish, from an inoculum suspension containing 10^5 spores mL^{-1}. The plates were maintained in growth chambers at 23 °C with photoperiod of 12 h, and mycelial growth measurements of each colony were taken every two days, in two perpendicular directions (diameter in cm). Mycelial growth inhibition was measured by the formula PI (%) = [(*Growth of the Control - Growth of the Treatment*)/ *Growth of the Control*] x 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 the development of *B. cinerea* visible at naked eye. The data referring to MIC and PI were evaluated with the program Statistical Analysis System model 9.4 (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 experimental design was randomized in factorial scheme, 8 x 5 with eight treatments (Control; *L. sidoides; E. staigeriana; P. pseudocaryophyllus* and the binary and ternary mixtures) and five concentrations, had five repetitions per treatment and was conducted three times.

4.2.4. Effects of the EO on *Botrytis cinerea* morphology

The effect of the EO with the highest antifungal activity on *B. cinerea* morphology was evaluated by Scanning Electron Microscopy (SEM), according to Yu, Wang, Shao, Xu, & Wang (2015), with modifications. For this, 150 mL of potato-dextrose broth (PDB) with the addition of 1 mL of a spore suspension (10^6 spores mL^{-1}) of the pathogen was incubated for five days, at 23 °C and photoperiod of 12 h.

Subsequently, the volume of EO corresponding to the MIC determined in the experiment *in vitro*, by the method of direct contact, was emulsified with soy lecithin and added to the broth, with the incubation continuing for further six hours in the same conditions described. A treatment with PDB without the addition of the EO was used as control. The samples were processed according to Escanferla, Moraes, Salaroli, & Massola Jr (2009), and the observations were performed in a Scanning Electron Microscope LEO 435 (Zeiss, England). This analysis was performed twice, with three repetitions each.

4.2.5. Chemical composition of the EO

The chemical composition was determined only for the EO that presented the best result in the *in vitro* test. The characterization was performed by gas chromatography coupled to mass spectrometry, using the equipment CGMS 2010 (SHIMADZU) and capillary-column gas chromatography diphenyl dimethylpolysiloxane (5 % diphenyl and 95 % dimethylpolysiloxane). The reactions were performed at 50 °C for 1.5 minute, then elevated at 4 °C min^{-1} until 200 °C, followed by 10 °C min^{-1} 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. The 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^{-1}. The mass detector was run on scan mode with a 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 for identification.

4.2.6. *In vivo* antifungal activity

The antifungal effect of the best treatment *in vitro* (by contact) was also evaluated *in vivo*, preventively and curatively, in association with CMC coating, resulting in the treatments: "C" – fruit without the application of CMC and EO; "COP"

– fruit treated preventively with EO + CMC and "COC" – fruit treated curatively with EO + 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, Mygind, & Meyer (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 (Cambuí - MG, Brazil), were selected and sanitized in 2.5 % sodium hypochlorite. For the preventive application, strawberries were immersed for two minutes in CMC associated with EO and, after drying, 30 µL of a *B. cinerea* spore suspension (10^5 spores mL^{-1}) were deposited on a 3-mm deep wound. The fruit were stored for 24 h in a growth chamber at 23 °C and 95 % of relative humidity, under a twelve-hour photoperiod. For the curative application, fungus inoculation was performed 24 h before the application of CMC associated with the EO. The further procedures were the same as described for the preventive application. The control (C) consisted only in the immersion of the strawberries in sterilized distilled water.

The antifungal activity of the treatments was determined by the incidence and severity of the disease in the fruit. Incidence was calculated after the seventh day of storage, from the number of symptomatic fruit in relation to the total number of fruit in each treatment, with the results expressed in percentage (%) (Ali, Wee Pheng, & Mustafa, 2015). Severity was evaluated daily by a scale of scores with six degrees (0 = absence of symptoms; 1 = 1 to 20 % of wounded area; 2 = 21 to 40 %; 3 = 41 to 60 %; 4 = 61 to 80 % and 5 = more than 81 % of the area wounded), with the results expressed in Disease Index (DI) (Cia, Benato, Pascholati, & OLIVEIRA GARCIA, 2010): DI (%)=[(1x$n1$) +(2x$n2$) +(3x$n3$) +(4x$n4$) +(5x$n5$)] x100/5xN, in which ni is the number of fruit infected in the scale of scores and N is the total number of fruit. Based on the DI values over time, in each repetition, the Area Under the Disease

Progress Curve (AUDPC) was calculated for severity (Campbell & Madden, 1990): AUDPC = $\sum$ [(y_{i+} y_{i+1}) / 2 x (t_{i+1} - t_i)], being y_i the DI of the wound at time t_i, y_{i+1}; DI at time, t_i the initial reading time and t_{i+1} the time in days of each read.

The experimental design used was in a 3 x 7 factorial scheme, involving three treatments and seven days of evaluation. Six repetitions of each treatment were used, each repetition composed of 12 strawberries. The experiment was performed twice and the data relative to AUDPC were evaluated by the Statistical Analysis System model 9.4 (Institute, 2010) and subjected to the analysis of variance (ANOVA) for the F test in randomized blocks (each block represented by each of the two repetitions of the experiment *in vivo*). The standard deviation of the means was calculated and the evaluation of the statistical difference among the means of the treatments at the level of significance of 5 % was determined by the Tukey test.

4.2.7. Effect of CMC associated with EO on the postharvest quality of strawberries

In this experiment, the strawberries were visually selected regarding plant health and appearance and sanitized in 2.5 % sodium hypochlorite. The fruit were immersed for two minutes in either sterilized distilled water ("C") or in EO + CMC ("POE"). Subsequently, the fruit were dried at room temperature and placed in polyethylene containers with 150 g of strawberries and stored at 4±1 °C and 90±1 % relative humidity. The fruit were analyzed in terms of their physicochemical, physiological, and appearance characteristics, every three days, starting on the day of experiment setup until eighteen days after refrigerated storage. The experimental design employed was a 2 x 7 factorial scheme, involving two treatments (C and POE) and seven periods of analyses (0, 3, 6, 9, 12, 15 and 18 d) with five repetitions of each treatment.

4.2.7.1. Physicochemical, physiological, and appearance analyses

Pericarp coloration was determined by the parameters Luminosity (L), Hue angle (°Hue) and Chromaticity (Chroma) using a colorimeter (Minolta Chroma Meter - CR-400) with the illuminant "C", in opposite sides of the fruit. The weight loss (WL %)

was calculated by the difference between the initial and final weight of the fruit. Disease index (DI) was evaluated following the method described in item 4.2.6.

For determination of total anthocyanin, a strawberry extract (SE) was prepared by homogenizing 2.5 g of strawberries in 25 mL of 80 % acetone. After 24 hours at 5±1°C, the mixture was centrifuged (12,000 g for 15 min at 4 °C) and the supernatant was kept under refrigeration. For the determination of total phenolic compounds and antioxidant activity, SE was prepared the same way; however, 0.5 g of strawberries were homogenized in 10 mL of 80 % acetone. Total anthocyanin determination (mg cyanidin-3-glucoside 100 g^{-1} of strawberries) followed the method of AOAC (2005). Total phenolic compounds (mg galic acid 100 g^{-1} of strawberries) were determined according to (Singleton & Rossi, 1965). The antioxidant activity (DPPH, µM trolox equivalent g^{-1} of strawberries) was determined folowing the method of Brand-Williams, Cuvelier, & Berset (1995) with modifications.

Respiration (nmol CO_2 kg^{-1} s^{-1}) was evaluated by placing 200 g of strawberries in hermetically closed glass containers that were kept at 4 °C for 1 h. Aliquot samples of the internal atmosphere (1 mL) were collected in triplicate. The analyses were conducted by gas chromatography (Trace 2000 GC, TheAOACrmo Finnigan) coupled to a flame ionization detector (FID), with nitrogen gas used as a carrier (33.3 mL min^{-1}) and column temperature at 200 °C.

For the statistical evaluation of the results, we used the multivariate method Principal Components Analysis (PCA) at SAS 9.3 (Institute, 2010). The data were subjected to normality and homogeneity tests for multivariate treatment analysis.

4.2.8. Effect of CMC associated with EO on the sensory quality of strawberries

The sensory quality of strawberries was evaluated for the same treatments described in item 4.2.7 ("C" and "POE") and also for the fruit immersed in CMC in order to verify if the CMC has any sensory effect on the strawberries.

The Simple Ranking test using the Friedman test (Meilgaard, Civille, Carr, 2007) was performed in a non-controlled environment during the day. The experimental design was the completely randomized 3 x 3, involving three treatments (C, POE, and CMC) and three sessions (1, 6, and 8 days of refrigerated storage).

Each taster received three samples simultaneously and randomly, coded with three-digit numbers. At least 45 non-trained panelists participated on each sensory panel.

The tasters classified the samples based on attributes of appearance (brightness, characteristic color of the strawberry fruit, aroma ("strawberry" and "weird") and overall preference. The lowest classification (=1) corresponded to the less intense attribute or the less preferable sample, and the highest classification (=3) corresponded to the most intense attribute or the most preferable sample. The tasters attributed only one sample for each classification. The sum of classifications was calculated, and data were analyzed by the Friedman test (Meilgaard, Civille, Carr, 2007).

4.3. Results

4.3.1. *In vitro* antifungal activity of the EOs

L. sidoides EO applied individually presented the highest antifungal activity, with total *B. cinerea* inhibition at the concentrations between 62.5 and 125 µl L^{-1} (Table 1). The mixtures that contained this EO (M1 and M3) also presented high inhibition rates. *P. pseudocaryophyllus* EO and the other mixtures also presented a dose-dependent antifungal activity, but the total pathogen inhibition was only possible at 500 µl L^{-1}. *E. staigeriana* EO presented a low potential for pathogen control, with a maximum inhibition of 4.6 % observed.

Table 1 – Mycelial growth (MG), in cm, percentage of mycelial growth inhibition (PI) of *Botrytis cinerea* isolated from strawberry, and minimum inhibitory concentration (MIC) at seven days of incubation, after the exposure by contact at different concentrations (μl L^{-1}) of essential oils incorporated to the medium PDA (mean values ± SD, n = 10).

Treatments	Concentrations (μl L^{-1})	MG (cm)	PI[1] (%)	MIC[2]
Eucalyptus staigeriana	0.0	8.54±0.00[a]	0.00±0.0[b]	
	31.0	8.29±0.34[a]	0.64±1.3[a]	
	62.5	8.28±0.40[a]	3.03±4.6[ab]	
	125.0	8.36±0.41[a]	2.10±4.6[ab]	**MIC> 500**
	250.0	8.38±0.34[ab]	1.80±4.0[a]	
	500.0	8.14±0.55[b]	4.60±6.4[a]	
Lippia sidoides	0.0	8.54±0.00[a]	0.00±0.0[d]	
	31.0	4.07±1.08[b]	52.32±12.6[c]	
	62.5	1.51±0.60[c]	82.34±7.1[b]	
	125.0	0.00±0.00[d]	100±0.0[a]	**62.5 < MIC ≤ 125**
	250.0	0.00±0.00[d]	100±0.0[a]	
	500.0	0.00±0.00[d]	100±0.0[a]	
Pimenta pesoudocaryop hyllus	0.0	8.54±0.00[a]	0.00±0.0[d]	
	31.0	8.30±0.39[b]	0.00±0.0[d]	
	62.5	8.32±0.31[c]	1.04±0.7[dc]	
	125.0	7.55±0.72[d]	11.58±8.4[bc]	**250 < MIC ≤ 500**
	250.0	6.10±0.38[e]	28.59±4.48[b]	
	500.0	0.00±0.00[f]	100±0.0[a]	
M1	0.0	8.54±0.00[a]	0.00±0.0[c]	
	31.0	8.21±0.50[b]	0.00±0.0[c]	
	62.5	7.10±1.19[c]	21.09±11.8[b]	
	125.0	2.61±0.17[d]	69.38±2.0[a]	**125 < MIC ≤ 250**
	250.0	0.00±0.00[e]	100±0.0[a]	
	500.0	0.00±0.00[e]	100±0.0[a]	
M 2	0.0	8.54±0.00[a]	0.00±0.0[d]	
	31.0	7.45±0.51[b]	12.12±5.4[d]	**250 < MIC ≤ 500**

	62.5	7.00±0.84[c]	17.99±9.8[d]	
	125.0	4.99±0.48[d]	41.55±5.6[c]	
	250.0	4.02±0.47[e]	52.86±5.6[b]	
	500.0	0.00±0.00[f]	100±0.0[a]	
M3	0.0	8.54±0.00[a]	0.00±0.0[e]	
	31.0	7.28±1.07[b]	9.91±1.5[d]	
	62.5	3.53±0.35[c]	58.59±4.2[c]	
	125.0	2.20±0.46[d]	74.20±5.5[b]	**125 < MIC ≤ 250**
	250.0	0.00±0.00[e]	100±0.0[a]	
	500.0	0.00±0.00[e]	100±0.0[a]	
M4	0.0	8.54±0.00[a]	0.00±0.0[c]	
	31.0	6.40±0.88[b]	24.71±8.3[b]	
	62.5	5.56±0.92[b]	34.81±10.8[b]	
	125.0	2.96±0.59[c]	65.26±6.9[a]	**250 < MIC ≤ 500**
	250.0	0.66±0.62[d]	92.27±7.3[a]	
	500.0	0.00±0.00[d]	100±0.0[a]	

[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. 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. Distinct letters represent a significant difference between concentrations of treatments by the Tukey test ($P < 0.05$). The original data (X) of some variables were transformed by the equations: *E. staigeriana* - CM and PI by X^3 and log X, respectively; *L. sidoides* - CM and PI by X^{-3} and $X^{0,5,}$ respectively; *P. pseudocaryophyllus* - CM by X^{-2}; M1 -CM and PI by X^{-3} and $X^{0,5,}$ respectively; M2 and M3 – CM by X^{-3}; M3 – PI by $X^{0,5}$.

4.3.2. Effects of the EO on the morphology of *Botrytis cinerea*

In the absence of EO, the mycelia of *B. cinerea* exhibited homogeneous and regular tubular hyphae, besides a smooth and long external surface (Figure 1A). The opposite occurred in the hyphae exposed to the EO of *L. sidoides*, which presented structural alterations such as superficial wrinkles, desquamation, distortion and destruction (Figure 1B).
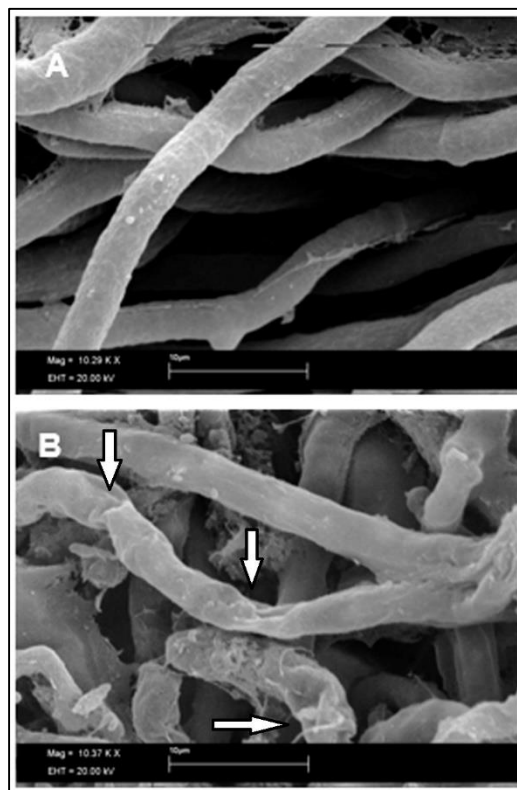
Figure 1 – Scanning electron microscopy (SEM) of the hyphae of *Botrytis cinerea* from strawberry, cultivated in potato broth for five days and subjected (B) or not (A) to *Lippia sidoides* EO at 125 µl L^{-1} after six hours. Arrows show the main points of destruction caused in the pathogen hyphae by the EO.

4.3.3. Chemical composition of the EO

The chemical composition was performed for the EO of *L. sidoides*. In this oil, 16 compounds had area with chromatographic peaks higher than or equal to 0.5 % of the total area of the peaks present in the chromatogram. The major compound present was thymol (58.8 %), followed in decreasing order by p- cimene and β-caryophylline, with 10.2 and 8 % of area, respectively (Table 2).

Table 2 – Chemical composition of the EO extracted from *Lippia sidoides* leaves.

Compounds[1]	*L. sidoides*[2] (%)	LRI[3]
α- thujene	1.15	930
Myrcene	2.00	995
α-terpinene	1.72	1021
p-cymene	10.02	1029
1.8 cineol	1.36	1036
γ-terpinene	5.76	1063
Ipsdienol	1.00	1154
Terpene-4-ol	0.58	1184
UC	0.51	1186
Ether methyl thymol	1.86	1240
Thymol	58.18	1304
β- caryophyllene	8.00	1431
Aromadendrene	0.50	1450
Biciclogermacrene	1.24	1508
δ-cadinene	0.54	1533
Oxide of caryophyllene	1.17	1597

[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. UC = unidentified compounds.

4.3.4. *In vivo* antifungal activity

Disease severity was lower in strawberries treated with EO associated with CMC, confirming the antifungal activity of the EO in the *in vivo* test, either when applied preventively or curatively (Figure 2). The incidence of gray mold reached 100 % on the seventh day of evaluation in all treatments, without statistical difference among them.
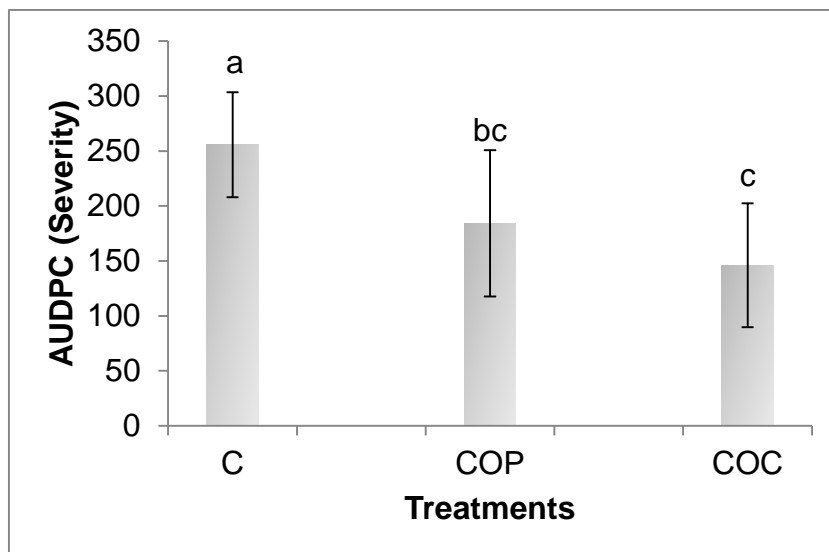
Distinct letters represent a significant difference among the treatments by the Tukey test (P <0.05). Vertical bars indicate the standard error of the mean (number of repetitions used in the experiment =12). C: fruit without the application of CMC and EO; COP: fruit treated preventively with CMC and EO; COC: fruit treated curatively with CMC and EO.

Figure 2 - Area under the disease progress curve (AUDPC) for gray mold severity, caused by *Botrytis cinerea*, in "Oso Grande" strawberries.

### 4.3.5. Physicochemical, physiological, and appearance analyses

The Principal Component Analysis extracted three main components from the total data set (Table 3), explaining 82.83% of the total variance.

Table 3 - Total data group (mean, ± standard deviation, n = 5)

Treatments	Storage period (Days)						
	0	3	6	9	12	15	18
Luminosity							
C	34.35 ± 1.03	34.19 ± 0.97	33.02 ± 2.01	33.59 ± 1.36	33.43 ± 1.13	35.47 ± 1.78	33.78 ± 1.69
POE	32.80 ± 1.75	33.65 ± 1.30	32.06 ± 0.77	35.30 ± 1.08	35.22 ± 1.06	34.87 ± 0.93	35.90 ± 0.68
Hue Angle (degrees)							
C	37.35 ± 0.94	37.15 ± 2.30	33.81 ± 2.29	34.75 ± 2.09	34.85 ± 1.28	36.84 ± 2.40	37.50 ± 2.85
POE	35.05 ± 1.57	37.04± 1.85	34.23 ± 1.54	36.71 ± 1.79	35.95 ± 1.37	35.99 ± 1.74	35.85 ± 1.67
Chromaticity							
C	43.03 ± 0.70	42.15 ± 1.47	39.99 ± 2.42	40.75 ± 1.67	41.18 ± 1.37	41.79 ± 1.04	38.88 ± 1.69
POE	38.87 ± 1.25	39.92 ± 1.64	37.49 ± 0.82	40.65 ± 1.25	40.14 ± 0.96	39.72 ± 0.93	38.87 ± 0.70
Weight Loss (%)							
C	0.00±0.00	2.29±0.32	5.94±1.49	8.44±1.77	10.84±2.05	13.36±2.49	15.40±2.24
POE	0.00±0.00	2.79±1.84	5.56±2.26	7.32±2.32	9.47±2.78	13.55±2.68	13.71±3.50
Disease Index (%)							
C	0.00±0.00	0.00±0.00	1.13 ± 1.03	2.33 ± 2.21	3.89 ± 2.49	4.29 ± 2.26	5.45 ± 3.01
POE	0.00±0.00	0.00±0.00	0.36 ± 0.81	0.36 ± 0.81	0.36 ± 0.81	1.09 ± 2.44	2.91 ± 2.44
Anthocyanin (mg cyanidin-3-glucoside 100 g-1 of strawberry)							
C	2.41±0.30	2.33±0.47	2.67±0.46	2.90±0.49	2.54±0.30	2.07±0.22	2.33±2.61
POE	3.18±0.48	2.08±0.56	2.29±0.14	1.97±0.41	2.38±0.28	2.36±0.27	1.90±0.66
Phenolic Compounds (mg gallic acid 100 g-1 of strawberry)							
C	1.57 ± 0.36	1.80 ± 0.22	1.45 ± 0.23	1.48 ± 0.18	1.11 ± 0.16	1.52 ± 0.28	1.68 ± 0.20
POE	1.91 ± 0.35	1.98 ± 0.13	1.62 ± 0.19	1.66 ± 0.16	1.33 ± 0.26	2.02± 0.34	1.83 ± 0.11
DPPH (µM trolox equivalent g-1 of strawberry)							
C	24.71±3.25	26.09±2.59	25.41±2.90	21.69±1.84	24.84±2.08	20.22±4.21	23.24±3.56
POE	28.96±0.71	28.42±0.78	27.22±1.34	24.04±1.49	26.40±3.12	27.36±1.57	23.91±1.50
Respiration (nmol CO2 kg-1 s-1)							
C	0.47±0.03	0.28±0.08	0.16±0.08	0.16±0.01	0.14±0.04	0.31±0.04	0.26±0.05
POE	0.37±0.04	0.31±0.04	0.16±0.01	0.16±0.00	0.13±0.00	0.24±0.03	0.18±0.05

n= number of repetitions used in the experiment.

The first principal component (PC1) explained 35.88 % of the statistical variance and was positively correlated with DPPH, and negatively correlated with Disease index and weight loss. The second principal component (CP2) explained 29.22 % of the statistical variance and was positively correlated with L and °Hue, and negatively correlated with anthocyanin. The third principal component (CP3) explained 17.73 % of the statistical variance and was positively correlated with phenolic compounds, and negatively correlated with chromaticity. Respiration was not correlated in the PCs because it did not obtain relevance in the scientific basis of the results (Figure 3).
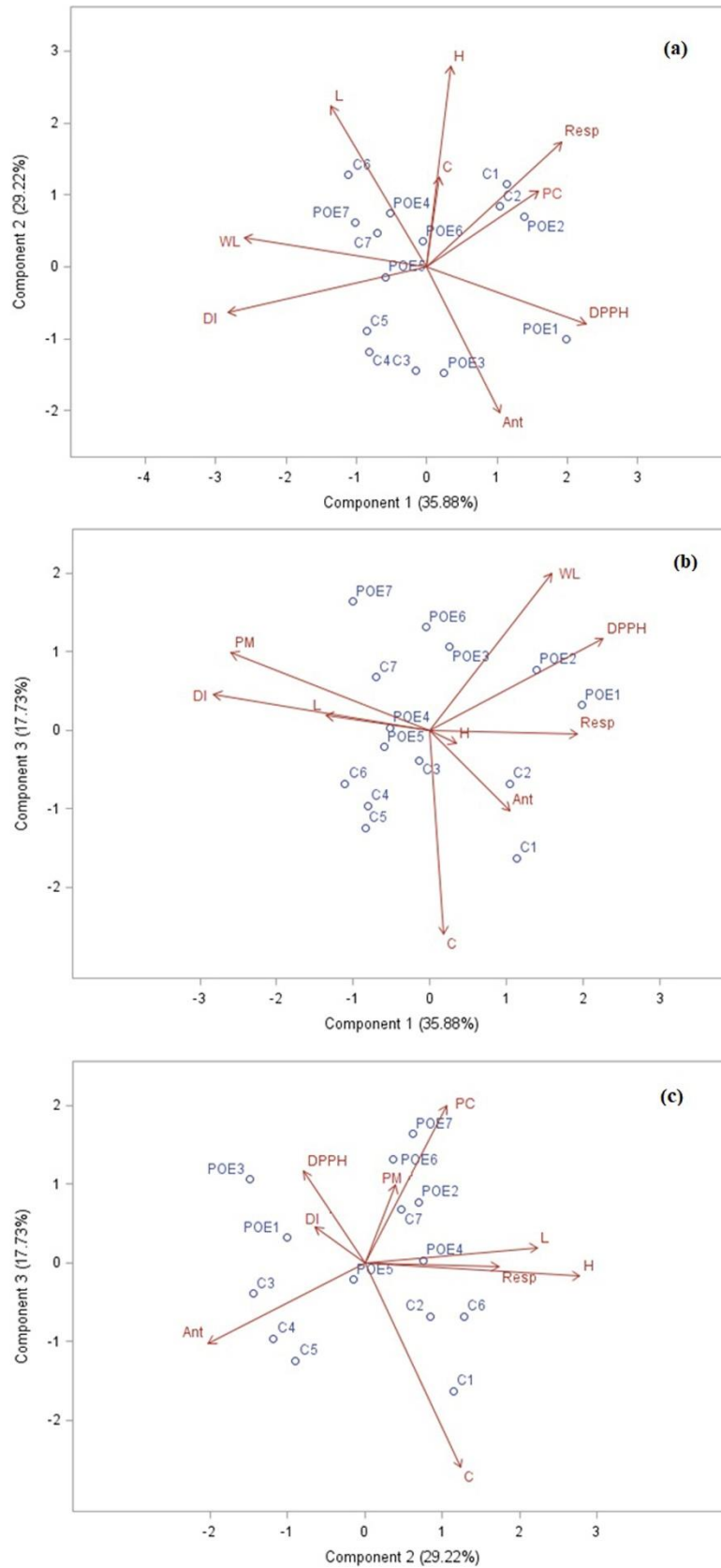
Component 1 = Principal Component 1; Component 2 = Principal Component 2; Component 3 = Principal Component 3. Variables: C = Chromaticity; L = Luminosity; Resp = Respiration; H = Hue angle; WL = Weight loss; PC = Phenolic Compounds; DPPH = Free-radical-scavenging activity by 2,2-diphenyl-picryl-hydrazil; Ant = Anthocyanin Content; DI = disease

index. Observations: C1 = control treatment analyzed on the same day of treatment application; C2; C3; C4; C5; C6, and C7 = strawberries stored for 3, 6, 9, 12, 15, and 18 d, respectively, after treatment application; POE1 = fruit treated with *L. sidoides* EO associated with CMC analyzed on the same day of treatment application.; POE2; POE3; POE4; POE5; POE6, and POE7 = strawberries treated with *L. sidoides* EO associated with CMC and stored at (temperature) for 3, 6, 9, 12, 15, and 18 d, respectively, after treatment application.
Figure 3 - Graph of the distribution of the cloud of variables and observations obtained in the Principal Components Analysis of strawberry postharvest parameters.

The variable DPPH was the most important in PC1, in which the observations POE1, POE2 and POE3 presented the most elevated means, followed by C1, C2 and C3, indicating that the EO increased the antioxidant activity. Observations POE4 and POE5 were similar to C4 and C5, with levels close to the general mean of antioxidant activity (DPPH). We have verified that the strawberries stored for a longer period (POE6, POE7, C6 and C7) presented a reduced antioxidant activity; nevertheless, those which contained EO were characterized by higher means.

Another variable of importance in CP1 was WL, in which the strawberries that contained EO (POE2, POE3) were characterized by a lower mass loss until six days of storage when compared to the control strawberries, since on the third day (C2), they presented a similar WL to the strawberries with EO at 6 days of storage. Therefore, the film with EO had a positive action on fruit quality and time for commercialization. Additionally, the strawberries treated with EO were characterized by lower DI during storage, indicating that the EO reduced the incidence of fungal diseases.

The variable °Hue had the highest importance in PC2. In the beginning of the storage period, the strawberries containing EO (POE1 and POE3) were characterized by color tone tending to red, whereas the tone of the control samples (C1 and C2) tended to a reddish orange. Regarding Anthocyanins, the strawberries treated with EO in the first and third periods of storage (POE1 and POE3) were characterized the highest contents of the pigment. From the fifth period of analysis, the anthocyanin content of the strawberries was similar among the treatments. It is possible to identify in the Graph of the distribution of the cloud of variables and observations (Figure 3a) a strong and inverse correlation between the variables °Hue and Anthocyanins, indicating that in the strawberries with high anthocyanin contents, the color tone will tend to red (lower °Hue). The results of the analysis of Luminosity are opposite to those observed for anthocyanins and °Hue, in which the strawberries

treated with EO were characterized by a lower L, indicating the presence of anthocyanin pigments.

The variable Chromaticity was represented in PC3 (Figure 3 c), and shows us that the control strawberries presented a more saturated color along the storage. In relation to the variable Phenolic compounds (PC), we have verified that the CMC coating with EO maintained these compounds, since the strawberries of this treatment were characterized by a higher amount of PC.

These results indicate that the coating based on CMC associated with *L. sidoides* EO exerted a positive effect on the maintenance of the quality of the strawberries along the refrigerated storage.

4.3.6. Effect of CMC associated with EO on the sensory quality of strawberries

Regarding the appearance attributes, samples POE and CMC presented a more intense brightness than the control ones (P <0.05) on the first day of evaluation (Table 4). Samples POE and CMC were classified with a characteristic color of strawberry that was more intense until the sixth day of storage, because of the presence of CMC, which might have preserved the original color of the fruit. The more intense characteristic color of strawberry and brightness may indicate fruit freshness, with *L. sidoides* EO helping in the improvement of the fruit appearance.

The tasters have noticed a different aroma in the strawberry samples treated with *L. sidoides* EO, which were classified as having a less intense strawberry aroma (Table 4). Furthermore, in the evaluation of the attribute weird aroma, these strawberries had a weird aroma with the highest intensity (P <0.05) during the eight days of refrigerated storage.
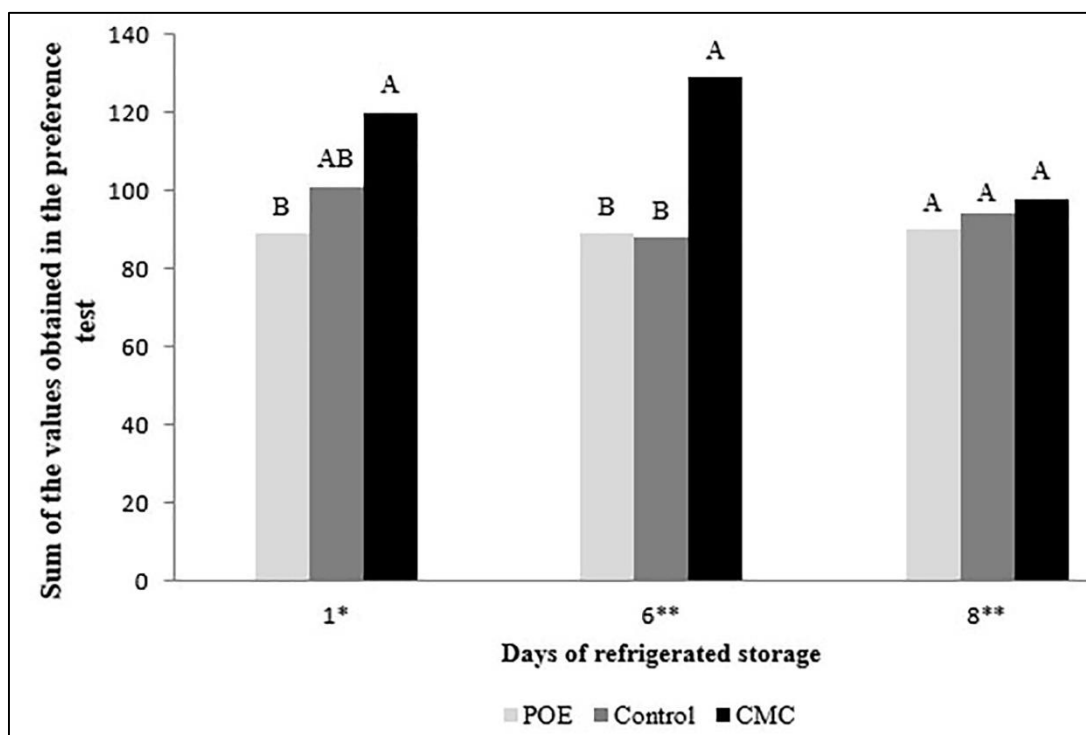
Table 4 – Sum of the values obtained in the test related to the sensory attributes of strawberries treated with CMC, *L. sidoides* EO associated with CMC, or non-treated control stored at 4±1 °C for one, six, and eight days.

Attributes	Treatments	1 d*	6 d**	8 d***
Appearance: Brightness	Control	78 B	83 B	91 A
	CMC	122 A	118 A	104 A
	POE	112 A	105 B	93 A
Appearance: Characteristic color of strawberry	Control	83 B	70 B	95 A
	CMC	122 A	123 A	93 A
	POE	108 A	113 A	101 A
Aroma: "strawberry"	Control	114 A	97 B	109 A
	CMC	117 A	127 A	102 A
	POE	82 B	80 B	69 B
Aroma: "weird"	Control	88 B	107 A	80 B
	CMC	91 B	81 B	77 B
	POE	132 A	112 A	119 A

Sum values followed by different letters in the same column indicate significant difference ($P < 0.05$) among treatments, according to the Friedman method.
* Sum of 52 evaluation judgements; ** Sum of 51 evaluation judgements.; *** Sum of 48 evaluation judgements.

The most described terms for weird aroma present in the strawberries treated with EO were "of a chemical product", "undefined", "of a strawberry that is too ripe", "of medicine", "artificial" and "of eucalyptus". Although the tasters have noticed a different aroma in the strawberry samples in which the EO was applied, at eight days of refrigerated storage this sample had a similar preference to the others ($P < 0.05$) (Figure 4).

Control – strawberries immersed in sterile distilled water; CMC – strawberries treated with carboxymethylcellulose; POE – strawberries treated with *L. sidoides* EO associated with CMC. Sum values with different letters for the same period (storage period, in days) indicate significant difference (P < 0.05) among treatments, according to the Friedman method.
* Sum of 52 evaluation judgements; ** Sum of 51 evaluation judgements; *** Sum of 48 evaluation judgements.
Figure 4 - Sum of the values obtained in the test related to the sensory attributes of strawberries treated with CMC, *L. sidoides* EO associated with CMC, or non-treated control stored at 4±1 °C for eight days.

4.4. Discussion

4.4.1. *In vitro* antifungal activity

L. sidoides EO, which presented the highest antifungal activity in the *in vitro* test, had in its composition elevated contents of thymol, which is a phenolic compound that belongs to a class of natural antioxidants and which can act on the fungal cell wall (Chavan & Tupe, 2014). Nevertheless, EOs are a complex mixture of active chemical compounds, which might present a synergistic effect of their components, this synergism being then responsible for their activity (Khoury et al., 2014). EOs can an alteration in the permeability and integrity of cell membranes, leading to the release of nucleic acids and proteins, affecting the growth and shape of the pathogens (Guo et al., 2017).

The antifungal potential of *L. sidoides* EO on pathogens, including those of postharvest, has been proven in other studies (Aquino, Sales, Soares, Martins, & Costa, 2014; Zillo, da Silva, de Oliveira, da Glória, & Spoto, 2018; Oliveira et al., 2019). Therefore, this EO has the potential either for isolated use or for the development of new formulations for the control of numerous pathogenic fungi. The antifungal activity of *E. staigeriana* EO, despite having been positive in other filamentous fungi (Herculano et al., 2015), still had not been evaluated for *B. cinerea* and presented a low inhibition rate (4.6 %).

4.4.2. Effects of the EO on *Botrytis cinerea* morphology

The alterations of the fungus in contact with the EO suggest that the action of the EO might include an attack to the cell wall, with a resulting loss of the ability of pathogenic infection and disease progress. *L. sidoides* EO is rich in thymol, a compound that can alter the structure of the fungal cell wall and facilitate ion exchange, increasing its permeability and hampering cell survival, since it affects essential processes (Sharma & Tripathi, 2008). These alterations were similar to those observed in studies conducted with *Rhyzopus stolonifer* and *Botrytis cinerea* exposed to EO(s). EOs can act in different ways depending on the pathogen, interfering in microbial respiration, reducing the total contents of lipids and ergosterol of the cell membrane, which perform important functions, including its stability and fluidity, cell growth and adhesion and its permeability (Soylu, Kurt, & Soylu, 2010; Tao, Jia, & Zhou, 2014; Li et al., 2016).

4.4.3. Chemical composition of the EO

L. sidoides EO presented thymol as major compound (58.18 %), followed by p-cimene (10.20 %). Many studies exhibited thymol as a major compound in the EO of *L. sidoides* from several locations of Brazil, with concentrations varying between 30.24 and 84.09 % (Marco et al., 2012; Aquino et al., 2014; Oliveira et al., 2019). Carvacrol, which is an isomer of thymol, has also been highly observed in some varieties of *L. sidoides*, as a major component of the EO of this species (Guimarães, Cardoso, Souza, Zacaroni, & Santos, 2014). However, it was not observed in this

work, probably because the material used for EO extraction was from a variety without the ability of production of this compound.

4.4.4. *In vivo* antifungal activity

The fruit treated with EO presented lower disease severity. The incorporation of EOs to polymer matrices reduce the diffusion of antimicrobial agents, maintaining the proper concentration of active compounds on the surface of the fruit, with a consequent rise in antimicrobial activity and extension of fresh products shelf life (Bautista-Baños, Sivakumar, Bello-Pérez, Villanueva-Arce, & Hernández-López, 2013).

The reduction in the severity of postharvest diseases in strawberries treated with coatings associated with EOs has also been observed by other authors *(Perdones et al., 2016;* Dong & Wang, 2017; Oliveira et al., 2019). The efficacy of EOs in the control of postharvest decay can be improved by the combination of other postharvest treatments in controlled conditions. The method of application must also be considered, and according to Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer (2011), the application of coatings associated with EO by immersion is more beneficial than the applications by pulverization, since there is a reduction in the inoculum of the postharvest pathogens present in the fruit surface.

Although disease severity was lower in the fruit subjected to CMC associated with EO, disease incidence was high in all treatments, because the experiments were performed under favorable conditions of inoculum pressure for ripe fruit, and without the use of low temperatures, providing ideal conditions for disease development. Furthermore, EOs are less efficient when applied to the food than when evaluated *in vitro*. For instance, the EO from thyme inhibited the growth of *Alternaria alternata in vitro*, but only reduced its growth in cherry tomatoes (Feng, Chen, Zheng, & Liu, 2011). Similar results were reported for citric EO against the same pathogen in tomatoes, with the growth *in vitro* completely inhibited, but not when assayed *in vivo* (Phillips, Laird, & Allen, 2012). The complexity of the food matrix, which is an environment rich in nutrients, provides an excellent means for fungal growth, as well as the repair and regeneration of fungal cell components (Espitia et al., 2012).

Consequently, it can be expected that *B. cinerea* exhibits a lower sensitivity to *L. sidoides* EO on the surface of the strawberries.

4.4.5. Physicochemical, physiological, appearance and sensory analyses

Mass loss, which has as main causes respiration and water evaporation by the epidermis, was lower in treatment POE. The coatings act as a semipermeable barrier against the loss of moisture, reducing mass loss in fruits (Guerreiro et al., 2015). The addition of EO to CMC decreases the hydrophilic nature of CMC, since it produces an effect in the coating reticulation, which can reduce the availability of hydroxyl groups for the interaction of with water molecules, making it more resistant to water (Dashipour et al., 2015; Dong & Wang, 2017). In relation to fruit sanity, several studies show a lower disease index in fruit treated with coatings associated with EOs, which is in agreement with our results and emphasizes the antimicrobial activity of these compounds (Mohammadi et al., 2015; Khalifa, Barakat, El-Mansy, & Soliman, 2016). The incorporation of the EOs in polysaccharide coatings can increase the shelf life of the fresh product, since the compounds released from the EOs are more adequately concentrated on its surface, with a consequent increase in the antimicrobial activity of these coatings (Sivakumar & Bautista-Baños, 2014). In this context, the application of CMC incorporated with *L. sidoides* EO in papayas was effective in the rise of their shelf life, with the preservation of their postharvest characteristics (Zillo et al., 2018). Regarding the attribute phenolic compounds, the reduction in their content until the sixth storage period in both treatments might have occurred because of the break in the cell structure derived from the natural senescence of the fruit (Gol, Patel, & Rao, 2013). Furthermore, the application of a coating enriched with antimicrobial agents, such as the EOs, can be considered a stress for the fruit (Peretto et al., 2017); thus, the rise or reduction of the antioxidant compounds during storage might depend on the severity of this stress, time and storage conditions. Nonetheless, in all periods, the strawberries treated with POE presented the highest values of phenolic compounds in relation to the other treatments, which can be justified by the fact that the CMC associated with EO forms a compact coating, which has probably protected the fruit, acting against the oxygen supply, thus avoiding the enzymatic oxidation of these compounds (Aminifard &

Mohammadi, 2013). In other studies involving fruits with edible coatings associated to EOs, a higher concentration of phenolic compounds in relation to the coating without the addition of these components was also observed (Dashipour et al., 2015; Shao et al., 2018).

The antioxidant activity was higher in the POE strawberries. The antioxidant activity of the EOs is mainly because of their phenolic compounds (Tongdeesoontorn et al., 2011). The phenolic compounds of *L. sidoides* EO, such as thymol, γ-terpinene and p-cymene, are capable of extinguishing the free radicals (Dimitrios, 2006; Hadian, Ebrahimi, & Salehi, 2010). Additionally, edible coatings with the addition of these compounds have their antioxidant activity related to the concentration of the EO present (Embuscado & Huber, 2009; Shojaee-Aliabadi et al., 2013). Dashipour et al. (2015) and Shojaee-Aliabadi et al. (2013) observed, among the different concentrations of EOs incorporated to edible coatings, an increase in the antioxidant activity of the coating according to the rise in the amount of the EO incorporated to the coating.

The lower luminosity observed in treatment POE can be explained by the change in the surface reflection properties when the fruit is coated, and CMC associated with EO is an opaque coating. The same was reported by (Vargas, Albors, Chiralt, & González-Martínez, 2006), in which the addition of oleic acid to the chitosan coating led to a luminosity reduction in strawberries. The color of strawberry is a quality attribute and indicative of consumer acceptability. In this sense, the instrumental analysis evidenced that the color tone (°Hue) of treatment POE tended to red, whereas that of the control tended to a reddish orange. This effect was also evidenced in the analysis of anthocyanins, in which the coated fruit (POE) had the highest values of this reddish pigment at 6 days of storage. The same was verified in the results if the sensory evaluation, in which the POE strawberries had a more intense color. Furthermore, this sensory attribute can be confirmed by the values of Chromaticity (color intensity), which were also higher for this treatment. However, Dong & Wang (2017) observed an opposite action, since the incorporation of garlic EO to the CMC coating delayed the accumulation of anthocyanins in strawberries.

The tasters identified a "weird" aroma and not that characteristic of strawberry in the samples that contained EO, which can be justified by the aromatic profile of this species of EO, which is characterized by phenols, aromatic and non-aromatic monoterpenes such as thymol and its biosynthetic precursors p-cymene

and γ-terpinene, respectively. Nevertheless, this differentiated aroma given by the EO did not impact the preference for this sample by the tasters.

4.5. Conclusions

L. sidoides EO presented presents the highest capacity of *B. cinerea* growth inhibition *in vitro* when applied by direct contact, causing the morphologic degeneration of the hyphae, suggesting its action on the fungal cell wall. The incorporation of this EO to CMC reduces disease severity in strawberries. Thus, the association of *L. sidoides* EO with CMC coating can be a potential alternative to the synthetic fungicides for the postharvest control of gray mold, caused by *B. cinerea* in strawberries, maintaining their physicochemical characteristics for eighteen days under refrigerated storage, improving fruit color, and preserving the sensory quality of fruit, with the exception of aroma.

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5. *IN VITRO* AND *IN VIVO* ANTIFUNGAL ACTIVITY OF THE FORMULATION 'ACQUATIV AGRO' AGAINST STRAWBERRY-DETERIORATING PATHOGENS IN THE POSTHARVEST.

Abstract

Strawberry, a pseudofruit with importance in several countries, presents a short postharvest shelf life, mainly because of the occurrence of diseases. With the absence of antifungals registered in the market for the postharvest application in this fruit, and because of the emergence of pathogen strains that are resistant to many active principles, alternative formulations have been developed that are less aggressive to the health and environment. Therefore, this study investigated the antifungal potential of the pre-commercial formulation from Orion Industrial Ltda. named 'Aquativ Agro' against *Colletotrichum acutatum*, *Botrytis cinerea* and *Rhizopus stolonifer.* The efficiency of the product *in vitro* was evaluated by the method of dilution in Potato-Dextrose-Agar, with fungal inhibition measured with 0; 31; 62.5; 125; 250 and 500 µl/L of the product, determining the minimum inhibitory concentration. Its capacity of control *in vivo* was evaluated in artificially inoculated 'Oso Grande' strawberries after immersion in the product. The action of the product on pathogen morphology was observed by scanning electron microscopy. 'Aquativ Agro' presented a dose-dependent antifungal activity, with 500 µl/L completely inhibiting the mycelial growth of *Colletotrichum acutatum* and *Botrytis cinerea*. *In vivo*, it was capable of reducing the severity of the diseases anthracnose and soft rot. The product caused wilting and rupture of the hyphae of the pathogens, suggesting action in the cell wall, resulting in a decrease in the efficacy of infection establishment and, consequently, disease progress. Thus, the formulation 'Aquativ Agro' can represent and innovative and efficient strategy for the control of these postharvest diseases in strawberries.

Keywords: Alternative antifungals; Organic salts; Organic acids; Essential oil

5.1. Introduction

The postharvest diseases, caused by fungal pathogens, are the main causes of economic losses in the global industry of fresh fruits and vegetables (Palou, Ali, Fallik, & Romanazzi, 2016). The high economic value of the world trade in this sector, coupled to the important problems related to the conventional fungicides, make the development of physical, biological and chemical methods innovative and ecologically correct for the control of postharvest diseases, becoming an active research field in several public sectors and private institutions worldwide. The adaptability of the pathogenic agents, leading to the resistance to fungicides, as well as the growing public concern about the negative side effects of the chemical

products in the environment and in human health, contribute to increase the demands of the consumers and employees to reduce the use of toxic synthetic fungicides (Rotolo et al., 2018).

As an alternative to the treatments with fungicides, the use of food preservatives is a trend for the control of plant pathogens. These agents are natural or synthetic compounds with known toxicity or low toxicity and classified as food quality additives or compounds generally recognized as safe (GRAS) (Karaca, Pérez-Gago, Taberner, & Palou, 2014). Among them, the most important are the inorganic or organic salts (carbonates, sorbates, benzoates, paraben salts, etc.) and edible coatings formulated with antifungal ingredients. Hydrocolloids (polysaccharides, such as cellulose derivatives, alginates, pectins or gums, and several vegetable proteins) and food-grade lipids are the main components of the matrix of coatings, since the antifungal ingredients included as GRAS are salts, essential oils and antagonistic microorganisms (Palou et al., 2016).

Because of the low general toxicity of these compounds, the application of these chemical alternatives alone might not always provide a commercially acceptable level of control of the postharvest diseases comparable to that obtained with conventional toxic fungicides. For this reason, the compounds with potential for isolated treatments are increasingly more evaluated and recommended, in combination with other ingredients and postharvest treatments of the same nature or different, as part of the control strategies (Palou et al., 2016). The lack of residual effect to protect the fruit against subsequent infections, the risks of adverse effects on fruit quality during the long-term storage and, above all, the lower efficacy and persistence of the treatments are general disadvantages of the treatments with only GRAS salt solutions in comparison with conventional fungicides (Usall et al., 2008).

In strawberries, the contamination by fungi is especially dangerous in the packing-houses during the procedures of storage, transport and commercialization, since even a small number of infected fruit can spread the disease to adjacent healthy strawberries. The presence of fungal diseases on the surfaces of the fruit leads to food sensory defects, such as visual deterioration, and noticeable changes of aroma, flavor or texture, besides reducing the safety of the final product, since some fungal genera and species can produce mycotoxins, toxic substances to humans (Sallas et al., 2017).

Among the diseases of great importance for the strawberry culture is anthracnose, which can be caused by several fungal species of the genus *Colletotrichum,* with *C. acutatum* being one of the most detrimental from an economic point of view (Zhang et al., 2016). This pathogen can infect different parts of the plant, including leaves, stem, root, crown, flower and fruit. The spores form appressoria which penetrate the cutaneous layer of the fruit, producing differentiated infectious hyphae, resulting in the development of typical anthracnose wounds (S. M. Yu, Ramkumar, & Lee, 2013). The symptoms of the disease, dark and depressed wounds with conidia in an orange mucilage, can become very evident in the postharvest step, since the causal agent is quiescent, in other words, needs a certain period of time, remaining inactive in the host, before restarting an active phase. Thus, the physiological and biochemical changes during the process of fruit ripening can activate different routes, which are important to maintain or facilitate the transition from a quiescent lifestyle to the necrotrophic lifestyle of the pathogen (Prusky, Alkan, Mengiste, & Fluhr, 2013).

High rates of damages and outbreaks of *C. acutatum* in strawberry have already been reported and in favorable conditions for the disease, the loss of productivity can reach up to 40% (Daugovish, Su, & Gubler, 2009). The application of synthetic fungicides, which represents 15 % of the total operational costs, and the use of less susceptible cultivars, are important tools for the control of this disease (Legard, MacKenzie, Mertely, Chandler, & Peres, 2005; Forcelini, Gonçalves, & Peres, 2017). Nevertheless, the chemical control has become the most difficult, since several effective fungicides are not sold anymore or have lost their record for use in strawberries and some pathogenic agents have developed resistance to the most commonly used fungicides.

Botrytis cinerea, the causal agent of gray mold, is another pathogen that causes great losses for the culture. This necrotrophic pathogen can secrete enzymes and phytotoxic metabolites which degrade the cell wall, facilitating the infection and development of the disease in strawberries (Choquer et al., 2007; Qin, Xiao, Cheng, Zhou, & Si, 2017). The symptoms of the disease can be observed in strawberries that are ripe or not, leaves, pistils and flowers. Some infections that started in the field remain quiescent during the growth stage, manifesting symptoms, usually, after harvest. This pathogen is also able to infect plant tissues by superficial wounds inflicted during harvest and further handling, sometimes developing during storage,

even at 0 °C, spreading to the healthy fruit by the aerial mycelial growth and conidia (Romanazzi, Smilanick, Feliziani, & Droby, 2016). Despite being a classic "high risk" pathogen in the sense of resistance management, its control is also performed mainly with synthetic fungicides (Hu, Dai, Wang, & Zhang, 2017). Most of the fungicides recorded in the pre-harvest against *B. cinerea* in strawberries have specific modes of action and, during the last decade, isolates of this species in strawberries and other cultures have developed resistance to most of the fungicides available (Amiri, Heath, & Peres, 2013).

Rhizopus stolonifer, another important pathogen for strawberry, is a fungal species that can cause 'soft rot' in vegetables, flowers, bulbs, rhizomes, seeds and succulent fruits, producing short branches (rhizoids) inside its food substrates and hyphae stolons which cross on the surface. This pathogen is able to degrade the cell walls of the host by the production of cellulases and pectins, which release nutrients, making the infected areas soaked and soft. This pathogen usually inhabits soil, being frequently associated to the decomposition of vegetables and fruits (Trigiano, Windham, & Windham, 2016). In the step of strawberry postharvest, soft rot, caused by *R. stolonifer*, is one of the diseases considered the most important (Costa & Ventura, 2006). It occurs from wounds caused in the steps of harvest and postharvest and prevails with the rise in temperature and moisture. As a characteristic, there is the presence of a white-colored mycelium with dark sporangia (Zawadneak, Schuber, & Mógor, 2014).

These three pathogens of great importance for the culture are distributed worldwide and require an intensive chemical control during the stage of flowering and fructification, which occur simultaneously in strawberry production (J. Mertely, MacKenzie, & Legard, 2002). Nevertheless, the decreasing efficiency and the increasing concern regarding the adverse environmental effects of these products have brought the need for developing new alternatives of selective control and methods for culture protection without, or with a reduced usage, of toxic conventional fungicides (Romanazzi et al., 2016). Thus, the development of alternative formulations that are safe and do not leave toxic residues, is extremely important.

Therefore, the present study investigated the antifungal potential of the pre-commercial formulation from Orion Industrial Ltda. called 'Aquativ Agro' against *Colletotrichum acutatum*, *Botrytis cinerea* and *Rhizopus stolonifer*. Its action on the

morphology of the pathogens, as well as its application, in a preventive and curative way, were also evaluated.

5.2. Materials and Methods

5.2.1. Formulation

The formulation Acquativ Agro was prepared in the company Orion Industrial, located in Fortaleza, Ceará, Brazil, from a mixture of organic acids and salts, potassium hydroxide, essential oil and carboxymethylcellulose. Information on the formulation are presented in the technical information (Appendix) of the product.

5.2.2. Isolation and identification of the fungal isolates

The method for isolation and identification of the pathogens was described in the previous chapters (II, III and IV). The isolates of *Colletotrichum acutatum*, *Rhizopus stolonifer* and *Botrytis cinerea*, used in the previous experiments, were the same used in the experiments of this chapter.

5.2.3. *In vitro* antifungal activity of the formulation Acquativ Agro

The antifungal activity of the formulation was evaluated by measuring the growth restriction of *C. acutatum*, *B. cinerea* and *R. stolonifer*, by the direct contact of the pathogen with the culture medium potato dextrose agar (PDA) containing the product, at the concentrations of 31; 62.5; 125; 250 and 500 µl/L (Plaza, Torres, Usall, Lamarca, & Vinas, 2004). A control treatment, containing only the culture medium, was also employed.

After solidification of the medium PDA, at the central point of the dish, the pathogen of interest was inoculated, from a suspension containing 10^5 spores mL^{-1}, with the aid of a previously sterilized metal rod. The fungal inoculum was prepared from colonies of the fungus of interest, growing in PDA culture medium for 10 to 15 days for *C. acutatum* and *B. cinerea* and 5 days for *R. stolonifer*. Thus, an inoculum suspension from each pathogen was obtained by scratching the surface of the colonies, with the aid of a metal loop, and their immersion into a Becker containing

47.5 mL of sterile water and 2.5 mL of Dimethyl Sulfoxide (DMSO). The concentration of spores in the solution, standardized to 1 to 10 x 10^5 spores mL^{-1}, was determined by counting on a Neubauer chamber.

After pathogen inoculation, the dishes were maintained in growth chambers with a photoperiod of 12 hours, at 25 °C for *C. acutatum* and *R. stolonifer* and at 23°C, for *B. cinerea*. The readings of pathogen growth were performed every two days of incubation for *C. acutatum*, daily for *B. cinerea* and every eight hours for *R. stolonifer*. For this, colony growth diameter was measured in two perpendicular directions, using a digital caliper (Zaas Precicion). The arithmetic mean of these two measures was considered representative of the colony growth diameter. Fungal growth was measured until the development of the colony in the control treatment reached the borders of the dish.

Mycelial growth inhibition at the different concentrations of the product was measured using the formula (Plaza et al., 2004):

$$PI\ (\%) = \frac{(Growth\ of\ the\ control - Growth\ of\ the\ treatment)}{Growth\ of\ the\ control} \times 100$$

The Minimum Inhibitory Concentration (MIC), when present, was considered as the lowest concentration of the treatment, among the concentrations evaluated, capable of completely inhibiting pathogen development visible to the naked eye.

The experimental design was the randomized in factorial scheme, comprising 1 product in 6 concentrations. The experiment contained five repetitions per concentration, with the experimental unit represented by one Petri dish. The data on colony diameter (cm) and growth inhibition were adjusted to regression models, considering as the most appropriate the one with the highest coefficient of determination (R^2).

5.2.4. Effects of Acquativ Agro on pathogen morphology

The damages caused by the product on the morphology of the pathogens were evaluated by Scanning Electron Microscopy (SEM) according to D. Yu, Wang, Shao, Xu, & Wang (2015) with modifications, as described in the previous chapters. To evaluate the effects of the product on the hyphae of *C. acutatum* and *B. cinerea*,

the concentration used was the MIC determined in the test *in vitro* (500 µl/L), and for *R. stolonifer*, the concentration used was 10,000 µl/L (1% v/v), since at the concentrations evaluated in the test *in vitro*, it was not possible to determine the MIC for this pathogen; therefore, the maximum concentration evaluated was increased to investigate possible damages to the morphology of the pathogen.

5.2.5. *In vivo* antifungal activity of the formulation Acquativ Agro

The *in vivo* antifungal activity of the formulation Acquativ Agro was evaluated at the concentration of 20,000 µl/L (2 %), 40 times higher than the minimum inhibitory concentration observed in the *in vitro* tests for *C. acutatum* and *B. cinerea* (500 µl/L). To perform this experiment, strawberries from the Oso Grande variety, from an organic farming located in Cambuí (MG, Brazil, 22° 36' 43" S 46° 03' 28" O), were visually selected regarding appearance and sanity, being then sanitized, by immersion for 10 minutes, in 2.5 % sodium hypochlorite. The fruit were subjected to the following treatments:

- "C" – fruit immersed in only distilled water;
- "Preventive" - fruit treated preventively with Acquativ Agro;
- "Curative" - fruit treated curatively with Acquativ Agro.

For the evaluation of the preventive action, strawberries were immersed in a solution of the product at 2 %, for 2 minutes, and after the natural drying, for 1 hour, they were distributed in excavated PVC trays (23 x 18 x 1.8 cm), so that each one would be individually accommodated, with twelve fruit per tray. The strawberries were marked with a ballpoint pen in the exact place of inoculation, thus avoiding possible confusions with naturally-occurring infections, derived from other sources of inoculum. Pathogen inoculation was performed by the deposition of 30 µL of a spore suspension of the fungus of interest (10^5 spores mL^{-1}) on a 3 mm-deep wound, which was performed with the help of a sterilized insulin needle of 0.25 mm in diameter (BD Ultra-Fine^{TM}).

After the steps of treatment and inoculation, the strawberries were stored for 24 hours in a wet chamber with 95 % of relative humidity, in a growth chamber at 25 °C for the pseudofruit inoculated with *C. acutatum* and *R. stolonifer*, and at 23 °C, for those inoculated with *B. cinerea*, both under a photoperiod of 12 hours. To constitute

the wet chamber, the trays containing the twelve strawberries inoculated were placed inside larger plastic trays, which received portions of cotton soaked in sterile distilled water and covered and closed with the help of a plastic bag.

To evaluate the curative mode of action, pathogen inoculation was performed 24 hours before product application. The further procedures were the same as described for the preventive mode of action.

The experimental design of this assay, for each pathogen evaluated, was in a 3 x 7 factorial scheme, involving three treatments ("C"; "Preventive" and "Curative") and seven days of evaluation. Six repetitions were used for each treatment, each one represented by a tray composed of 12 strawberries.

The antifungal activity of the treatments was evaluated by the incidence and severity of the disease in the fruit. Incidence, calculated from the number of symptomatic fruit in relation to the total number of fruit in each treatment, was evaluated after the seventh day of storage in the strawberries inoculated with *C. acutatum* and *B. cinerea,* and after 3 days, in those inoculated with *R. stolonifer*, with the results expressed in percentage (%) (Ali, Wee Pheng, & Mustafa, 2015).

Severity was evaluated daily in the fruit inoculated with *C. acutatum* and *B. cinerea,* and every 8 hours in the fruit inoculated with *R. stolonifer*, using a scale of scores composed of six degrees (0 = absence of symptoms; 1 = 1 to 20% of wounded area; 2 = 21 to 40%; 3 = 41 to 60%; 4 = 61 to 80% and 5 = more than 81% of wounded area) (Figure 1), with the results expressed in Disease Index (DI), according to Cia, Benato, Pascholati, & Oliveira Garcia (2010), where DI(%) = [(1xn1) + (2xn2) + (3xn3) + (4xn4) + (5xn5)] x100/5xN, in which ni is the number of fruit infected in the respective scale of scores and N is the total number of fruit.

Based on the DI values over time, in each repetition, the Area Under the Disease Progress Curve (AUDPC) was calculated for severity according to Campbell & Madden (1990): AUDPC = $\sum$ [(y_i + y_{i+1}) / 2 x (t_{i+1} - t_i)], being y $_i$ the DI of the wound at time t $_{i;}$ y $_{i+1}$; DI at time, t $_i$ the initial reading time and t $_{i+1}$ the time in days of each reading.
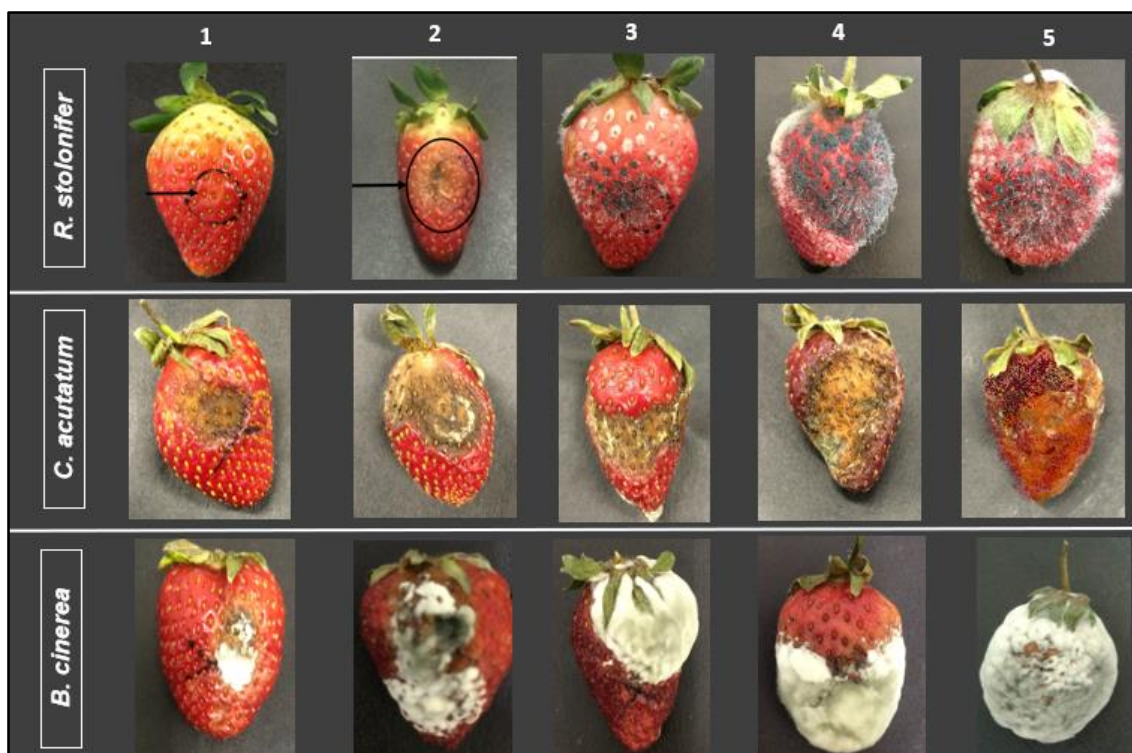
Figure 1 - Scale of scores composed of six degrees of severity of the diseases soft rot, anthracnose and gray mold, caused respectively by *Rhizopus stolonifer*, *Colletotrichum acutatum* and *Botrytis cinerea* (1 = 1 to 20% of wounded area; 2 = 21 to 40%; 3 = 41 to 60%; 4 = 61 to 80% and 5 = more than 81% of wounded area) in strawberries.

The data referring to the AUDPC were evaluated by 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 the level of significance of 5 % ($p<0.05$), was determined by the Tukey's test.

5.3. Results

5.3.1. *In vitro* antifungal activity of the formulation Acquativ Agro

The product Acquativ Agro presented a dose-dependent activity, with a capacity of total mycelial growth inhibition of *C. acutatum* and *B. cinerea*, observed between the concentrations of 250 and 500 µl/L (Figures 2 and 3).
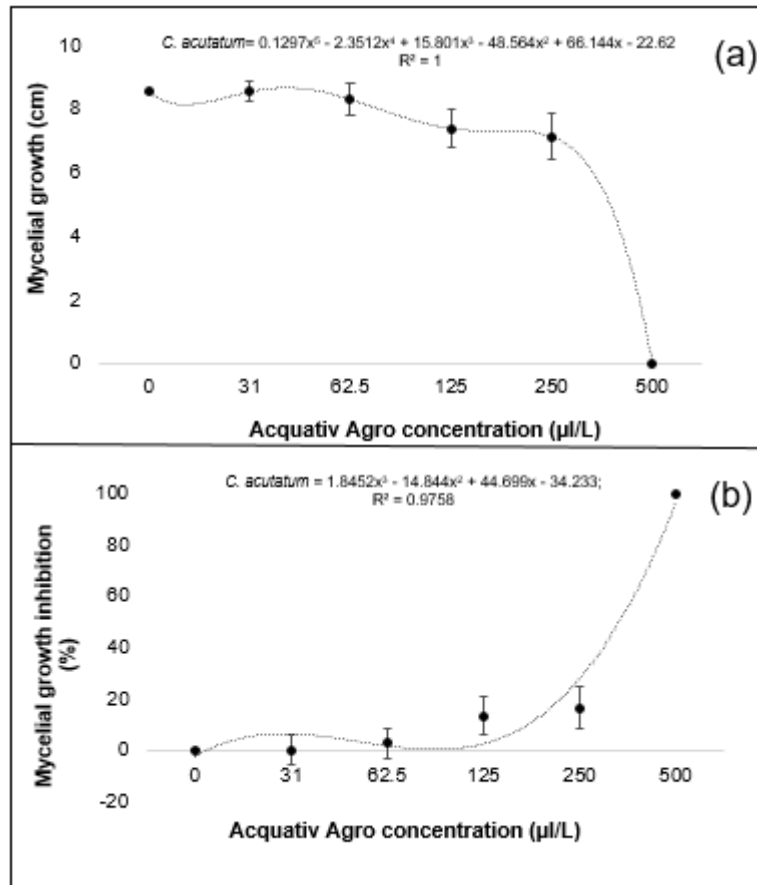
Figure 2– Mycelial growth (a) and percentage of mycelial growth inhibition (b) of *Colletotrichum acutatum*, isolated from strawberry, at 12 days of incubation, after exposure by contact to different concentrations (µl/L) of Acquativ Agro incorporated to the medium Potato-Dextrose-Agar (PDA). Vertical bars indicate the standard error of the mean (n=5).
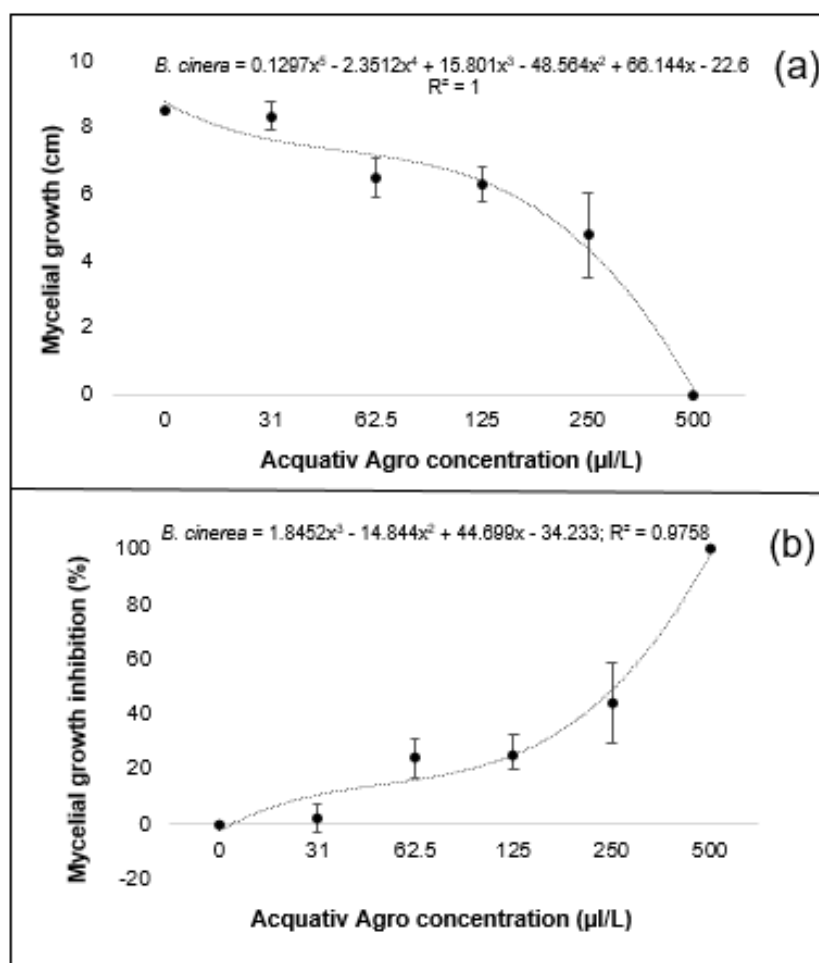
Figure 3 – Mycelial growth (a) and percentage of mycelial growth inhibition (b) of *Botrytis cincerea*, isolated from strawberry, at 7 days of incubation, after exposure by contact to different concentrations (µl/L) of Acquativ Agro incorporated to the medium Potato-Dextrose-Agar (PDA). Vertical bars indicate the standard error of the mean (n=5).

The concentrations of Acquativ Agro evaluated in this study were not able to inhibit 100 % of the mycelial growth of *Rhizopus stolonifer*, with a maximum inhibition of 19.90 % observed, at the concentration of 500 µl/L (Figure 4).
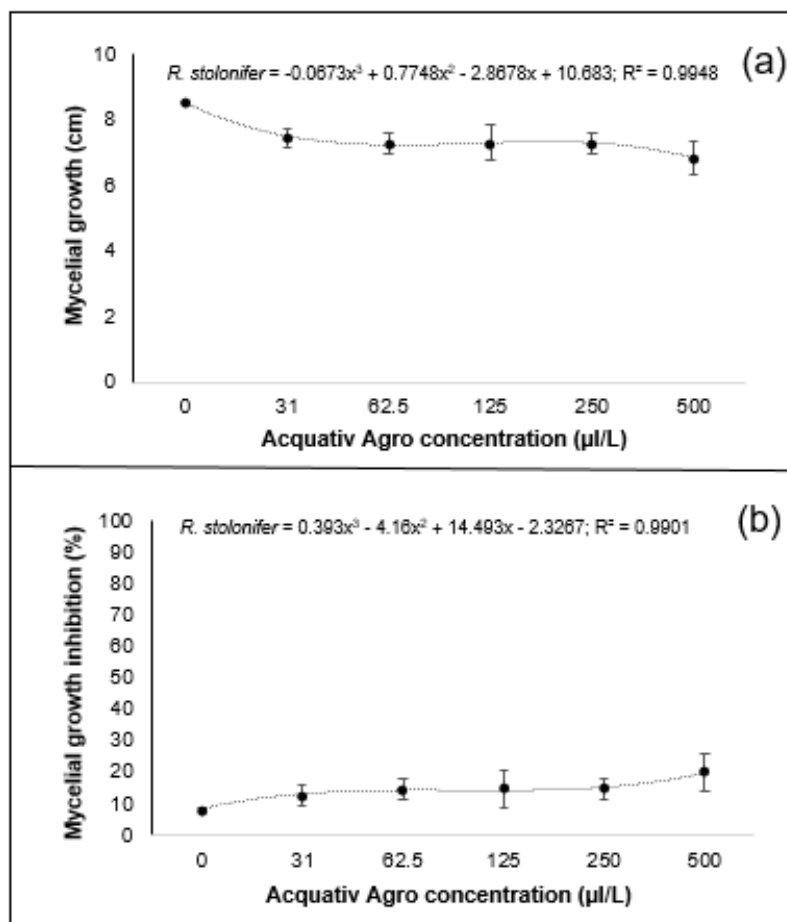
Figure 4 – Mycelial growth (a) and percentage of mycelial inhibition (b) of *Rhizopus stolonifer*, isolated from strawberry, at 3 days of incubation, after exposure by contact to different concentrations (µl/L) of Acquativ Agro incorporated to the medium Potato-Dextrose-Agar (PDA). Vertical bars indicate the standard error of the mean (n=5).

5.3.2. Effects of Acquativ Agro on the morphology of the pathogens

In the absence of Acquativ Agro, the mycelia of *C. acutatum, B. cinerea* and *R. stolonifer* exhibited homogeneous tubular hyphae, regular and with defined septa, besides a smooth and long outer surface (Figures 5A, 6A, 7A). The opposite occurred in the hyphae exposed to the product, which presented structural alterations, such as superficial wrinkles, desquamation, distortion and destruction (Figures 5B, 6B, 7B).
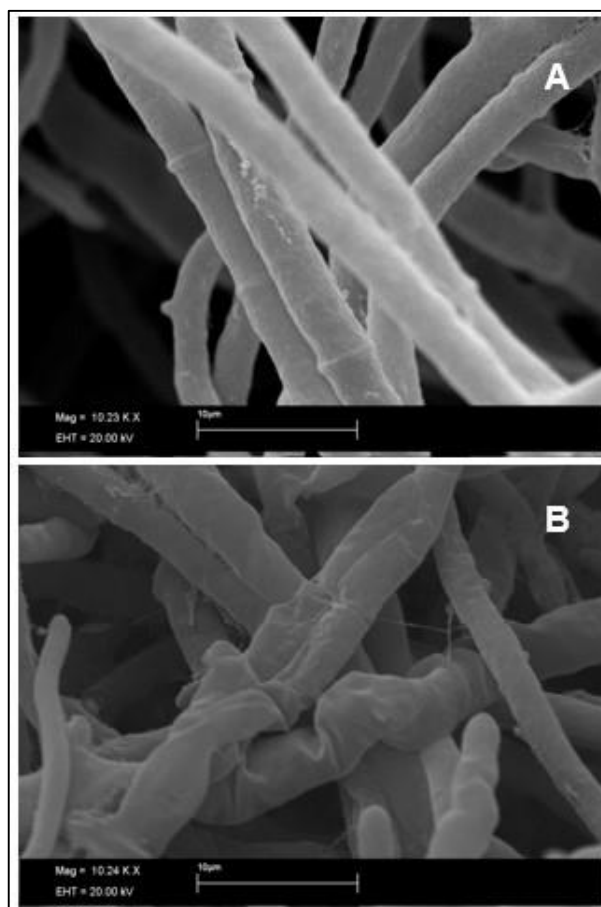
Figure 5 - Scanning electron microscopy of *Colletotrichum acutatum* hyphae, isolated from strawberry, cultivated in potato broth for two days, subjected (B) or not (A) to the product Acquativ Agro at the concentration of 500 µl/L.
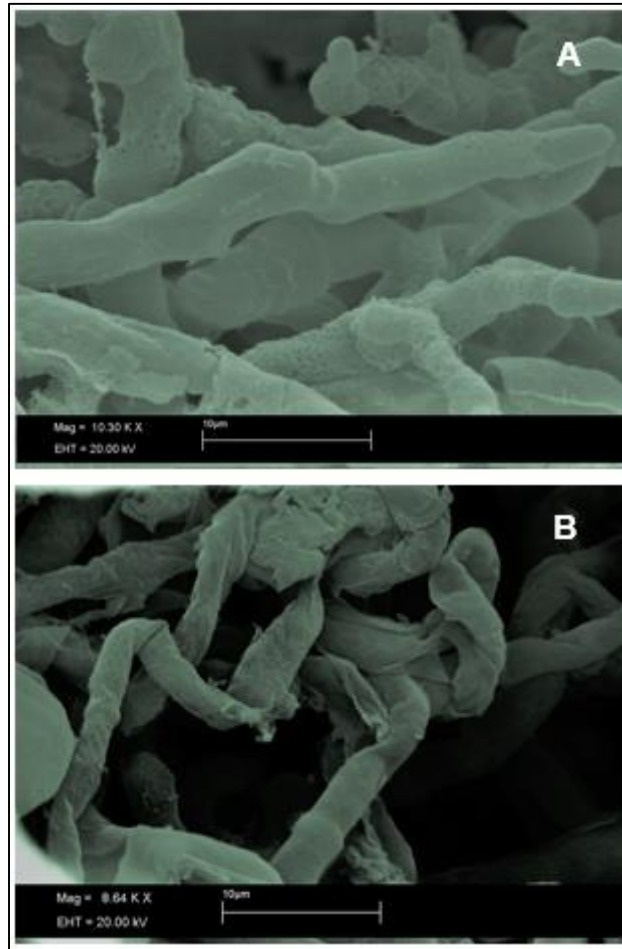
Figure 6 - Scanning electron microscopy of *Botrytis cinerea* hyphae, isolated from strawberry, cultivated in potato broth for seven days and subjected (B) or not (A) to the product Acquativ Agro at the concentration of 500 µl/L.
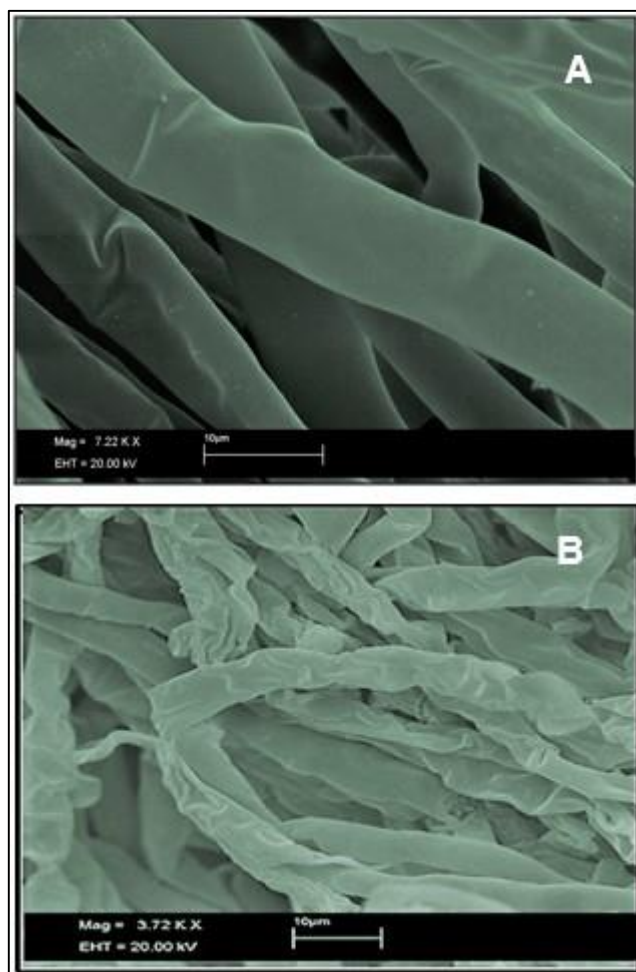
Figure 7 - Scanning electron microscopy of *Rhizopus stolonifer* hyphae, isolated from strawberry, cultivated in potato broth for two days and subjected (B) or not (A) to the product Acquativ Agro at the concentration of 10,000 µl/L.

5.3.3. *In vivo* antifungal activity of the formulation Acquativ Agro

The severity of anthracnose was lower in the strawberries treated preventively with Acquativ Agro (Figure 8). The product did not present a significant curative action for this disease, since this treatment did not differ statistically from the control treatment. The incidence of anthracnose reached 100% at the seventh day of evaluation, with similarity among the treatments.
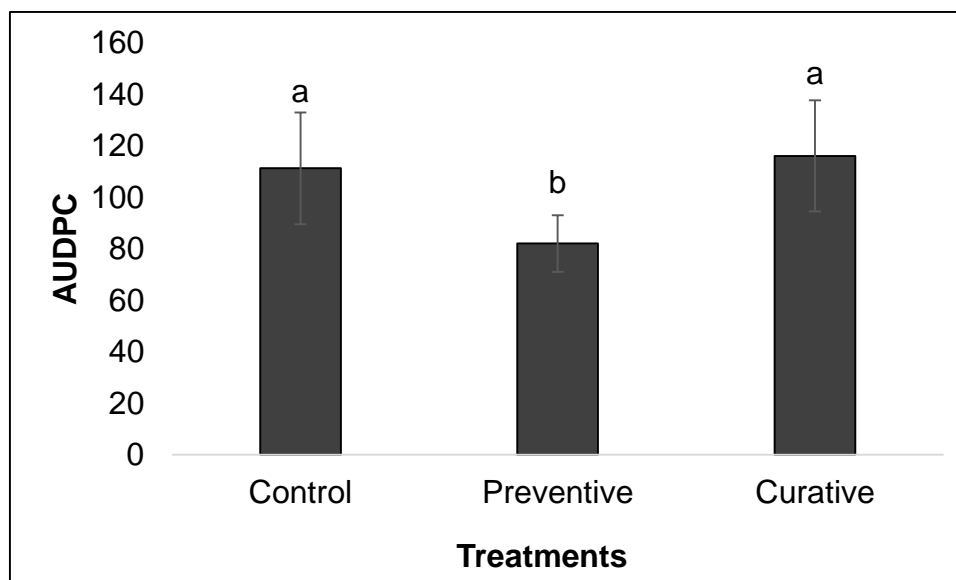
Figure 8 - Values of the Area Under the Disease Progress Curve (AUDPC), for the severity of the disease anthracnose, caused by *Colletotrichum acutatum,* in strawberries.
Distinct letters represent a significant difference among the treatments by the Tukey's test (P<0.05). Vertical bars indicate the standard error of the mean (n=12). Control: fruit without the application of Acquativ Agro; Preventive: fruit treated preventively with Acquativ Agro (2%); Curative: fruit treated curatively with Acquativ Agro (2%).

The product Acquativ Agro, at the concentration of 2%, was not efficient in reducing Gray mold severity in strawberries, since the treatments preventive and curative did not present significant statistical differences from the untreated strawberries (Figure 9). The incidence of this disease reached 100% at the seventh day of evaluation, with similarity among the treatments.
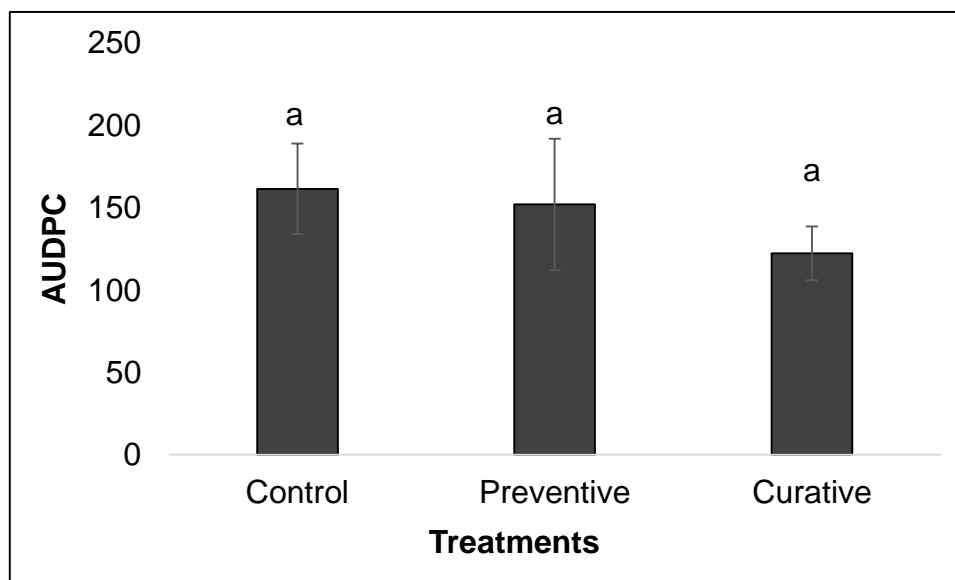
Figure 9 - Values of the Area Under the Disease Progress Curve (AUDPC), for the severity of the disease Gray Mold, caused by *Botrytis cinerea*, in strawberries.
Distinct letters represent a significant difference among the treatments by the Tukey's test (P<0.05). Vertical bars indicate the standard error of the mean (n=12). Control: fruit without the application of Acquativ Agro; Preventive: fruit treated preventively with Acquativ Agro (2%); Curative: fruit treated curatively with Acquativ Agro (2%).

The severity of the soft rot, caused by *Rhizopus stolonifer*, was lower in the strawberries treated with Acquativ Agro (Figure 10), regardless of the mode of action. The incidence of this disease reached 100% at the seventh day of evaluation, with similarity among the treatments.
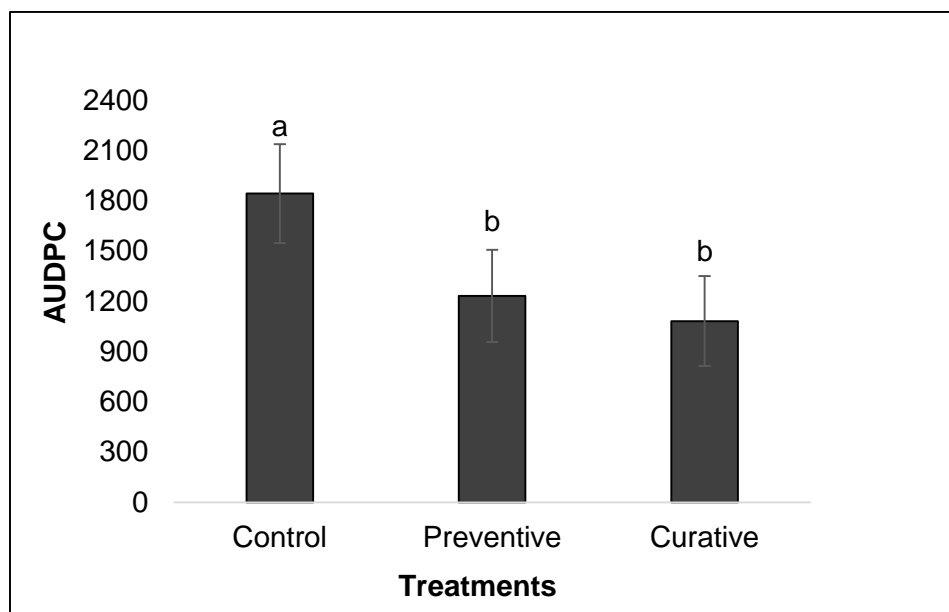
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Figure 10 - Values of the Area Under the Disease Progress Curve (AUDPC), for the severity of the disease caused by *Rizopus stolonifer* in strawberries.
Distinct letters represent a significant difference among the treatments by the Tukey's test (P<0.05). Vertical bars indicate the standard error of the mean (n=12). Control: fruit without the application of Acquativ Agro; Preventive: fruit treated preventively with Acquativ Agro (2%); Curative: fruit treated curatively with Acquativ Agro (2%).

5.4. Discussion

5.4.1. *In vitro* antifungal activity of the formulation Acquativ Agro

The formulation was effective in inhibiting the mycelial growth of *C. acutatum* and *B. cinerea (250 < MIC ≤ 500* µl/L); however, for *R. stolonifer*, the concentrations evaluated were not enough for a significant inhibition (MIC > 500 µl/L), which can be justified by the difference in pathogen severity. The good performance of the formulation on *C. acutatum* and *B. cinerea* is justified by the action of its ingredients, such as salts and acids with antifungal potential. Several inorganic and organic salts, classified as food additives or GRAS substances, have been reported as efficient manners for the control of postharvest diseases in fresh horticultural products, when applied as aqueous solutions (Kowalczyk, Kordowska-Wiater, Złotek, & Skrzypek, 2018). Other food preservatives, such as weak organic acids, are also employed separately or in combination with the physical treatments to guarantee the safety and stability of the products during storage (Kocić-Tanackov & Dimić, 2013), since they are capable of inhibiting the growth of bacteria and fungi (Sofos & Busta, 1981).

According to Brul & Coote (1999), in the solutions of weak acids there is a balance dependent on the pH between the inseparable and dissociated states. This kind of preservative presents an optimum inhibitory action at low pH, since this favors the unbound state, being able to enter the pathogen cell by the plasma membrane. Therefore, it is classically believed that the inhibitory action occurs because of the compound which crosses the plasma membrane in the inseparable state. Subsequently, when the higher pH in the interior of the cell is found, the molecule will be dissociated, resulting in the release of charged anions and cations, which cannot cross the plasma membrane, causing an imbalance in the cell.

The antifungal activity of the formulation can also be justified by the presence of essential oil, a compound that can be extracted from several aromatic plants. Many studies have been demonstrating that its activity is mainly related to the interaction with the cell membrane of the different microorganisms, being able to cross it, causing cell disturbance and death of the microorganism (dos Santos et al., 2012; Kumar et al., 2016).

5.4.2. Effects of Acquativ Agro on pathogen morphology

The exposure of the mycelium of the three pathogens to the product Acquativ Agro resulted in morphological alterations which suggest that its antifungal action might include an attack to the plasma membrane of the hypha, resulting in a disorder in the cell, with a consequent mycelial death. The essential oil, one of the ingredients of the formulation, is rich in phenolic compounds belonging to the class of natural antioxidants, which can act on the fungal cell wall and plasma membrane (Chavan & Tupe, 2014; Guimarães, Cardoso, Souza, Zacaroni, & Santos, 2014), facilitating ion exchange, increasing its permeability and hampering cell survival (Moreira et al., 2010; Rao, Zhang, Muend, & Rao, 2010).

According to Rao et al. (2010), these compounds facilitate ion exchange in the cellular environment of the fungi, such as the entrance of $H+$ and $K+$, thus increasing membrane permeability and making cell survival difficult, since it hampers the essential processes, such as electron transportation; furthermore, they also block $Ca2+$ influx into the cell compartments and inhibit the biosynthesis of ergosterol, which is an important component of the fungal cell membrane and whose inhibition

leads to the exit of ions and several molecules from the cell, causing its death (Ahmad et al., 2011; Wang et al., 2018).

The plasma membrane has a vital role in the maintenance of a homeostatic environment for the cell, exchanging materials and transferring energy and information (Cox et al., 2000; Shao, Wang, Xu, & Cheng, 2013). According to Booth & Kroll (1989), preservatives such as weak acids also have the capacity of spreading in the cell, until the balance is reached, according to the pH gradient through the membrane, resulting in the accumulation of anions and cations inside the cell, making pathogen survival difficult.

5.4.3. *In vivo* antifungal activity of the formulation Acquativ Agro

In the test *in vivo*, the formulation only did not present an effective control, in the doses evaluated, for the disease gray mold. This can be justified by the difference in aggressiveness among the pathogens, requiring tests with higher concentrations of the product, since the results *in vitro* and the analyses of scanning electron microscopy have indicated an antifungal activity of the formulation on *B. cinerea*. According to Palou et al. (2016), the *in vitro* studies tend to be positive for alternative compounds, but this is not always the case for the application *in vivo*, since the treatment can affect fruit physiology many times requiring an appropriate transporter for an efficient application on the fruit surface.

There was a significant reduction in anthracnose severity when the strawberries were subjected to Acquativ Agro in a preventive way, and a reduction of soft rot when subjected to the product in a preventive and curative way. The formulations of preventive effect are those effective if applied before the occurrence of pathogen penetration in the host, preventing or reducing the chances of occurrence of the disease. Thus, when the formulation was applied to the surface of the strawberries, it acted as a toxic barrier, preventing the penetration of the fungi by inhibiting the germination of spores and the germ tube. Given its non-specific mode of action, the preventive formulations can be highly toxic to the host cells (Garcia, 1999); nevertheless, in this study no symptom of phytotoxicity was observed in the strawberries. On the other hand, the curative effect, observed in the control of *R. stolonifer*, is the one where there is an attenuation of the symptoms of repair of the

damages caused by the pathogen, as an action directed against the pathogen, after the establishment of its effective contact with the host (KIMATI, 1995).

The mode of action of some ingredients of the formulation, such as organic salts, has not been completely explained. There are evidences that its inhibitory capacity depends on the presence of salt residues in the areas of infection occupied by the pathogen, typically wounds in the fruit peels, and interactions between this residue and fruit tissue components. Additionally, there might be a certain direct toxic action of the different anions and cations and pH alterations of the fruit (Palou, Smilanick, Usall, & Viñas, 2001; Dore, Molinu, Venditti, & D'Hallewin, 2010). It was demonstrated that the immersion of peaches and nectarines, naturally infected, in a solution of organic salt, reduced brown rot in more than 80% (Gregori, Borsetti, Neri, Mari, & Bertolini, 2008), this kind of treatment was also efficient in the control of postharvest diseases in pears (El-Eryan & El-Metwally, 2014). A coating formulation with the addition of organic salts can reduce the fast degradation of the surface of some fruits and can also be successfully used for the control of microbial growth (Karaca et al., 2014; Mehyar, Al-Qadiri, & Swanson, 2014; Junqueira-Gonçalves, Alarcón, & Niranjan, 2016).

The *in vivo* antifungal activity of the formulation can also be attributed to the presence of essential oil. This ingredient has been approved by the Food and Drug Administration (FDA) as "generally recognized as safe" (GRAS) and can be used as a food additive (Marchese et al., 2016). The antifungal activities of this compound on *P. digitatum* and *P. italicum* were reported by tests *in vitro* and *in vivo* as capable of reducing fruit degradation in a concentration-dependent manner (Castillo et al., 2014; Pérez-Alfonso et al., 2012). The low solubility in water and the high volatility of the essential oils might restrict their isolated application on disease control (Ceborska et al., 2015); however, chemical processes and the preparation of formulations can enable their use.

This work shows that an alternative formulation, which combines different preservative additives, is promising for the control of the main pathogens of strawberries in the postharvest; nevertheless, these results must be validated by assays in large scale, to demonstrate the value of this treatment at a commercial level.

5.5. Conclusion

The evaluation, *in vitro* and *in vivo*, of 'Acquativ Agro' enabled the observation that this formulation presents antifungal activity on the main postharvest pathogens of strawberries: *Botrytis cinerea*, *Colletotrichum acutatum* and *Rhizopus stolonifer*, causal agents of the diseases gray mold, anthracnose and soft rot, respectively. Furthermore, it was verified that this product causes a morphological degeneration on the hyphae of the pathogens, suggesting its action on the fungal cell wall. It is then concluded that this alternative formulation, which combines different preservative additives, is promising for the control of the main strawberry pathogens in the postharvest; nonetheless, studies on the validation of the efficacy in large scale are necessary.

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APPENDIX

ORION	TECHNICAL INFORMATION	FT: 09
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PHYSICOCHEMICAL		
	TEST	SPECIFICATIONS
01	Color	Dark yellow
02	Appearance	Viscous liquid
03	pH	6.00 – 7.50 (pHMetro QUIMIS REF. Q400AS)
04	Density	1.140 - 1.180 g/cm^3 (volumetric flask PN. 1716 - °C room temperature)
05	Solubility	In water

Ingredients

Water, organic acids and salts, potassium hydroxide, essential oil and carboxymethylcellulose.

Product expiry

The product remains stable for 12 months keeping the packages closed, stored in a dry and ventilated place, protected from the weather, far from heat sources.

Observations

Product for exclusive use in tests.