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ALTERNATIVE SUPPRESSION OF *Botrytis cinerea* IN STRAWBERRY USING ESSENTIAL OILS

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ABSTRACT - Strawberry (*Fragaria x ananassa*) is a perishable fruit with a short post-harvest life and a high incidence of fungal diseases, especially *Botrytis cinerea*. For control and prevention, synthetic products are often used which negatively influence in the food and environmental security. The present work was carried out with the objective of evaluating the inhibitory potential of essential oils in the growth and control of the *B. cinerea in vitro* and *in vivo*. The essential oils of thyme (*Thymus vulgares* L.), cinnamon (*Cinnamomum cassia* L.), lemongrass (*Cymbopogon flexuosus* L.), peppermint (*Mentha piperita* L.) and tea tree (*Melaleuca alternifolia* Maiden & Betche) were tested, in addition to control made of autoclaved water, using a completely randomized design with 4 replications. The fungus growth was evaluated in vitro through grading. Strawberry fruits were evaluated for the presence of *B. cinerea*, firmness and total soluble solids content. The growth of the *B. cinerea* in vitro, the percentage of gray mold infection, the total soluble solids content and the texture of strawberry were evaluated. The *in vitro* growth of the fungus received lower scores with the essential oils of thyme and cinnamon, with values of 0.32 and 0.92, respectively, with greater suppression. The percentage of strawberry with symptoms were lower than the treatments with tea tree (8,33%), thyme (25%), lemongrass (25%) and peppermint (26,66%). The essential oils of thyme and cinnamon have potential for use in the control of the *Botrytis cinerea* in *in vitro* test and the essential oils of peppermint, thyme, tea tree and lemongrass have potential for use in the suppression in strawberry fruits.
Keywords: *Fragaria* x *ananassa*, alternative control, post-harvest.

CONTROLE ALTERNATIVO DE Botrytis cinerea EM MORANGO COM USO DE ÓLEOS ESSENCIAIS

RESUMO - O morango (*Fragaria x ananassa*) é um pseudofruto perecível, com curta vida pós-colheita e alta incidência de doenças fúngicas, com destaque para o *Botrytis cinerea*. Para seu controle e prevenção, frequentemente são utilizados produtos sintéticos, que influenciam negativamente a segurança alimentar e ambiental. O presente trabalho foi conduzido com o objetivo de avaliar o potencial inibitório de óleos essenciais no crescimento e controle do fungo *B. cinerea in vitro* e *in vivo*. Foram testados os óleos essenciais de tomilho (*Thymus vulgares* L.), canela (*Cinnamomum cassia* L.), capim-limão (*Cymbopogon flexuosus* L.), hortelã-pimenta (*Mentha piperita* L.) e melaleuca (*Melaleuca alternifólia* Maiden & Betche), além da testemunha constituída de água autoclavada, utilizando o delineamento experimental inteiramente casualizado, com 4 repetições. Avaliou-se o crescimento do fungo *in vitro* por meio de atribuição de notas. Os pseudofrutos foram avaliados quanto à presença de *B. cinerea*, à firmeza e teor de sólidos solúveis totais. O crescimento *in vitro* do fungo recebeu as menores notas com os óleos essenciais de tomilho e canela, com valores de 0,32 e 0,92, respectivamente, indicando maior controle. A porcentagem de pseudofrutos de morango que apresentaram sintomas de mofo cinzento foi inferior os tratamentos com melaleuca (8,33%), tomilho (25%), capim-limão (25%) e hortelã-pimenta (26,66%). Os óleos essenciais de tomilho e canela apresentam potencial para uso no controle do fungo *Botrytis cinerea* em teste *in vitro* e os óleos essenciais de hortelã-pimenta, tomilho, melaleuca e capim-limão apresentam potencial para uso no controle do fungo em pseudofrutos de morango.
Palavras-chave: *Fragaria* x *ananassa*, controle fitossanitário, pós-colheita.

INTRODUCTION

The strawberry (*Fragaria* x *ananassa* Duch) is an interspecific hybrid belonging to the Rosaceae family (HUMMER et al., 2012). The eatable part is a pseudofruit of reddish coloration, rich in antioxidants, fibers and source of vitamin A and C, responsible for its anti-inflammatory activity and for the antineurodegenerative and anticarcinogenic potential (MORAES et al., 2008). It stands out amongst the commercialized little fruits for presenting the biggest economic expression (OLIVEIRA et al., 2016).

The strawberry is a non-climacteric perishable fruit that presents a short post-harvest shelf life, due to its high susceptibility to pathogenic microorganisms' attack,

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mainly from *Botrytis cinerea*, the gray mold-causing fungus (BRAGA, 2012; CAMPOS et al, 2015; NASERZADEH et al., 2019). The disease caused by this fungus is responsible for innumerable losses in the strawberry production and other vegetables, which presents as typical symptoms the complete rotting or mumification of the fruit (BRAGA, 2012; TOFOLI et al, 2011).

The most efficient method for controlling *B. cinerea* is based on the use of synthetic antifungal agents. However, the resistance of the fungus, the environment contamination threat and the food security highlight the need of seeking safer and more efficient methods (CHENG; SHAO, 2010). In this perspective, the market of natural products with potential of application in the food conservation, with essential oils, is growing strong compared to the synthetic chemical additives (ALMEIDA et al., 2020).

The essential oils are obtained from the secondary metabolism, constituted of complexes mixes of volatile substances with characteristic odor, usually derived from terpenes, being possible of trespassing 300 compounds, with lipophilic features, low molecular weight and liquid physical state (MORAIS, 2009). They are synthetized by plants in order to attract pollinizers, to protect against predators and pathogens, to prevent against water waste and temperature increase, turning them into sources of biocides agents, widely studied in the agroecosystems, constituting of an alternative for the substitution of the synthetic fungicides (KNAAK; FIUZA, 2010; MAIA et al., 2015).

The fungicides actions of the essential oils are directly related with the mycelial growth inhibition and/or indirectly with the induction of phytoalexins production and other plant defense compounds (MORAES, 2009). In fungi, essential oils can affect the spore protection barrier or act before its formation, promoting abnormal development and/or disruption of germ tubes (DANTIGNY; NANGUY, 2009). The hydrophobicity of the essential oils allows the interaction between oil and fungal cell membrane, causing alterations in its structure, being able to dissolve it, to penetrate in the cell interior and to cause the extravasation of cellular content (BRAGA, 2012; COSTA et al., 2011; PEREIRA et al., 2011).

Allied with the antimicrobial effect, the essential oils present the advantage of low toxicity for mammals, being easily degradable, not being persistent in the environment and showing relative low production cost (ISMAN, 2000). These characteristics make them be studied and utilized in the food industry for avoiding food oxidation and prolonging the postharvest shelf life of innumerable fruits and vegetables, through its inhibitory effect in the development of rot-causing pathogens (BORGES et al., 2013; LIMA; CARDOSO, 2007; SCATAZZINI, 2018).

In order to elucidate a natural method that enhances the strawberry postharvest shelf life, the present study was conducted with the aim of evaluating the inhibitory potential of essential oils in the growth and control of the fungus *Botrytis cinerea*, *in vitro* and *in vivo*, in strawberry pseudofruits.

MATERIAL AND METHODS

Place of execution and collection of vegetal material

The work was realized in two stages – *in vitro* and *in vivo*, conducted at the UERGS Teaching Laboratory, at the Unity in Cachoeira do Sul/RS (geographic coordinates: latitude: 30°0’45” South and longitude: 52°55’11” West), in the period between April and November of 2018. For both stages, the fungus *B. cinerea* was isolated from strawberry pseudofruits from the Albion cultivar, acquired from organic farmer from the municipality of Paraíso do Sul/RS (geographic coordinates: latitude: 29°39’50” South, longitude: 53°8’54” West).

Preparation of the PDA culture medium

By means of direct isolation, fungal structures were transferred to 70mm Petri dishes with PDA (potato, dextrose, agar) culture medium, with the aid of a platinum loop, in aseptic conditions in the laminar flow cabinet, and taken to the growth chamber, where they stood until the reproductive structures reached all the plate. These strains were kept under refrigeration in refrigerator (5-6°C) until the use in the tests. The PDA medium was prepared with 200 g of potato with peel, 20 g of dextrose, 15 g of agar and 1 L of distilled water. Potatoes were washed, sliced and boiled for 30 min. in distilled water and after, the broth was filtered in Becker, to which it was slowly added the agar and the dextrose and completed with distilled water for the volume of 1 L. The pH was measured and adjusted to the value of 6,0, using NaOH.

In vitro test

The in vitro test was conducted in completely randomized design, with 6 treatments and 3 repetitions. Tests were made with commercial essential oils of thyme (*Thymus vulgaris*), with 48% of thymol; cinnamon (*Cinnamomum cassia*), with 83,9% of cinnamic aldehyde; lemon grass (*Cymbopogon flexuosus*), with 45% of geranial and 34% of neral; peppermint (*Mentha piperita*) with 48% of menthol and 28% of menthone; and tea tree (*Melaleuca alternifolia*), with 37% of terpineol and 25% of terpinene, plus the control constituted of autoclaved water. The essential oils were prepared and kept in stock solution, diluted at 1% in grain alcohol and conditioned in amber flasks to keep its properties. In the moment of application, they were diluted at 0.001% in distilled water.

The reproductive structures of *B. cinerea* isolated from fruits were distributed in Petri dishes containing the PDA medium. Another plate containing filter paper soaked with 5 mL of the treatments solution was juxtaposed to the one containing the fungus. Plates were sealed with plastic film and taken to the growth chamber at the temperature of 23°C. Evaluations were taken on the fungal growth at 7 days after installation of each test, attributing grade 0 (zero) for the absence of growth; grade 1 for intermediate growth, when mycelia occupied half the plate and grade 2 for total growth, when mycelia covered the totality of the plate. Tests were realized in triplicate.

In vivo test

Two *in vivo* tests were conducted, both in completely randomized design, with 6 treatments and 4 repetitions. The strawberry cultivar Albion was used, conventional and acquired at the local market for the test 1, and without agrochemicals acquired from farmer from municipality of Paraíso do Sul, for the test 2.

Pseudofruits were previously sanitized with sodium hypochlorite at 1%, washed with distilled water, dried with paper towel, taken to laminar flow cabinet, submitted to UV light during 5 min. in the first test and 9 min. in the second one. Treatments were sprayed at the amount of 1 mL and, after 1 min., were dried with paper towel and then inoculated with the fungus *B. cinerea* by direct inoculation, with aid of a swab and then taken to the growth chamber with temperature of 24°C where they stood for three days, until showing signs of deterioration.

For the *in vivo* experiments, the six types of essential oils were tested in two evaluation moments, at zero and three days after inoculation, with 4 repetitions. Pseudofruits were evaluated for the presence of *B. cinerea*, firmness and total soluble solids content. The occurrence of gray mold was determined by counting the pseudofruits that presented any sign of mycelial development, converting posteriorly the numbers to percentage. Firmness destructive analysis and total soluble solids content were made in one pseudofruit per repetition. Firmness was measured at the equatorial region of the pseudofruits using portable digital penetrometer (3020 - Homis®), with an 8 mm tip, selecting the measurement unit in N, and the total soluble solids content using a portable analogical refractometer with ATC.

Statistical Analysis

Data were submitted to analysis of variance and the means were evaluated by the Scott-Knott test, with aid of the Sisvar software (FERREIRA, 2011).

RESULTS AND DISCUSSION

The grades attributed to the mycelial growth of the fungus *B. cinerea* cultivated in vitro were influenced by the applied treatments (Table 1). The minor growth and major suppression of the fungus were observed with the essential oils of thyme and cinnamon, with grades 0.32 and 0.57, respectively, while the major growth and minor suppression were observed with the essential oil of tea tree and water (control), with grades of 1.70 and 1.82, respectively. Another study with strawberries observed that the essential oil of *Solidago canadensis* directly inhibited the growth of *B. cinerea in vitro* (LIU et al., 2016). The percentage of *in vivo* infected strawberries by the fungus was influenced by the treatments, with better results for the essential oils of tea tree, thyme, lemongrass and peppermint, whereas cinnamon did not differ from control, in experiment 1 (Table 1).

TABLE 1 - Attributed grades to the mycelial growth of the fungus *B. cinerea in vitro* culture and percentage of infected strawberries by the fungus *in vivo* culture.

Treatments	Grades for mycelial growth *in vitro****	Infected strawberries *in vivo*	
		Test 1	Test 2
		------------%------------	
Control	1.82 d*	45.00 b	80.0
Cinnamon	0.57 a	38.33 b	75.0
Peppermint	0.92 b	26.66 a	65.0
Thyme	0.32 a	25.00 a	55.0
Tea tree	1.70 d	8.33 a	25.0
Lemongrass	1.25 c	25.00 a	65.0
F	**	*	ns
CV (%)	28.02	91.95	53.55

*Means followed by the same letters in the column do not differ from each other by the Scott-Knott test at 5% probability of error, ns= not significant, at 5% probability of error, ** = significant at 5% probability of error, *** = grade 0 (zero): absence of growth, grade 1: intermediate growth, grade 2: total growth.

The essential oil of thyme controlled the mycelial growth of the fungus in the plates and in the pseudofruit in the first test, reducing in 20% the number of infected strawberries in comparison with control. Positive effects using the essential oil of thyme (*Thymus vulgaris*) in the inhibition of mycelial growth and germination of *B. cinerea* spores have been registered in the literature (DAFERARA et al., 2003; PLOTTO et al.; 2003; VITORATOS et al., 2013). Thyme is an aromatic plant, medicinal and condimental, belonging to the Lamiaceae family and its essential oil presents antifungal, antibacterial and antioxidant activities (NASCIMENTO et al., 2000; JAKIEMIU et al., 2010). Its use in the proportion of 0,5 mL directly applied as a compress over the packing cover containing strawberry, presented control action over the fungus *Botrytis* (CAMPOS et al., 2015). The antifungal action is resultant of the presence of thymol and carvacrol, that possess antimicrobial activity against a wide spectrum of microorganisms (ROMERO et al., 2009).

The cinnamon essential oil significantly reduced the mycelial growth of *B. cinerea in vitro*, however this effect was not observed when directly applied in the strawberry. Similar results were observed *in vitro* by Garcia et al. (2019), starting from concentration of 250 mg L^{-1}, and by Lorenzetti et al. (2011), in concentration of 1%. The effect of cinnamon essential oil also inhibited the development of fungi *A. niger*, *A. flavus*, *Rhizopus* sp. and the severity of fungi in treated nuts (LORINI et al., 2016) and the development of *Penicillium* spp., green mold-causing fungus in pear-orange fruits (SILVA et al., 2018). Contrasting the above-mentioned, the cinnamon nanoemulsion in 0.2% concentration showed reduction of 13.50% in another fungus, *Rhizopus stolonifera* in strawberry (NASERZADEH et al., 2019).

The efficiency of cinnamon in controlling fungi occurs due to the majoritarian compounds of its composition (LORENZETTI et al., 2001), such as cinnamic aldehyde, which is considered as the main antimicrobial agent present in the cinnamon extracts, exhibiting inhibition capacity against various bacteria and fungi (CHENG et al., 2006; CHENG et al., 2008; JANTAN et al., 2008). This compound represents 83.9% of active principles of the utilized oil. However, this effect could not be observed in the reduction of infected strawberries, maybe by not inducting the defense compounds in the fruit, in the used concentration.

The tea tree essential oil did not show significative effect in the development of the fungus in vitro, compared with control. However, when applied directly over the pseudofruits, demonstrated major efficiency in the suppression, reducing in 37% the infected strawberries, in comparison with control (Table 1). A possible explanation for this result is that the essential oils do not act only as fungicides, but also as resistance inductors (GARCIA et al., 2019; MORAIS, 2009). However, Garcia et al. (2019) observed that this essential oil applied in the post-harvest of fruits of grape “Rubi” was not enough for reducing the gray mold in the berries, probably by not activating the defense mechanisms in the fruit.

The peppermint essential oil was efficient in the mycelial growth suppression of *B. cinerea* in vitro, as well in the first evaluation of the direct application in the strawberry. In the literature, works with diverse results are found. The antifungal effect of the peppermint essential oil over *B. cinerea* was verified by Aminifard e Mohammadi (2012) in *in vivo* tests realized in plum and *in vitro*, by inhibiting the growth of post-harvest pathogenic fungus in cherry-tomato (GUERRA et al., 2015). However, the antimicrobial effect of the essential oil applied on packs of eatable strawberries was not clear in studies developed by Leite et al. (2015) and Vu et al. (2011). Menthol is the responsible component by the antifungal properties of the peppermint essential oil (EDRIS; FARRAG, 2003; SCARTAZZINI, 2018), and is found as the main component in the essential oil used in this work (48%).

The lemongrass essential oil was efficient in the mycelial growth suppression *in vitro*, and in the percentage of strawberries that developed the disease in the first evaluation, compared with control. Lorenzetti et al. (2011) also verified the suppression efficiency of *Botrytis* with lemongrass essential oil in strawberries. This essential oil presents 45% of geranial, responsible for the antiseptic and antibacterial activity, inhibiting the growth of fungi and bacteria, with the advantage of having low toxicity and biodegradable nature (AHMAD; VILJOEN, 2015; CHEN; VIJOEN, 2010; DUARTE et al., 2007; TATSADJIEU et al., 2009).

It was not observed significative difference between the treatments, in the experiment 2, where the infection percentage was higher (Table 1), which can be related to the pre-harvest factors, specially related to the use of synthetic amendments for crop protection, what did not happen with the strawberries acquired for this experiment. Although there occurred no significative difference, it was possible to observe that those treated with essential oil of tea tree had minor infection. As the used oil concentration was of 0.001%, it’s possible that bigger concentrations reflect in positive results.

There was no significative difference for the total soluble solids content in strawberry pseudofruits in function of the treatments (Table 2). In a similar way, in study developed by Braga (2012), the total soluble solids content did not vary significantly in strawberry treated with essential oils of lemongrass (*Cymbopogon citratus*), palmarosa (*C. martinii*), cinnamon (*Cinnamomum* sp.), basil (*Ocimum basilicum*), cordia (*Cordia verbenacea*), clove (*Eugenia caryophyllata*), anise (*Pimpinella anisum*), thyme (*Thymus vulgaris*) e chamomile (*Matricaria recutita*). In study with grape, the use of pitanga (Brazilian cherry) essential oil increased in 20% and that of tea tree by 27.1% the soluble solids content in fruits contaminated with *B. cinerea* (GARCIA et al., 2019).

The total soluble solids content of the strawberry utilized in the experiment are below the minimum of 7.5° Brix, according to the Normative Instruction n. 19, from June 19th, 2013 (BRASIL, 2013). However, according to Kader (1991), these values can vary between 4.6% and 11.9%, in conformation with pre-harvest factors and cultivar, as well as registered by Casonatto et al. (2016) for the cultivar Albion, that in the maturation showed 6.5° Brix.

These contents reduced over the storage period, except for the pseudofruits treated with peppermint, which had increase of 0.4° Brix. The variations are, probably, due to the dehydration associated with the senescence and growth of microorganisms (BORGES et al., 2013). The non-climacteric fruits are harvested in the physiological maturity and as a consequence, present small modifications in the sugars content, being possible to occur, in some of them, an increase in the initial content, as a result of the polysaccharides metabolism of the cell walls (CHITARRA; CHITARRA, 2005).

TABLE 2 - Mean values of total soluble solids and firmness of strawberry pseudofruits in function of essential oils over days after storage.

Treatments	Total soluble solids		Pseudofruit firmness	
	Days after storage			
	0	3	0	3
	----------º Brix----------		----------N----------	
Control	6.65 a*	6.25 a	0.54 a	0.10 a
Cinnamon	6.98 a	5.83 a	0.44 a	0.09 a
Peppermint	6.13 a	6.53 a	0.45 a	0.11 a
Thyme	6.28 a	5.28 a	0.39 a	0.08 a
Tea tree	5.95 a	5.68 a	0.31 a	0.06 a
Lemongrass	5.85 a	5.80 a	0.33 a	0.05 a
F	ns	ns	ns	ns
CV (%)	21.41	13.72	57.58	56.64

*Means followed by the same letters in the column do not differ from each other by the Scott-Knott test at 5% probability of error, ns= not significant, at 5% probability of error.

There was no significative difference between treatments for strawberry texture, with mean values from 0.41 N at 0 (zero) days after storage (DAS), reducing to 0.17 N at 3 DAS, with major reductions in the control, in the order of 0.44 N. These alterations can be attributed to the major mass loss and fungal development, accelerating the pseudofruits senescence. However, significative effect with the use of menthol essential oil and superior textures values were registered by Scartazzini (2018) in strawberry pseudofruits, inoculated with *B. cinerea*, in which the mean values of firmness in the time zero of storage were 1.11 N, reducing to 0.9 N in fruits without essential oil treatment, independently of having coating or not, and 1.05 N in fruits with eatable coating and essential oil application.

Evaluating the essential oils of citronella, *pitanga*, *patchouli*, *guaçatonga* and tea tree, Vismara (2019) observed that these last two differed significantly from control, not differing from the others, keeping major firmness of the strawberry pseudofruits, suggesting that the oils allowed minor metabolic activity and, consequently, minor degradation of the cell wall. The maintenance of the pulp firmness is an important factor of the post-harvest quality of strawberries, because the visual aspect and the fruits integrity are determining factors in the acquisition by the customers. However, the firmness reduced over the days, reflecting the pseudofruits degradation, but with less variation in those treated with tea tree (0.25 N), probably due to the minor development of the gray mold (Table 1). The firmness loss in the strawberries can be attributed to the changes that occurred in the cell tissue structure of the fruits during its maturation and senescence, that can be related to the loss of water and to the fungal development (CHIRALT et al., 2001). Also, the strawberry is a climacteric fruit, but with a high respiratory rate at 20°C (CHITARRA; CHITARRA, 2005), what contributes to its fast deterioration.

It's important to consider that the majoritarian constituents of the essential oil can vary in function of varieties and access, besides biotic and abiotic factors where the aromatic plants were cultivated, what can reflect in different results when applied for crop protection. As the secondary metabolites represent a chemical interface between plants and the surrounding environment, their synthesis is frequently affected by the environmental conditions. Thereby, variations may occur in the total content and/or in the proportions of secondary metabolites in the plants (GOBBO-NETO; LOPESS, 2007). Thus, more important than the essential oil, is the composition of the majoritarian active principles. Novel tests *in vitro* and *in vivo* with strawberry pseudofruits must be done, inclusive with higher concentrations, to confirm its inhibition capability, because the showed results are promising.

CONCLUSIONS

The essential oils of thyme and cinnamon showed potential of use for suppression of the fungus *Botrytis cinerea* in test *in vitro*.

The essential oils of peppermint, thyme, tea tree and lemongrass showed potential of use for suppression of the fungus *Botrytis cinerea* in strawberry pseudofruits.

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