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Effect of essential oils on the development of *Colletotrichum* sp. fungus in fragments of *Feijoa sellowiana* fruits

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ABSTRACT. The aim of this work was to evaluate the effect of the essential oils of *Syzygium aromaticum*, *Cymbopogon citratus*, *Eucalyptus citriodora* and *Rosmarinus officinalis* on the mycelial development of the fungus *Colletotrichum* sp. in fragments of *Feijoa sellowiana* fruits. The essential oils were incorporated in the PDA (Potato-Dextrose-Agar) medium in the concentrations of 250, 500 and 1000 ppm, 0 ppm (PDA only) (negative control), and fungicide fluazinam 1% (positive control). The area under the mycelial growth curve (AUMGC) and percent inhibition of mycelial growth (PIMG) were calculated. In the second evaluation, fruits fragments bordering the disease symptom were immersed in essential oils aqueous solution of *S. aromaticum*, *C. citratus*, and *E. citriodora*, at the concentration of 5000 ppm, 0 ppm (water only - negative control) and fluazinam 1% (positive control). The immersion times in the treatments were: 2, 4, 8, 12 and 24 hours, with subsequent incubation in Agar-Agar medium at 25°C. This evaluation was performed daily for 15 days, observing the moment of fungal germination through the emission of the mycelium. It was verified from the obtained results that all treatments reduced the fungal growth, and the essential oils of *C. citratus* and *S. aromaticum* totally inhibited its growth from the dose 500 and 1000 ppm, respectively. Regarding the test on fruit fragments, the essential oil of *S. aromaticum* at the immersion times of 12 and 24 hours was effective in inhibiting the fungus until the 15th day of evaluation.

Keywords: alternative control; *Syzygium aromaticum*; *Cymbopogon citratus*; *Eucalyptus citriodora*; *Rosmarinus officinalis*.

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Introduction

In Brazil, the abundance of autochthonous food species is substantial; nevertheless, this biodiversity is scarcely used compared to its potential. For this reason, it is the great relevance the encouragement of strategies aimed to increase the biological diversity, managing native species, as well as, to generate an alternative income. In this scenario, the South region of Brazil is home to several food species, among which *Feijoa sellowiana* O. Berg (sin. *Acca sellowiana*) stands out. This species is native to the southern Brazilian plateau and northeast of Uruguay, with dispersions in Argentina and Paraguay (Keller & Tressens, 2007). The interest in the study of this myrtaceae is because this species is adapted to the edaphoclimatic conditions of south Brazil colder regions, and for presenting fruits with significant organoleptic potential (Ducroquet, Hickel, & Nodari, 2000).

However, among the challenges that this species offers, the phytosanitary issues are mentioned, and the most significant disease of *F. sellowiana* is anthracnose (Andrade & Ducroquet, 1993). This disease is caused by the fungus *Colletotrichum* sp., reported as one of the most important pathogens that infect the fruit trees (Agrios, 2005). In *F. sellowiana*, this fungus can cause plantlets tipping, loss of large numbers of seedlings, partial or total drying of branches, even causing the death of adult plants. In addition, in conditions where this disease is more intensified, it may damage 100% of the fruits near maturation and juveniles (Andrade & Ducroquet, 1993; Ducroquet et al., 2000). The control of anthracnose is carried out basically by choosing genotypes and resistant cultivars, management and cultural treatments, sanitization measures and mainly using chemical products (Agrios, 2005).

The large-scale consumption of pesticides to control pests and diseases has increased significantly; nevertheless, its excessive use has been raising concerns for the population due to problems for human health

and for the environment. Moreover, the society seeks healthier lifestyles (Ghini & Kimati, 2000; Bettiol & Ghini, 2003; Bettiol, Maffia, & Castro, 2014).

Based on this context, studies with alternative methods in the control of plant diseases have been standing out, since these methods have the objective of controlling diseases without having the same problematic effect of agricultural pesticides. Among them, the use of medicinal plants has been exploited due to its importance in the contribution as a natural source of molecules in the control of diseases and pests in agriculture (Schwan-Estrada & Stangarlin, 2005).

Medicinal plants have secondary compounds that can either present direct activity through crude extracts, hydroalcoholics and plant essential oils on phytopathogens, or indirectly, by activating plant defense mechanisms to pathogens (Hammerschmidt & Dann, 1997). Several crude extracts and essential oils have already been tested on phytopathogens in several studies (Fiori et al., 2000; Itako, Tolentino Júnior, & Schwan-Estrada, 2013; Cruz et al., 2013; Mattos, Povh, Rissato, Schwan, & Schwan-Estrada, 2019). Promising results regarding the fungus of the genus *Colletotrichum* using plant compounds have been obtained both, in the *in vitro* conditions on its development, and in postharvest protection in some crops of agricultural importance (Anaruma et al., 2010; Perumal, Sellamuthu, Nambiar, & Sadiku, 2016; Andrade & Vieira, 2016).

Thus, the purpose of this study was to evaluate the effectiveness of essential oils of *Syzygium aromaticum*, *Cymbopogon citratus*, *Eucalyptus citriodora* and *Rosmarinus officinalis* in the *in vitro* development of *Colletotrichum* sp. and in diseased fragments of *Feijoa sellowiana* fruits.

Material and methods

Fungal isolation and extraction of essential oils

The experiment was conducted at the Phytopathology Laboratory of the *Universidade Federal de Santa Catarina* (UFSC), Curitibanos Campus. The fungus *Colletotrichum* sp. was isolated from diseased fruits and stored in PDA (Potato-Dextrose-Ágar) culture medium and incubated at 24°C for 12 hours of photoperiod.

Healthy leaves of *C. citratus* (DC) Stapf, Poaceae family, *E. citriodora* Hook, family Myrtaceae, and *R. officinalis* L. Family: Lamiaceae were collected in the University's Medicinal and Aromatic Plants Garden. The essential oils were obtained by steam distillation using a Clevenger apparatus. After the extraction, they were stored in amber glasses at room temperature. Only the essential oil of dried floral of *S. aromaticum* (L. Merr. & L. M. Perry, Myrtaceae family) was purchased commercially.

Evaluation of mycelial growth on the fungus *Colletotrichum* sp.

In order to evaluate each essential oil in the mycelial growth of the fungus *Colletotrichum* sp., the aliquots of oil were incorporated in the PDA melting medium in the doses of 250, 500 and 1000 ppm and distributed in Petri dishes. As negative control the dose used was 0 ppm (PDA only). As a positive control, the chemical fungicide fluazinam at 1% concentration was used. In all treatments 1% (v v^{-1}) of Tween20® was added to the culture medium to facilitate the emulsification of each of the essential oils.

After the medium solidification, a 6 mm diameter disc of the mycelial fungus, with 7 days growth, was deposited in the center of the Petri dish with the culture medium. The plates were sealed and incubated at 24ºC for a photoperiod of 12 hours. The mycelial growth was evaluated by measuring two opposite diameters of every colony at 24 hours intervals, and these measurements were finished when the control had the fungus colony established on 80% of the growing media surface.

The mycelial growth data obtained by the daily measurements of the colonies were used to calculate the area under the mycelial growth curve (AUMGC). The equation proposed by Campbell and Madden (1990) was used Equation 1:

$$AUMGC = \sum \left(\frac{y_{i+1} + y_i}{2}\right) \cdot (t_{i+1} - t_i) \qquad (1)$$

where: y_i and y_{i+1} are the colony growth values observed in two consecutive evaluations, ti+1 and ti are the periods of the evaluations.

The AUMGC was used in the calculation of percentage of inhibition of mycelial growth (PIMG) using the formula described by Bastos (1997): PIMG (%) = t*100/T, where T is the AUMGC of the control (dose 0 ppm) and t is the AUMGC of the treatments.

The experimental design was completely randomized (DCR) with five replicates. Each Petri dish was considered a repetition. Data were submitted to analysis of variance and then to the non-linear regression analysis using package '*drc*' of software R by the model of Gompertz with 3 parameters, $\left(y = D \cdot e^{-e^{B \cdot (\log(x) - E)}}\right)$, where D is the upper limit parameter, B is the relative slope of the curve and E parameter is the logarithm of the inflection point.

Tests in fruits fragments of *Feijoa sellowiana* fruits

Feijoa sellowiana fruits (cv. Alcântara) with symptoms of anthracnose were collected from plants belonging to the active germplasm bank of *Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina* (Epagri), Experimental Station of São Joaquim, located in São Joaquim, state Santa Catarina.

For the evaluation of the treatments with the essential oils in different immersion times, fragments of 10 mm were removed in the lesioned area of the fruits presenting symptoms of the disease. The fruit fragments were previously disinfested with 70% alcohol (1 min.) and then with 2% sodium hypochlorite (2 min.) and rinsed in sterile distilled water (Punja & Rahe, 1993). After the surface disinfection, the fruit fragments were immersed in aqueous solution of the essential oils of *S. aromaticum*, *C. citratus* and *E. citriodora* at the concentration of 5000 ppm. In addition to the essential oils, distilled water was used as a negative control and as a positive control the chemical fungicide fluazinam at the concentration of 1%. In all treatments 1% (v v^{-1}) of Tween20® was added to facilitate emulsification.

The immersion times in the previously described treatments were: 2, 4, 8, 12 and 24 hours. After this period four fragments were arranged equidistantly in Petri dishes on the surface of the agar-agar (AA) culture medium. The plates were sealed and incubated at 24°C and for a photoperiod of 12 hours. The evaluations were performed daily, for 15 days, observing the fungal germination time through the emission of mycelium around the fragment of the fruit.

The experimental design was completely randomized with four replicates. Each Petri dish with four fruit fragments was considered a repetition. The data were submitted to survival analysis using the 'survival' package from statistical software R. Survival curves of Kaplan-Meier (*survfit* function) were calculated. The curves were compared by the log-rank test Mantel-Haenszel (G-rho Family test) (*survdiff* function) against the control and the fungicide fluazinam (Dudley, Wickham, & Coombs, 2016).

Results and discussion

Fungi toxic activity of the essential oils

Regarding the percentage of inhibition of mycelial growth (PIMG) (Table 1), all treatments with oils significantly reduced fungal growth as compared to negative control, including the fungicide treatment (positive control), which inhibited 100% of mycelial growth (data not shown). The response was dose-dependent adjusted to the Gompertz model with 3 parameters, except for the essential oil treatment of *R. officinalis* which did not fit the model.

Table 1. Inhibition of mycelial growth (PIMG - %) of the fungus *Colletotrichum* sp. treated with essential oils at different doses and coefficients of nonlinear regression of Gompertz model.

Dose	Inhibition of mycelial growth (%)			
	Syzygium aromaticum	*Cymbopogon citratus*	*Rosmarinus officinalis*	*Eucalyptus citriodora*
0 ppm	0.0	0.0	0.0	0.0
250 ppm	61.8	21.0	-9.3[1]	-0.4[1]
500 ppm	91.3	100.0	11.2	47.7
1000 ppm	100.0	100.0	49.1	70.6
	Gompertz[2] Model coefficients			
B	-0.0086	-0.0254	-	-0.0115
D	98.61	100.14	-	70.66
E	164.93	267.52	-	418.99

[1]Negative values indicate higher growth than control. [2]Gompertz Model with 3 parameters $\left(y = D \cdot e^{-e^{B \cdot (\log(x) - E)}}\right)$ where D is the upper limit parameter, B is the relative slope of the curve and E parameter is the logarithm of the inflection point.

The essential oil of *C. citratus* completely inhibited the mycelial development of the fungus from the 500 ppm dose (Table 1). Also, *S. aromaticum* inhibited from the 1000 ppm dose. The *E. citriodora* essential oil inhibited mycelial development in 70.6% at 1000 ppm dose. The essential oil of *R. officinalis* at the dose of 250

ppm had higher mycelial growth than the control. There was only inhibition of mycelial development at doses of 500 to 1000 ppm by 11.2 and 49.1%, respectively.

The efficacy of *C. citratus* and *S. aromaticum* oil has been proven in several studies in other pathosystems. Fiori et al. (2000) assessed several essential oils and verified that *C. citratrus* oil completely inhibited spore germination and growth of the fungus *Dydimella bryoniae*. Ranasinghe, Jayawardena, and Abeywickrama (2002) verified the fungistatic and fungicidal effect of volatilization of the oils of *S. aromaticum* and *Cinnamomum zeylanicum* in the development of the fungi *Lasiodiplodia theobromae*, *Colletotrichum musae* and *Fusarium proliferatum*. Itako et al. (2013) evaluated the effect of increasing doses of *C. citratus* on the development of *Alternaria solani* and the induction of pathogenesis-related enzymes. They also observed the fungitoxic effect of the oil and its ability to induce the peroxidase and polyphenoloxidases enzymes in *Solanum lycopercisi* L. Perumal et al. (2016) investigated the antifungal effect by volatilization of five essential oils on *Mangifera indica* L. and observed that the oil of *T. vulgaris* and *S. aromaticum*, entirely inhibited the development of the fungus *C. gloeosporioides*. Oliveira, Oliveira, Vieira, Câmara, and Souza (2018) evaluated the effect of *C. citratus* oil, associated with a chitosan to control five pathogenic species of *Colletotrichum* in guava (*Psidium guajava* L.) and *in vitro*, and observed an inhibition of mycelial growth of all tested fungal species. These results corroborate with those obtained in the present work, since the oil of *S. aromaticum* and *C. citratus* completely inhibited the development of the fungus.

Salgado et al. (2003), evaluating the oils of different eucalyptus species, verified that the essential oil of *Eucalyptus urophylla* presented greater fungitoxic action and this was attributed to the presence of the compound globulol, absent in *Eucalyptus camaldulensis* and *E. citriodora*. According to Silva (2006), the chemical composition and quantity vary depending on the age of the plant, the type of tissue, its habitat and the type of soil. This partially explains the discrepancy found among the studies conducted at different locations using the same methodology and same plant species.

Regarding the results with essential oil of *R. officinalis* similar results were obtained by Daferera, Ziogas, and Polissiou (2003) in which evaluated six essential oils including *R. officinalis* on the development of fungi *Botrytis cinerea*, *Fusarium* sp. and observed among the evaluated oils, the *R. officinalis* showed lower inhibitory activity against fungi.

Test on fruits fragments

In preliminary tests, doses of 1000, 2000 and 4000 ppm were tested, and these did not show results in inhibiting the fungus in the fruits fragments. Thus, only 5000 ppm was used in the experiment.

Regarding the germination of the fungus in the treatments by immersion of *F. sellowiana* fruits fragments in solution of the essential oils, the 2 and 4 hours immersion times were not efficient in inhibiting fungus development for any of the essential oils tested. However, the other times presented variable responses of 50 to 90% inhibition (Table 2).

Table 2. Inhibition of fungal germination (%), evaluated on the 15th day in fragments of *Feijoa sellowiana* fruits treated with the essential oils of *Cymbopogon citratus*, *Syzygium aromaticum* and *Eucalyptus citriodora* at 5000 ppm dose, fungicide fluazinam (positive control) and pure water (negative control).

Immersion time	Inhibition of fungal germination (%)				
	Cymbopogon citratus	*Syzygium aromaticum*	*Eucalyptus citriodora*	Fungicide	Pure water*
2 hours	0	0	0	100	0
4 hours	0	0	0	100	0
8 hours	60	70	90	100	0
12 hours	60	90	50	100	0
24 hours	70	90	50	100	0

*Germination of all the fruits fragments in the 4th day of evaluation.

Due to the low efficiency in inhibition of mycelial growth of the essential oil of *R. officinalis*, this oil was not used in the assay with the fruit fragments. In relation to the treatment of the negative control (Figure 1A), there was no inhibition of fungal germination in all fruit fragments from the 4th day of evaluation. Yet, the positive control treatment (Figure 1B) totally inhibited germination until the 15th day. In the 8 hour immersion time (Figure 1C, D and E), the fruit fragments treated with the essential oils of *S. aromaticum* and *E. citriodora* remained without hyphae formation until the 4th day, and for the oil of *C. citratus* until the 5th day, similar to the negative control treatment. The percentage of fruit fragments without hyphae formation, from 6th day until

15th day, was gradually reduced. All treatments with essential oils presented percentages lower than 40% in the inhibition of germination, observing the lowest percentage of 10% in the treatment with *E. citriodora* oil.

In the case of 12 hours immersion time (Figure 1F, G and H), the fruit fragments treated with the essential oils of *C. citratus* and *E. citriodora* maintained inhibition close to the negative control, and from the 6 to the 12th day, they had inhibitory effect of 73 and 58%, respectively. The treatment using *S. aromaticum* oil (Figure 1G) maintained 100% inhibition until the 8th day, and from that period to 15th day the inhibition was 90%.

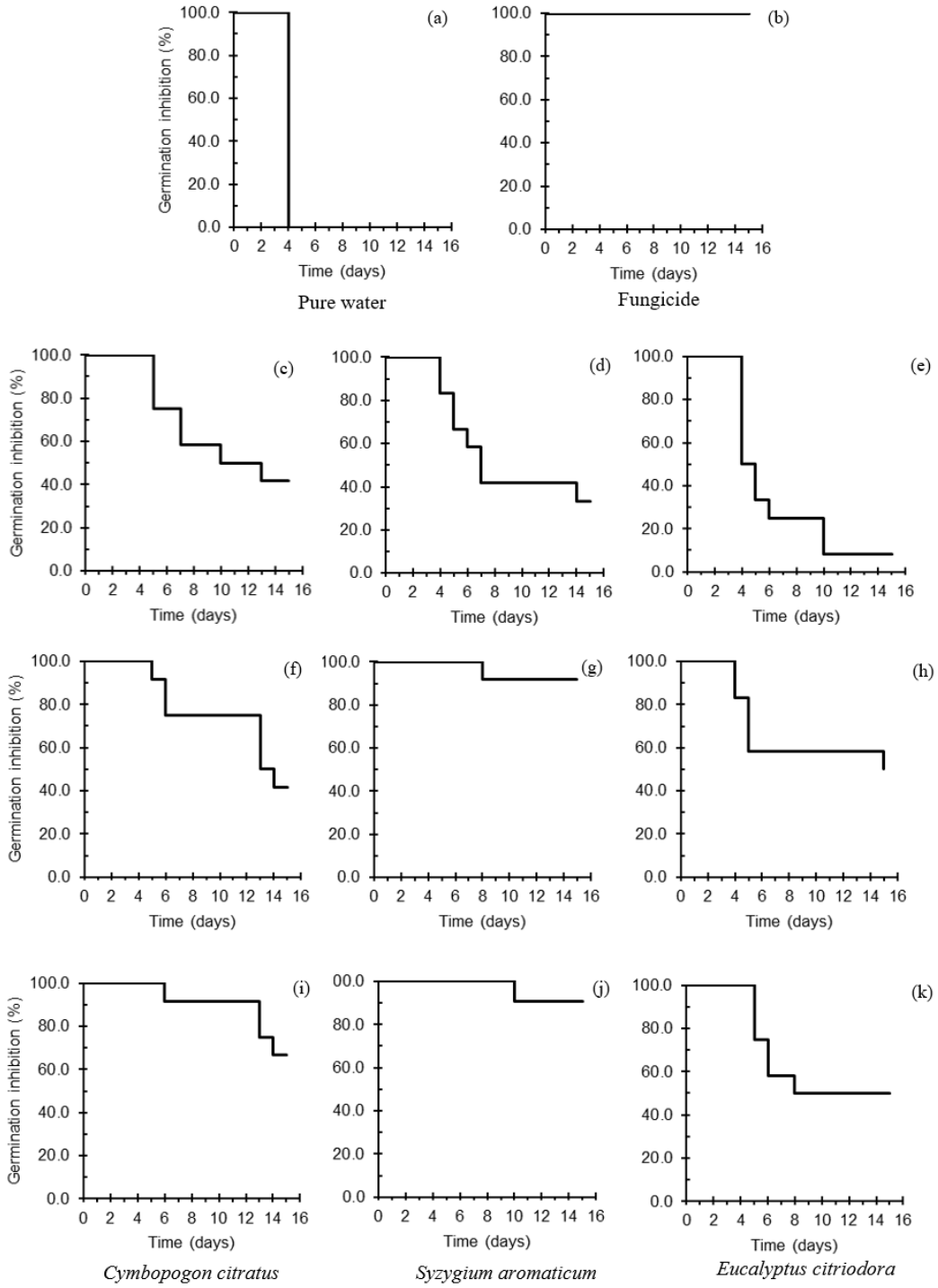

Figure 1. Kaplan-Meier survival curve for the percentage inhibition of the fungus *Colletotrichum* sp. germination in fragments of *Feijoa sellowiana* fruits until 15 days of evaluation for: (a) Pure water (negative control); (b) Fungicide fluazinam (positive control); (c, d and e) at 8 hours of immersion; (f, g and h) 12 hours of immersion; (i, j and k) 24 hours of immersion with the essential oils of *Cymbopogon citratus*, *Syzygium aromaticum* and *Eucalyptus citriodora*, respectively, at the dose of 5000 ppm.

And subsequently, in the 24 hours immersion time (Figure 1I, J and K) of the fruit fragments, the treatments with the oils of *S. aromaticum*, *C. citratus* and *E. citriodora* inhibited the formation of hyphae for 8,

6 and 5 days respectively. The *C. citratus* oil inhibited up to 70% germination of the fungus until the last day of evaluation. The oil of *S. aromaticum* showed to be efficient in the inhibition of the fungus until the 15th day, inhibiting in up to 90% of the fungus in the fruit fragments.

Regarding to the comparison test between the survival curves (Table 3), it was verified that all treatments with essential oils at immersion times of 8, 12 and 24 hours were higher than the negative control, indicating better efficiency in controlling of the growth by the essential oils. Compared with the positive control, most treatments were lower, except for the treatment with essential oil of cloves in the immersion times of 12 and 24 hours. In this time, the treatment using the *S. aromaticum* essential oil, the efficacy observed was similar to the positive control.

These results reaffirm the potential of *C. citratus* and *S. aromaticum* to possess antifungal and antimicrobial compounds. Barrera-Necha, Bautista-Banos, Flores-Moctezuma, and Estudillo, (2008) evaluated the effectiveness of nine essential oils, including *S. aromaticum* oil on the *in vitro* development of *C. gloeosporioides* isolated from *Carica papaya* L. and observed that increasing doses (200, 250 and 300 µg mL^{-1}) efficiently inhibited mycelial development and spore germination. From the *in vitro* results, it a test in papaya fruits was performed, and its efficacy was verified through the smaller percentage of infection. However, the treatments did not exceed the activity of the synthetic fungicide. Anaruma et al. (2010), evaluated 28 essential oils of medicinal plants on the development of *C. gloeosporioides* and observed that the oil of *C. citratus*, *Coriandrum sativum*, *Cymbopogon flexuosus* and *Lippia alba* inhibited mycelial growth of the fungus, and the oil of *C. citratus* reduced the rate of anthracnose disease in fruit of *Passiflora edulis*.

The essential oil of *C. citratus* has antifungal and antibacterial therapeutic properties, and the main component is citral (Nascimento, Innecco, Marco, Mattos, & Nagao, 2003). The main constituent of *S. aromaticum* oil is eugenol, a very efficient aromatic compound, with nematicidal, insecticidal, bactericidal and fungicidal activity (Lorenzi & Matos, 2002). Partly, one of the explanations of the action on fungal inhibition in both mycelial growth and sporulation of the fungus *Colletotrichum* sp. it may be due to the major presence of these components in the essential oil.

Analyzing the results, it was verified the highest efficiency of *S. aromaticum* oil compared to the other oils tested to inhibit germination of the fungus from the fruits fragments. According to Costa et al. (2011) the antifungal effect of the essential oil of *S. aromaticum* is related to its hydrophobicity, which allows them to interact with the cell wall constituents (lipids), altering the permeability, causing disturbances in these structures. The same authors studied the action of the *S. aromaticum* oil in the *in vitro* mycelial growth of phytopathogenic fungi and evidenced several morphological alterations (disorganization of the cellular contents, less turgidity of the hyphae, decrease in cell wall sharpness) on fungi *F. oxysporum*, *F. solani* and *R. solani*.

Table 3. Comparison test of Kaplan-Meier survival curves in the immersion time of 8, 12 and 24 hours by log-rank Mantel-Haenszel test (G-rho Family test).

	Cymbopogon citratus		*Syzygium aromaticum*		*Eucalyptus citriodora*	
	χ^2	p-value	χ^2	p-value	χ^2	p-value
			Immersion time of 8 hours			
Against the negative control	23.0**	< 0.001	16.4**	< 0.001	7.7**	0.006
Against the positive control	9.7**	0.002	11.8**	< 0.001	21.5**	< 0.001
			Immersion time of 12 hours			
Against the negative control	23.0**	< 0.001	23.0**	< 0.001	16.4**	< 0.001
Against the positive control	9.6**	0.002	1.0	0.317	7.7**	0.005
			Immersion time of 24 hours			
Against the negative control	23.0**	< 0.001	22.0**	< 0.001	23.0**	< 0.001
Against the positive control	4.6**	0.032	1.1	0.296	7.8**	0.005

**Significant by the Mantel-Haenszel log-rank test at the 5% probability level. Negative control: 0 ppm; positive control: fungicide fluazinam.

Conclusion

The essential oil of *S. aromaticum* was effective in inhibiting total mycelial growth and germination (hypha formation) in the *F. sellowiana* fruit fragments in 12 and 24 hours immersion time.

The other essential oils tested showed partial inhibition in the development of the fungus.

The results demonstrate the potential of essential oils in the control of plant diseases in fruits.

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