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Antifungal activity of *Baccharis trimera* and *Foeniculum vulgare* essential oils for control of bitter rot in apple

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Abstract: The present study aimed was to determine the chemical composition of *Baccharis trimera* and *Foeniculum vulgare* essential oils (EOs) and their *in vitro* and *in vivo* effect against *Colletotrichum fructicola*, the causal agent of bitter rot in apple. Chemical composition of EOs was determined by GC-MS and their effect against *C. fructicola* was evaluated *in vitro* by mycelial growth and conidia germination and *in vivo* by reducing disease symptoms on postharvest apples. The major compound found in *B. trimera* EO was carquejyl acetate (74.71%) and in *F. vulgare* EO was estragole (88.47%). In the *in vitro* tests, *B. trimera* EO showed a fungistatic action, while *F. vulgare* EO presented fungicidal action against mycelial growth. Both EOs showed complete inhibition of conidia germination. In the *in vivo* assay, both EOs tested were inefficient in reducing disease severity caused by *C. fructicola,* in preventive and curative treatment. These results are promising and indicate that the EOs might be further investigated as a natural alternative to synthetic fungicides for apple bitter rot control due to its *in vitro* fungicidal effect.

Keywords: *Colletotrichum fructicola,* alternative control, essential oil, *Malus domestica*

Resumo: *O objetivo do presente estudo foi determinar a composição química dos óleos essenciais (OEs) de Baccharis trimera e Foeniculum vulgare e seu efeito in vitro e in vivo no controle de Colletotricum fructicola, agente causador da podridão amarga em maçãs. A composição química dos OEs foi determinada por CG-EM, e seu efeito contra C. fructicola foi avaliado in vitro pelo crescimento micelial e germinação de conídios, e in vivo pela redução dos sintomas da doença no pós-colheita de maçãs. O principal composto identificado no OE de B. trimera foi o acetato de carquejila (74,7 %) e no OE de F. vulgare o estragol (88,47%). Nos testes in vitro, o OE de B. trimera apresentou ação fungistática, enquanto que o OE de F. vulgare apresentou ação fungicida sobre o crescimento micelial. Ambos OEs inibiram completamente a germinação de conídios de C. fructicola. No teste in vivo, os OEs testados foram ineficientes na redução da severidade da doença causada por C. fructicola, tanto no tratamento preventivo quanto no tratamento curativo. Estes resultados são promissores e indicam que os OEs podem ser melhor investigados como uma alternativa natural aos fungicidas sintéticos para o controle de podridão amarga em maçãs devido ao seu efeito fungicida in vitro.*

Palavras-Chaves: *Colletotrichum fructicola, controle alternativo, óleo essencial, Malus domestica*

1. INTRODUCTION

The use of postharvest fungicides is not always efficient to control the major rots and their use in -pre and postharvest constitute environmental and toxicological risks [1]. Growing public concerns about health and environmental hazards associated with pesticide use have shown considerable interest in developing alternative non-polluting and health safe control methods [2]. Among the possibilities of alternative control, it is the use of essential oils (EOs). The EOs are well known for their antifungal and biodegradable properties and for not leave any residual effect on fresh produce [3]. In this context, several studies explore the potential of EOs of several plant species as antifungal agents and their use in food preservation [4] [5] [6] [7].

Apple (*Malus domestica* Borkh.) is one of the most important temperate fruit crops worldwide and the second most important temperate fruit crop in Brazil [8]. 'Fuji' is a popular apple variety due to its taste, good hardness, and long shelf-life [9]. However, this apple variety is susceptible to bitter rot caused by *Colletotrichum fructicola* Prihast., L. Cai & K.D. Hyde [10] [11]. Bitter rot is a fruit rot disease that causes significant losses in most countries whit apple commercially cultivated. Symptoms are described as initial light brown lesions in the fruit that enlarge over time, becoming brown and sunken. Each lesion usually progresses into the core of the fruit in a V-shaped pattern [12]. During favorable environmental conditions for the disease, losses up to 50% may occur during the different harvest, field handling, packing operations, transportation, and storage - pre and postharvest stages [13] [14].

Baccharis trimera (Less) is a native species widely distributed in Brazil with significant popular use in South America, mainly as natural medicinal products [15], and has been studied regarding its chemical composition and biological activity against phytopathogens [16] [17] [18]. *Foeniculum vulgare* Mill. is widely cultivated throughout the temperate and tropical regions for its aromatic fruits, which are used as a culinary spice [19] and have several biological properties, including antifungal activity against phytopathogens [20] [21] [22].

This study evaluates the effectiveness of *B. trimera* and *F. vulgare* EOs on *in vitro* on antifungal activity on mycelial growth and conidia germination of *C. fructicola* and their *in vivo* control of bitter rot disease in postharvest of apples.

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2. MATERIAL AND METHODS

Colletotrichum fructicola (A004/17) was isolated from apple at Vacaria (Serra Gaúcha, RS, Brazil) and kept in the fungal collection of the Laboratory of Phytopathology, University of Caxias do Sul. For molecular identification, the DNA was extracted from fungal hyphae according to the methodology described by Tappia-Tussell et al. [23]. After the DNA was extracted, PCR-based techniques were used to amplify the internal transcribed spacer region (ITS-5.8S rDNA) and its products were sequenced according to the methodology described by Echeverrigaray et al. [24]. Finally, the DNA sequences obtained were compared with those deposited in the GeneBank Database using the nBLAST algorithm (NCBI).

Leaves of *B. trimera* were collected at Bento Gonçalves, RS, Brazil (voucher specimen is deposited in the Herbarium of the University of Caxias do Sul with access number 43211). The fruits of *F. vulgare* were purchased through the company Temperabem Comércio de Produtos Alimentícios Ltda, located in the city of São Paulo – SP, under the manufacturing lot 7316291.

B. trimera EO was extracted by steam distillation from dried leaves, according to Pedrotti et al. [25]. *F. vulgare* EO was extracted by hydrodistillation according to Pedrotti et al. [22]. For the identification and quantification of EO compounds, the method described in Pedrotti et al. [25] was used. The chromatographic peaks of each component were analyzed and identified using the Wiley library [26] and based on comparing the registered linear retention indices with literature data (NIST). The linear retention index was calculated with the Van den Dool and Krats [27] equation using a standard solution (C8-C26 hydrocarbons).

The antifungal properties of EOs were assessed for their effects on the mycelial growth of *C. fructicola* according to Pedrotti et al. [28]. Different concentrations of EO (0.0; 1.0; 2.0 and 3.0 $\mu L \cdot mL^{-1}$) were emulsified with Tween 20 (1:1) and added to the PDA culture medium. These emulsions were poured into 9 cm (⌀) Petri dishes and inoculated with 5 mm (⌀) agar disks colonized by *C. fruticola* obtained from 7 day-long pre-cultures. Tests were conducted in triplicate with fifteen plates for each treatment. Incubation was conducted in a growth chamber at 25 ºC with a 12 h photoperiod for 14 days. The mycelial growth was recorded on the 3rd, 5th, 7th, 10th, and 14th days by measuring the orthogonal diameter. Transfer experiments were performed to distinguish the fungicidal and fungistatic effects of EOs [28].

The antifungal activity of EOs on conidia germination evaluation used conidia of *C. fructicola* harvested from 14 day old fungal colonies grown in PDA at 25 ºC under 12 h photoperiod. Conidial suspensions with a concentration of 1□10^6 conidia/mL^{-1} were used. Aliquots of conidia suspension (50 µL) were placed in microtubes containing Potato Dextrose Broth medium (500 µL) with different EOs concentrations (0.0; 0.5; 1.0; 2.0; 3.0 and 4.0 $\mu L\ mL^{-1}$), emulsified with Tween 20 (1:1). The microtubes were incubated at 25 ºC, and the evaluation was performed after 24 h. Samples were placed on a hemocytometer chamber and observed under the microscope (40×) for conidia germination. Tests were conducted in triplicate with fifteen replicates per treatment and, for each replicate, a hundred conidia were evaluated. The conidia were considered germinated when the germ tube's length equaled or exceeded the length of the conidia [28].

To evaluate the antifungal activity of *B. trimera* and *F. vulgare* EOs in postharvest on apples it was carried out experiments with curative and preventive treatments. Mature and asymptomatic apples 'Fuji' were obtained from the local trade of Bento Gonçalves, RS, Brazil. The apples were thoroughly washed with a hypochlorite solution of 1.5% to remove the possible remains of chemical applications and rinsed with distillate water. The treatments with different EOs were based on results from the *in vitro* test. Thus, the concentration of 3.0 $\mu L\ mL^{-1}$ was defined. The EOs were emulsified with Tween 20 (1:1) and added to sterile water. Conidial suspensions were obtained by dislodged from the surface of cultures using sterile water and, the suspension was diluted to obtain a suspension of 1 x 10^6 conidia/ml^{-1}. The curative treatment consisted of inoculation of apples with one drop of 10 µl of the conidia suspension placed over a wound measuring 1 to 2 mm deep made with a sterile needle and, after 4 h, the application of EOs was carried out. The preventive treatment consisted of applying EOs on apples and inoculated after 24 h with one drop of 10 µl of the conidia suspension placed over the wound. The control treatment was inoculated with 10 µL of sterile water, and the diseased treatment was inoculated with 10 µL of conidia suspension. Apples were placed in plastic boxes and kept at 25 ± 2 °C with 80-90% relative humidity in the dark for ten days. At the end of this period, the disease's severity was assayed by measuring of the lesion's diameter, using a digital caliper. Tests were conducted with fifteen apples for each treatment.

For statistical analysis, data normality was determined by Kolmogorov-Smirnov test, and the homogeneity of variances was determined using Levene's test. Data were analyzed by ANOVA and the threshold for statistical significance was set at $p \leq 0.05$. In the case of statistical significance Dunnett's T3 or Tukey test was applied to separate the means. All statistical analyses were performed using SPSS 22.0 program.

3. RESULTS AND DISCUSSION

The compositions of EOs varied according to plant species (Table 1). The major compound found in *B. trimera* EO was carquejyl acetate (74.71%), with eighteen other compounds present in lower concentrations. In general, 87.82% of *B. trimera* EO compound were monoterpenes (11.06% hydrocarbons and 76.76% oxygenated), and only 11.24% were sesquiterpenes (2.50 % hydrocarbons and 8.74% oxygenated). These results are within the range reported by Simões-Pires et al. [29] and Besten et al. [30] found carquejyl acetate (35.5 to 68% and 40.7 to 73.5%, respectively) as the major compound of EO from *B. trimera* collected in different places of southern Brazil. Carquejyl acetate

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is a compound present specifically in the *B. trimera* species, often differentiating this species with others of the genus *Baccharis* [31]. Moreover, previous studies reported the antifungal activity and medicinal properties of this compound [17] [18] [31].

The major compound identified in *F. vulgare* EO was estragole (88.47%), and thirteen other compounds were present in lower concentrations, of which 99.07% corresponded to monoterpenes (5.34% hydrocarbons and 93.73% oxygenated) and only 0.56% were sesquiterpene oxygenated. Differently from the data obtained in this study, where the estragole was identified as a major compound, Roby et al. [32] and Pedrotti et al. [22] found trans-anethole (65.4 and 79.14%, respectively) as the major compound of *F. vulgare* EO. The literature search revealed variations in the composition and proportion of the major compound of *F. vulgare* EO. These differences in components and the content of them in EOs may be related to the geographical origins [33], cultivated varieties, seasons [29], as well as extraction methods [34] [35].

As expected, the *in vitro* antifungal activity of EOs differed for each plant species and concentration tested (Figure 1). The effect of *B. trimera* EO on the mycelial growth resulted in complete inhibition at concentration 3.0 µL mL^{-1}. At lower concentrations (1.0 and 2.0 µL mL^{-1}) significant mycelial growth reduction was observed until the 10^{th} and 14^{th} day, respectively (Figure 1 A). Moreover, even at the highest concentrations, the *B. trimera* EO exhibited fungistatic activity, as mycelial growth was evidenced after transfer to the BDA medium. This result differs from that obtained by Caneschi et al. [17] and Pedrotti et al. [18] that reported fungicide activity of *B. trimera* EO against *Trichophyton rubrum* and *Microsporum canise* and *C. acutatum* and *Botrytis cinerea,* respectively.

The effect of *F. vulgare* EO also varied according to the concentration (Figure 1 B). At the lowest concentration (1.0 µL mL^{-1}), the EO exhibited significant inhibition of mycelial growth until the 7^{th} day, while at 2.0 µL mL^{-1}, significant inhibition was evidenced until the 10^{th} day. High concentration of *F. vulgare* EO (3.0 µL mL^{-1}) resulted in complete inhibition of mycelial growth all over the experiment. Moreover, no growth was detected on the transfer experiment, indicating fungicidal activity. The EO of *F. vulgare* has been reported to reduce the mycelial growth of *Sclerotinia sclerotiorum* [21] and to inhibit *Aspergillum niger, A. flavus, Fusarium graminearum* and *F. moniliforme* [20], *C. acutatum,* and *B. cinerea* [18].

The evaluation of the antifungal activity of EOs on conidia germination showed that *B. trimera* EO inhibited the conidia germination of *C. fructicola* at concentration 0.5 µL mL^{-1} completely. Conversely, *F. vulgare* EO showed a significant reduction in conidia germination at concentrations 0.5, 1.0, and 2.0 µL mL^{-1} and completely inhibited germination at concentration 3.0 µL mL^{-1} (Figure 2). Several studies also demonstrated the capabilities of several EOs to inhibit conidia germination of *Colletotrichum* species [22] [36] [37].

Previous studies suggest that the antifungal properties of EOs are due to the inhibition of ergosterol biosynthesis, the main steroid component of the cell membrane in fungi, and leads to the loss of membrane integrity and necrotic death. Moreover, the conidial germination inhibition is associated with a loss of membrane integrity, decreased cell metabolism, and accumulation of reactive oxygen species [38] [39].

Table : Chemical composition of essential oils obtained from leaves of *Baccharis trimera* and fruits of *Foeniculum vulgare.*

Compounds	**RI** [1]	**RA** [2]	
		B. trimera	*F. vulgare*
Monoterpenes		**11.06**	**5.34**
Hydrocarbons			
α-pinene	14.209	-	0.41
α-thujene	14.576	6.86	-
Camphene	15.768	1.09	-
β-pinene	19.302	0.34	-
Sabinene	21.383	1.61	0.14
Myrcene	21.895	0.74	0.11
Limonene	24.024	-	4.44
Cis-β-ocimene	26.960	0.42	-
ρ-cymene	27.896	-	0.24
Oxygenated monoterpenes		**76.76**	**93.73**
1,8-cineole	24.350	-	0.34
Fenchone	34.167	-	3.58
β-isophorone	34.169	0.19	-
Camphor	39.776	1.86	-
Terpinen-4-ol	43.203	-	0.14
Estragole	46.233	-	88.47
Carquejyl acetate	47.993	74.71	-
Carvone	48.663	-	0.76
Anethole	52.164	-	0.44
Sesquiterpenes		**2.50**	**0.00**
Hydrocarbons			
δ-cadinene	49.432	2.50	-
Oxygenated sesquiterpenes		**8.74**	**0.56**
Palustrol	55.669	3.45	-
Caryophylene oxide	56.964	0.20	-
Ledol	57.971	0.74	-
Globulol	58.238	2.67	-
Viridiflorol	58.838	0.54	-
Spathulenol	59.563	0.49	-
β-eudesmol	61.700	0.64	0.56
Others		**0.94**	**0.37**
Trans-pinocarvyl acetate	45.179	0.16	-
Cryptone	46.205	0.79	-
Anisaldehyde	57.751	-	0.23
Cis- α-copaene-8-ol	58.525	-	0.14

[1] RI, retention index determined relative to *n*-alkanes (C_8–C_{26}). [2] RA, Relative amounts of the compounds identified based on the area of each peak in the total chromatogram area.

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The postharvest apple symptoms consisted of sunken, brown lesions, each with a V-shaped pattern extending to the fruit core. Fruiting bodies with orange to brown conidial masses developed over the lesions. The *in vivo* experiments showed that the EOs of *B. trimera* and *F. vulgare* were inefficient to control bitter rot (Table 2), not reducing the severity of disease caused by *C. fructicola,* both in preventive and curative treatment. Similarly, Lopez-Reyes et al. [40] and Vieira et al. [41] evaluated the efficiency of different EOs on apples of different varieties in the postharvest to control *Botrytis cinerea* and *Penicillium expansum* and revealed that only a few EOs showed antifungal action against both fungi in all apple cultivars tested. Moreover, according to Allaniz et al. [11], *C. fructicola* species is the most aggressive one causing bitter rot of apple and, when permitted, chemical treatments remain the primary method for controlling postharvest diseases of fruit.

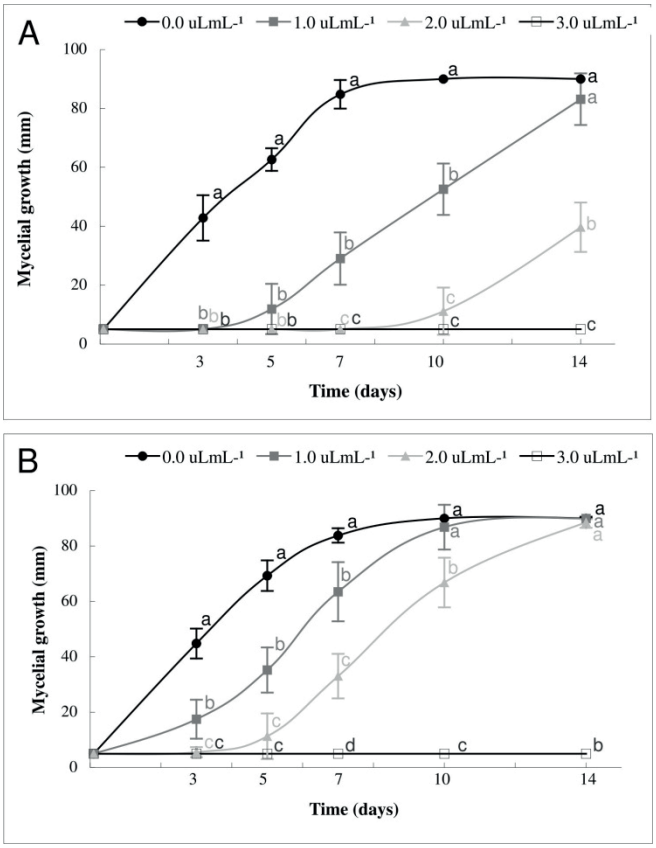

Figure 1: Effect of different concentrations of *Baccharis trimera* (A) and *Foeniculum vulgare* (B) essential oils, added on the solid media, on the mycelial growth of *Colletotrichum fructicola*. Values are the average of fifteen replicates per treatment ± SE. The letters indicate the comparison among the different essential oil concentrations evaluated in each day. Means followed by same letter do not differ by Tukey test ($p<0.05$).

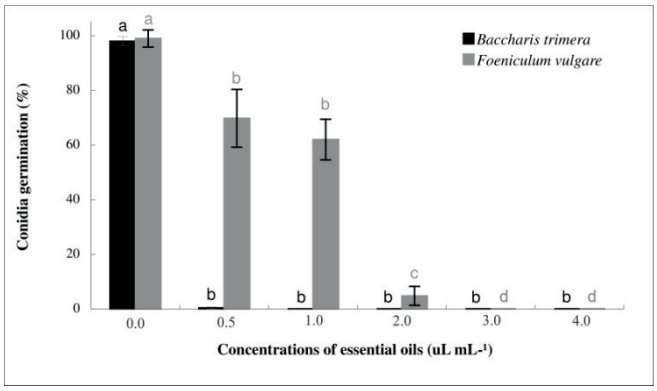

Figure 2: Effect of different concentrations of *Baccharis trimera* and *Foeniculum vulgare* essential oils on conidia germination of *Colletotrichum fructicola*. Values are the average of fifteen replicates per treatment ± SD. Means followed by same letter do not differ by Tukey test ($p < 0.05$).

In this way, this work demonstrated the antifungal activity *in vitro* of both OEs, but from the results obtained in the *in vivo* assay, it is clear the need to develop new studies. This effect is clearly due to environmental factors distinct between the *in vitro* and *in vivo* conditions. Under *in vivo* conditions, the efficacy of natural products as the EO is often limited by their sensitivity to more significat variability of environmental factors, which favor the rapid volatilization of compounds present in EO, unlike *in vitro* conditions which are more stable with a lower variability of conditions. Thus, we propose that studies evaluating different concentrations, combining EOs with other products, and incorporating them in an edible coat should be carried out looking for the bitter rot control. These methods can protect EOs from environmental factors and improve the stability and bioavailability of these compounds, increasing their efficiency.

Table 2. Effects of *Baccharis trimera* and *Foeniculum vulgare* essential oils in the severity of bitter rot on postharvest apples.

	Diameter of the lesions (mm)	
	Baccharis trimera (3.0 µL mL^{-1})	*Foeniculum vulgare* (3.0 µL mL^{-1})
Control	00.00 ± 00.00 b	00.00 ± 00.00 b
Diseased	14.25 ± 02.44 a	18.44 ± 00.83 a
Preventive treatment	14.20 ± 01.66 a	16.68 ± 01.49 a
Curative treatment	12.95 ± 01.63 a	21.21 ± 00.52 a

Values are the average of ten replicates per treatment ± SE. Means followed by same letter do not differ by Tukey test ($p < 0.05$).

4. CONCLUSIONS

The results *in vitro* demonstrated antifungal activity of *B. trimera* and *F. vulgare* EOs against *C. fructicola* (mycelial growth and conidia germination). *In vivo* results demonstrated that the EOs could not control bitter rot in postharvest of "Fuji" apple. More studies are required before these EOs can be recommended

as commercial and natural antifungal agents to increase apple's postharvest storage life.

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