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CROP SCIENCE

Antifungal activity of essential oil from *Eucalyptus staigeriana* against *Alternaria alternata* causing of leaf spot and black rot in table grapes

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Abstract: *Alternaria alternata* causes leaf spot and black rot diseases in leaves and grapes of grapevines, respectively, and leads to huge economic losses in table grapes production. As natural antifungal agents, essential oils (EOs), which are generally recognized as safe substances, shows strong antifungal activity against fungal phytopathogens. The aim of this study was to determine the chemical composition of *Eucalyptus staigeriana* EO and its *in vitro* and *in vivo* effects against *A. alternata*. The major compounds of *E. staigeriana* EO were citral (34.32%, of which 21.83% geranial and 12.49% neral), limonene (20.60%) and 1,8-cineole (12.33%). *E. staigeriana* EO exhibited the highest inhibitory activity on mycelial growth and conidial germination at 1 µL mL^{-1}. Moreover, the EO was able to reduce the incidence and severity of leaf spot disease in leaves and black rot disease in table grapes caused by *A. alternata*. These results represent a possible alternative to reduce the use of synthetic molecules for the control of diseases in postharvest of table grapes and in vineyard.

Key words: *Alternaria alternata,* Eucalypts, Alternative control, Grape, Essential oil.

INTRODUCTION

Pathogenic species, as the genus *Alternaria,* are found in several agronomically important plants, including grapevines. *Alternaria alternata* (Fr. Keissler) has frequently been isolated from leaves in the vineyard and grapes in pre and postharvest samples (Trinidad et al. 2015, Kassemeyer 2017).

A. alternata causes leaf spot in leaves of grapevines, leading to the development of necrotic lesions that evolve rapidly through the leaf blade. This causes the premature fall of leaves, reducing the agricultural production by impairing plant photosynthesis and directly affecting the quality grapes (Sônego et al. 2005, Troncoso-Rojas & Tiznado-Hernández 2014).

This fungus is also causal agent of black rot in grapes during fruit development in the vineyard and postharvest storage (Prendes et al. 2016, Kassemeyer 2017). Postharvest decay in the supply chain results in significant economic losses and, has been identified as a significant cause of fruit damages (Prusky 2011). Moreover, *A. alternata* has been linked to food poisoning and a great variety of adverse effects on human health due to the production of mycotoxins (Dall'Asta et al. 2014).

Treatments with synthetic fungicides represent more than half of pesticides applied in viticulture, and some of them are also used

for postharvest disease control (Troncoso-Rojas & Tiznado-Hernández 2014). However, the use of fungicides is not always efficient to control of the disease and, their use in pre and postharvest constitute environmental and toxicological risks (Neri et al. 2006, Vieira et al. 2018). Thus, there is considerable interest in developing alternative control methods (Youssef & Roberto 2014). Essential oils (EOs) could be used as alternatives for synthetic fungicides, for are natural biodegradable products, with antimicrobial properties, low environmental impact, and low mammalian toxicity (Isman 2000, Burt 2004, Pedrotti et al. 2019a).

Eucalyptus, a genus native to Australia, belongs to the Myrtaceae family and comprises about 900 species, some of which are extensively distributed worldwide (Brooker & Keing 2004). More than 300 species of this genus contain volatile oils in their leaves and have been commercially used for the production of EOs by industries (pharmaceutical, toiletries, cosmetics and food) (Marzoug et al. 2011). Several studies have shown the antifungal properties of some *Eucalyptus* EOs against phytopathogens (Tomazoni et al. 2017, 2018, Pedrotti et al. 2019a).

The aim of this study was to evaluate the chemical composition of the EO obtained from *E. staigeriana* leaves and their *in vitro* effect on the mycelial growth and conidia germination of *A. alternata*. Its ability to control leaf spot in leaves of grapevines and, its *in vivo* potential to control black rot disease during the postharvest of table grapes.

MATERIALS AND METHODS

Fungi isolation and DNA extraction

The strain of *Alternaria alternata* (A41/17) was isolated from grapevine leaves collected in Bento Gonçalves (Serra Gaúcha, RS, Brazil), and maintained in the fungal collection of the Laboratory of Phytopathology, University of Caxias do Sul, RS, Brazil. The isolate was taxonomically classified by Internal Transcribed Sequence (ITS-5.8S rDNA) sequencing, and comparison to sequences deposited in the GeneBank Database using nBLAST algoritm (NCBI) (Murray & Thompson 1980, White et al. 1990). For all purposes, fungal isolate was cultivated on PDA (Potato Dextrose Agar) medium at 25ºC.

Plant material

Leaves of *Eucalyptus staigeriana* were collected from plants located in the University of Caxias do Sul, Caxias do Sul, RS, Brazil, in September 2018, between 8:30 am and 9:30 am. The climatic conditions during the month of collection were an average temperature of 17°C, precipitation of 182 mm, and relative humidity of 79.7%. After collection, the leaves were oven-dried at 30ºC until constant mass was obtained. A voucher specimen of the plant species was deposited in the University of Caxias do Sul Herbarium (accession n°. 37937).

Extraction and analysis of essential oil

EO was extracted by steam distillation from dried leaves for 1 hour (Cassel et al. 2009). The identification and quantification of compounds in the EO, was performed using an HP 6890 gas chromatograph (GC) coupled with a Hewlett Packard MSD5973 mass selective (MS) detector, equipped with HP Chemstation software and Wiley 275 mass spectra data. The analyses were conducted using an HP-Innowax fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness, Hewlett Packard, Palo Alto, USA) with following conditions: column temperature, 40°C (8 min) to 180°C at 3°C/ min, 180–230°C at 20°C/min, 230°C (20 min); interface 280°C; split ratio 1:100; carrier gas He (56 KPa); flow rate: 1.0 mL/min; ionization energy 70 eV; mass range 40–350. Volume injected was 0.4 µL (diluted in

hexane 1:10). Analytical gas chromatography was carried out in a Hewlett Packard 6890 gas chromatograph with a flame ionization detector (FID) equipped with a HP Chemstation software. A HP Innowax bonded phase capillary column (30 m × 0.32 mm i.d., 0.50 µm film thickness, Hewlett Packard, Palo Alto, USA) was used with following conditions: column temperature, 40°C (8 min) to 180°C at 3°C/min, 180–230°C at 20°C/min, 230°C (20 min); injector temperature 250°C, detector temperature 250°C; split ratio 1:50; carrier gas H2 (34 KPa). Injection volume was 1 µL (diluted in hexane 1:10). The constituents of the EO were identified by comparing their mass spectra with those of the Wiley library (GC/MS) and comparing the practical linear retention index with literature data (Nist). The linear retention index was calculated using the Van den Dool and Krats equation using a standard solution of C8 to C26 hydrocarbons. The relative percentage of each component was obtained from chromatographic peak areas, assuming the sum of all eluted peaks to be 100% (Pedrotti et al. 2019a).

Evaluation of *in vitro* antifungal activity of essential oil

Mycelial growth

The antifungal properties of EO were assessed both for its contact and volatile phase effects against mycelial growth of *A. alternata*. The contact phase effect of EO was tested according to Pedrotti et al. (2019a). EO concentrations ranged between 0.25 and 1.5 µL mL^{-1}. The EO was emulsified with Tween 20 (1:1) and added to the PDA culture medium. The control treatment consisted of PDA medium and Tween 20 at the highest concentration used to emulsify the EO. These emulsions were poured into 9 cm (⌀) Petri dishes and inoculated with a 5 mm (⌀) agar disks colonized by *A. alternata* obtained from 7 day-long pre-cultures.

To assess the fungicidal action of the volatile phase of EO on the mycelial growth of *A. alternata,* the method adopted by Pedrotti et al. (2019a) was used. Briefly, agar disks with 5 mm (⌀) colonized by *A. alternata* were placed in the center of Petri dishes containing PDA medium. A 100 µL EO aliquot at the concentrations of 12.5, 25 and 50% (v/v) emulsified with 0.1% Tween 20, and pure EO (100%, devoid of Tween 20) were applied onto a cotton ball attached to the inner face of a Petri dish lid. The control treatment consisted of 100 µL of a 0.1% Tween 20 solution. For both tests, at each concentration, ten replicates were used. Incubation was performed at 25°C and 12 h photoperiod for fourteen days. Fungal growth was recorded on the 3rd, 5th, 7th, 10th, and 14th days by measuring the orthogonal diameter of the mycelia.

Transfer experiments

For provide a distinction between the fungistatic and fungicidal effects of EO on the fungi, transfer experiments were performed. Mycelial plugs that did not grow were transferred to Petri dishes containing PDA medium to assess their viability and growth after 5 days at 25°C (Pedrotti et al. 2019b).

Conidia germination

Antifungal activity of EO on conidia germination was tested according to Pedrotti et al. (2019 a). Briefly, *A. alternata* conidia were harvested from 14 day old fungal colonies grown in PDA at 25°C under 12 h photoperiod. Five milliliters of sterile water were added to a Petri dish culture. Conidial suspensions were obtained by displacement from the surface of cultures using sterile water. Suspensions were diluted to obtain a concentration of 1 ⌀ 10^6 conidia mL^{-1}. Aliquots of conidia suspension (50 µL) were placed in

microtubes containing 500 μL of Potato Dextrose Broth medium with different EO concentrations (0.25 to 1.5 μL mL^{-1}), emulsified with Tween 20 (1:1). The tubes were incubated at 25°C, and the evaluations were performed after 6, 12, and 24 h. Samples were placed on a hemocytometer chamber and observed under the microscope (100×) for conidia germination. The conidia were considered to be germinated when the length of the germ tube equaled or exceeded the length of the conidia. All experiments were conducted in ten replicates and for each replicate a hundred conidia were evaluated.

Antifungal activity in leaves

Leaves of *Vitis* spp. (*V. labrusca* × *V. vinifera*) 'Isabela' conventionally grown in Bento Gonçalves, RS, Brazil were used in experiments. Leaves were collected in the morning and the test conducted on the same day. Leaves were sanitized with 70% ethanol (1 min) and then 1.5% sodium hypochlorite (3 min), followed by washing with sterile distilled water. After drying, the leaves were placed in 9 cm (⌀) Petri dishes containing agar-water culture medium, with the abaxial face in contact with the culture medium. The antifungal activity of EO on leaves was evaluated both as curative and preventive treatments. For the curative treatment, the center of leaves was inoculated on the abaxial face with 5 mm (⌀) agar disks colonized by *A. alternata* obtained from 7 day-long pre-cultures. After 24 h, the leaves were sprayed with EO concentrations of 1 and 2 μL mL^{-1}. As for the preventive treatment, the same concentrations of EO were sprayed in leaves and inoculated after 24 h with agar disks colonized by *A. alternata*. For both tests, at each concentration, ten replicates were used. Incubation was performed at 25°C and 12 h photoperiod for seven days. After the incubation, disease incidence was evaluated. For disease severity measurements decayed areas on the surface of leaves were quantified using the ImageJ software.

In vivo antifungal activity in grapes

Conidia of *A. alternata* were harvested from a 14-day-old fungal colony grown on PDA at 25°C with a 12 h photoperiod as described above. The suspension was diluted with sterile water to obtain a concentration of 1 10^{6} conidia mL^{-1}. Conventionally grown *Vitis* spp. (*V. labrusca* × *V. vinifera*) 'Isabela' grapes from Bento Gonçalves, RS, Brazil, were used in experiments. Grapes were collected in the morning, and the test conducted on the same day. Collection was followed by sanitization with 1.5% sodium hypochlorite (3 min), after which the fruit were washed with sterile distilled water. The antifungal activity of EO on grapes was evaluated both as curative and preventive treatments according to the method described by Pedrotti et al. (2019b). Wounds approximately 2 mm deep were made on ten berries in grape clusters. After wounding, in the postharvest curative treatment, a conidia suspension of *A. alternata* was inoculated, and after 24 h, grape clusters were sprayed with EO at the concentrations of 1, 2, and 3 μL mL^{-1}. For the preventive treatment, after wounding, the same EO concentrations were sprayed on grape clusters, and after 24 hours, inoculated with a conidia suspension of *A. alternata*. For both experiments, the grapes were placed in plastic boxes (30 cm wide × 40 cm long × 15 cm high) and incubated at 25 ± 1°C / 80-90% relative humidity with a 16 h photoperiod for seven days. After incubation, disease severity was assessed, and the superficial decayed area on the grape berry was visually evaluated using a scale from 0 to 100% (Supplementary Material- Figure S1).

Statistical analysis

All statistical analysis was performed using SPSS 22.0. 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 $p < 0.05$. In the case of statistical significance, Dunnett's T3 test or Tukey's test was applied to separate the means.

RESULTS AND DISCUSSION

Chemical composition of essential oil

EO extracted from *E. staigeriana* dried leaves of yielded 5.20% (i.e., mL 100 g^{-1} of dried leaves). The analyses identified 21 compounds (Table I). The major compounds in the EO of *E. staigeriana* were identified as citral (34.32%; of which 21.83% geranial and 12.49% neral), limonene (20.60%) and 1,8-cineole (12.33%). Overall EO composition consisted of 88.07% monoterpenes (30.36% hydrocarbons and 57.71% oxygenated) and 11.75% ester compounds, and was found to be similar to those reported in the literature (Macedo et al. 2010, Tomazoni et al. 2017, Pedrotti et al. 2019a), indicating that EO composition of is highly species sensitive, with low influence of environmental factors.

Antifungal activity of essential oil *in vitro* tests

For *in vitro* testing, preliminary assays were performed to define which OE concentrations should be tested. Firstly, the concentration that completely inhibited *A. alternata* mycelial growth was identified, and this concentration to be used to defined the other concentrations used in the experiments.

In the contact phase (Figure 1a), the effect of *E. staigeriana* EO on the mycelial growth of *A. alternata* resulted in complete inhibition at concentration 1 μL mL^{-1}. The fungicidal action if this concentration was confirmed by the transfer experiment, where no mycelial growth could be observed. In lower concentrations (0.25 and 0.5 μL mL^{-1}) we observed mycelial growth, but a significant inhibition compared to control during 7th and 10th day (Figure 1a).

Volatiles compounds of *E. staigeriana* EO exerted a significant inhibition of mycelial growth at the concentrations of 12.5%, all over the experiment. At higher concentrations of 25, 50 and 100 % granted total inhibition of the mycelial growth of *A. alternata* was observed, and the fungistatic action was confirmed by the transfer experiment, where mycelial growth could be observed (Figure 1b).

Application of *E. staigeriana* EO at 1 μL mL^{-1} resulted in complete inhibition of conidia germination of *A. alternata* all experiment, and concentrations of 0.25 and 0.5 μL mL^{-1} conferred a significant reduction in the germination compared to control (Figure 2).

Some studies reported the fungicidal action of *E. staigeriana* EO against the phytopathogens *A. solani* (Tomazoni et al. 2017), *Stemphylium solani* (Tomazoni et al. 2018), *Botrytis cinerea* and *Colletotrichum acutatum* (Pedrotti et al. 2019a). Considering the antifungal properties of different EOs, previous studies suggest that the inhibition of fungal growth is associated to mitochondrial morphological and function modifications that affect the respiratory metabolism, decreasing the activities of tricarboxylic acid cycle related enzymes and changing metabolic abilities. EOs can also affect cell membrane permeability, increase intracellular accumulation of reactive oxygen species, and interfere with growth-related gene expression. Moreover, compounds of EOs also affect the enzymes responsible for conidia germination and interfere with amino acids that are necessary for the germination processes (Nychas 1995, Tian et al. 2012, Zheng et al. 2015, Tang et al. 2018).

Antifungal activity of essential oil in leaves

Leaf spot caused by *A. alternata* in grapevine leaves affects the quality of the grapes directly, causing the premature fall of leaves and reducing fruits production, by impairing plant

Table I. **Chemical composition of *Eucalyptus staigeriana* essential oil.**

Compound	RI[1]	RA[2]
Monoterpenes Hydrocarbons		**30.36**
α-pinene	13.942	1.10
α-phellandrene	21.895	0.27
Myrcene	21.995	0.64
Limonene	23.861	20.60
γ-terpinene	26.309	0.62
Cis-β-ocimene	26.704	0.36
ρ-cymene	27.677	0.73
δ-terpinene	28.285	6.04
Oxygenated Monoterpenes		**57.71**
1,8-cineole	24.268	12.33
Linalool	40.518	0.62
Terpinen-4-ol	42.987	1.05
Neral	46.356	12.49
Geranial	48.349	21.83
Citronellol	49.295	1.31
Nerol	50.664	3.01
Geraniol	52.347	5.07
Esters		**11.75**
Citronellyl acetate	45.338	0.60
Terpinyl acetate	46.787	6.64
Neryl acetate	47.931	2.43
Geranyl acetate	49.038	2.08
Others		**0.20**
Geranic acid	63.270	0.20

[1] RI, retention index determined relative to *n*-alkanes (C_8–C_{20}). [2] RA, Relative amounts of the compounds identified based on the area of each peak in the total chromatogram area.

photosynthesis (Sônego et al. 2005, Troncoso-Rojas & Tiznado-Hernández 2014). Leaves treated with EO of *E. staigeriana* exhibited a significant reduction of disease severity caused by *A. alternata* in both, preventive and curative treatments, demonstrating the efficiency of the EO in the control of leaf spot disease (Table II). Treatments with *E. staigeriana* EO in leaves reduced more than 80% the severity of disease caused by *A. alternata* both in preventive

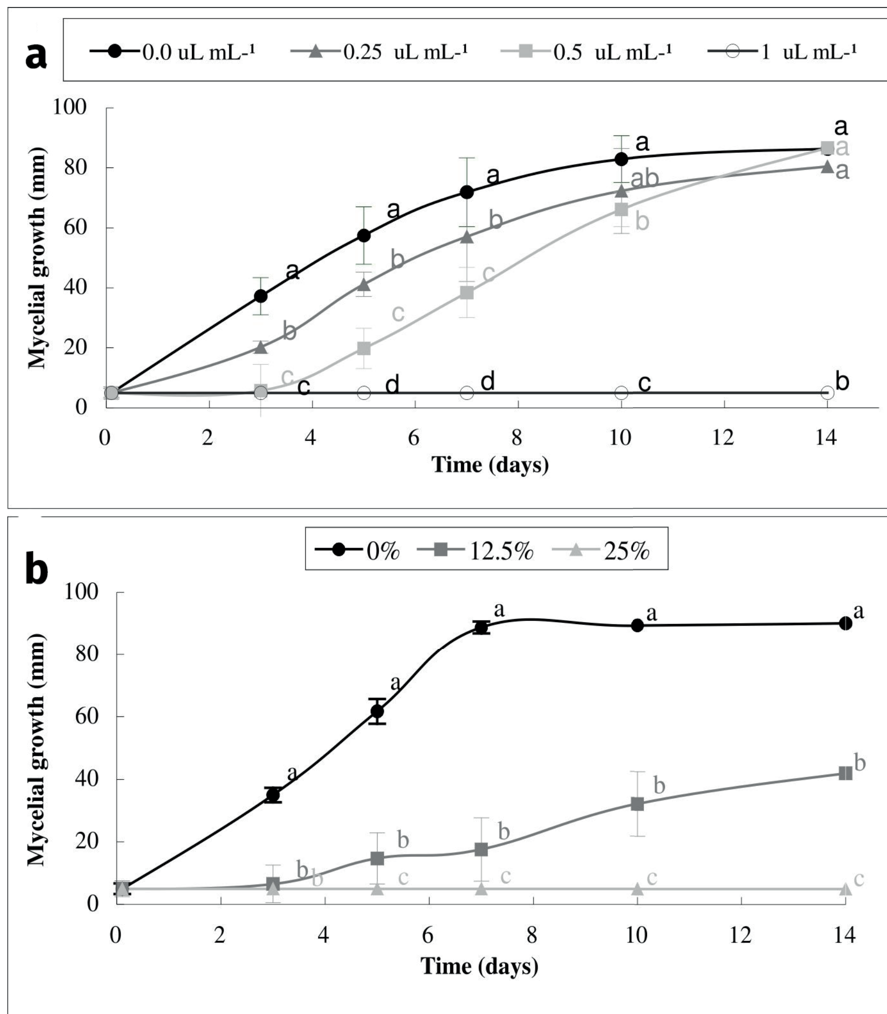

Figure 1. Effect of different concentrations of *Eucalyptus staigeriana* essential oil added to the solid media – contact phase (a), and on the lid – volatile phase (b), on the mycelial growth of *Alternaria alternata*. Values are the mean of ten replicates per treatment ± standard deviation. The letters indicate the comparison among the different essential oil concentrations evaluated in each day. Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

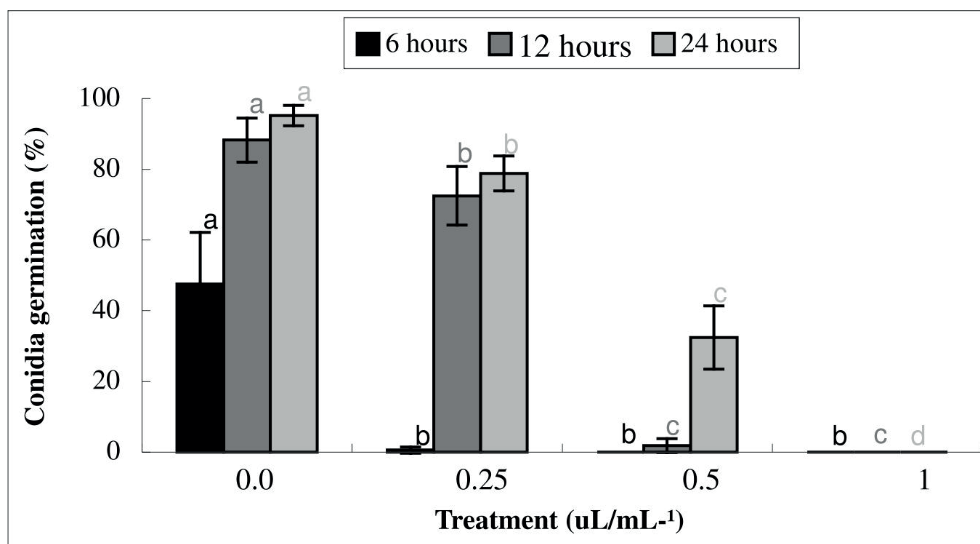

Figure 2. Effect of different concentrations of *Eucalyptus staigeriana* essential oil on conidia germination of *Alternaria alternata* evaluated at different times. Values are the mean of ten replicates per treatment ± standard deviation. Means followed by same letter do not differ by Dunnett's T3 test ($p < 0.05$).

Table II. **Effect of different treatments of *Eucalyptus staigeriana* essential oil *in vitro* applied of *Vitis labrusca* × *Vitis vinifera* 'Isabela' leaves on severity of disease caused by *Alternaria alternata* (leaf spot disease).**

	Preventive treatment	**Curative treatment**
Treatments	Severity (%)	Severity (%)
Absolute control	0.00 ± 0.00 c	0.00 ± 0.00 c
Control with essential oil	0.00 ± 0.00 c	0.00 ± 0.00 c
Control with inoculum	3.89 ± 1.18 a	3.89 ± 1.18 a
1 µL mL^{-1} essential oil	0.66 ± 0.33 b	0.63 ± 0.42 b
2 µL mL^{-1} essential oil	0.49 ± 0.34 b	0.62 ± 0.45 b

***Values represent the mean of ten replicates per treatment ± standard deviation. Letters indicate the comparison among the different treatments. Means followed by same letter do not differ by the Tukey test ($p > 0.05$).**

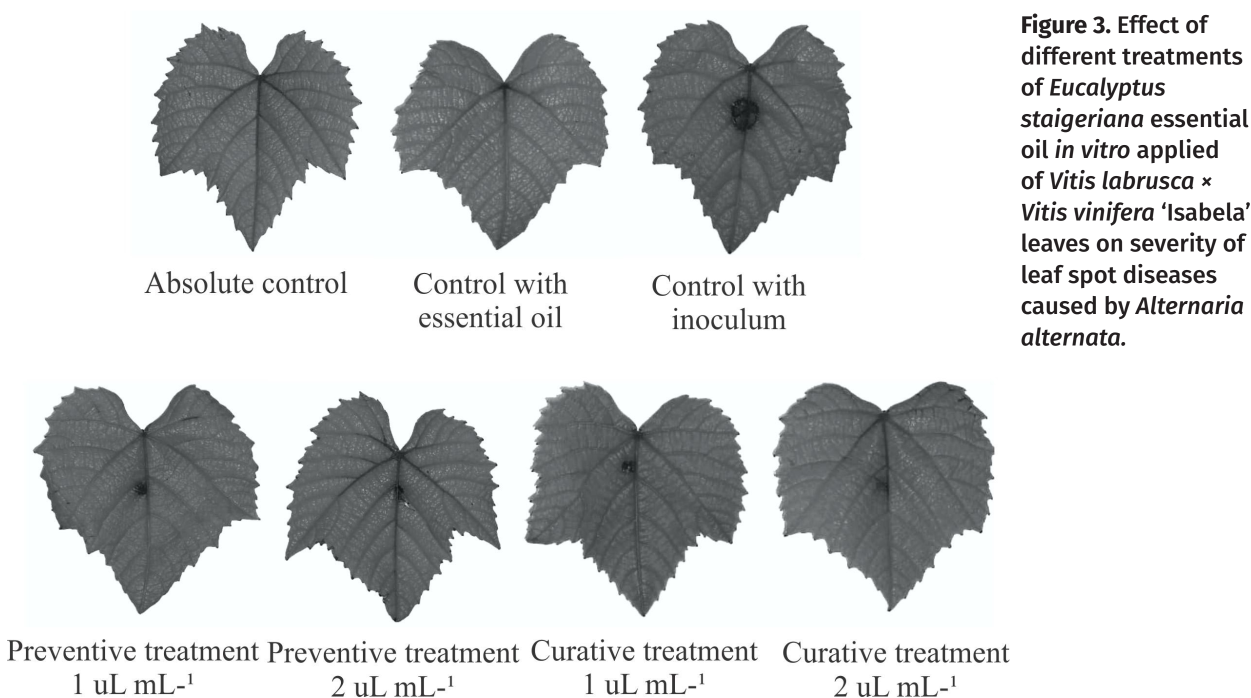

Figure 3. **Effect of different treatments of *Eucalyptus staigeriana* essential oil *in vitro* applied of *Vitis labrusca* × *Vitis vinifera* 'Isabela' leaves on severity of leaf spot diseases caused by *Alternaria alternata.***

and curative treatment, demonstrating their effectiveness (Table II, Figure 3).

In vivo antifungal activity of essential oil on grapes

The effects of *E. staigeriana* EO in postharvest of grapes are presented in Table III. Different OE concentrations (1, 2, and 3 µL mL^{-1}) were efficient, reducing the incidence and severity of black rot disease caused by *A. alternata* in preventive and curative treatments compared to control. Demonstrating that *E. staigeriana* EO was efficient in the control of postharvest black rot diseases in table grapes, indicating that it can be applied in the postharvest chain in the storage or packaging process of grapes. Similarly, Pedrotti et al. (2019a) showed that *E. staigeriana* EO was able to reduce the incidence and severity of gray rot caused by *B. cinerea* and the severity of ripe

Table III. Effect of different treatments of *Eucalyptus staigeriana* essential oil *in vivo* applied of *Vitis labrusca* × *Vitis vinifera* 'Isabela' grapes on incidence and severity of disease caused by *Alternaria alternata* (black rot disease).

	Preventive treatment		Curative treatment	
Treatments	**Incidence (%)**	**Severity (%)**	**Incidence (%)**	**Severity (%)**
Control	67.00 ± 2.05 a	12.57 ± 4.46 a	67.00 ± 2.05 a	12.57 ± 4.46 a
1 μL mL^{-1}	07.70 ± 1.04 b	05.00 ± 0.51 b	08.70 ± 1.07 b	08.50 ± 2.26 b
2 μL mL^{-1}	06.30 ± 0.93 b	04.00 ± 0.50 b	07.70 ± 0.94 b	05.00 ± 0.51 b
3 μL mL^{-1}	04.30 ± 0.68 b	03.70 ± 0.59 b	08.30 ± 1.29 b	05.00 ± 0.51 b

Values represent the mean of ten replicates per treatment ± standard deviation. Letters indicate the comparison among the different treatments. Means followed by same letter do not differ by the Tukey test ($p < 0.05$).

rot caused by *C. acutatum* when applied in the field in grapevines (*V. vinifera* 'Tannat').

CONCLUSIONS

Considering the *in vitro* and *in vivo* results of *A. alternata* growth inhibition, we can conclude that *E. staigeriana* EO could be used as a possible biofungicide for control of field and postharvest fungal disease caused by *A. alternata* on grapevine leaves and grapes. However, additional studies are required before this EO can be recommended as a commercial and natural antifungal agent in the postharvest treatment of table grapes and in grapevines on the field. For this, we suggest the development of a formulation containing EO, the evaluation of its stability and durability and with that, define the frequency of its application.

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SUPPLEMENTARY MATERIAL

Figure S1. Scale for assessing the severity of diseases, from 0 to 100 % according to the grape berries area affected by the disease.

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