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Alternative control of bacterial leaf blight of eucalypt using essential oils

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Abstract

Bacterial leaf blight, attributed to *Xanthomonas citri* pv. *eucalyptorum* comb. nov., poses a significant threat to eucalypt nursery and field conditions in Brazil, resulting in substantial annual losses. While various disease management practices are employed, the quest for alternative control methods and environmentally friendly antimicrobial agents remains imperative. Thus, this project was developed to assess the potential efficacy *in vitro* of essential oils derived from tea tree (*Melaleuca alternifolia*), copaíba (*Copaifera officinalis*), and lemongrass (*Cymbopogon* spp.*)* against *X. citri pv. eucalyptorum.* The sensitivity of the bacteria to these oils was examined via antibiogram testing, with each antibiogram subjected to different oil dilutions (6.25 %, 10.00 %, 12.50 %, 25.00 %, 50.00 %, and 100.00 %). Inhibition zones were assessed to determine the inhibitory capacity of each compound on microbial growth, and statistical analysis was conducted using the Mann-Whitney test. Copaíba oil did not exhibit any inhibitory effect on the pathogen growth *in vitro*. Conversely, both lemongrass and tea tree oils displayed inhibitory effects on the target bacteria, with a minimum inhibitory concentration of 12.50 %. Furthermore, no significant distinctions were observed between the utilization of 12.50 % and 25.00 % concentrations of tea tree, neither between 25.00 % of lemongrass or 50.00 % of tea tree dilutions. These findings may pave the way for new strategies in pathogen management.

Keywords: Plant Pathology. *Xanthomonas citri* pv. *eucalyptorum* comb. nov. *Eucalyptus* spp. Disease management.

Introduction

Xanthomonas is a large genus that includes at least 35 species causing diseases in economically important crops worldwide, such as tomato, rice, citrus fruits, teak, and eucalypt (ARAÚJO; TEBALDI, 2019; JIANG et al., 2020; ZAREI; TAGHAVI; OSDAGHI, 2022; MONTEIRO; MELO; ROSSATO, 2023; FERRAZ et al., 2024). Diseases caused by *Xanthomonas* can result in substantial economic losses in forestry and agricultural; therefore, controlling this pathogen is essential to ensure crop health and productivity (MONTEIRO; MELO; ROSSATO, 2023). On *Eucalyptus* spp., bacterial leaf blight (BLB) is a major disease, especially under nursery conditions, associated with the recently proposed patovar *Xanthomonas citri* pv. *eucalyptorum* comb. nov. (FERRAZ et al., 2024).

Management of bacterial diseases generally involves an integrated approach, with the application of copper-based fungicides being a widely adopted strategy for controlling foliar diseases. In eucalypt, the management of BLB in nursery conditions includes the avoidance of inoculum sources, the use of genetically resistant clones, and the prevention of foliar wetting. However, the disease remains a significant concern, causing considerable losses in susceptible clones during rainy periods in open-field nurseries. Therefore, it is crucial to explore alternative strategies for the pathogen management.

Essential oils have emerged as promising alternatives for pest and disease management in plants due to the presence of chemical agents harmful to pathogens in their composition (PEREIRA et al., 2012; LUCAS et al., 2012;

BRUN et al., 2019; RAVEAU; FONTAINE; SAHRAOUI, 2020; GUPTA et al., 2023). The application of essential oils not only facilitates disease control but also triggers biochemical defense mechanisms across a wide range of plant species, thereby enhancing their overall resistance to pathogens (BANANI et al., 2018; YANG et al., 2022; BINIAZ; KAVOOSI; AFSHARIFAR, 2024).

In the agricultural field, extensive research has been conducted on a variety of essential oils to assess their effectiveness in managing diverse plant diseases. For example, tea tree oil successfully controlled spore germination of pathogens like *Hemileia vastatrix*, which causes rust in coffee plants. It also induced systemic resistance against *Fusarium* wilt in bananas and *Xanthomonas* spp. in tomatoes (DALIO et al., 2020; REUVENI; SANCHES; BARBIER, 2020). Additionally, lemongrass oil reduced the development of *Botrytis cinerea* in grapes (LOPES et al., 2023), and had potential to control *Xanthomonas axonopodis* pv. *punicae* in pomegranate (CHOWDAPPA et al., 2018). Copaíba oil has also been highlighted as a promising agent for controlling of range of plant pathogens, including the fungus *Colletotrichum musae* (NÓBREGA et al., 2019) and the bacterium *Pectobacterium carotovorum* subsp. *carotovorum* (GUERRA et al., 2014).

Given the limited methods available for controlling BLB in eucalypt and the potential microbicidal properties of copaiba (*Copaifera officinalis* (Jacq.) L.), lemongrass (*Cymbopogon* sp.) and tea tree (*Melaleuca alternifolia* Cheel), this study was developed to evaluate the efficacy of these oils in controlling the pathogen *Xanthomonas citri* pv. *eucalyptorum*.

Material and methods

The experiments were carried out at the Plant Pathology Laboratory of the Federal Institute of Minas Gerais *campus* São João Evangelista, located in São João Evangelista, Minas Gerais, Brazil, which is situated at an average altitude of 689 meters, with a latitude, of 18º 32' S and longitude of 42º 45' W.

In the assays, one isolate of *X. citri pv. eucalyptorum* was used. The isolate was obtained from symptomatic eucalypt leaves collected in southern Bahia, Brazil, and provided by the Laboratory at Forest Pathology of Federal University of Viçosa. The culture was growth in Luria-Bertani (LB) at 28ºC for 48 hours.

Essential oils of *M. alternifolia*, *Cymbopogon* spp., and *C. langsdorffii* were purchased commercially and diluted in dimethyl sulfoxide (DMSO) to obtain concentrations of 100 %, 50 %, 25 %, 12.50 %, and 6.25 %. The dilutions were defined based on previous tests.

The inhibitory capacity of bacterial growth was assessed using an antibiogram methodology for phytobacteria analysis described by Romeiro (2001), which employs two-layer culture media to perform the test. The isolate was cultured on Luria-Bertani (LB) medium for 24 hours with agitation at 28ºC. The resulting bacterial suspension was then adjusted to a concentration of $1x10^8$ CFU mL^{-1} using a spectrophotometer. From this suspension, 100 μL aliquots were transferred to 20 mL test tubes, which were filled with a semisolid water-agar medium (0.6 to 0.8 %) to achieve a final volume of 8 mL, ensuring the temperature remained below 50°C (Figure 1, step 1). These tubes were set aside to form the upper layer of the culture media.

The bottom layer consisted of a 2 % water-agar medium, which was allowed to cool to 50º C before being poured into the plates to form a uniform layer. The bottom layer was poured directly into the Petri dish to ensure a uniform thickness, correcting any unevenness or irregularities in the dish's base, which was crucial for creating a proper foundation for the upper layer, that contained the bacterial

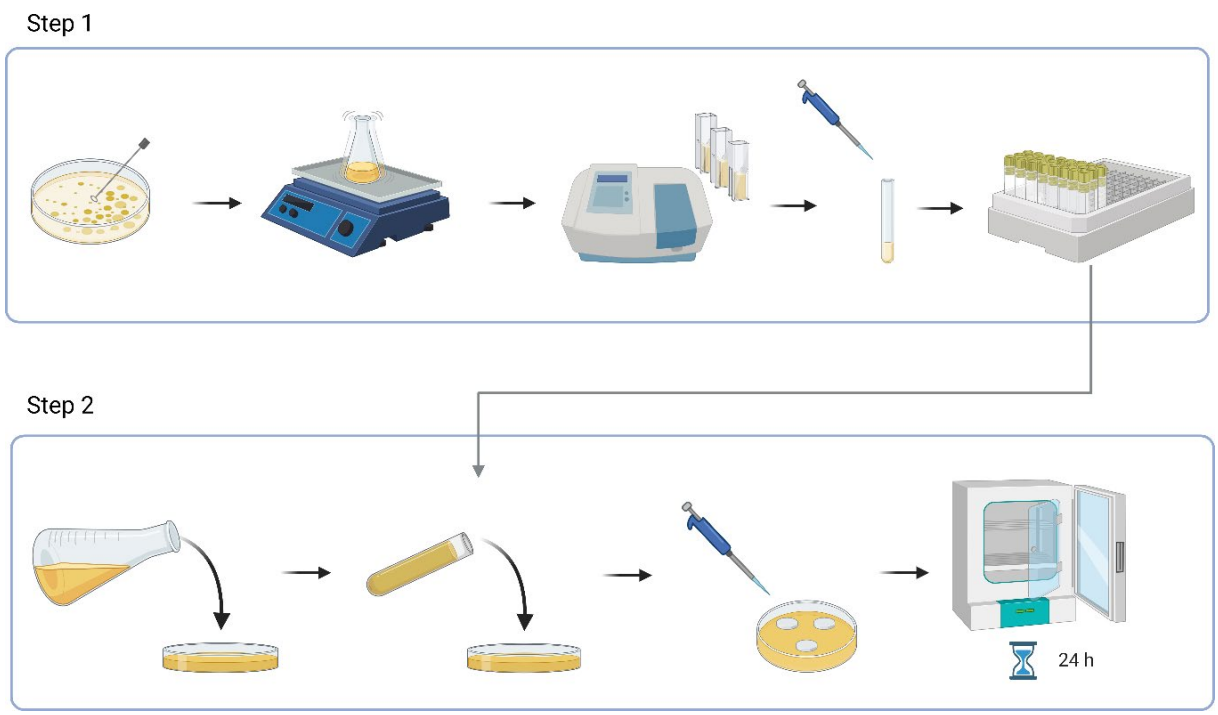
suspension. The tubes containing the semisolid water-agar media with bacteria were poured over the bottom layer, forming the upper layer. The dishes were then placed into a refrigerator for 10 to 20 minutes to accelerate the solidification process of the medium.

Following the solidification of the medium, three sterile paper disks each impregnated with 10 μL of essential oils at the specified concentrations were placed equidistantly on the surface of the upper layer (Figure 1, step 2). The paper disks were prepared using a 6 mm diameter punch and sterilized in and dry heat sterilizer at 100°C for 4 hours before use. The dishes were sealed with plastic wrap and incubated in a Biochemical Oxygen Demand (B.O.D.) incubator at 28°C.

The diameter of the inhibition zones was measured daily, beginning 24 hours after setting up the assay and continuing until complete growth of the colonies on the Petri dishes. The measurements of each inhibition zone were adjusted by subtracting the diameter of the corresponding disk. The diameters of the inhibition zone were then interpreted according to the criteria established by the committee for Clinical Laboratory Standards International (2024).

The experiments were performed independently using a completely randomized design, which included three essential oils (*M. alternifolia*, *Cymbopogon* sp., and *Copaifera langsdorffii*) tested at five concentrations (100 %, 50 %, 25 %, 12.50 %, and 6.25 %), with five replicates per concentration. Additionally, a positive control (antibiotic rifamycin) and a negative control (DMSO) were included. The Mann-Whitney test ($\alpha = 0.05$), recommended for non-parametric data, was employed to compare the effects both within and between the different essential oil concentration.

Figure 1. Schematic representation of the methodology used in this study. The diagram outlines the step-by-step process, including sample preparation and treatment applications.



Source: Prepared by the authors.

Results and discussion

Among the oils tested, copaiba oil (*C. officinalis*) had performance comparable to the negative control (DMSO), with no detectable bactericidal activity against *X. citri pv. eucalyptorum*. In contrast, the positive control (rifamycin) proved to be highly effective in bacterial inhibition, with an average inhibition zone of 26.93 mm. Among the essential oils tested, tea tree oil (*M. alternifolia*) promoted the highest average inhibition (6.67 mm), followed by lemongrass oil (*Cymbopogon* sp.) with an average inhibition of 3.73 mm. Statistical comparison showed that rifamycin significantly differed from the average performance of all tested oils, proving superior to all of them. As expected, the negative control (DMSO) had no inhibition, confirming its neutral effect on the phytobacteria (Table 1). Due to the ineffectiveness of copaiba oil in controlling the target organism, it was not included in further statistical analyses.

The greatest inhibitory effect at concentrations of 50 % and higher (Figure 2) was identified for tea tree. As the concentration increases, the oil's effectiveness also intensifies. In contrast, lemongrass oil showed an inhibitory effect on the pathogen appearing only at concentrations of 12.5 % and 25 % (Figure 2). Concentrations above 25 % and below 12.5 % were ineffective in controlling the bacteria. The lack of effectiveness at higher concentrations is probably due to the rapid evaporation of undiluted lemongrass oil, as observed by Agnolin et al. (2014). Research indicates that lemongrass oil's antimicrobial and insecticidal effects are primarily attributed to volatile compounds (ALVES et al., 2023) with citral being the most significant (GAO et al., 2020). Due to this volatility, the compounds evaporate from the medium before they can impact bacterial growth. This rapid evaporation of the compounds and its lack of effectivity at different concentrations contributed to the low average inhibition zone observed for this oil.

The pairwise comparison within each oil, evaluated using the Mann-Whitney test (0.05 %), revealed significant differences for all lemongrass concentrations (Table 2). Concentrations that exhibited an inhibitory effect (12.50 % and 25 %) significantly differed from those that did not, with a p-value < 0.05. A significant difference was observed between the 12.5 % and 25 % concentrations (p-value < 0.05). For tea tree oil, significant differences were noted between all tested concentrations, except between 12.5 % and 25 %, where the p-value exceeded 0.05.

Comparison of the mean inhibition zones among the tested oils reveals that 25 % lemongrass oil and 50 % tea tree oil do not differ significantly. All other comparisons reveal differences, except when both oils exhibited no inhibitory capacity (p-value = 1). Overlaying Table 3 with Figure 2 shows that lemongrass oil was only significantly superior when comparing 25 % lemongrass with 12.5 % tea tree oil. This concentration differences can guide the selection of oils, considering factors such as cost, extraction difficulty, and desired results.

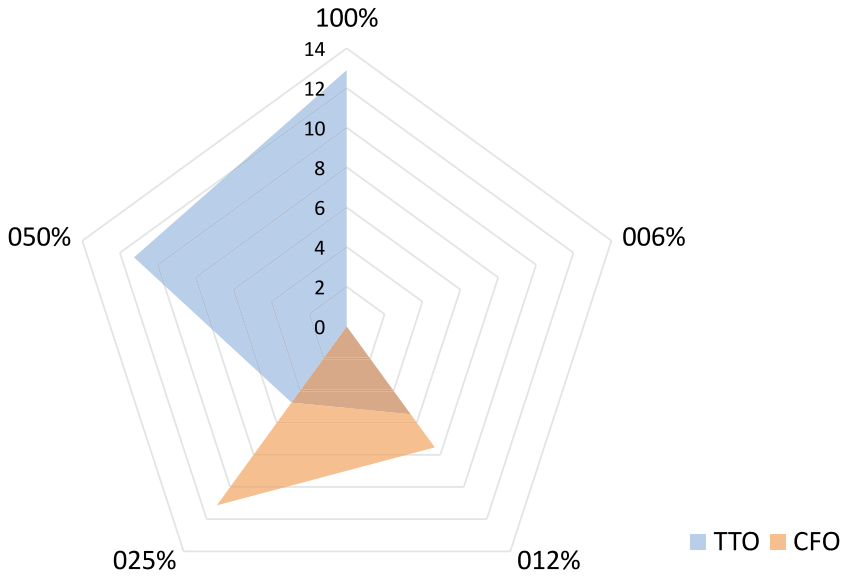
Table 1. P-values from the Mann-Whitney test (0.05) comparing the effect of rifamycin and DMSO with the oils tested, concerning the diameter of the inhibition halo formed (cm).

Testedcompound	Rifamycin	DMSO
Tea Tree	<0.0001	<0.0001
Lemongrass	<0.0001	<0.0001
Copaíba	<0.0001	1.000^{NS}

^{NS} not significant.

Source: Prepared by the authors.

Figure 2. Effectiveness of *X. citri pv. eucalyptorum* control using *Melaleuca alternifolia* (TTO) and *Cymbopogon sp.* (CFO) oils at the tested concentrations. The levels on the radar chart represent the diameter of the inhibition halo in millimeters.



Source: Prepared by the authors.

Table 2. Comparison of the tested concentrations of lemongrass (below diagonal) and tea tree (above diagonal). Values refer to the p value.

Conc.%	6.25	12.50	25.00	50.00	100.00
6.25		0.046	0.011	0.012	0.007
12.50	1.000*		0.011	0.012	0.007
25.00	0.001	0.001		0.219*	0.015
50.00	0.007	0.007	0.011		0.015
100.00	1.000*	1.000*	0.001	0.001	

Mann Whitney test (0.05). * = No difference. Values presented refer to p-values. [1]Comparison between two concentrations with zero inhibitory effect; Conc= Concentration. The values in the table refer to the P-values of the peer-to-peer comparison.

Source: Prepared by the authors.

Table 3. Comparison of the tested concentrations between lemongrass and tea tree oils.

		Lemongrass				
	Conc. (%)	**100**	**50**	**25**	**12.5**	**6.25**
Tea Tree	**100**	0.007	0,024	0.012	0.007	0.007
	50	0.007	0.007	0.664*	0.012	0.007
	25	0.007	0.007	0.011	0.012	0.007
	12.5	0.007	0.007	0.011	0.021	0.007
	6.25	1.000*	1.00*	0,007	0.007	1.000*

Mann Whitney test (0.05). * = No difference. Values presented refer to p-values. [1]Comparison between two concentrations with zero inhibitory effect. The values in the table refer to the P-values of the peer-to-peer comparison.

Source: Prepared by the authors.

Research has demonstrated the success of lemongrass oil in controlling several species of *Xanthomonas* (LUCAS et al., 2012; GAO et al., 2020; CHUNG et al., 2022; MARTINAZZO; BRAGA; TEODORO, 2022; KOLOZSVÁRINÉ NAGY et al., 2023; MARIN et al., 2024). Similarly, tea tree oil has been frequently studied for its effects on the *Xanthomonas* genus (RAMOS; BORGES; TEBALDI, 2017; DALIO et al., 2020; MAČIONIENĖ et al., 2021; VISHAKHA et al., 2022). However, in contrast to the results presented here, Ramos et al. (2017) and Mačionienė et al. (2021) reported a low efficacy in controlling *Xanthomonas*, which may be due to a pathogen-specific effects related to the oil used.

Differences in the effectiveness of the oils at various concentrations for controlling *X. citri pv. eucalyptorum* may be related to variations in the chemical composition and concentration of specific components in each oil. The bactericidal activity of lemongrass oil is primarily attributed to citral, whereas terpene-4-ol is the main bactericidal compound suggested in tea tree oil (CARSON; HAMMER; RILEY, 2006; CORDEIRO et al., 2020; JOHANSEN; DUVAL; SERGERE, 2022; MONDELLO et al., 2022). The phytobacteria analyzed in this study seems to exhibit greater sensitivity to citral, evidenced by the more pronounced control effect at concentrations of 12.5 % and 25 % for lemongrass oil compared to tea tree oil. Consequently, it is plausible to speculate that the plant pathogen tested in this study may lack sensitivity to β-caryophyllene, the primary bactericidal agent in copaiba oil.

These findings elucidate the potential of essential oils in controlling *X. citri pv. eucalyptorum.* However, future research should be performed *in situ* by directly applying these compounds to the plants intended for protection. This approach will enable a comprehensive analysis of the efficacy of essential oils under conditions that mimic the natural cultivation environment, thereby providing precise data on their impact on plant health and pathogen suppression. Furthermore, this approach will offer valuable insights into the longevity and action of the compounds, especially considering that essential oils may undergo photodegradation, which compromises the stability and efficacy of the active compounds over time (AY et al., 2019). This aspect is crucial for evaluating the practical durability and effectiveness of these oils. Additionally, essential oils may induce plant resistance to pathogens, potentially acting through different mechanisms across various metabolic pathways (BANANI et al., 2018; YANG et al., 2022; BINIAZ; KAVOOSI; AFSHARIFAR, 2024).

Conclusions

Copaiba oil had no inhibitory effect controlling the phytobacterium *X. citri pv. eucalyptorum in vitro.* However, lemongrass and tea tree oils showed inhibitory effectiveness at concentration above 12.5 %. No significant differences were observed between the 12.5 and 25 % concentrations of tea tree oil. Additionally, no differences were found between the 25 % concentrations of lemongrass oil and the 50 % concentration of tea tree oil in terms of bacteria control.

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