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Potency of Eucalyptus Oil and Citronella Oil in Suppressing Virulence Factors of *Xanthomonas oryzae* pv. *oryzae*

Potensi Minyak Kayu Putih dan Minyak Serai Dapur dalam Menekan Faktor Virulensi Bakteri *Xanthomonas oryzae* pv. *oryzae*

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ABSTRACT

Bacterial leaf blight, caused by the bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is an important disease of rice and can cause yield losses of 10-50%. Efforts to control bacterial leaf blight in rice using eucalyptus oil and citronella oil have not been widely reported. This study aims to evaluate the potential inhibition of eucalyptus oil and citronella oil against *X. oryzae* pv. *oryzae* isolate code BaK_2 *in vitro*, focusing on *X. oryzae* pv. *oryzae* virulence factors. The virulence factor assay consisted of biofilm formation, exopolysaccharide (EPS) production and motility. The results showed that eucalyptus oil and citronella oil could inhibit the growth of *X. oryzae* pv. *oryzae in vitro*. The minimum inhibitory concentration (MIC) was 15% for eucalyptus oil and 5% for citronella oil. Virulence factor tests showed that eucalyptus oil and citronella oil had no significant effect on biofilm formation, but could reduce EPS formation and limit the movement of *X. oryzae* pv. *oryzae* by both swimming motility and motility.

Key words: biofilm, exopolysaccharide, *minimum inhibitory concentration,* motility

ABSTRAK

Penyakit hawar daun yang disebabkan oleh bakteri *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), merupakan salah satu jenis penyakit yang penting pada tanaman padi dan dapat menyebabkan kehilangan hasil mencapai 10-50%. Upaya pengendalian penyakit hawar daun bakteri pada tanaman padi dengan minyak kayu putih dan minyak serai dapur belum banyak dilaporkan. Tujuan penelitian ialah mengevaluasi daya hambat dan penekanan faktor virulensi dari minyak kayu putih dan minyak serai dapur terhadap *Xoo* kode isolat BaK_2 secara *in vitro*. Pengujian daya hambat dilakukan dengan metode *double layer*. Pengujian faktor virulensi dilakukan terhadap pembentukan biofilm, pembentukan eksopolisakarida (EPS), serta motilitas *X. oryzae* pv. *oryzae*. Hasil penelitian menunjukkan bahwa minyak kayu putih maupun minyak serai dapur dapat menghambat pertumbuhan *X. oryzae* pv. *oryzae* secara *in vitro*. Potensi *minimum inhibitory concentration* (MIC) pada masing-masing yaitu 15% pada minyak kayu putih dan 5% pada minyak serai dapur. Faktor virulensi pada tiga pengujian, menunjukkan bahwa minyak kayu putih dan minyak serai dapur tidak berpengaruh signifikan terhadap pembentukan biofilm, namun dapat menurunkan pembentukan EPS dan mampu membuat pergerakan bakteri *minimum inhibitory concentration* menjadi terbatas baik secara *swimming motility* maupun secara *twitching motility*.

Kata kunci: biofilm, eksopolisakarida, *minimum inhibitory concentration*, motilitas

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INTRODUCTION

Rice (*Oryza sativa*) is widely cultivated in Indonesia due to its role as the main staple food. Rice production in Indonesia were always fluctuated as reported by Badan Pusat Statistik (BPS) (2023). One of the problems in rice cultivation is the presence of plant diseases, including bacterial leaf blight disease caused by *Xanthomonas oryzae* pv. *oryzae*. Yield losses caused by this pathogen range from 10%–30%, whereas under favorable conditions for the development of the disease on susceptible varieties, yield losses may reach 50%. Yield losses can even reach 80% if infection occurs at the tillering stage, which causes reduction of grain yield (Ansari *et al.* 2018; Reinke *et al.* 2018).

Virulence factors in plant bacterial pathogens comprise several components that enable these bacteria to cause diseases in their host plants. These factors also play a role in bypassing plant defenses, infecting plants, and reproducing to defend themselves. The primary virulence factors such as biofilm formation, exopolysaccharide (EPS) production, and motility, are crucial for *X. oryzae* pv. *oryzae* to survive and infect rice plants as their hosts. The formation of biofilm and EPS depends on the motility of *X. oryzae* pv. *oryzae*. Inhibiting these virulence factors is essential to reduce *X. oryzae* pv. *oryzae* infection and the resulting symptoms (Antar *et al.* 2020; Maryam *et al.* 2020; Mishra *et al.* 2020).

Essential oils, such as eucalyptus and citronella oil with antibacterial mode of action has been reported for its potential to reduce plant pathogenic bacteria. Eucalyptus oil, renowned for its antimicrobial properties with aromadendrene, 1,8-cineole, citronellal, and citronellol as its key components (Mulyaningsih *et al.* 2011). Conversely, citronella oil has been demonstrated to induce the destruction of bacterial biofilms and inhibit the growth and development of bacteria. The main components of citronella oil include neral, isoneral, geranial, isogeranial, geraniol, geranyl acetate, citronellal, citronellol, germacrene-D, and elemol, which collectively constitute approximately 60–80% of the oil (Mukarram *et al*. 2022). The objective of this research was to determine the inhibitory potency of eucalyptus oil and citronella oil on *X. oryzae* pv. *oryzae in vitro*, as well as to ascertain the suppression of these essential oils on biofilm formation, exopolysaccharide (EPS) production, and bacterial motility as the virulence factors of *X. oryzae* pv. *oryzae*.

MATERIALS AND METHODS

This research was conducted from January to September 2023 at the Phytopathology Laboratory, Gadjah Mada University. The isolate of *X. oryzae* pv. *oryzae* BaK_2 was obtained from rice plants of the Ciherang variety and is a collection of the Plant Disease Laboratory, Gadjah Mada University. The BaK_2 bacterial isolate was stored in glycerol and subsequently regrown on wakimoto media (Ou 1985; Anonim 2007). The culture was incubated at 28 °C. The eucalyptus and citronella oils were obtained from CV Agung Jaya Solo, Central Java.

Effect of Essential Oils on The Colony Growth of *X. oryzae* pv. *oryzae*

The essential oils (eucalyptus and citronella oils) were prepared in a series of concentrations. The concentrations were set at 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%. The essential oils are diluted using 30% ethanol as a solvent. The initial step involved preparing a suspension of *X. oryzae* pv. *oryzae*, which had been grown on wakimoto media for 48 hours, with a density of 108 cfu mL^{-1}. Subsequently, 200 µL of *X. oryzae* pv. *oryzae* suspension was added to 5 mL of wakimoto media with 0.6% agar (6 g L^{-1} agar). This mixture was then poured and spread onto a petri dish that had been previously filled with 10 mL of wakimoto media. The solidified wakimoto's medium was pierced with a cork drill until it touched the bottom of the petri dish. A total of 10 µL of essential oil was applied to the hole and incubated at 28 °C for 5 days. The inhibition zone formed

around the hole was measured and used as the minimum inhibitory concentration (MIC). In this study, the control group consisted of treatment without essential oil, application of 30% ethanol, and application of zinc thiazole active ingredient bacteria at a concentration of 1.895 mL L^{-1} (0.1895%).

Effect of Essential Oils on the Formation of *X. oryzae* pv. *oryzae* Biofilms *in Vitro*

An experiment on biofilm formation was conducted in accordance to the methodology outlined by Thepbandit *et al*. (2021), with modifications pertaining to the media and essential oil treatment. The essential oils were applied at four different concentrations: ½MIC, 1MIC, 2MIC, and 4MIC. The control group received the same treatment as in the previous experiment.

The *X. oryzae* pv. *oryzae* isolate was cultured in liquid wakimoto media and incubated on a shaker for 24 hours at 28 °C. A total of 55 µL of the bacterial suspension was added to each well of the microplate, followed by the addition of 55 µL of the essential oil. The microplates were then incubated for 24 hours. Additionally, 110 µL of a 0.1% crystal violet solution was added to each well and incubated for a further 15 minutes at 28 °C. Subsequently, the microplate was washed twice with sterile water to remove the crystal violet solution and bacterial suspension. The biofilm bound to the crystal violet was dissolved with 100 µL of 96% ethanol, and its absorbance value was measured at a wavelength of 560 nm (with a bandwidth of 10 nm) using a microplate reader.

Effect of Essential Oils on the Formation of *X. oryzae* pv. *oryzae* Exopolysaccharides (EPS) *in Vitro*

An experiment on EPS formation was conducted following Thepbandit *et al*. (2021) protocol with several modifications related to the use of media and essential oil treatments. The concentration of essential oils and controls employed in this experiment were identical to those utilized in the biofilm experiment. The *X. oryzae* pv. *oryzae* isolate was cultured in liquid wakimoto media and incubated on a shaker for 24 hours at 28 °C. A total of 1.5 mL of *X. oryzae* pv. *oryzae* suspension was transferred to a 2 mL Eppendorf tube, and 500 µL of essential oil was added in accordance with the specified treatment concentration. The Eppendorf tube was incubated for 24 hours at 28 °C, then subjected to centrifugation at 9000g for 20 minutes. The formed EPS pellet was separated from the supernatant, and 1 mL of cold 95% ethanol (0 °C) was added. The EPS pellet was then subjected to another centrifugation at 9000g for 20 minutes, after which the ethanol was discarded. The EPS pellets were subsequently dried at 28 °C until a constant weight was achieved.

Effect of Essential Oils Toward the Motility of *X. oryzae* pv. *oryzae* in Vitro

Two methods were employed to conduct motility experiments, i.e. swimming motility and twitching motility. These methods were adapted from those described by Thepbandit *et al*. (2021), with several modifications related to the use of media and essential oil treatments. The concentrations of essential oils and controls employed in the biofilm experiment were replicated in subsequent experiments. The swimming motility experiments were conducted on wakimoto media with 0.3% agar (3 g of agar per liter). The initial step was to prepare a suspension of *X. oryzae* pv. *oryzae* in sterile water. A total of 10 mL of test media was combined with 100 µL of essential oil, corresponding to the specified treatment concentration, and then poured into a petri dish. Once the media had solidified, 3 µL of *X. oryzae* pv. *oryzae* suspension was placed in the center of the test media and then incubated at 28°C. Twitching motility experiments were conducted on wakimoto media with 1.5% agar (15 g L^{-1} agar). A total of 10 mL of test media was combined with 100 µL of essential oil, in accordance with the specified treatment concentration, and subsequently poured into a Petri dish. Once the media had solidified, the 48-hour-old *X. oryzae* pv. *oryzae* isolate was smeared onto the central surface using a sterile toothpick.

The media was then incubated at 28 °C, and bacterial motility was observed after 72 hours by measuring the diameter of the movement area. The observation of bacterial motility was carried out 72 hours after incubation by measuring the diameter of the movement area.

Data Analysis

The study was conducted using a completely randomized design with five replicates for each treatment. All data were subjected to analysis using an ANOVA (analysis of variance) test, followed by Tukey's HSD (honestly significant difference) test to ascertain significant differences between treatments.

RESULTS

Culture of *X. oryzae* pv. *oryzae*

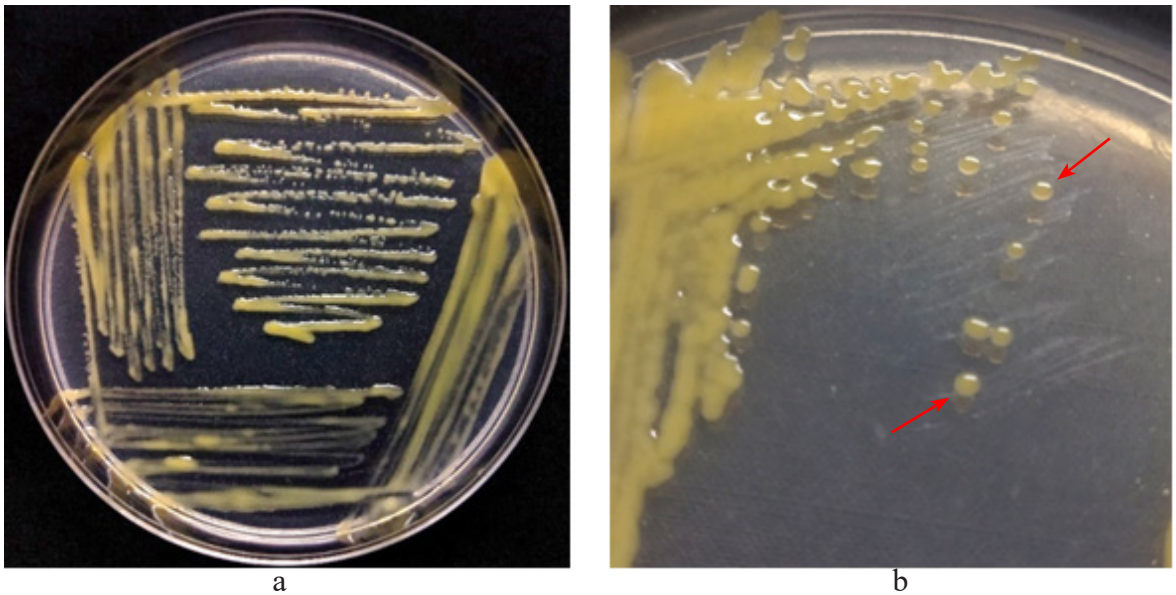
The BaK_2 is *X. oryzae* pv. *oryzae* pathotype IV isolated from Ciherang rice variety grown in Kasihan, Bantul, Yogyakarta with symptoms of bacterial leaf blight disease (Irpawa 2023, unpublished results). The *X. oryzae* pv. *oryzae* colonies have distinctive characteristics, including yellow color, mucoid or slimy texture due to its ability to form EPS, and round shape with a raised surface and smooth edges (Figure 1).

Growth Inhibition of *X. oryzae* pv. *oryzae* by Essential Oils

Application of eucalyptus oil at concentrations of 1%, 2%, 3%, 4%, 5% and 10% did not significantly inhibit the growth of *X. oryzae* pv. *oryzae* compared to the positive control, i.e. 30% ethanol application and treatment without essential oils. However, there was a significant difference between the 15% concentration and the control group, as well as between the 15% concentration and the bactericide containing the active ingredient zinc thiazole. This shows that eucalyptus oil at a concentration of 15% has the lowest inhibitory concentration or MIC (Table 1). The application of citronella oil at concentrations of 1%, 2%, 3%, and 4% did not result in notable differences when compared to the positive control group (Table 1). However, a significant discrepancy was observed at a concentration of 5%, which was subsequently utilized as MIC.

Formation of *X. oryzae* pv. *oryzae* Biofilms *in Vitro*

The 30% ethanol control treatment did not cause significant differences in absorbance compared to the control without application. Significant differences in absorbance were observed between the application of



a b

Figure 1 *Xanthomonas oryzae pv. oryzae* isolate BaK_2. A, Bacterial colonies on wakimoto media; and B, Single colony

Table 1 Formation of inhibition zone following application of eucalyptus and citronella oil on *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)

Treatment concentration (%)	Diameter of inhibition zone (cm)	
	Eucalyptus oil	Citronella oil
1	0.00 e	0.00 f
2	0.00 e	0.11 f
3	0.00 e	0.00 f
4	0.00 e	0.21 f
5	0.14 e	2.32 d
10	0.24 e	3.93 c
15	2.98 c	6.12 b
20	7.85 b	8.35 a
30	8.40 a	8.50 a
40	8.50 a	8.50 a
50	8.50 a	8.50 a
60	8.50 a	8.50 a
70	8.50 a	8.50 a
80	8.50 a	8.50 a
90	8.50 a	8.50 a
100	8.50 a	8.50 a
Application of ethanol 30% (with *Xoo* inoculation)	0.00 e	0.00 f
Without application of essential oil (with *Xoo* inoculation)	0.00 e	0.00 f
Without application of essential oil (without *Xoo* inoculation)	8.50 a	8.50 a
Application of bactericide, active ingredient Zinc Thiazole (0.1895%)	1.35 d	1.35 e

Note: Numbers in the same column followed by different letters are significantly different with HSD Tuckey test and α value of 0.05.

eucalyptus oil and citronella oil and the control treatment without application. The application of eucalyptus oil and citronella oil did not result in notable differences in the reduction of *X. oryzae* pv. *oryzae* biofilm formation when compared to the application of bactericides. No significant differences were observed in biofilm formation between the concentrations of eucalyptus oil and citronella oil, including ½MIC, 1MIC, 2MIC, and 4MIC. These results indicate that variations in essential oil concentration do not significantly affect the reduction of biofilm formation of *X. oryzae* pv. *oryzae* bacteria (Table 2).

Formation of *X. oryzae* pv. *oryzae* Exopolysaccharides *in Vitro*

Significant differences in EPS production were discerned between the applicationof eucalyptus oil and citronella oil in comparison to the control group. Furthermore, a notable discrepancy was observed in EPS production when eucalyptus oil and citronella oil were applied, in comparison to the bactericide Zinc Thiazole. The concentration of essential oils, whether at ½MIC, 1MIC, 2MIC, or 4MIC, resulted in significantly disparate values when compared to the application of bactericide. The application of ½MIC eucalyptus oil and citronella oil resulted in higher values than the bactericide, while other concentrations resulted in lower values than the bactericide. This indicates that the application of eucalyptus oil and citronella oil at the optimal MIC effectively reduces the formation of EPS of *X. oryzae* pv. *oryzae* (Table 2).

Motility of *X. oryzae* pv. *oryzae in Vitro*

On Table 3, it was shown a noticeable distinction on the *X. oryzae* pv. *oryzae*

motility between the application of eucalyptus oil and citronella oil at ½MIC, 1MIC, 2MIC, and 4MIC compared to the control group. Additionally, there was a significant difference on the motility between the essential oil treatment and the comparison involving bactericide Zinc Thiazole. The concentrations of essential oils, whether ½MIC, 1MIC, 2MIC, or 4MIC, caused significantly different values compared to bactericide application. When applied at ½MIC, both eucalyptus oil and citronella oil showed lower bactericidal values. This implied that the application of eucalyptus oil and citronella oil at ½MIC effectively restricted the movement of *Xoo* bacteria based on the motility experiment.

A significant difference on motility was evident between the application of eucalyptus oil and citronella oil at ½MIC, 1MIC, 2MIC, and 4MIC in comparison to the control group. Additionally, a substantial difference on motility was noted between the essential oil treatment and the comparison bactericide Zinc Thiazole. The concentrations of essential oils, whether ½MIC, 1MIC, 2MIC, or 4MIC, exhibited significantly different values compared to bactericide application. When applied at ½MIC, both eucalyptus oil

Table 2 Formation of biofilm and exopolysaccharides by *X. oryzae* pv. *oryzae* after treatment using essential oils

Treatment concentration (%)	Biofilm[a] (OD)	Exopolysaccharides (EPS) (mg)
Eucalyptus oil ½MIC[b]	0.157 c	14.22 c
Eucalyptus oil 1MIC	0.184 bc	11.00 f
Eucalyptus oil 2MIC	0.229 bc	9.82 h
Eucalyptus oil 4MIC	0.133 bc	7.96 j
Citronella oil ½MIC	0.168 bc	14.94 b
Citronella oil 1MIC	0.162 bc	11.90 e
Citronella oil 2MIC	0.142 bc	10.46 g
Citronella oil 4MIC	0.197 bc	8.54 i
Without application	0.544 a	18.90 a
Application of ethanol 30%	0.371 ab	19.00 a
Application of bactericide, active ingredient Zinc Thiazole (0.1895%)	0.157 c	12.80 d

[a]Measured using a microplate reader;
[b]MIC, minimum inhibitory concentration;
Note: Numbers in the same column followed by different letters are significantly different with HSD Tuckey test and α value of 0.05.

Table 3 Diameter movement of *X. oryzae* pv. *oryzae* in the swimming and twitching motility experiment.

Treatment concentration (%)	Swimming motility (cm)	Twitching motility (cm)
Eucalyptus oil ½MIC[b]	0.96 c	0.82 b
Eucalyptus oil 1MIC	0.40 e	0.75 b
Eucalyptus oil 2MIC	0.30 f	0.33 d
Eucalyptus oil 4MIC	0.30 f	0.28 d
Citronella oil ½MIC	0.52 d	0.33 d
Citronella oil 1MIC	0.10 g	0.30 d
Citronella oil 2MIC	0.10 g	0.00 e
Citronella oil 4MIC	0.00 h	0.00 e
Without application	2.02 a	1.00 a
Application of ethanol 30%	0.02 a	1.00 a
Application of bactericide, active ingredient Zinc Thiazole (0.1895%)	1.07 b	0.58 c

Note: Numbers in the same column followed by different letters are significantly different with HSD Tuckey test and α value of 0.05.

and citronella oil showed lower bactericidal values. This indicated that the application of eucalyptus oil and citronella oil at just ½MIC effectively limited the movement of *X. oryzae* pv. *oryzae* based on the twitching motility experiment (Tabel 3).

DISCUSSION

The lowest inhibitory concentrations of eucalyptus oil (15%) and citronella oil (5%) indicate the potential concentration as minimum inhibitory concentration (MIC) for each essential oil. The findings of this study suggest that eucalyptus oil and citronella oil exert antimicrobial effects, particularly in the inhibition of *X. oryzae* pv. *oryzae* growth *in vitro*. The diffusion method on PSA media has been demonstrated to effectively inhibit the growth of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* by citronella oil at a dilution of 1:5 (v/v) (Wonni *et al*. 2016). As stated by Naveena *et al*. (2020), eucalyptus oil at a concentration of 2000 ppm demonstrated the highest inhibitory activity. Conversely, citronella oil at a concentration of 500 ppm produced an inhibition zone of 12.1 mm, while at 1000 ppm it produced an inhibition zone of 13.4 mm.

The determination of MIC for natural materials such as essential oils has not been standardized internationally. Consequently, no established unit of measurement exists for the concentration or dose of essential oils, in contrast to pesticides. The MIC of essential oils is dependent upon the specific microorganisms being tested. It has been observed that different bacterial strains exposed to the same essential oil can produce varying MICs. Additionally, the culture medium utilized can influence the resulting MIC. Furthermore, factors such as the plant species, growing environment, extraction of plant parts, and extraction techniques employed can contribute to variations in the compound components present in essential oils (Hulankova 2022).

Virulence factors of *X. oryzae* pv. *oryzae* that discuss in this research consist of biofilm formation, EPS production, and motility. Our results show that the concentration of both essential oils does not significantly affect the reduction of *X. oryzae* pv. *oryzae* biofilm formation. This implies that eucalyptus oil or citronella oil can replace the use of bactericides in reducing biofilm formation. The application of eucalyptus oil and citronella oil at a concentration of 15% and 5%, respectively has effectively reduced the formation of bacterial EPS. Biofilms are produced by bacteria, which can serve as a protective environment for the bacteria themselves (Ham and Kim 2018). In *X. oryzae* pv. *oryzae*, biofilm and EPS have a close relationship because both play important roles in the adaptation of bacteria to the environment and the colonization of host cells. Biofilms are bacterial communities attached to surfaces, consisting of bacterial cells and an extracellular matrix composed of EPS. Meanwhile, EPS plays a role in forming the biofilm matrix and protecting bacteria from unfavorable environmental conditions. EPS is also a crucial component in biofilms, contributing to bacterial virulence properties. Furthermore, EPS production in *X. oryzae* pv. *oryzae* is regulated by cyclic diguanylate monophosphate (c-di-GMP) signals. These signals are common secondary molecules in bacteria, controlling various biological functions, including the cell cycle, motility, biofilm formation, and expression of virulence factors in pathogenic bacteria (Wang *et al.* 2018; Xue *et al.* 2018).

The application of 15% eucalyptus oil and 5% citronella oil demonstrated effective inhibition of *X. oryzae* pv. *oryzae* movement, as evidenced by the inhibition of both swimming and twitching motility. This demonstrates the beneficial impact of essential oil application in inhibiting bacterial movement, suggesting its potential as an alternative method for pathogen control. As defined by Palma *et al.* (2022), swimming motility refers to the ability of bacteria to move in a liquid environment using flagella for propulsion. In contrast, twitching motility involves the movement of bacteria on solid surfaces through type IV pili, protein structures on the surface of bacterial cells. These pili also play a role in regulating

bacterial movement, biofilm formation, and EPS production in *X. oryzae* pv. *oryzae* (Li *et al.* 2020).

The differences in concentration and test results for eucalyptus oil and citronella oil indicate that the concentration of essential oils can vary according to the method, type of essential oil, and even the origin of the essential oil. However, their use can be beneficial in replacing the role of bactericides, with one of the advantages being that they are easily degraded in nature when applied to rice plants. This aligns with the characteristics of essential oils, namely that they volatilize easily. Therefore, when applying them, attention must be paid to the application interval and concentration (Misra *et al.*, 2022).

Based on the experiments conducted in this research, it can be concluded that eucalyptus oil and citronella oil effectively inhibit the growth of *X. oryzae* pv. *oryzae* in vitro, with a MIC potency of 15% and 5% for eucalyptus oil and citronella oil, respectively. While these essential oils do not significantly impact biofilm formation, they can reduce EPS production and restrict the movement of *X. oryzae* pv. *oryzae* through both swimming and twitching motility.

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