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Inhibition of Cellulase Activity *Fusarium oxysporum* f. sp. *lycopersici* Pathogens in Tomato Plants Using Essential Oils from Citronella in Vitro

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Abstract: The purpose of this study is to determine the ability of essential oils from citronella plants to inhibit cellulase enzyme activity in the fungus Fusarium oxysporum f. sp. lycopersici. and evaluate its potential as a disease-control material in tomato plants. This research was carried out in August 2023 until completed in February 2024. The method used in this study is experimental which consists of the process of exploring the fungus that causes disease in tomato plants, microscopic characterization of fungal isolates, molecular identification, inhibition test of fungal colony growth using essential oils from citronella plants, cellulase enzyme activity test, and cellulase enzyme activity inhibition test using essential oils from citronella plants. The variables observed in this study consisted of inhibiting the growth of *Fusarium oxysporum* f. sp. *lycopersici*. By essential oils, test the activity of quantitative cellulase enzymes, and cellulase enzymes activity inhibition test using essential oil from citronella plant. The results of microscopic characterization and molecular tests show that the fungal isolate used in this study has 99% similarity with *Fusarium oxysporum* f. sp. *lycopersici*. Essential oils extracted from citronella plants effectively inhibit the formation of fungal colonies. This is evidenced by the results of observation at the age of 7 HSI mushrooms with the growth diameter of fungal colonies of 5.06 cm, 3.56 cm, and 2.44 cm after applying essential oils with concentrations of 333 ppm, 666 ppm, and 1000 ppm. Results of observation and calculation of cellulase enzyme activity values in *Fusarium oxysporum* f. sp. *lycopersici isolate*. Cellulase enzyme activity of 0.831 U/mL was obtained. The application of essential oils with sequential concentrations (333 ppm, 666 ppm, and 1000 ppm) was able to decrease the activity of cellulase enzymes, with activity inhibition of 31%, 39%, and 44% in *Fusarium oxysporum* f. sp. *lycopersici* isolates.

Keywords: essential oils; *fusarium oxysporum cellulase*; inhibition; tomato plants

INTRODUCTION

Tomato plant (*Lycopersicon esculentum* Mill.) is one of the important types of horticultural commodities in Indonesia that has various benefits for living needs, both used as food management, development of medicinal ingredients or vitamins, and inseparable from its potential which has a fairly stable economic value (Da Rato *et al.*, 2021). Tomato commodity production in Indonesia in 2022 reached 1.16 million tons. This amount is a production covering 34 provinces throughout Indonesia. In 2023, Indonesia only managed to produce 1.14 million tons of tomatoes. Based on these data, tomato commodity production in Indonesia decreased by 1.12 tons in 2023. The decrease in tomato production was certainly influenced by several factors, one of which was the disruption of plant pests (OPT) in the form of *Fusarium oxysporum* f. sp. *lycopersici.* Causes of fusarium wilt disease in tomato plants (Central Statistics Agency, 2024).

Fusarium wilt disease caused by the fungus *Fusarium oxysporum* f. sp. *Lycopersici.* is an important disease that often hinders the cultivation of tomato plants. This pathogen infects the vascular tissue of plants, causing inhibition of the absorption of water and nutrients (Soesanto, 2006). The appearance of fusarium wilt on tomato plants is the result of the pathogenicity process. This process is influenced by the activity of enzymes secreted by disease-causing fungi in their hosts, the most significant enzymes that play a role in the pathogenicity process of *Fusarium oxysporum* f. sp. *lycopersici*. namely cellulase (Freeman *et al.*, 2000). Cellulase is a catalyst that can hydrolyze cells found in plant cell walls, then convert them into glucose that can be used as a source of carbon for growth. This enzyme also plays a role in the degradation process

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of the host cell wall, making it easier for organelles in the form of asperium or fungal hyphae to carry out the process of penetration, injection, and inflation into plant cells and tissues (El Boustani *et al.*, 2000).

The use of chemical pesticides to control plant diseases has had many negative impacts on the biological environment. Certain plants can produce secondary metabolites that can be used for the development of medicines and botanical pesticides. One type of plant whose essential oil can be utilized is citronella (Yanti *et al.*, 2020). The use of essential oils extracted from citronella plants as botanical pesticides can reduce negative impacts on the biological environment (Suprapta *et al.*, 2003). The results of the identification of the main compound components contained in the essential oil of citronella plants by Alma *et al.*, (2007) consisted of citronellal (34.60%), geraniol (23.17%), and citronel (12.09%). These components have been studied to inhibit cellulase enzyme activity in cellulolytic microbes, with a decrease in activity of 4.41% after the addition of essential oil from citronella plants with a concentration of 0.5% (Putu *et al.*, 2019). Based on the description above, this study needs to be conducted with the aim of determining the ability of essential oil from citronella plants to inhibit cellulase enzyme activity in *Fusarium oxysporum* f. sp. *lycopersici*. and evaluating its potential as a control material for fusarium wilt disease in tomato plants.

METHODS

This research was conducted from August 2023 to February 2024 at the Plant Disease Laboratory, Faculty of Agriculture, Brawijaya University, Malang. Several test variables in this study were arranged using a Completely Randomized Design (CRD) consisting of 4 treatments and 4 replications.

Exploration and sampling of disease-causing fungi were obtained from farmers' fields, Sumber Brantas Village, Bumi Aji District, Batu City. Parts of tomato plants showing symptoms of *Fusarium oxysporum* f. sp. *lycopersici.* were taken to the laboratory for the isolation process. After isolation, the next step is the purification of the fungal isolate which is carried out on PDA media by taking a little or part of the mycelium using an ose needle, then transferring it to a petridish containing new PDA media aseptically (Andi Murniati *et al.*, 2022).

Pure cultures of isolates were observed for macroscopic and microscopic morphological characteristics. Macroscopic characteristics were observed based on the color, shape, and growth of the colony. Meanwhile, microscopic characteristics are observed based on the characteristics of fungal hyphae and conidia (Afriani *et al.*, 2018). In observing the characteristics of fungi microscopically, observations were made using a microscope with a magnification of 400x (Syamyuktha *et al.*, 2020). Molecular identification of fungi was carried out by officers at PT. Genetika Science Indonesia (GSI) in Tangerang City, Indonesia.

The inhibition of fungal colony growth using essential oils was carried out using several treatments, namely: control (without adding essential oil concentration), treatment with the addition of 333 ppm, 666 ppm, and 1000 ppm essential oils. Each treatment was mixed evenly in PDA media poured into a petri dish. After the mixture hardened, the purified fungal isolate was drilled using a 5 mm diameter drill, then taken using an ose needle and placed right in the middle of the petri dish. Furthermore, it was incubated at room temperature for 7 days after incubation (HSI). The power of essential oils on fungal colony growth was calculated using the equation (Elfina *et al.*, 2015) is presented in Equation 1.

$$D = \frac{d1-d2}{2} \text{.....(1)}$$

Note:
D : Mushroom diameter

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D1 : Horizontal diameter of the mushroom colony
D2 : Vertical diameter of the mushroom colony

Cellulase enzyme activity test on *Fusarium oxysporum* f. sp. *lycopersici* isolates. Performed by preparing two micro tube units. One tube for the standard and one for the cellulase sample. In the standard tube, 100 µL of crude extract enzyme, 100 µL of 1% Carbomexthyl Cellulase (CMC) substrate solution, 259 µL of pH 6 phosphate buffer, 400 µL of 10% standard glucose, and 100 µL of DNS were added. This standard was then heated to 100°C for 5 minutes until the Dinitrosalisilat (DNS) color changed. In the sample tube, 100 µL of crude extract enzyme, 100 µL of substrate solution (1% CMC), and 259 µL of pH 6 phosphate buffer were added. The sample was incubated at 37°C for 30 minutes. Next, 400 µL of 10% standard glucose and 100 µL of Dinitrosalisilat (DNS) were added, then heated at 100°C for 5 minutes until the DNS color changed. To obtain the cellulase activity value in the test isolate, the Equation 2 was used (IBPE Putra *et al.*, 2019).

$$AE = \frac{C}{BM\ glucose \times t} \times \frac{H}{E} \text{.....(2)}$$

Note:
AE : Enzyme Activity (Unit/mL)
C : Glucose Concentration
BM : Molecular weight of glucose (180 g/mol)
t : Incubation Time
H : Total Enzyme-Substrate Volume (mL)
E : Enzyme Volume (mL)

The working procedure of the *Fusarium oxysporum* f. sp. *lycopersici.* cellulase enzyme activity inhibition test is the same as the cellulase enzyme activity test. It's just that sample tubes are added with each essential oil concentration treatment (Control, 333 ppm, 666 ppm, and 1000 ppm). Furthermore, the standard and sample on both divortex tests are up to a homogeneous color. The tube and standard sample are centrifuged at 9000 rpm for 4 minutes. Supernatants were transferred as much as 600 µL to the cuvette and aquades were added as much as 2400 µL. then absorbance was measured at a wavelength of 540 nm using a UV-Vis spectrophotometer. To determine the percentage of inhibition of cellulase enzyme activity in the tested isolate, calculations are carried out with the Equation 3.

$$\%\ Inhibition = \frac{(sample\ blank)}{blank} \times 100\% \text{.....(3)}$$

Where blanks as enzyme activity in isolates and samples of essential oil tampers are enzyme activity with essential oil inhibition.

The results of in vitro tests in the form of observations of the diameter of fungal colony growth, and inhibition of cellulase enzyme activity using essential oils were analyzed quantitatively. Statistical analysis was carried out using analysis of variance (ANOVA) with the help of Software Minitab 2017.

RESULTS AND DISCUSSION

1. Identification of *Fusarium oxysporum* f. sp. *lycopersici.*

- Microscopic characterization of isolates

Morphological observations of *the fungus Fusarium oxysporum* f. sp. *lycopersici.* Microscopic **(Figures 1)** show that fungal isolates have two types of conidia including microconidia and macroconidia. Microconidia have oval, hyaline and single- or double-celled characteristics of smaller size. Macroconidia are shaped like a crescent moon and have many cells, about three to five septa. This is in line with the results of observations by those who revealed that microscopic observations were obtained of three types of spores, namely macroconidia, microconidia and chlamydospores. Macroconidia has a crescent-like shape with a total of three to five septa. The length of macroconidia is

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between 17.08 µm – 26.87 µm and can be longer. Microconidia are smaller in size than macroconidia and oval in shape. (Manici *et al.*, 2017).

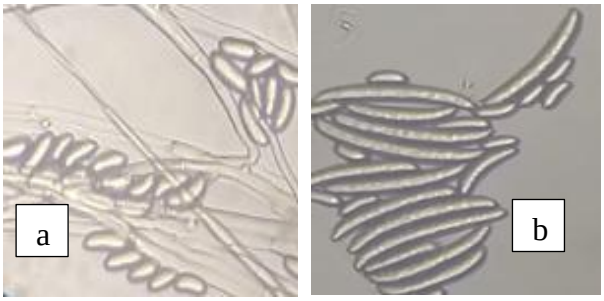

Figure 1. Observation of the microscopic morphology of fungal isolates: a). Microconidia, b). Macroconidia - Identification of *Fusarium oxysporum* f. sp. *lycopersici.* isolate.Molecular

The results of DNA isolation based on the electrophoresis process show that DNA can be isolated well, this can be seen from the resulting band looking clear, bright and thick. In line with the opinion that reveals that the electrophoresis process that produces a qualitatively clear and thick band can show that the concentration of DNA obtained is quite high while the thin band shows a low concentration of DNA. The mushroom isolate used had a DNA concentration of 13.8 µg/µl. Based on the results of gel electrophoresis visualization, the mushroom isolate has a DNA band size of 450 bp **(Figure 2)**.

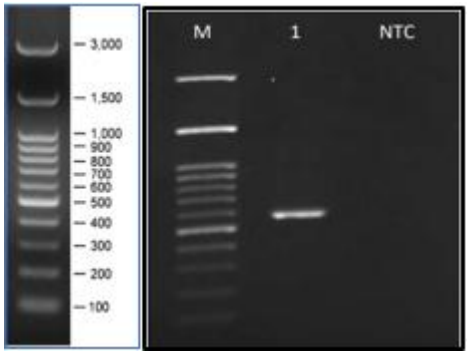

Figure 2. DNA electrophoresis results: M) markers, 1) Fungal targets

Fungal isolates are identified molecularly to determine the identity of the isolate down to the species level. The isolates are identified based on ribosomal DNA (rDNA) sequences in the ITS region. Based on the results of sequence analysis using the Basic Local Alignment Search Tool (BLAST) program accessed on http://www.ncbi.nml. Nih.govv/BLAST found that the isolate with the code G3131-1 identified had a 99% similarity to *Fusarium oxysporum* f. sp. *lycopersici*. Therefore, the results of BLAST with a homological value of ≥99% indicate that the isolate is considered the same species **(Table 1)**.

Table 1. Identification results of *Fusarium oxysporum* f. sp. *Lycopersici.*

Isolate	Separate Code	Species name	Accession number	% Identity of BLAST results
Mushroom	G3131-1	*Fusarium oxysporum* f. sp. *lycopersici.*	KU671029.1	99%

2. Test the inhibition of the growth of fungal colonies using essential oils

The parameter used in the fungal colony growth inhibition test using essential oils is the diameter of the mushroom colony growth. Data on the growth diameter of fungal colonies at observations 1 to 7 HIS is presented in Table 2.

Based on the results of variety analysis (ANOVA) presented in the form of a table **(Table 2)**, it is shown that all treatments with the addition of essential oil concentrations have a real effect in inhibiting the growth of *Fusarium oxysporum* f. sp. *lycopersici*. Treatment with an additional concentration of essential oils ranging from 333 to 1000 ppm had a

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significantly different effect with treatment without an increase in essential oil concentration (control), while treatment with concentrations of 333, 666 to 1000 ppm did not differ significantly.

Table 2. Growth diameter colony of *Fusarium oxysporum* f. sp. *lycopersici*.

Treatment	Diameter of Everyday Mushroom Colony						
	1	2	3	4	5	6	7
P0 (control)	1.14a	1.48a	4.13a	5.80a	7.91A	8.60a	8.96a
333 ppm	0.00b	1.66b	2.75b	3.80 b	5.03b	5.06b	6.18b
666 ppm	0.00b	0.96b	1.76c	2.43c	3.06c	3.56c	4.47c
1000 ppm	0.00b	0.78b	0.93c	1.43d	2.06c	2.44c	2.80 c

Note: Numbers followed by the same letter in the same column show very different differences based on Duncan's Multiple Range Test of 5%.

The growth diameter of fungal colonies in weeks 1-6 of the highest HSI observation was found in the control treatment, which was 1.14 cm to 8.60 cm, while the growth diameter of the fungal colony in the treatment varied between 0.00 to 5.06 cm. Observations made in the 7th week of MSI, found the smallest diameter of fungal colony growth in treatment with an essential oil concentration of 1000 ppm, which is 2.80 cm. Treatment starting with an essential oil concentration of 333 ppm is the most efficient concentration in inhibiting the growth of fungal colonies with an inhibitory diameter of 6.18 cm. Followed by treatment with a concentration of 666 ppm to 1000 ppm with a smaller growth diameter of 4.80 to 2.80 cm.

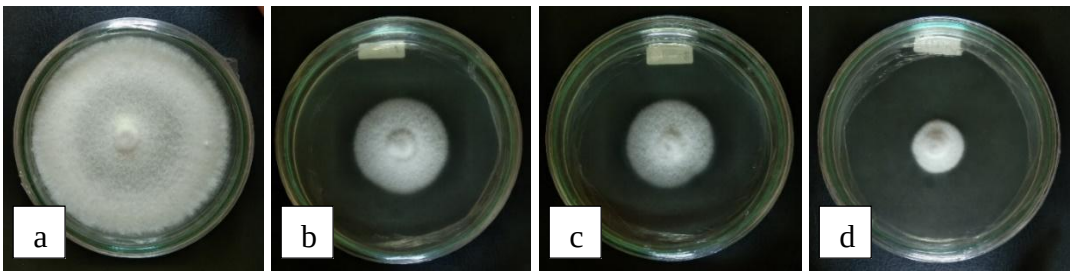

Figure 3. Comparison of the results of the treatment of essential oil concentration on the growth of Fol fungi: a) Control treatment, b) 333 ppm treatment, c) 666 ppm treatment, d) 1000 ppm treatment.

Results of the growth inhibition test of *Fusarium oxysporum* f. sp. *lycopersici.* using essential oils (**Figure 3**), shows that essential oils are able to inhibit the growth of fungal colonies. This is evidenced by the shrinking size of the diameter of the mushroom colony along with the increase in the concentration of essential oils. Of the four treatments, the control treatment (without the addition of essential oils) had the largest diameter size and the treatment with the addition of an essential oil concentration of 1000 ppm had the smallest colony diameter in all replicates. This shows that the essential oil of citronella contains active substances that act as antifungals against *Fusarium oxysporum* f. sp. *lycopersici.* In line with the opinion of Septikahady *et al.*, (2024) revealed that citronella plant extract has the potential to be a plant-based fungicide, citronella plant extract with a concentration of 4% is able to inhibit the growth of fungal colonies *of Collectrotrichum capsici* with a growth

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diameter of 2.94 cm in 7 DAP and *Fusarium oxysporum* by 87.18% after adding the concentration of essential oils extracted from citronella plants with concentration 2000 ppm. revealed that the antimicrobial properties of the dominant compound components in essential oils are due to their ability to interact with sterols on microbial cell membranes causing the leakage of certain proteins and enzymes. Dominant compounds can also inhibit pathogenic activity (enzyme activity) by interacting at the active site of cellulase enzymes, these interactions can interfere with substrate binding by enzymes or alter enzyme conformations so that catalytic activity is disrupted. (Mahalwal *et al.*, 2003).

3. Test the activity of quantitative cellulase enzymes

The cellulase enzyme activity test was carried out using the *Dinitrosalisilat* (DNS) method, which is based on the estimated amount of glucose (sugar reduction) as a result of cellulose hydrolysis. DNS is an aromatic compound that will react with reduced sugars to form 3-amino-5-dinitrosalicylic acid, a compound capable of absorbing strong electromagnetic wave radiation at a certain wavelength (Thiruneelakandan *et al.*, 2015). Therefore, the measurement of cellulase activity in this study was carried out using a spectrophotometer at a wavelength of 540 nm.

The glucose solution was chosen to create the standard curve because glucose is a reduced sugar resulting from the process of hydrolysis of cellulose by cellulase enzymes. The standard curve is created with a variation in glucose concentration of 0; 5; 10; 20; 40; 50; 75, and 100 ppm. The standard glucose curve result has a linear equation y = 0.058x – 0.0696 with a correlation value (R^2) of 0.9899 The higher the reduction sugar content contained in the sample, the more 3-amino 5-nitrosalicylic acid molecules are formed, so the higher the absorbance of the sample will be. The results of the observation of the corrected absorbance value in the test sample in this study are 0.075 so that when included in the equation y = 0.058x – 0.0696, the reduced sugar content is obtained of 2.493 mg/mL.

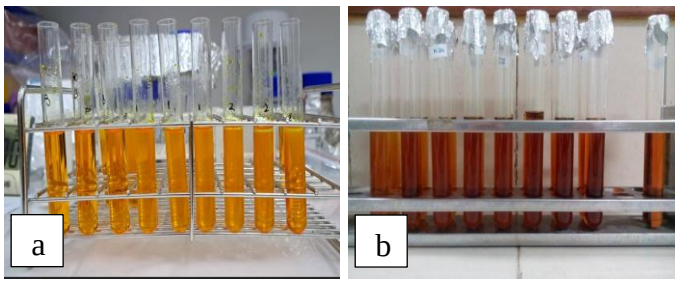

Figure 4. Reaction of reduced sugar with DNS: a) before being reacted with DNS, b) after being reacted with DNS.

The reaction between reduced sugar and DNS is a redox reaction in the sugar aldehyde group and oxidized into a carboxyl group. Meanwhile, DNS as an oxidizer will be reduced to 3-amino acids and 5-nitrosalicylic acids. The reaction takes place in an alkaline atmosphere and high temperature of about 90-100 °C. In observing the reaction between the reduced sugar and DNS, a discoloration was found in the tested samples. The color of the sample before being reacted with DNS is yellow, after being reacted with DNS and heated at 90-100 °C, the color of the sample changes to a slightly reddish-orange (**Figure 4**). This is suspected to be due to the presence of reduced sugar contained in the tested sample. In line with the opinion of the

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Prophet Rasul *et al.*, (2015) who revealed that if there is reduced sugar in the tested sample, then the DNS solution that was originally yellow will react with the reduced sugar so that it causes a reddish-orange color. the darker the DNS color, the less sugar there is.

Table 3. Cellulase enzyme activity in *Fusarium oxysporum* f. sp. *lycopersici*.

Example	Absorbance	Glucose Concentration (mg/mL)	Cellulase Enzyme Activity (U/mL)
1	0,075	2,493	0, 831 reviews

The results of cellulase enzyme activity measurements obtained in *Fusarium oxysporum* f. sp. *lycopersici*. which is 0.831 U/mL (Table 3). One of the factors that affects the production of cellulase enzymes by microbes is the incubation time. The incubation time used in this test is 72 hours. In line with research conducted by Mrudula *et al.*, (2011), the production of cellulase enzymes in *Aspergillus niger*. produces the highest activity within 72 hours. Hamid *et al.*, (2015) explained in their research with selected strains obtained the highest cellulase enzymes using *Aspergillus niger* from the first day to the fifth day, the best results were obtained from cellulase enzyme activity during the incubation period for 3 days with a cellulase enzyme activity value of 0.525 U/mL.

4. Cellulase Enzyme Activity Inhibition Test Using Essential Oil from Citronella Plant

This test was carried out to determine the decrease in cellulase enzyme activity after essential oil treatment. The results of the cellulase enzyme activity test from the addition of essential oils are shown in Table 4 which shows that there is a decrease in enzyme activity in the isolates to which essential oils are added.

Table 4. Percentage inhibition of cellulase enzyme activity by essential oil

Treatment	Enzyme activation (U/ml)	Inhibition of enzyme activity (%)
Control (Empty)	0.831A	0%
333 ppm	0.572b	31%
666 ppm	0.509b	39%
1000 ppm	0.463b	44%

Note: Numbers followed by the same letter in the same column show very different differences based on Duncan's Multiple Range Test of 5%.

The results of the variety analysis (ANOVA) presented in the form of a table (**Table 4**) showed that all essential oil treatments ranging from concentrations of 333 ppm to 1000 ppm had significantly different effects from the control treatment (without the addition of essential oils), while treatments with concentrations of 333, 666 to 1000 ppm did not have significantly different effects between treatments. The cellulase enzyme activity obtained in the isolate with control treatment was 0.831 U/mL. In the treatment with a concentration of 333 ppm, the essential oil was 0.572 U/mL with an activity inhibition percentage of 31%. In the treatment with essential oil 666 ppm of 0.509 U/mL with an inhibition percentage of 39%. Meanwhile, at an

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essential oil concentration of 1000 ppm, cellulase enzyme activity of 0.643 U/mL was obtained with an activity inhibition percentage of 44%. From the results obtained, the essential oil of the citronella plant has the potential to inhibit the activity of the cellulase enzyme released by the fungus *Fusarium oxysporum* f. sp. *lycopersici*.. This is in line with the opinion of (Jumepaeng *et al*., 2013) which revealed that the activity of cellulase and α-amylase in cellulite microbes decreased after being tested by increasing the concentration of essential oil from citronella plants. The ability of essential oils as inhibitors of cellulase enzyme activity is related to the natural compounds contained in them. Marei *et al*., (2018) revealed that natural compounds in essential oils such as alcohol, citranellal, geraniol, limonene, citranelol, ci-monoterpenes, aldehydes, trans-Cinnamaldehyde, p-Cymene, eugenol, mentone, phenol and other esters are known to inhibit cellulase and pectinase enzyme activities in *F . oxysporum, F. solani, P. infestan, A. solani, A. niger* and four other types of fungal species.

Furthermore, Cui *et al*., (2018) revealed that the dominant compounds contained in essential oils are able to affect microbes in nucleic acid synthesis and gene expression, inhibit the protein synthesis process, inhibit the activity of ATPase and β-galactosidase enzymes that cause death in microbes, and generally play a role in changing the structure or interaction of active residues in cellulase enzymes by interfering with catalytic activity.

CONCLUSION

Application of essential oil concentrations extracted from citronella plants with concentrations of 333 ppm, 666 ppm, and 1000 ppm effectively inhibited the growth of fungal colonies and potentially inhibited the activity of the cellulase enzyme *Fusarium oxysporum* f. sp. *lycopersici.*, with inhibition values on the diameter of fungal colony growth of 22.75%, 47.41%, and 67.05%, respectively, and were able to inhibit the activity of the cellulase enzyme in *Fusarium oxysporum* f. sp. *lycopersici*. isolates by 31%, 39%, and 44%, respectively.

Further research is needed on the degradation of cellulose content in plant cell tissues by cellulase enzymes using Scanning Electron Microscope (SEM).

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