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Growth Inhibition of *Botrytis cinerea* Fungus on Strawberry (*Fragaria* sp.) Using Kaffir Lime (*Citrus hystrix*) Leaf Essential Oil Emulsion

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Abstract. Post-harvest damage is caused by biological factors such as the attack of *Botrytis cinerea* pathogenic fungi. causes gray mold rot disease to be a source of problems in strawberry production. This study aims to determine the potential of kaffir lime leaf essential oil emulsion as an antifungal against *Botrytis cinerea*. The fungus was isolated from strawberry fruit samples that showed symptoms of gray mold disease. Fungal isolation was carried out through the direct planting method and antifungal inhibitory ability test by food poisoned method with potato extract agar media. This experiment used a completely randomized design consisting of 6 treatments with 5 repetitions. The results showed that kaffir lime leaf essential oil emulsion contains secondary metabolites that can inhibit 100% of the growth of *Botrytis cinerea* pathogenic fungal colonies. Essential oil concentrations of 0.10%, 0.15%, and 0.20% had a significant effect on the growth of *Botrytis cinerea* pathogenic fungi.

Keywords: *Botrytis cinerea*; *Citrus hystrix*; essential oil; inhibition; strawberry

INTRODUCTION

One of the agricultural sub-sectors that has the potential to improve farmers' welfare, the regional economy, and the national economy and even be able to increase foreign exchange through exports is horticultural commodities (Santosa et al., 2016). Horticultural commodities have great potential because they have high economic value. However, there are fundamental problems that can affect the low quality of horticultural commodities so that it impacts their economic value, one of which is pathogen attacks in the post-harvest phase. In developing countries such as Indonesia, attention to post-harvest diseases in horticulture is still lacking (Azmi et al., 2023). These pathogen attacks are the cause of yield loss and a decrease in product quality of 20-25% (Waryat & Handayani, 2020). Among all types of post-harvest pathogens, pathogens from the fungal group are the main cause of post-harvest damage to vegetable and fruit commodities (Angraeni, 2019). Post-harvest diseases can occur starting in the plant, during harvest, post-harvest handling in the field, packaging, transportation, and storage (Angraeni, 2019). Post-harvest diseases, especially those caused by fungi such as *Botrytis cinerea* Persoon (1801), can affect vegetable and fruit commodities from planting to storage. *Botrytis cinerea* can cause damage to strawberries and various other products. *B. cinerea* can cause up to 50% yield loss in strawberries (Mertely et al., 2018).

One of the solutions to protect especially horticultural commodities such as strawberries from biological damage caused by pathogens is the use of natural products, such as essential oils, which can be applied through edible coatings. Edible coatings made of hydrocolloids, lipids, and a combination of both, can be added with antimicrobial ingredients such as essential oils from spices to improve the quality and shelf life of the fruit. Essential oil is an organic compound contained in aromatic plants obtained from all parts of the plant or certain parts such as flowers, leaves, stems, fruits, roots, and seeds (Agustina & Jamilah, 2021). The application of this technology can reduce post-harvest losses by 15-40%, including damage during distribution and storage (Azmi et al., 2023).

Research on edible coating emulsion utilizing kaffir lime leaf essential oil was conducted to protect strawberry fruit from *B. cinerea* attack. Kaffir lime leaf essential oil has an antimicrobial potential that can inhibit fungal growth causing fruit rot. Antimicrobial compounds in kaffir lime leaf essential oil are citronellal, citronellol, and geraniol which are proven to inhibit the growth of *Aspergillus niger*, *Fusarium oxysporum*, *Penicillium digitatum*, and *Trichophyton mentagrophytes*

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var. Interdigitale (Pumival et al., 2020). Therefore, a study was conducted on the edible coating emulsion of kaffir lime leaf essential oil used to inhibit the growth of *B. cinerea* in vitro, so that it can be used as a biological control on post-harvest pathogens. This research also has an important role in developing the potential of regional commodities, especially Tulungagung Regency, known as the largest kaffir lime producer in East Java.

METHODS

The research was conducted in the Plant Disease Laboratory, Faculty of Agriculture, Universitas Brawijaya. The research was conducted for 7 months, starting from March to October 2023. This study used a complete randomized design with 6 different treatments. Each treatment was repeated 5 times on the inhibitory activity test of kaffir lime leaf essential oil edible coating emulsion carried out in vitro. The following are the stages of the research:

Isolation and Identification of *Botrytis cinerea* Pathogenic Fungi

The source of isolates suspected to be *B. cinerea* was obtained from symptomatic strawberry fruit in the field. Furthermore, isolation of pathogenic fungi that grow on the surface of strawberry fruit and inoculated on PDA media. Isolates were incubated for 7 days, purified for further rejuvenation and incubated again for 7 days and the fungal culture was stored on PDA media at 25°C. Isolates were identified macroscopically and microscopically.

The identification of fungi was carried out morphologically using the identification key (Mirzaei & Goltapeh, 2014). Observations were made on the morphology of colonies, conidiophores, and conidia. The molecular identification was carried out through the stages of DNA isolation, DNA amplification, electrophoresis, and DNA visualization. Pure Botrytis culture was rejuvenated in 250 mL of potato dextrose solution medium and incubated for 4 days. The mycelial mass of the fungus was extracted.

DNA was extracted and purified with DNeasy Plant Minikit (Qiagen, Germany). Amplification was performed on a Thermo Cycler PCR machine using universal primers, forward primer ITS1 (5'-TCCG TAGG TGAA CCTG CGG-3') and reverse primer ITS4 (5'-TCCT CCGC TTAT TGAT ATGC-3') with an amplified target size of ± 550 pb (White et al., 1990). DNA amplification reactions were performed with a total volume of 25 µL consisting of 12.5 µL go Taq green (Thermo Scientific), 1 µL forward primer 10 µM, 1 µL reverse primer 10 µM, 0.5 µL MgCl, 9 µL ddH2O, and 1 µL DNA template.

Amplification conditions are divided into several stages, namely denaturation initiation at 94°C for 3 minutes, followed by 29 amplification cycles, each cycle consisting of DNA denaturation at 94°C for 30 seconds, primer attachment at 53°C for 30 seconds, and elongation at 72°C for 1 minute, followed by final elongation 72°C for 3 minutes (Nielsen & Protection, 2002). Furthermore, the sequencing process or reading process of the DNA nucleotide strands was carried out by PT Genetika Science Indonesia. The bioinformatics data analysis stage is carried out on fungal isolates that have been sub-cultured, and identified at the species taxa level using the Basic Local Alignment Search Tool (BLAST) bioinformatics method online at https://blast.ncbi.nlm .nih.gov/Blast.cgi.

Preparation of Kaffir Lime Leaf Essential Oil Edible Coating Emulsion

Kaffir lime leaves were extracted by using a modification of the steam distillation method (Warsito et al., 2017). Fresh kaffir lime leaves were weighed 2.3 kg, then steam distilled for 4 hours using distilled water solvent (distilled water). The extraction results (pure essential oil) were collected in glass bottles and stored in a refrigerator. Furthermore, the content of chemical compounds in the essential oil of kaffir lime leaves was tested using GC-MS using a GCTSQ quantum mass spectrometer (Thermo Scientific, Austin, TX, USA) with a TG-5MS direct capillary column (30 m×0.25 mm×0.25 µm film thickness). Column oven temperature and chemical separation and

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identification conditions can be found in previous studies. Components were identified by comparing their retention times and mass spectra with the WILEY 09 and NIST 11 mass spectral databases. The fit factor with the X Calibur 3.0 GC/MS data system, where the value≥ 650 was acceptable to confirm the compounds and measurements were made.

The preparation of edible coating emulsion was carried out according to research (Ardiansyah & Mulia, 2016) with modifications. Modifications method is edible coating emulsion was made by preparing *Carboxymethyl Cellulose* (CMC) with a concentration of 1% (m/v) dissolved in 100 ml of distilled water and heated using a hot plate and stirred constantly using a magnetic stirrer at 70°C for ± 45 minutes. Furthermore, 50% (m/v) glycerol was added and kept stirring using a magnetic stirrer with a fixed temperature of 70°C for ± 10 minutes. The addition of glycerol as a plasticizer can increase the flexibility of the coating, smoother surface, and increase the ability of an edible coating to reduce the rate of water vapor transmission. After mixing well, the essential oil of kaffir lime leaves was added according to the different concentrations (0,05%; 0,10%; 0,15% and 0,20%) treatments as well as the control treatment and added with tween 80 (0.1% of the essential concentration) which was mixed using a magnetic stirrer at 30°C for 6 minutes and homogenized. The use of tween 80 in the edible coating emulsion mixture functions as a surfactant. Then the emulsion was cooled at room temperature ± 25°C and ready for application.

Antifungal Inhibitory Test of Kaffir Lime Leaf Essential Oil Edible Coating Emulsion Against Pathogenic Fungi

Antifungal activity test on edible coating emulsions that have been made is carried out using the food poisoned technique method. PDA media was sterilized using an autoclave, mixed with essential oil edible coating emulsion according to the treatment, and homogenized. The Kaffir Lime leaf essential oil emulsion treatment 0,05% (5% emulsion was added to PDA), 0,10% (10% emulsion was added to PDA), 0,15% (15% emulsion was added to PDA), and 0,20% (5% emulsion was added on PDA). The mixed PDA media with essential oil emulsion was then poured into a sterile Petri dish with a diameter of 9 cm. When the treated PDA media is solid, then inoculate the *B. cinerea* pathogenic fungi by taking a fungal isolate that has previously been incubated for 7 days and taken using an ose that has been marked with a cork borer with a hole diameter of 5 mm and inoculated in the center of a petri dish containing PDA media that has been treated. Then incubated for 7 days at a room temperature of 25^{o} C until the pathogenic fungus grows filling the cup on control media (Erhonyota et al., 2023).

The growth rate of fungal colonies was observed for 1 to 7 days after incubation in each treatment by calculating the colony diameter. The growth of *B. cinerea* fungal colonies was measured based on the growing colonies diameter and measured using a caliper. The colony diameter is calculated based on the formula 1 (Elfina et al., 2015):

$$D = \frac{(D1 + D2)}{2} \quad \ldots (1)$$

Description:
D : Diameter of *B. cinerea* fungal colonies
D1 : Vertical diameter of *B. cinerea* fungal colonies
D2 : Horizontal diameter of *B. cinerea* fungal colonies

The next calculation is the percentage of growth inhibition of *B. cinerea* fungi observed and calculated using the formula 2 (Sudania et al., 2023).

$$P = \frac{(D1 - D2)}{D1} \times 100\% \quad \ldots (2)$$

Description:
P : Percentage inhibition
D1 : Diameter of *B. cinerea* fungal colonies in control treatment
D2 : Diameter of *B. cinerea* fungal colonies on plant extract treatment media

Categories of fungal growth inhibition activity (Diana & Khotimah, 2014) are inactive

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(0); weak (0% < P ≤ 25%); moderate (25% < P ≤ 50%); strong (50% < P ≤ 75%) and very strong (P > 75%). Furthermore, the dry weight of mycelium was measured at the end of in vitro observation. The measurement of mycelium dry weight was carried out by adding 10 ml of 1% HCl, then heating in a water bath until it melted, then poured on filter paper that had been known by weight, and sprayed with sterile water to remove the agar and HCl that was still attached. The fungal colonies left on the filter paper were dried in an incubator at 30°C for 24 hours and then weighed using an analytical balance (Hapsari et al., 2021).

RESULTS AND DISCUSSION

The source of fungal pathogen inoculum was obtained from the strawberry farm of Lumbung Stroberi in Pandanrejo Village, Bumiaji, Batu City, East Java. The pathogen was obtained from symptomatic strawberry fruit samples in the field on ripe strawberry fruit ready for harvest. The pathogen *B. cinerea* damages the old tissue of the host plant, but the pathogen can also enter the tissue during plant development.

Morphological Characteristics of the *Botrytis cinerea* Pathogenic Fungi

Pathogenic fungi were obtained from symptomatic strawberry fruit in the field found on ripe strawberry fruit ready for harvest (Figure 1.a). The morphological characteristics of *B. cinerea* species are indicated by the colony growth pattern, which resembles fingers (radial), colony color: white, whitish gray, then turns gray or brown (Figure 1.b). Hyphae are shaped like bubbles bordered by white, gray, to brown partitions and then form a branching and insulated mycelium. Furthermore, the conidiophores emerge perpendicularly from the mycelium, insulated, branching at the ends. The older the conidiophores are, the brighter the color at the tip and the lighter closer to the branching (Figure 1.c). The shape of the conidiophores is perpendicular with the tip of the conidium producing many conidia. The shape of the conidia produced is ovate with various sizes ranging from 0.56-4.5 µm (Figure 1.d). Hyphae have partitions, in the form of long bubbles. The tip of the conidiophore swells to form an ampulla and there is a denticle as a place to attach the conidium, the shape of *B. cinerea* conidia, ovate and pear-like (pyriform). Conidia are 6.00- 18.70 × 4.00-10.50 µm and appear gray in bundles (Holz et al., 2007).

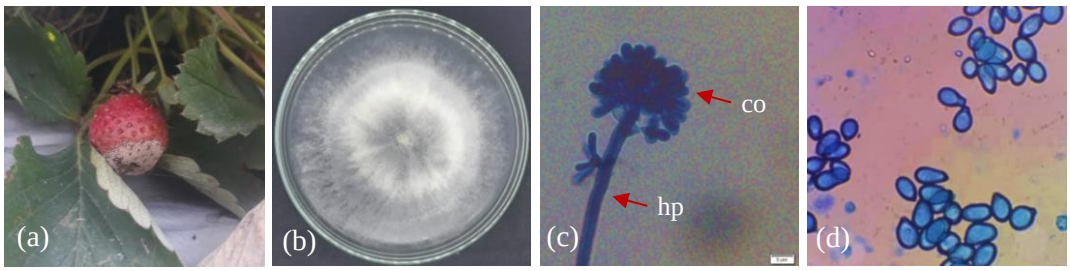

Figure 1. Isolation of *Botrytis cinerea* from Strawberry. a) Symptoms of Gray Mold disease on Strawberry Fruit, b) Colony morphology of pathogenic fungus *Botrytis cinerea* 7 DAI (Day After Inoculation), c) Conidiophores of *B. cinerea*, d) Conidia of *B. cinerea* Pathogenic Fungi 14 DAI. **co**: conidia, **hp**: hyphae

Molecular Identification of *Botrytis cinerea* Pathogenic Fungi

The results of molecular analysis using ITS1 forward primer (5'-TCCG TAGG TGAA CCTG CGG-3') and ITS4 reverse primer (5'-TCCT CCGC TTAT TGAT ATGC-3') that amplified 550 bp (Figure 2) showed that isolate sample (*B. cinerea*) had a similarity level of 99.81% with *B. cinerea* CF95 in GenBank data.

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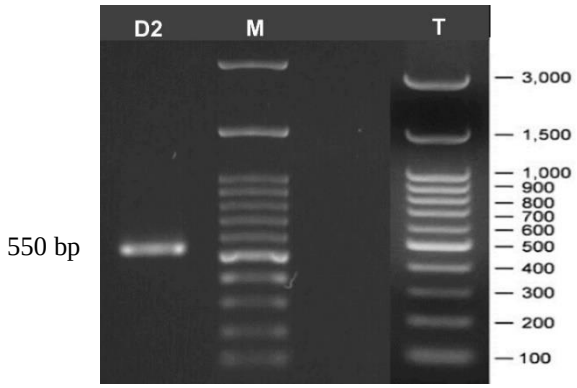

Figure 2. Visualization of *Botrytis cinerea* fungal DNA bands amplified using the ITS1/ITS4 universal primer pair at 550 bp band size. **D2**: *B. cinerea* pathogenic fungi sample, **M**: 100 bp DNA marker (Thermo Scientific, US), **T**: 1 bp DNA marker (Thermo Scientific, US).

The similarity percentage indicates the percentage of the same number of nucleotides between the sample and the comparison sequence. Therefore, it can be stated that the nucleotide sequence data in this research sample has a very high level of similarity with the comparison nucleotide data from GenBank, and can be considered as the same species as the comparison sequence (Newell et al., 2013).

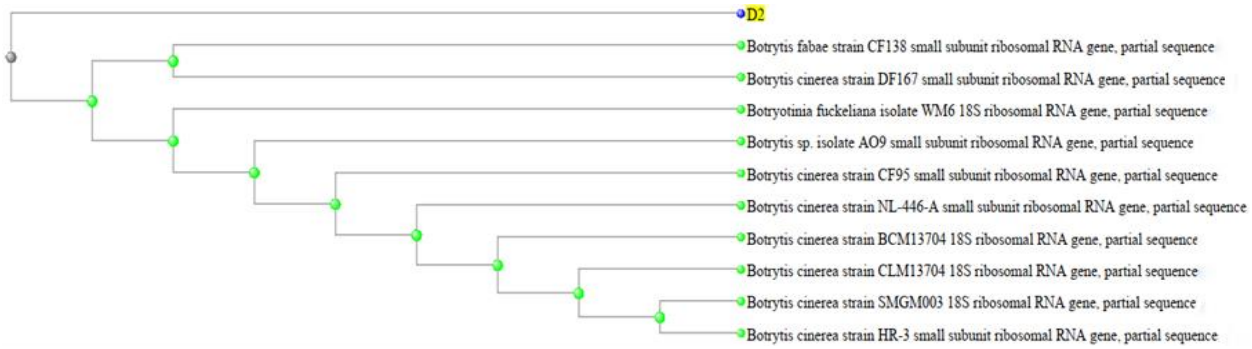

Figure 3. Phylogenetic tree of Gray Mold pathogenic fungal isolates

Phylogenetic analysis showed that isolate D2 was similar to *Botrytis fabae strain* CF138, *B. cinerea* strain DF167, *Botryotinia fuckeliana* isolate WM6, *B. cinerea* strain AO9, *B. cinerea* CF95, *B. cinerea strain* CLM13704, *B. cinerea* strain BCM13704, *B. cinerea* strain CLM13704, *B. cinerea strain* SMGM003, and *B. cinerea* strain HR3 (Figure 3). The branching of the phylogenetic tree shows the closeness of a sample as seen from its genetic distance value.

Effect of Kaffir Lime Leaf Essential Oil Edible Coating Emulsion on the Growth of *Botrytis cinerea*

B. cinerea fungus in all treatments except the positive control treatment experienced growth from the first day to the seventh day. Fungal colonies treated with kaffir lime leaf essential oil had a smaller average colony diameter when compared to colonies in the emulsion control without essential oil. The growth of *B. cinerea* fungus in the treatment of kaffir lime leaf essential oil with a concentration of 0.05% showed an increase in the diameter of fungal colonies when compared to the treatment of essential oil with concentrations of 0.10%, 0.15%, and 0.20% (Figure 4). The growth of *B. cinerea* fungi in

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the essential oil treatment was smaller when compared to the positive control treatment (PDA media only and emulsion without essential oil).

Based on the test results of compound content analysis using GC-MS, the results show that there are several compounds in kaffir lime leaf essential oil, namely Citronellal (96.49%), beta-Myrcene (0.65%), Alpha-Terpinolene (0.96%), Citronellyl acetate (0.61%), Bicyclo[3.1.0] Hexane (0.93%) and Geranyl acetate (0.27%). According to the results of research (Warsito et al., 2017) regarding the content of compounds in kaffir lime essential oil, there are several main components which include sabinene (9.21%), α-pinene (21.44%), limonene (12.59%), and citronellal which is the most dominant component reaching (46.40 - 85.07%).

The content of compounds in kaffir lime leaf essential oil in this study is the largest, namely citronellal (96.49%). Citronellal is a monoterpene compound with high anti-fungal properties. The compound can suppress the growth of plant pathogens by disrupting cell walls or inhibiting cell wall permeability so that important components such as proteins leave the cell and the cell gradually dies (Nugraheni et al., 2014). Citronellal also causes the condition of hyphae to be damaged and folded, there are many holes and almost no cytoplasm is found. Essential oils can penetrate the fungal cell wall to the cell membrane and change cell permeability and cause cytoplasmic leakage because it interacts with the cytoplasmic membrane of *Aspergillus niger* (Li et al., 2013).

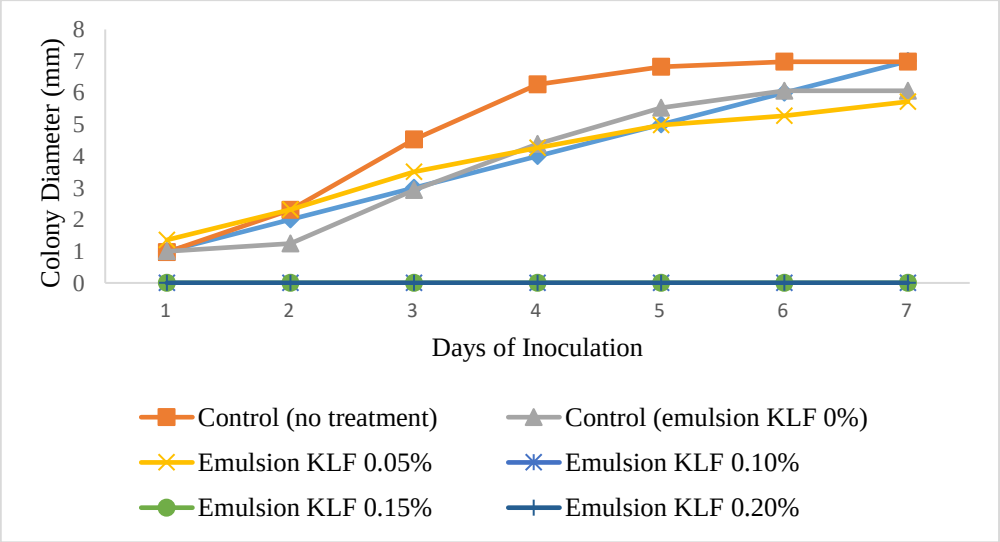

Figure 4. Growth rate curve of *Botrytis cinerea* fungi for 7 days incubation

The growth of *B. cinerea* fungus in each treatment (Figure 4) showed different mean diameters of fungal colonies during the 7-day incubation period. Essential oil *edible coating* emulsion at concentrations of 0.10%, 0.15% and 0.20% had a significant effect on the mean diameter of *B. cinerea* fungal colonies with essential oil emulsion treatment with a concentration of 0.05% ((F3,16=80.990; *p=0*.000; ANOVA) and the percentage of *B. cinerea* fungal growth inhibition activity (F3,16=154.548; *p=0*.000; ANOVA) on the seventh day of incubation.

Concentrations of 0.10-0.20% are concentrations with a percentage level of fungal growth inhibition of up to 100%. Inhibition of *B. cinerea* fungal growth by 100% is assumed that the kaffir lime leaf essential oil emulsion has been able to inhibit the growth of *B. cinerea* fungi observed from the diameter and structure of the fungal mycelium (Diana & Khotimah, 2014).

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Table 1. Mean colony diameter of *B. cinerea* fungi and activity of kaffir lime leaf essential oil emulsion on inhibition of *B. cinerea* fungi growth on day 7

Treatment	Average Fungal Colony Diameter (mm)	Percentage of Antifungal Activity (%)	Level of Inhibitory Activity	Mycelium Dry Weight (gr)
Control (PDA)	6,98 c			19,60 b
Control (PDA + Emulsion KLF 0%)	6,06 b			13.60 ab
PDA + Emulsion KLF 0.05%	5,72 b	22,39 a	Weak	23,60 b
PDA + Emulsion KLF 0.10%	0,00 a	100 b	Very strong	0,00 a
PDA + Emulsion KLF 0.15%	0,00 a	100 b	Very strong	0,00 a
PDA + Emulsion KLF 0.20%	0,00 a	100 b	Very strong	0,00 a

Notes: Numbers indicated by the same letter do not show significantly different results according to Duncan's test at the 5% significance level (KLF: Kaffir Lime Leaf)

The fungal colony diameter growing in petridish each treatment (Figure 5) determines the ability level of kaffir lime leaf essential oil emulsion in inhibiting the growth of *B. cinerea* fungi. The greater the concentration of essential oils contained in the media, the amount of essential oils that diffuse into the fungal cells increases, causing disruption of fungal growth and can even cause fungal death (Diana & Khotimah, 2014)

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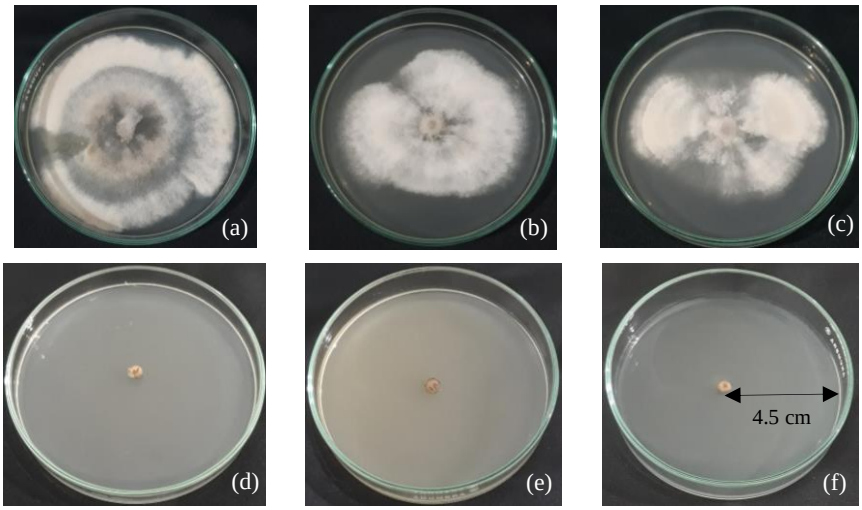

Figure 5. Growth of *B. cinerea* fungi on Kaffir Lime leaf essential oil Edible Coating emulsion treatment with different concentrations (7 DAI); (a) Control (PDA); (b) Control (PDA mixed with emulsion without KLF essential oil); (c) PDA with KLF 0.05%; (d) 0.10%; (e) 0.15% and (f) 0.20%

The dry weight of mycelium calculated at the end of observation (7 DAI) in the treatment of kaffir lime leaf essential oil emulsion with a concentration of 0.10% showed results that were not significantly different from the concentration treatments of 0.15% and 0.20%, but the three treatments were significantly different from the control treatment and the concentration of 0.05% essential oil emulsion. The treatment with a 0.05% concentration of kaffir lime leaf essential oil emulsion showed the highest mycelium dry weight value of 23.60 grams.

The growth of *B. cinerea* fungi is influenced by the concentration of kaffir lime leaf essential oil emulsion given in each treatment. The greater the concentration of kaffir lime leaf essential oil emulsion given, the smaller the growth of *B. cinerea* fungi in vitro. Research results (Yanti et al., 2017) at a

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concentration of 0.20% (2000 ppm) of kaffir lime leaf essential oil, there was no visible formation of fungal mycelia *Aspergillus flavus* and *Aspergillus parasiticus* during 7 days of observation. The higher the concentration of essential oil added, the longer the delay in spore germination.

This essential oil is very effective in inhibiting Aspergillus species, especially *Aspergillus candidus*, *A. tamari* and *A. niger*. Meanwhile, *A. parasiticus* and *A. flavus,* which are two types of Aspergillus that often cause problems with the appearance of aflatoxins in foodstuffs, can also be inhibited, but with a lower inhibitory ability. This indicates that the high concentration of essential oil is followed by the amount of compounds in it. Thus causing the ability to inhibit mold growth also to increase. If the extract used is higher, the zone of inhibition formed is even greater (Saputera et al., 2019).

The main component of kaffir lime essential oil in the study was citronellal (87.91%). Citronellal is an anti-fungal compound that can inhibit the growth of fungal pathogens. Based on research (Wu et al., 2016) showed that the treatment using citronellal with a concentration of 0.20 µl/ml inhibited spore germination visually. Hyphae morphology in the treatment using citronellal microscopically looks abnormal (irregular hyphae shape, squiggly surface), while the spore condition shows an irregular shape, the shape is also abnormal and the surface is rough.

The antifungal activity of kaffir lime leaf extract is related to the secondary metabolite contents in kaffir lime leaves such as flavonoids. Flavonoid compounds are classified as anti-fungal since they can form complex compounds associated with proteins that are directly soluble and bind to the fungal cell wall. Furthermore, it can cause replication and transcription process of microbial cells to be inhibited (Anitasari & Sari, 2021). The 80% concentration has the largest inhibition zone diameter on *Candida albicans* growth compared to other concentrations. This indicates that the 80% concentration has higher active compounds and can affect the permeability of cell membrane so that the intracellular fluid from the fungus is pulled out, the cell shrinks and is destroyed leading to cell death.

CONCLUSION

The study showed that the treatment of kaffir lime leaf essential oil *edible coating* emulsion *in vitro* with concentrations of 0.10%, 0.15%, and 0.20% was able to inhibit *Botrytis cinerea* growth up to 100%. This value is classified as a very strong inhibition category. The results of this study significantly affected the inhibition percentage of pathogenic fungal growth against the control treatment. It indicates the need for further research on the application of kaffir lime leaf essential oil *edible coating emulsion* treatment on fruit to determine the effectiveness of *edible coating* emulsions that have been made when applied to post-harvest handling.

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