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Inhibitory Effect of *Syzygium aromaticum* L. Essential Oil Against the Fungal Pathogens of *Capsicum annuum* L.

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ABSTRACT

Fungal pathogens are causative agent of pepper diseases that affect the yield loss. At least 10% of food loss in the developing country caused by plant diseases. Clove oil (*Syzygium aromaticum* L.) was reported to be able to control pathogenic fungi. In order to evaluate the effect of clove oil in inhibiting the growth of the pepper disease, three types of major causal agent of pepper diseases were used namely *Colletotrichum acutatum*, *Phythopthora capsici*, and *Pythium* sp. The experiment was evaluated in vitro. The three pathogens were treated with the essential oil in different concentration according to IC50 result on each fungi. The clove oil showed inhibitory effect against tested fungi. The *Syzygium aromaticum* L. essensial oil showed the best inhibitory effect at concentration 340 µl/L, 180 µl/L and 100 µl/L for *C. acutatum*, *P. capsici*, and *Pythium* sp. respectively.

1. INTRODUCTION

Pepper (*Capsicum annuum* L.), a member of the genus Capsicum, is one of the essential horticultural crops, widely cultivated in lowland or highland. It has high economic value and has potential as an export commodity because of its functions and character as a tropical plant [1]. *Colletotrichum acutatum*, *Phythopthora capsici.*, and *Pythium* sp. are the primary pathogenic agents attacking root, leaves, stem, and fruit pre- or post-harvest stage [2, 3].

C. acutatum, known as anthracnose, sporulates then spreads rapidly throughout the crop, causing yield loss up to 100% [2]. *P. capsici* is estimated to affect vegetable loss up to 50% [4]. On the other hand, Majeed et al. [5] stated that *Pythium* spp. damping-off is in charge of 90% causing plant death at pre- and post-emergence of seedlings in nurseries and fields. The use of various kinds of synthetic fungicides to control pathogens has been discouraged due to their effect on the resulting resistant strain and harmful to the environment and human health [6]. Plant-based fungicides are more eco-friendly and less hazardous than synthetic fungicides due to their low toxicity, high degradability, and multiple action mechanism [7].

Essential oil is one of a plant's secondary metabolites recognized for its antimicrobial and antifungal properties [8]. Clove oil is one of the essential oil, derived from the clove plant (*Syzygium aromaticum* L.), extracted from fallen leaves offers valuable options for plant protection management [9]. Clove oil has an antimicrobial activity due to its significant compounds, such as eugenol, β-caryophyllene, and eugenol acetate [10]. It has shown clove oil effectively controls *Penicillium italicum* (blue mold) disease incident in citrus [11]. Furthermore, clove oil extract showed high potency as bio fungicide with its effectiveness in suppressing the growth of *Botrytis cinerea* (grey mold) on detached strawberry leaves which was evaluated in vitro on PDA under different concentration [12]. Another report stated that clove oil successfully inhibited several fungal pathogens causing root rot and wilt on tomato. The essential oil showed disruption of fungal growth and conidial malformation as well [13].

Several authors have shown the positive effects of this clove oil against the pathogen [10-13]. However, the literature on its effect against three major pepper pathogens is still limited. This current study determined the impact of clove oil against *C. acutatum*, *P. capsici*, and *Pythium* sp. In addition, investigation of pathogenicity and clove oil compounds.

2. MATERIALS AND METHODS

2.1 Material

The materials used for this study were *C. acutatum*, *P. capsici.*, and *Pythium* sp. isolates obtained from Indonesian Vegetable Research Centre (BALITSA), clove essential oil, PDA (*Potato Dextrose Agar*) media, alcohol 70%, tween 20, sodium hypochlorite (NaOCl), soil, pepper fruits, pepper leaves, and pepper seeds.

2.1.1 Clove oil preparation

Before carrying out the test, dilution of clove oil. According to Dadang & Prijono [14], water cannot dissolve vegetable pesticides but as much as 0.2 percent in an emulsifier. It produced tween 20 solutions with a concentration of 0.2 percent (v/v) and diluted clove oil to a concentration of 1 percent. Proposed that can blend clove oil with PDA until homogenous. Made the concentration of 1 percent to facilitate the usage of clove oil easier because the concentration used in the test was less than 1 percent. The dilution, according to Saridewi et al. [15], is computed using the formula:

$$M_1V_1 = M_2V_2$$

Keterangan: M_1 = concentration before dilution; M_2 = concentration after dilution; V_1 = volume before dilution; V_2 = volume after dilution.

2.2 Preliminary test

2.2.1 Analysis of clove oil compound

GC-MS analyses by Lembaga Ilmu Pengetahuan Indonesia (Indonesian Institutes of Sciences) using 5% diphenyl / 95% dimethylpolysiloxane.

2.2.2 Pathogenicity test

Fruits, leaves, and seeds of pepper were sterilized with 3% sodium hypochlorite for 3 min and 70% alcohol for 3 min, then washed three times using distilled water for 1 min [16]. Phatogenecity test was carried out by different methods adjusting to phatogen nature to explore phatogenic potential of the isolated that used in this study. The investigation used the method of Ivey et al., Dagani & Ceramica, and Hodiyah et al. [2, 17, 18] with slight modifications. Inoculate pepper fruits by 20µl of the *C. acutatum* conidial suspension on the surface injured site. Inoculate the leaves in four locations across its underside with agar disk cut of *P. capsici* colonies. Inoculum of *Pythium* sp. was infested on sterilized soil and then incubated for four days. After that, planted pepper seed in the media. Observation of the symptoms were according to Ivey et al. [2, 3, 5].

2.2.3 IC50 analysis

IC50 value was analyzed to determine clove oil concentration for further in vitro assay. The four days old pathogen mycelial disk (5 mm) were inoculated on PDA medium with different clove oil concentrations (0 µl/L, 200 µl/L, 400 µl/L, 600 µl/L, 800 µl/L, and 1000 µl/L) and then incubated at 25℃, the record of colony diameter every day [19]. Calculate the MGI (mycelia growth inhibition) percentage using the formula:

$$Mycelia\ Growth\ Inhibition = \frac{(dc - dt)}{dc} \times 100$$

where, *dc* is colony diameter for control and *dt* is colony diameter for treated sample [20]. The MGI result was analyzed using probit analysis to obtain an IC50 value.

2.3 In vitro antifungal activity assay

The poisoned food method determined the effect of different concentrations of clove oil against *C. acutatum*, *P. capsici*, and *Pythium* sp. [21]. Obtained incorporated clove oil into PDA medium at desired concentration (Table 3), the concentration of clove oil from IC50 value, 1.5x IC50 value, and 2x IC50 value. Furthermore, the four days of each mycelia disk (5 mm) were inoculated into media and deposited into the plate's center. After incubation, measured the diameter of fungal growth and the antifungal effect was estimated by the formula:

$$\text{Antifungal activity (\%)} = \frac{Dc-Ds}{Dc} \times 100$$

where, Dc = diameter of growth of control.
Ds = diameter of growth of the sample containing clove oil.

2.4 Statistical analysis

A complete randomized design was used, consisting of at least six replicates. Data were analyzed using one-way ANOVA followed by Duncan's multiple range test at 5% of the P-value level.

3. RESULTS AND DISCUSSION

3.1 Result

3.1.1 Clove oil composition

The composition of clove oil is presented in Table 1. The main components were eugenol (69.45%) and caryophyllene (21.76%), while the other elements such as 1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z, Z, Z-; Copaene; and Caryophyllene oxide contained less than 4%.

Table 1. Five active compounds of clove oil with the highest composition analyzed by GC-MS

No	Retention Time	% Area	Active Compound
1	14,728	69.5	Eugenol
2	15,626	21.8	Caryophyllene
3	16,071	3.6	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
4	14,905	1.6	Copaene
5	17,875	1.3	Caryophyllene oxide

3.1.2 Pathogenicity test

The pathogenicity test was conducted to determine the pathogenicity potency of the strain. This test also aims to prove that the pathogen shows the same symptoms as the symptoms in the field [18]. The result of the pathogenicity test is shown in Table 2. The symptoms that appear are matched with [2, 3, 5].

Figure 1 compares the condition of the sample that was inoculated with *C. acutatum* for eight days and control.

Table 2. Pathogenicity test results of *C. acutatum*, *P. capsici*, and *Pythium* sp.

Pathogen	Symptoms
C. acutatum	Conidial masses occur in concentric rings on circular sunken lesions
P. capsici	Small round or irregular leaf spots, white hyphae on the leaf surfaces, the leaves dry
Pythium sp.	62% of the seeds mortality

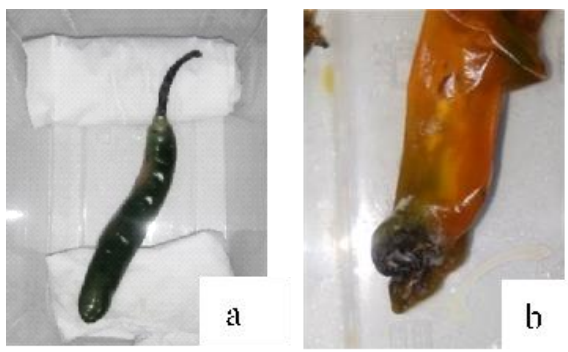

Figure 1. *C. acutatum* pathogenicity: a. Control at 8 day; b. Inoculated fruit at 8 day

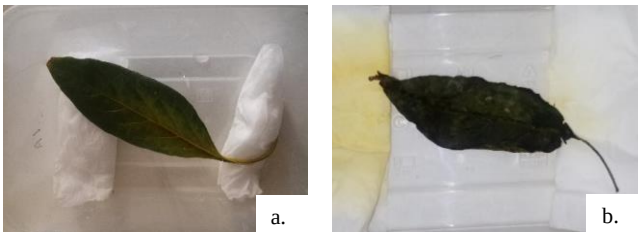

Figure 2. *P. capsici* pathogenicity: a. Control at 6 day; b. Inoculated leaf at 6 day

Figure 2 compares the condition of the sample that was inoculated with *P. capsici* for six days and control.

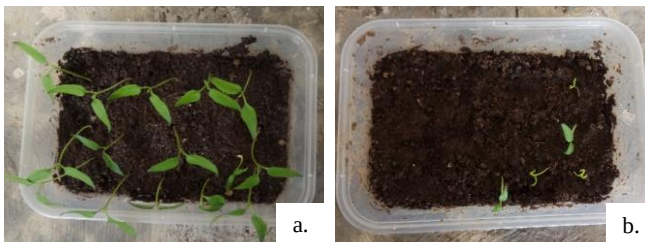

Figure 3. *Pythium* sp. Pathogenicity: a. Control at 13 day; b. Infested soil at 13 day

Figure 3 compares the condition of the sample that was inoculated with *Pythium* sp. for thirteen days and control.

3.1.3 IC_{50} analysis

The data in Table 3 shows the value of IC50 analysis, which is furthermore used as a benchmark for determining concentration for in vitro assay. The data of mycelia growth inhibition were tested by probit analysis (regression analysis). Different pathogens show different values; therefore, the in vitro assays' concentration differs depending on the pathogen.

Table 3. IC_{50} value (µl/L) for *C. acutatum*, *P. capsici*, and *Pythium* sp.

Treatment	Pathogen		
	C. acutatum	*P. capsici*	*Pythium* sp.
A (Control)	0	0	0
B (IC_{50})	170	90	50
C (1 ½ IC_{50})	255	135	75
D (2 IC_{50})	340	180	100

3.1.4 In vitro antifungal activity assay

The antifungal effect of clove oil against *C. acutatum*, *P. capsici*, dan *Pythium* sp. are given in Table 4 and Figure 4. All the concentrations of clove oil showed inhibitory effect against a given pathogen.

Table 4. Inhibitory effect of clove oil against *C. acutatum*, *P. capsici*, and *Pythium* sp.

Pathogen	Clove oil concentration (µl/L)	Colony diameter (mm)	Growth rate (mm/day)	Inhibition rate (%)
C. acutatum	A (control)	37.53d	6.26d	0a
	B (170)	25.93c	4.32c	30.7b
	C (255)	16.68b	2.78b	55.5c
	D (340)	10.70a	1.78a	71.4d
P. capsici	A (control)	29.08c	7.27c	0a
	B (90)	10.08b	2.52b	64.6b
	C (135)	1.59a	0.4a	94.3c
	D (180)	0.39a	0.1a	98.6c
Pythium sp.	A (control)	39.93b	39.93b	0a
	B (50)	31.64b	31.64b	20.8b
	C (75)	17.50a	17.50a	55.9c
	D (100)	13.18a	13.18a	67c

*Values within each column for each pathogen followed by different letters are significantly different according to Duncan test (P < 5%).

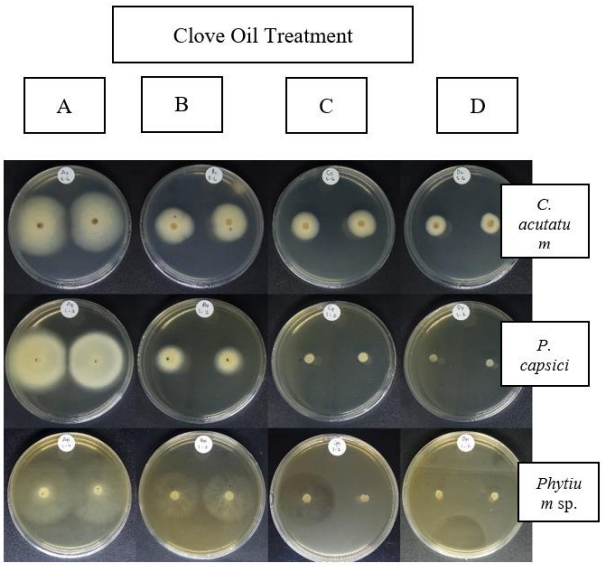

Figure 4. Mycelial growth on different isolates with different concentration

Table 1 and Figure 4 showed the inhibition rate of each pathogen is higher at the lowest concentration of clove oil compared with control (170 µl/L for *C. acutatum*; 90 µl/L for *P. capsici*; and 50 µl/L for *Pythium* sp.) with 30.7%, 64%, and 20% inhibition rate respectively. The difference of the number is due to the different virulence of each pathogen. The higher concentration of clove oil, the more pathogens are inhibited. The rate improved by increasing concentration up to 340 µl/L (71.4%), 180 µl/L (98.6%), and 100 µl/L (67%) for *C. acutatum*, *P. capsici*, and *Pythium* sp. respectively. Table 1 also noted that *Colletotrichum acutatum* had the highest tolerance to clove oil.

3.2 Discussion

Eugenol and caryophyllene were found as the main component of clove oil. This composition is comparable with data reported by Amelia et al. [22] and Jahiel et al. [10] for clove oil composition, where the major component was eugenol (70-80%) and caryophyllene (13-20%). Amelia et al. [22] stated that caryophyllene is the second primary compound

of clove oil. These components are responsible for the antibacterial and antifungal activity of clove oil [23].

The antifungal effect of clove oil can probably be due to eugenol and caryophyllene. The high amount of eugenol produced by clove oil inhibits the growth of each fungus. Eugenol inhibits pathogenic sporulation by deactivating essential enzymes and inhibiting ergosterol biosynthesis from test fungi [24]. Pereira et al. [25] reported that the eugenol contained in clove oil acts on cell membranes with a mechanism to inhibit the biosynthesis of essential components in fungal cell membranes that can damage cell membranes and reduce their function.

Based on research conducted by Latifah-Munirah et al. [26], the integrity of the pathogenic cell walls is damaged by the eugenol reaction against pathogens. An essential part of fungal cells, Ergosterol is a target for antifungal agents to damage cells. Eugenol phenolic compounds can change the nature of proteins and react with phospholipid cell membranes which change their permeability [27]. Jenie et al. [28] report that sesquiterpene compounds have a significant enough effect as antifungal compounds by changing the membrane function as surfactants and disrupting the work of the plasma membrane.

Our study found that *C. acutatum* has the highest tolerance against clove oil. Duduk et al. [29] reported clove oil inhibited *C. acutatum* mycelial growth with a 100% rate at a high concentration of 667 µl/L. This result can be because *C. acutatum* has melanin, contributing to stress tolerance and virulence [30]. Meanwhile, according to Wang et al. [31] Oomycete pathogens such as *Phytophthora spp.* and *Pythium* sp. lack membrane sterols; they acquire sterol from their environment (sterol auxotroph). The membrane cell condition of this pathogen helps the clove oil to react with the cell membrane then destroy it causing their vital intercellular material to be lost. Furthermore, the essential oil penetrates the cell cytoplasm then inhibits DNA synthesis, resulting in pathogen death [32].

The concentration of Clove oil in the current study does not exceed the appropriate concentration limit for bio-pesticide. The concentration should be less than 1%, as [14] stated. The results of clove oil research conducted by Harni et al. [33] in controlling rust disease (*Hemileia vastatrix*) on coffee leaves showed that clove oil at 5000 µl/L was phytotoxic to coffee leaves. At the same time, Pereira et al. [34, 35] stated that there were no phytotoxicity symptoms in a similar study due to clove oil (1000 µl/L) application.

Chen et al. [11] reported clove oil inhibited the development of blue mold disease in citrus fruit. The essential oil applied at 0, 0,05, 0.1, 0.2, 0.4, and 0.8%. Based on those statements, clove oil with the effective concentration, as shown in the results, can be considered economical and safe to use for peppers.

4. CONCLUSION

In doses ranging from 50 to 340 ppm, clove oil had a substantial effect on three species of fungi: *Colletotrichum acutatum, Phytophthora capsici,* and *Pythium* sp. *Pythium* sp. exhibited the best response at a concentration of 75 ppm.

It is suggested that additional study be conducted to determine the efficacy of clove oil against these infections in the field.

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