**Running title:** Clove Oil Nano Biofungicide Inhibits Urediniospores *H. vastatrix.*

**Clove oil Nano Biofungicide Formula as an inhibitor of Urediniospores *Hemileia vastatrix* that causes Coffee Leaf Rust Disease**

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**Novelty statement**

The composition of clove oil and surfactants produced a clove oil nano biofungicide formula with a particle size of 16.25-24.89 nm with a homogeneous and evenly distributed particle population. The resulting clove oil nano biofungicide formula can inhibit *H vastatrix* urediniospores up to 97.45%.

**Abstract**

Leaf rust disease caused by *H. vastatrix* causes the coffee leaves to fall, and the number of flowers formed decreases, so that coffee productivity decreases. Disease control using clove oil is environmentally friendly and safe for human health, but its solubility and effectiveness are relatively low. The nano oil emulsion formula can increase the effectiveness of essential oil-based biofungicide. Optimizing the use of surfactants Glycerol (10-15%), Tween 80 (10-15%), time (60 minutes), and speed (750 and 1000 ppm) in clove oil nano emulsions with smaller sizes and high stability. Six nano emulsion formulas of clove oil were clear color or without sediment, tiny droplets 16.25-24.89 nm, and had a typical mono dispersion graph with a PDI value < 0.2, containing a homogeneous and evenly distributed particle population. Clove oil nano emulsion formula has a high inhibitory effect on the germination of urediniospores *H. vastatrix*. It can be tested further on infected plants on a larger scale.

**Keywords:** Clove oil, Eugenol, Nano emulsion, Urediniospores.

**Introduction**

The disease that often attacks coffee plants is coffee leaf rust (*Hemileia vastatrix*). This disease causes many leaves to fall, so the number of flowers formed decreases, resulting in decreased productivity. Leaf rust has been reported since 1869 in Ceylon (Sri Lanka) then around 1920 spread to most of Africa and Asia. In Indonesia, leaf rust disease is reported to have attacked 14 provinces, with an attack area of 32.825.45 hectares throughout Indonesia (Agricultural Data Center and Information System 2021). Disease attacks on coffee plants cause a significant reduction in crop production of around 20%–40% per year (Worrall *et al.* 2018).

The limited knowledge of farmers and the extent of existing land causes disease control. Currently still using synthetic pesticides so that production costs are high and can leave residues on products that can harm consumer health and pollute the environment. Alternative disease control can be done by utilizing biofungicide. Biofungicide are fungicides whose active ingredients come from plants or other organic materials that are efficacious in controlling disease attacks on plants. Biofungicide is gaining popularity worldwide to manage crop diseases better.

The weakness of the antimicrobial active ingredients of essential oils is their low solubility in water because they are hydrophobic. Hence, their concentration is low in the solvent phase, and their effectiveness as an antimicrobial is lower (Ghosh *et al.* 2014). Essential oil nano emulsion can be a solution to overcome these weaknesses. Antimicrobial nano emulsions are highly stable oil-in-water emulsions of nanometer-sized droplets that act against viruses, fungi, and bacteria (Pant *et al.* 2014). According to Miastkowska *et al.* (2020), nanotechnology can increase the antifungal activity of oil tested against plant-pathogenic fungi.

Nanotechnology-based biofungicide provides disease control options with minimal risk to humans and are environmentally friendly. Nano emulsion biofungicide can transform global agriculture in terms of food protection. Bio-fungicide in the form of nano-emulsion has the advantage of being able to reduce the concentration of the active substance without reducing its biological effectiveness as an antifungal (Miastkowska *et al.* 2020), small particle size, more effective, high solubility, and low toxicity (Kumar *et al.* 2021), the ability to control disease on the target, increase crop productivity, product quality, consumer acceptance, and efficient use of resources (Scortichini 2022).

Based on the description above, a study was conducted to obtain a bio-fungicide formula for clove oil nano emulsion (*Syzygium aromaticum*) to control coffee leaf rust (*Hemileia vastatrix*).

**Materials and Methods**

**Research materials and locations**

The research was carried out at the Integrated Laboratory of the Industrial and Beverage Crops Research Institute, Sukabumi Regency, West Java province, Indonesia, from January to December 2021. The materials and tools used included: *Water Agar Media* (WA), *Potato Dextrose Agar* (PDA), *bacto agar*, hemocytometer, compound microscope, tween 80, *Medium Chained Triglycerides* (MCT), and clove oil (Qiandra, eugenol 73%).

**Preparation of nano biofungicide**

Manufacture of clove oil nano biofungicide using modified methods of using modified methods of Komaiko and McClements (2015); S. Yuliani and Noveriza (2019). Clove oil was put into a beaker and mixed with MCT and Tween 80 with concentrations according to treatment, stirred using a magnetic stirrer at a speed of 750 rpm for 30 minutes to form an organic phase. Separately, the aqueous phase is prepared by mixing water and glycerol under the same stirring conditions as preparing of the organic phase. The nano emulsion was then formed by adding the aqueous phase to the organic phase while stirring for 60 minutes at a speed of 750 rpm and continued for 60 minutes at 1000 rpm. The formed nano emulsion was placed into a sample vial for further analysis. Observation parameters: determination of droplet size, measurement of pH, and testing of the germination inhibition of urediniospores *H. vastatrix*. The composition of the clove oil nano emulsion formulation is shown in Table 1.

**Droplet size measurement**

Droplet and *Polydispersity Index* (PDI) measurements used a *particle size analyzer* (PSA) with dynamic light scattering type. The cuvette was cleaned, and 10 ml of the sample was put into the cuvette. The cuvette containing the sample was inserted into the sample holder and analyzed by the instrument (Yuliani *et al.* 2016).

**pH measurement**

The pH measurement was carried out at room temperature using a pH meter. Before use, the electrode was calibrated using standard solutions of pH 4 and 7. After the calibration process, the electrode was dipped into the preparation and the pH value of the preparation appeared on the screen (Yuliani *et al.* 2016).

**Preparation of *H. vastatrix* urediniospores inoculum**

*H. vastatrix* is a fungus that is an obligate parasite, a living organism that can only live or survive on living plants. So that urediniospores are collected directly from coffee leaves that are still fresh and naturally infected in the field to obtain urediniospores that are still active. Urediniospores were collected using a brush and stored in a test tube for 48 hours before use. Inoculation was carried out using 32 mg of urediniospores suspended in 2 ml of sterile water, then added tween 80 (0.025%).

**Antifungal assay of nano emulsion formula**

Test for inhibition of uredospore germination by conducting in vitro testing of *H. vastatrix* using the modified spore germination inhibition method. The experiment used a completely randomized factorial design consisting of a nano formula of clove oil (F1, F2, F3, F4, F5, and F6) and several concentrations (5%, 10%, 15%, and 20%). The tested formulas are presented in Table 1. Each treatment was dripped with 10 µl on a glass slide with WA media and then air-dried. Then, 10 µl of urediniospores *H. vastatrix* was added, covered with a cover slip, and incubated for 72 hours at room temperature in a tray lined with damp tissue in the dark. Each treatment was repeated five times. Observations using a compound microscope on germinated urediniospores for each treatment. Urediniospores were observed for 24 hours. The percentage inhibition of urediniospores germination (PIU) was calculated based on the following formula:

PIU is the percentage inhibition of urediniospores, Uk is the total number of germinated urediniospores, and Ub is the number of urediniospores observed.

**Data analysis**

The effect of treatment on the observed variables, and analysis of variance was carried out using the STAR (Statistical Tool for Agricultural Research) program. If there were differences, it was continued with Duncan's New Multiple Range Test further tested at a 5% level.

**Results**

**Physical properties of clove oil nano emulsion**

Six clove oil nano emulsion formulas have been produced from this study (Table 2). Clove oil composition with surfactant concentration (Glycerol and Tween 80) indicates that the surfactant mixing ratio is optimal to produce eugenol nanoparticles with stable and evenly distributed particle size.

**Clove oil nano emulsion stability**

The pH measurement was carried out to determine the acidity of the nano emulsion formulations, which had different processes (Table 2), and the stability of the formulations. The results showed that the pH value of the nano emulsion ranged from 4.87-5.31. Formulas F3, F4, and F5 each have relatively lower pH values ​​(4.89, 4.87, and 4.96) than formulas F1, F2, and F6.

All the resulting formulas were perfectly dispersed and showed no segregation, precipitate, or foam (Fig. 1). Formula F3 has the highest level of clarity compared to other formulas. Furthermore, the F4 and F5 formulas also have clear colors. The low pH value influences the transparent color in the formulas F3, F4, and F5. Then Formula F1 has a slight white color, and formulas F2 and F6 have a white or foggy color, with each having a pH value of >5.

**Particle size and Polydispersity Index (PDI)**

Clove oil nano formula produced droplet size diameter < 100 nm, with the smallest droplet size shown in F3 of 16.25 nm and F4 of 18.18 nm compared to other formulas and the largest in formula F6 (Table 2). The addition of Tween 80 concentration from 10% to 15% (Table 1.) influenced decreasing the particle size of the clove oil emulsion nanoparticles in formulas F3 and F4, followed by a decrease in the PDI value.

The analysis of the clove oil nano emulsion showed that the smallest PDI value in the F3 formula was 0.058, and then sequentially, the F4, F2, F1, F6, and F5 formulas (Table 2). These results indicate that the nano formula of clove oil produced has a mono dispersion standard or homogeneous particle size.

The clove oil nano formula studied has a single peak particle size chart that is typical (mono dispersion). It contains a homogeneous and evenly distributed particle population (Fig. 2). Formulas F3 and F4 have small particle population sizes and are homogeneous compared to other formulas. However, Formulas F1, F2, F5, and F6 are still included in the criteria with a small and homogeneous particle size population with a PDI value <0.2 (Table 2).

**Antifungal assay**

The test of clove oil nano emulsion to inhibit the germination of urediniospores *H. vastatrix* used the formulas F1, F2, F3, F4, F5, and F6 (Table 3). The percentage of inhibition ranged from 84.33%-97.45%. Formula F3 in the highest inhibition (97.45%) of the germination of urediniospores *H. vastatrix* at all concentrations applied. Likewise, the formulas F4, F1, and F5 are as good as F3. Meanwhile, formulas F2 and F6 produced a low level of inhibition on the germination of *H. vastatrix* urediniospores. This indicated that the high inhibitory power of formulas F3, F4, F1, and F5 on the germination of urediniospores *H. vastatrix* was influenced by the small particle size of the nano emulsion (16.25-21.70) with a PDI value <0.2 (Table 2).

The effect of clove oil nano emulsion concentration to inhibit germination of urediniospores was highest at 15% and 20% concentrations of 96.44%. While the concentration of clove oil nano emulsion was at a concentration of 5%, with the lowest inhibition reaching 81.86%.

In the results of the linear regression analysis, it was seen that the particle size influenced the inhibition rate of urediniospores H. *vastatrix* sprouts, which was 73.6% (Fig. 3). The obtained linear regression equation is y=124.86-1.6391x. The equation shows that each increase in the particle size level of 1 nm will cause a decrease in the inhibition rate of *H*. *vastatrix* urediniospores sprouts by 1.6391%. This indicated that clove oil nano emulsion effectively inhibited the germination of urediniospores.

**Discussion**

The nano emulsion formulation was made using a low-energy emulsification technique using phase inversion. In the phase inversion mechanism, nano emulsions form through two stages: the formation of water in oil (w/o) emulsion, which then reverses the phase to o/w. A w/o emulsion is formed when water is added to the mixed phase between the oil and the emulsifier.

The size of the emulsion produced from nano emulsion technology with low energy methods is influenced by the composition system (ratio and type of surfactant, oil-in-water ratio), emulsion preparation (additives, stirring speed), and environmental conditions (temperature) (McClements 2013). As stated by Ghosh *et al.* (2014) that the selection of the type of surfactant, surfactant concentration, and optimal emulsion time will result in smaller particle size and more excellent stability. This condition is related to the role of surfactants Glycerol and Tween 80 which results in smaller particle sizes and higher stability. Glycerol is a factor that affects droplet size and PDI nano emulsion (Chong *et al.* 2018).

The low pH value affects the transparent color of the formulas F3, F4 and F5. According to Liu *et al.* (2019), nanoparticles produced under acidic pH conditions are more stable. Yuliani *et al.* (2016) stated that the pH value of the nano emulsion was affected by temperature, type and concentration of the extract used, type of surfactant, other fillers, storage, aqueous phase, and oil phase.

One of the advantages of nano emulsions is that they have kinetic stability (McClements 2012). Due to the tiny particle size and the minor surface tension between the oil and water molecules, nano emulsions have almost no tendency to agglomerate or sediment, reducing the possibility of creaming or sedimentation formation. As a result, nano emulsions are much more stable than other emulsion systems and more translucent than conventional microemulsions and emulsions (McClements 2012). In addition, the absence of sediment is due to the small particle size of a nano emulsion system not affected by gravity (Sutradhar and Amin 2013). Therefore, when spraying using a sprayer, it will not block the nozzle.

The average particle size and polydispersity index (PDI) indicate the quality that determines a formula's safety, stability, efficacy, and in vitro and in vivo behavior (Danaei *et al.* 2018). These parameters are significant physical characteristics when manufacturing products. The characteristics of nanocarriers with small particle sizes can affect bulk properties, stability, product performance, encapsulation efficiency, bio-distribution, final product appearance, and cellular uptake (Bahari and Hamishehkar 2016; Maherani and Wattraint 2016).

The clove oil nano formula produces droplet sizes < 100 nm in diameter, influenced by the addition of Tween 80 concentration from 10% to 15%, as in Formulas F3 and F4, followed by a decrease in PDI values. In line with the research of Dhivya *et al.* (2019) that the addition of a surfactant concentration of 50% can reduce the droplet size of *Acorus calamus* oil up to 43.17% which is spontaneously emulsified. Thus, proving that the surfactant concentration affects the particle diameter. The results of Pathak *et al.* (2013) stated that the droplet size will decrease as the ratio of surfactant to cosurfactant increases.

Particle size distribution is a physicochemical characteristic of nano emulsion that significantly determines whether it accumulates in plant tissues. Therefore, safe, stable, and efficient nanocarrier formulation requires homogeneous (mono dispersion) particles of a certain size nanocarrier. The composition of the nano-carrier and the nature of the solvent and co-solvent used require reasonable consideration to control the particle size distribution during its preparation (Bulbake *et al.* 2017; Dong *et al.* 2017; Mozafari *et al.* 2017). After preparation, the resulting nano emulsion must be analyzed physicochemical to ensure its suitability. The parameters measured were nanoparticle size and polydispersity index (PDI).

The PDI value reflects a system's uniformity level, whereas the more minor the PDI value, the distribution of particles in a mono-dispersion system is more uniform (Luo *et al.* 2017). The size distribution algorithm lies between the two extreme PDI values, namely, 0.05-0.7 based on the standard documents ISO 13321:1996 E and ISO 22412:2008 (Sakho *et al.* 2017). Nanoparticles with a PDI value> 0.7 have a wide distribution and are thought to contain large droplets or clusters that can settle slowly (Azmy *et al.* 2019; Danaei *et al.* 2018). An index with a value less than 0.05 is seen with a highly monodispersed standard. The polydispersity index (PDI) shows the diversity of a dispersed system and the PDI value ranges from 0 to 1.

The resulting clove oil nano formula with a PDI value of <0.2 has a standard mono dispersion or homogeneous particle size. As Azmy *et al.* (2019) stated, PDI <0.6 indicates small and homogeneous droplet size, 0.6-0.7 medium droplet size, and >0.7 contains large droplets and wide/heterogeneous distribution. In line with the opinion of (Shahavi *et al.* 2016) that the optimum conditions of clove oil processed into nano emulsions, can produce a droplet size of 50 nm, and a polydispersity index of 0.49 mV. The droplet size and the small PDI value will reduce the occurrence of coagulation due to electrostatic repulsion between particles carrying the same electric charge and make the particles stable. In addition to droplet size, the stability of clove oil is influenced by the type of emulsifier used, pH, and storage temperature.

The inhibition of germination of urediniospores in each formula was significantly different. The high inhibition of the formula against uredinospora H. vastatrix germination was influenced by the small nano emulsion particle size, PDI value <0.2 and solution concentration. The more concentrated the concentration of the clove oil nano emulsion applied, the higher the germination inhibition of H. vastatrix urediniospores. In line with the research results of (Šernaitė *et al.* 2020) that clove extract with a eugenol content of 52.88% has a high potential to be applied in bio fungicide formulations and is effective in suppressing *Botrytis cinerea* fungal infection that causes gray mold disease in strawberries. Several eugenol derivatives have high antifungal activity, almost comparable to the commercial fungicide BC-1000. There are two mechanisms of action of eugenol compounds in fungi, namely (1) through accumulation in fungal membranes which is controlled by the lipophilic character of the molecules, and (2) through chemical reactions with unsaturated chains or reduction by enzymes due to the presence of electron-withdrawing solid groups on the fungal aromatic rings. Disrupts the fungal membrane and production of ROS (Olea *et al.* 2019).

The mechanism of toxicity of the clove oil nano emulsion is due to the active ingredient, eugenol, that enters the membrane and disrupts the fungal cell membrane. The destruction of the eugenol emulsion to the fungal cell membrane can be through the mechanism of inhibiting the biosynthesis of ergosterol and eugenol entering between the fatty acid chains to form a lipid bilayer membrane that changes the fluidity and permeability of the membrane (Abd-Elsalam and Khokhlov 2015).

Nano emulsions have higher antimicrobial activity than essential oils (Abd-Elsalam and  Khokhlov 2015; Ali *et al.* 2017). Nanoparticles attack phytopathogens by forming reactive oxygen species (ROS) antioxidants. The ROS formed can damage cell membranes and other cellular components such as fats, proteins, RNA, and DNA molecules (Al-Khattaf 2021). The nanoparticles' negative charge and the microbial membranes' electrostatic interaction can stick together. Depolarization of the membrane results in disruption of respiration and membrane permeability which in turn causes cell death. The spread of nanoparticles causes the formation of gaps around the cell wall (Alghuthaymi *et al.* 2015). Cell membrane damage and cell surface perforation caused by nanoparticles cause inflammation around the cell membrane and the release of cytoplasmic components (Ibrahim *et al.* 2020).

**Conclusion**

Optimizing the use of surfactants (Glycerol and Tween 80), surfactant concentration, rotation time, and speed in clove oil nano emulsions with smaller sizes and high stability. Clove oil nano emulsion formula that has been produced in as many as six formulas does not have lumps or deposits, has a droplet size of 16.25-24.89 nm with a typical one-peak particle size graph (mono dispersion) containing a homogeneous and evenly distributed particle population. Formula F3 produced the highest germination inhibition of urediniospores *H. Vastatrix* and was as good as formulas F4, F1, and F5. The inhibitory power of the formula on the germination of urediniospores *H vastatrix* was influenced by the small nano emulsion particle size with a PDI value <0.2. The more concentrated the concentration of clove oil nano emulsion applied, the greater the inhibition on the germination of urediniospores *H. vastatrix*.

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**Author contributions**

Conceptualization, K.; methodology, K., S.P., G.I.; preparation of materials N.H., R.; data curation, K.D.S., Y.H.; formal analysis, K.K.H.; original draft writing-preparation, S.P., G.I.; review and editing, K.K.H., Y.H., G., K.D.S.; supervision, Y.F. All authors comment on previous versions of the manuscript. All authors read and approved the final manuscript.

**Conflict of interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data Availability**

Data Availability Data presented in this study will be available on a fair request to the corresponding author.

**Ethics Approval**

Not applicable to this paper.

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**Table 1:** Composition of the clove oil nano emulsion formulation

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Formula | Clove oil (%) | MCT (%) | Gliserol (%) | Tween 80 (%) | Aquades (%) | Speed (rpm) |
| F1 | 5.0 | 5 | 10 | 10 | 60.0 | 750 and 1000 |
| F2 | 7.5 | 5 | 10 | 10 | 57.5 | 750 and 1000 |
| F3 | 5.0 | 5 | 10 | 15 | 55.0 | 750 and 1000 |
| F4 | 7.5 | 5 | 10 | 15 | 52.5 | 750 and 1000 |
| F5 | 5.0 | 5 | 15 | 10 | 55.0 | 750 and 1000 |
| F6 | 7.5 | 5 | 15 | 10 | 52.5 | 750 and 1000 |

MCT (*Medium Chained Triglycerides*)

**Table 2:** Characterization of the clove oil nano emulsion formulation

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Nano emulsion formula** | **Condition after storage** | **Particle size (nm)** | **PDI** | **pH** |
| F1 | No sediment | 21.28 | 0.079 | 5.31 |
| F2 | No sediment | 22.20 | 0.066 | 5.01 |
| F3 | No sediment | 16.25 | 0.058 | 4.89 |
| F4 | No sediment | 18.18 | 0.059 | 4.87 |
| F5 | No sediment | 21.70 | 0.121 | 4.96 |
| F6 | No sediment | 24.89 | 0.116 | 5.00 |

PDI (*Polydiversity Index*)

**Table 3:** Effect of clove oil formula and concentration on the percentage inhibition of urediniospores germination of *H. vastatrix*

|  |  |
| --- | --- |
| Clove oil nano emulsion formula | Inhibition (%) |
| F1 | 95.33 ab |
| F2 | 84.67 b |
| F3 | 97.45 a |
| F4 | 95.33 ab |
| F5 | 88.00 ab |
| F6 | 84.33 b |

The same letter shows no significant difference at the 5% level.

**Table 4:** Effect of several concentrations and formulas of clove oil nano emulsion on the percentage inhibition of urediniospore germination of *H. vastatrix*

|  |  |
| --- | --- |
| Clove oil nano emulsion concentration (%) | Inhibition (%) |
| 5 | 81.86 c |
| 10 | 88.67 b |
| 15 | 96.44 a |
| 20 | 96.44 a |

The same letter shows no significant difference at the 5% level.



**Fig. 1:** Clove oil formula after 24 hours of storage. The formulas were perfectly dispersed and showed no separation, sediment, or foam. F1 is slightly white, F2 is white or hazy, F3 has the highest level of clarity, formulas F4 and F5 have transparent colors, and F6 is white or hazy.

Size Distribution by Intensity

20

15

10

5

0

1

10

100

1000

10000

Size (d.nm)

Record 28: Average 17.

Intensity (Percent)

0.1

**F1**

20

15

10

5

1

10

100

1000

10000

Size Distribution by Intensity

Intensity (Percent)

0

Record 20: Average 15.

**F2**

Size (d.nm)

0.1

20

15

10

5

0

1

10

100

1000

10000

Size Distribution by Intensity

Intensity (Percent)

0.1

Size (d.nm)

Record 36: Average 19.

**F4**

20

15

5

0

1

10

100

1000

10000

Size Distribution by Intensity

Intensity (Percent)

0.1

Size (d.nm)

Record 24: Average 16.

**F5**

10

20

15

10

5

1

10

100

1000

10000

Size Distribution by Intensity

Intensity (Percent)

Size (d.nm)

**F3**

Record 32: Average 18.

0

0.1

16

14

12

10

8

6

4

2

0

0.1

1

10

100

1000

10000

Size (d.nm)

**F6**

Record 52: Average 21.

Intensity (Percent)

Size Distribution by Intensity

0.1

**Fig. 2:** Graphical representation of the particle size of clove oil formulations F1, F2, F3, F4, F5, and F6. The representation of clove oil formulations F1, F2, F3, F4, F5, and F6 from the particle size chart shows a mono dispersion sample (one peak) containing a homogeneous particle population.

**Fig. 3:** Linear regression curve of the relationship between particle size and inhibition percentage of *H.* *vastatrix* urediniospores sprouts