Molecular identification of white root fungal pathogens and in vitro effect of nanopesticide

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Abstract. White root is an important disease in plantation crops such as rubber, cashew, clove, coffee, and nutmeg. This study aims to molecularly identify white root fungi isolated from white root disease of rubber and nutmeg plants and in vitro effect of nano formulated citronella and clove oils to the fungi. Few fungi isolated from the rubber and nutmeg plants were identified using 4 pairs of universal primers. Effect of nano-pesticide citronella and clove oils was evaluated in invitro against mycelial growth of white root fungi isolates (K1 and P1). The result showed that DNA of the fungi was successfully extracted from fungi isolated from nutmeg and rubber plants using 4 pairs of universal primers. K1 and P1 isolates were identical to Marasmius palmivora (99.1%) and K2 was close to Rigidoporus microsporus (97.5%), while KP isolates could not be identified because the DNA was contaminated with Trichoderma harzianum. Nanoformulation of citronella and clove oils at 0.5% concentration inhibited mycelial growth of M. palmivora isolated from rubber and nutmeg plants. The study implies that nano formulation of clove and citronella oils are potential to be tested at green house scale and then in the field for controlling the white root fungal pathogen.

Keywords: Marasmius palmivora, Rigidoporus microsporus, botanical pesticide.

1. Introduction
White root disease (WRD) is a major disease in some plantations such as rubber [1], cashew [2], cloves [3], nutmeg [4], and coffee [5]. This disease has been reported to eradicate thousands of hectares of nutmeg plants in NAD [4]. This disease has been reported in nutmeg plantations in South Aceh since 1990. The symptoms of this disease are yellowing and wilting leaves, starting from the top of the shoot, continuing from one branch to another then all the leaves fall, and the plants die off. When peeled the cambium shows blackish and white net mycelia colonize the base of the stem. The white fungal hyphae are also seen on the diseased roots. The disease could reduce yield up to 70% [4].

Rigidoporus microsporus causes a white root disease is characterized with a thick fan-shaped fruit body with clear orange to brownish red and a white thread mycelia formation. The pathogen is primarily transmitted by contact between the diseased roots from the infected plants with the roots of healthy plants. The role of basidiospores contained in the fruiting body of the fungus is not known [6]. Until now, the
pathogen that causes white root disease in nutmeg is still unclear. And how to identify morphologically is very difficult and takes time, so that a faster technique is needed.

WRF disease control is carried out in various ways including technical, mechanical, biological, and chemical cultures. Plant products in the form of essential oils and extracts have the potential to be used as botanical pesticides. Botanical pesticides are believed to be more selective and less persistent compared to synthetic pesticides, therefore, they are safer to the environment [7]. Clove and citronella oils formulated with emulsifiers and adhesives inhibited the growth of *Phytophthora palmivora* both in vitro and on cocoa seedlings in greenhouses experiments, as well as in cocoa plantations [8,9]. Also, citronella oil controlled *Puccinia horiana* causing white rust disease in *Chrysanthemum* [10]. Clove oil was effective to control *Fusarium oxysporum* causes of rhizome rotten disease in ginger [11]. Eugenol, citronella, and geraniol, as well as a combination of rhizobacteria and botanical pesticides (citronella, geraniol, and eugenol) also inhibited mycelium growth of WRD [12]. This study aims to molecularly identify white root fungi isolated from white root disease of rubber and nutmeg plants and in vitro effect of nano formulated citronella and clove oils to the fungi.

2. Materials and methodology

This research was conducted at the Pests and Diseases Laboratory at the Bogor Spice and Medicinal Crops Research Institute from January to August 2019. WRF isolate was isolated from nutmeg (P1) that have been collected from Cibinong Experimental Garden. Rubber isolates (K1 and K2) have been collected from North and South Sumatra and KP isolate from the Liberika coffee plant in Tanjung Jabung Barat, Jambi. K1 fungus isolates are very pathogenic, because they can infect and cause clove, nutmeg and cashew disease (pathogenicity test have done and data not shown).

2.1. Molecular identification of WRF fungi.

2.1.1. Isolation of fungal DNA. Isolation was carried out on WRF derived from rubber plants (K1 and K2), Pala (P1) and coffee plants (KP) obtained from the purification of isolates. WRF DNA isolation was carried out by two methods, namely the Doyle method with modification [13], and the Bhat et al method [14]. First the mycelium is inoculated in 200 ml of GDP in Erlenmeyer, then grown in a shaker for 4-7 days at room temperature. Then the micellar is harvested by filtering using Whatman filter paper. The mycelia obtained was weighed as much as 0.2 g, then crushed using a sterile mortar by adding 1000 µl of the CTAB buffer [CTAB 2% (Sigma H-5882); 1.4 M NaCL; 0.2% 2 mercaptethanol, 100mM Tris-HCl pH 8; 20mM EDTA pH 8.0; PVP-40 2%] and 2% PVP-40 (Polyvinylpyrrolidone) to fine and then put in effendof 1.5 ml. The microtube is heated in a heat block at 65 °C for 60 minutes, every 10 minutes is reversed. CI solutions (Chloroform isoamyl alcohol - 24: 1, v / v) are added with the same volume, then vortexed. Samples were centrifuged at 10,000 rpm at room temperature for 10 minutes. DNA solution (clear colored at the top) or supernatant will separate from the chloroform solution mixed with other cell parts (white) in the lower phase. The upper phase is moved (± 500-600 µl) transferred to a new microtube, plus cold isopropanol (-20 °C) as much as 2/3 volume, mixed by pipetting or reversed to obtain DNA deposits. Then the DNA solution was centrifuged at 6,500 rpm for 1-2 minutes, then the supernatant was removed (DNA in the form of white deposits on the base of the microtube). DNA deposits were washed twice with 70% cold ethanol (-20 °C) as much as 150 µl, and each washing was centrifuged at 12,500 rpm for 10 minutes. The DNA pellets are then dried air at ± 15-20 minutes, and then dissolved using Nuclease-Free Water (Thermo Scientific) as much as 50 µl, then incubated at room temperature for 30 minutes [13]. Then measured the quality and quantity of DNA using the Nano Photometer.

2.1.2. Amplification of fungal DNA. The extracted DNA was then amplified using SensQuest Labl cycler (Germany). ITS gene amplified using ITS 5 and ITS primers 4 Total volume of PCR was made as much as
50 µl consisting of 2 µL forward primer; 2µL reverse primer; 4 µL template DNA; Red Mix (BIOLINE) as much as 25 µL; and 17 µL Aquabides as a solvent. There are 2 PCR programs, namely (1) run under 1 cycle of initial denaturation at 94°C for 3 minutes, 30 cycles consisting of denaturation 94°C for 30 seconds, annealing 55°C for 20 seconds, extension 72°C for 1 minute, final extension 72°C for 7 minutes and the storage temperature at the final stage 4°C, and (2) carried out under 1 cycle of initial denaturation at 95°C for 5 minutes, 35 cycles consisting of denaturation 95°C for 1 minute, annealing 57.1°C for 1 minute, extension 72°C for 1 minute, final extension 72°C for 7 minutes, and storage temperature at the final stage 4°C. The primary used is universal primary consisting of 4 primary pairs, namely (1) ITS4 / ITS5, ITS 4 as reverse primer and ITS 5 as forward primers. The ITS5 Primer is (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 primary is (5’- TCCTCCGCTTATTGATATGC-3’), (2) ITS2 / ITS1F, ITS 2 as reverse primer and ITS 1F as forward primer. The ITS1F primer is (5’-CTTGGTCATTTAGAGGAAGTAA-3’) and ITS2 primer is (5’-GCTGCGTTCTTCATCGATGC-3’), (3) ITS4 / ITS1F, ITS 4 as reverse primer and ITS 1F as forward primer, and (4) ITS2 / ITS5, ITS 2 as reverse primer and ITS 5 as forward primer.

2.1.3. Visualization of WRF fungus DNA amplification results. Amplification of WRF DNA produced using a PCR machine, then checked by electrophoresis. Markers measuring 100 bp were taken as 3 µl, then put in a well on 1.2% agarose gel with Red Safe coloring of 2 µl. PCR products from each sample were taken as 3 µl, then put into a well at 1.2% of the agarose gel, then electrophoresis for 50 minutes with a voltage of 50 volts. The finished agarose gel is then rinsed with sterile distilled water, and visualized with a gel document.

2.1.4. Sequencing of fungus DNA. Sequencing is done to get the base arrangement that forms the WRF DNA sequence. The sequencing process was conducted at the 1st base in Singapore. The results obtained from DNA sequencing are then traced through the Basic Local Alignment Search Tool (BLAST) data tracking program at the National Center for Biotechnology Information at the National Institute for Health (USA), so that we can find out the percentage homology of the isolates.

2.2. Effect of nanopesticide to mycelial growth of K1 and P1 isolates (Invitro Test)

2.2.1. Preparation of test media and White Root Fungus (WRF) isolates from rubber and nutmeg plants. WRF isolates (K1 and P1) are propagated on Potato Dextrosa Agar (PDA) media 7 days after incubation. Type colony of WRF isolates (K1 and P1) from rubber and nutmeg plants which are white in color and mycelia growth like cotton (Figure 1).

2.2.2. Effect of citronella and clove nano pesticides to mycelial growth of K1 and P1.
In vitro testing is done by mixing the nano pesticide formula as treatment: (1) nano citronella 0.1%; (2) nano citronella 0.2%; (3) nano citronella 0.5%; (4) nano clove 0.1%; (5) nano clove 0.2%; (6) nano clove 0.5%; and (7) control, then mixing into potato dextrose agar (PDA) media (temperature 40°C). The mixing media is homogenized by shaking that tube and poured into a petri dish (9 cm in diameter), then left it until the medium freezes. WRF pathogenic fungi originating from rubber and nutmeg with a diameter of 5 mm are taken from a 7-day-old culture and are placed in the center of a petri dish. After that, it is incubated at room temperature (29°C) for 7 days. Observations are made by measuring the diameter of the colony.
Figure 1. Type colony of WRF isolates (K1 and P1) from rubber and nutmeg plants which are white in color and mycelia growth like cotton.

3. Results and discussion
Isolation of fungal DNA is done successfully carried out with both methods of Doyle [13] with modification and Bhat et al. [14]. The results of measuring the total quantity of DNA of K1 fungus samples using Nano Photometer (IMPLEN, Korea) are 998.30 ng / µl and P1 are 1187.30 ng / µl. Amplification of fungi DNA in both samples (K1 and P1) is done successfully carried out with both PCR programs and produced a DNA band measuring 700 bp (Figure 4). The PCR program number 1 is used for next sequencing because the cycle is shorter.

Figure 2. Visualization of WRF DNA products from rubber plants (K1) and nutmeg plants (P1) on agarose gel 1.2%. Produce a DNA band measuring 700 bp with two method. Description: C = negative control. M = Marker 100 bp. Method 1: Doyle [14] and Method 2: Bhat et al.[15].
Furthermore, the PCR results of the two fungi is sequenced and obtained nucleotide sequences identical to *Marasmius palmivora*, homologies were 99.1% (GenBank: JQ653433.1). *Marasmius palmivora* is a pathogen that causes bunches rot disease in oil palm plants. This pathogen is also reported to infect rubber, coconut, pineapple and banana plants [15]. The identification results are different from the previous reports; therefore, PCR is carried out using other general primary pairs, namely (1) ITS5 / ITS4, (2) ITS1F / ITS2, (3) ITS1F / ITS4, and (4) ITS5 / ITS2 (Figure 2). The fungus DNA amplification of the basidiomycetes group is carried out in combination with several primary pairs to avoid irregularities in the part of amplified DNA [16]. The PCR results can be seen in Figure 3 and with different DNA sizes and continued with sequencing to obtain the nucleotide sequence.

![Visualization of WRD fungus DNA products from rubber plants (K1) and nutmeg plants (P1) on agarose gel 1.2% using 4 primary pairs namely (1) ITS4 / ITS5 (600 bp), (2) ITS2 / ITS1F (350 bp), (3) ITS4 / ITS1F (700 bp), and (4) ITS2 / ITS5 (300 bp). Description: C = negative control. M = Marker 100 bp.](image)

The total quantity of DNA of K2 and KP fungus samples are 234.30 ng / µl and 740.60 ng / µl respectively that are measuring using Nano Photometer (IMPLEN, Korea). Amplification of fungal DNA in both samples (K2 and KP) is successfully carried out with two pairs of primers (primary 2 and primary 3) and produced DNA bands measuring 300-350 bp and 600-700 bp (Figure 4). The PCR results is continued to the sequencing process, to obtain the nucleotide sequences.

Nucleotide DNA sequences K1 and P1 fungus isolates are identical to *Marasmius palmivora* (99.0% and 99.1%), then K2 fungus isolates is identical to *Rigidoporus microsporus* (97.5%) in Table 1, whereas KP fungus isolates are still contaminated because the results sequencing of its DNA sequences is identical to *Trichoderma harzianum* (99%). Especially for KP isolates from coffee plants, refinement is still carried out.
Figure 4. Visualization of DNA isolates from WRD from rubber (K1 and K2), nutmeg (P1) and coffee (KP) plants on agarose gel 1.2% using 2 pairs of primers namely ITS2 / ITS1F (350 bp) to the right and ITS4 / ITS1F (700 bp) to the left. Description: C = Negative control. M = Marker 100 bp.

Table 1: Nucleotide homology of white root fungus isolates from rubber and nutmeg with several isolates *M. palmivorus*, *M. roreri*, *R. microporus* and *R. ulmarius* from GenBank.

| No | Species                  | Number accession in GenBank | Country     | Host          | Rubber NS (K1) | Rubber SS (K2) | Nutmeg (P1) |
|----|--------------------------|-----------------------------|-------------|---------------|----------------|----------------|--------------|
| 1  | Marasmiellus palmivorus  | JQ653438, JQ653433, MF100964, MK788181, MG251431, JQ653444 | Malaysia, Africa, India | Elaeis guineensis, Arachis hypogea, Lagerstroemia speciosa | 97.1, 97.1, 99.0, 99.0, 98.0, 96.9 | 53.1, 53.1, 52.4, 51.6, 51.6, 53.0 | 97.2, 97.2, 99.1, 99.1, 98.2, 97.1 |
| 2  | Moniliophthora roreri    | JX515290, MH861051, EU047937 | Bolivia, Ecuador, USA | Theobroma cacao | 83.6, 84.7, 84.6 | 53.0, 52.6, 51.8 | 83.8, 84.9, 84.7 |
| 3  | Rigidoporus microporus  | KJ654611, MH855397, MN103604, HQ400708, KX090082 | Indonesia, Malaysia, Thailand | Eucalyptus pellita, Rubber, Rubber | 50.8, 54.3, 53.9, 52.8, 53.7 | 97.5, 93.7, 92.8, 93.9, 92.3 | 49.1, 54.3, 53.9, 52.8, 53.7 |
| 4  | Rigidoporus ulmarius     | KC414238, KL981333, AY593868 | China, Gabon, England | - | 49.1, 52.2, 53.2 | 82.3, 80.7, 83.4 | 49.1, 52.2, 53.2 |

Notes: Rubber NS: North Sumatra; Rubber SS: South Sumatra.

The relationship between K1, K2 and P1 isolates with the nucleotide sequences from *Marasmius palmivorus* and *Rigidoporus microporus* in the Bank Gen can be seen in Figure 5. Isolate K2 (Rubber-South Sumatra) has a very close relationship with isolates originating from Indonesia (KJ654611). This isolate was isolated from the *Eucalyptus pellita* plant. While K1 isolate (Rubber-North Sumatra) and P1 isolate clustered with *M. palmivorus* isolate from Africa. This result revealed that phylogenetic analysis confirmed the homology analysis.
Figure 5. Phylogenetic tree of white root fungus isolates from rubber and nutmeg using MEGA X software, neighbor-joining methods, by bootstrapping 1000 times. M. palmivorus: Marasmiellus palmivorus, M. roreri: Moniliophthora roreri, R. microporus: Rigidoporus microporus, R. ulmarius: Rigidoporus ulmarius.

The responses of isolates K1 and P1 to nano cloves and citronella at concentrations of 1% and 2% showed that the WRF tested were 100% inhibited and could not grow again when transferred to new media (data not shown). To get a more precise and efficient concentration, testing is done again at a lower concentration (Table 2, Figure 6 and 7). The results showed that on nano clove and citronella administration at a concentration of 0.5% the percentage of fungal inhibition of WRF was above 70 percent. Therefore, this concentration can be used for further testing at the greenhouse stage and in the field. Nano clove is more effective than nano citronella, this is indicated by a higher percentage of inhibition at a concentration of 0.5%.

Based on in vitro test, botanical pesticides with active ingredients eugenol can inhibit WRF mycelium growth in rubber reaching 20.93%, while citronella plus geraniol is 48.84% [12]. These results indicate there is an increase in the effectiveness of inhibition of eugenol which is formulated with nano technology. Therefore, it is necessary to conduct further testing in the green house scale and in the field by comparing the nano clove formula with non-nano formula to the WRF fungus.
Table 2. The diameter (mm) of the White Root Fungus (WRF) after 4 days of incubation and the percentage of inhibition.

| Type of isolate | Treatment Type of nano pesticide | Concentration (%) | Average colony diameter (mm) | Inhibition (%) |
|----------------|---------------------------------|-------------------|-----------------------------|----------------|
| K1             | Clove                            | 0.1               | 82.6                        | 8.22           |
|                |                                  | 0.2               | 62.0                        | 31.11          |
|                |                                  | 0.5               | 0                           | 100            |
|                | Citronella                       | 0.1               | 86.2                        | 4.22           |
|                |                                  | 0.2               | 83.6                        | 7.11           |
|                |                                  | 0.5               | 17.6                        | 80.44          |
|                | Control                          | 0                 | 90.0                        |                |
| P1             | Clove                            | 0.1               | 76.8                        | 14.67          |
|                |                                  | 0.2               | 55.0                        | 38.89          |
|                |                                  | 0.5               | 0                           | 100            |
|                | Citronella                       | 0.1               | 89.0                        | 1.11           |
|                |                                  | 0.2               | 84.6                        | 6.00           |
|                |                                  | 0.5               | 20.2                        | 77.56          |
|                | Control                          | 0                 | 90.0                        |                |

Figure 6. Testing the effect of citronella (left) and clove (right) nano pesticide on White Root Fungus (WRF) from nutmeg plants on PDA media after 4 days of incubation. Description: [1.] Concentration of 0.1 %, [2.] 0.2 %, [3.] 0.5 %, and [4.] Control.
**Figure 7.** Testing the effect of citronella (left) and clove (right) nano pesticides on White Root Fungus (WRF) from rubber plants on PDA media after 4 days of incubation. Description: [1.] Concentration of 0.1%, [2.] 0.2%, [3.] 0.5 %, and [4.] Control.

4. Conclusions

DNA isolation and identification of WRF from nutmeg and rubber have been successfully carried out using 4 pairs of universal primers for the fungus Basidiomyces and Ascomycetes. K1 and P1 are identical to *Marasmius palmivora* (99.1%), K2 fungus isolates are identical to *R. microsporus* (97.5%), while KP fungus isolates are still contaminated because DNA sequences is identical to *Trichoderma harzianum* (99%). Nano citronella and clove oils at 0.5 percent inhibited the growth of WRF fungus isolates invitro. The study implies that nano formulation of clove and citronella oils are potential to be tested at green house scale and then in the field for controlling the white root fungal pathogen.

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