

In vitro potential control of white root fungus in nutmeg (*Myristica fragrans*) based on botanical fungicide of coconut shell liquid smoke



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Abstract. Nutmeg (*Myristica fragrans*) is spice plant which has an important position as a source of essential oils. An important problem in nutmeg cultivation is white root disease which can reduce nutmeg production. Until now, white root disease is still a problem in nutmeg plantations in South Aceh district. One of the solution that can be explored as an environmentally friendly control measure is to use the botanical fungicide coconut shell liquid smoke. This research aims to determine the potential of coconut shell liquid smoke in controlling white root fungus in vitro. The research was carried out in the Plant Protection Laboratory, Faculty of Agriculture, Teuku Umar University. The research design used was a non-factorial Completely Randomized Design (CRD) consisting of nine levels of liquid smoke concentration treatment; control 0%, 0.5%, 1%, 2%, 3%, 4%, 6%, 8%, 10%. Each treatment involved three replications. Data analysis was carried out using analysis of variance and Tukey's least significant difference (HSD) test. The research stages carried out consisted of producing coconut shell liquid smoke, isolating white root fungus from the roots of infected nutmeg plants and potential assay of liquid smoke in inhibiting the growth of white root fungus. In vitro bioassay result showed that liquid smoke treatment 3% was the best treatment because it was the lowest concentration causing total inhibition of the growth of white root fungus.

Keywords: White root disease, Biofungicide, Liquid smoke, Nutmeg, Inhibition

INTRODUCTION

Nutmeg plant (*Myristica fragrans*) is original plants from Indonesia which has great potential as a trade commodity and is able to generate foreign exchange from exports. This plant is known as a spice plant and source of essential oils needed in various industries, such as the food, medicine, perfume and cosmetics industries. Indonesia is one of the world's largest producers and exporters of nutmeg seeds and mace, with a world market share of 75 percent. The position of Indonesia's nutmeg exports on the world market has an average trade specialization index value from 2007-2016 of 0.988. This value shows that the position or stage of Indonesian nutmeg exports is at the maturity stage [1]. Indonesian nutmeg products are favored by foreign markets because they provide a distinctive aroma and have a high oil yield. This is an indicator that Indonesian nutmeg products are quite in accordance with SNI and ISO requirements [2].

In the cultivation of nutmeg plants, there are problems that cause nutmeg plants to die in various regions of Aceh, including the South Aceh region. In 2017, the

nutmeg planting area in South Aceh district was recorded at 16,289 ha with production reaching 5,238 tons per year. The production centers are in Meukek and Tapaktuan Districts covering an area of 3,785 ha and 2,177 ha respectively. However, since the 1980s, this plant has been attacked by Stem Borer Pests (HPB) as well as White Root Fungus disease which has reduced the population and planting area. BPS data shows that the average area of new nutmeg planting in Aceh Province is 496.03 ha per year. Several business actors such as farmers, refiners and processed product entrepreneurs stated that white root fungus attacks have existed since the early 1980s and until now have not been able to be controlled optimally (Ismail et al., 2021). As a result of this disease attack, nutmeg production can be reduced by up to 70% [3]. The spread of white root disease can occur through direct contact with the roots of diseased plants or through the remains of infected plant roots.

Various efforts have been made to control white root disease, including using synthetic pesticides. Synthetic pesticides in their application have been proven to be able to reduce losses or damage, so that currently the role of pesticides cannot be ignored in achieving production



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targets. However, on the other hand, synthetic pesticides are generally toxic and have a negative impact on the environment [4]. Considering the possible side effects, it is necessary to develop pesticides that are easily degraded naturally, are toxic to target microorganisms, but are not toxic to humans and non-target organisms around them.

One alternative control to overcome white root fungus attacks is through the development of bioactive materials derived from plants such as liquid smoke. Liquid smoke is the result of distillation or condensation of steam resulting from indirect or direct combustion of materials containing carbon and other compounds. One of the raw materials that can be used as a source to obtain liquid smoke is coconut shells. Brown liquid smoke is produced from the pyrolysis process with an acidity degree (pH) of around 2.5 [5]. The antibacterial and antifungal effects of liquid smoke are due to the presence of phenol compounds and the low pH of liquid smoke which causes lysis and disruption of cell wall permeability, thereby inhibiting metabolism and microbial growth [6]. High levels of organic acid and functional phenol components combine to prevent and regulate microbial growth in a synergistic way [7]. Therefore, this liquid smoke has the potential to control microorganisms, including the fungus that causes white root disease in nutmeg plants. This research aims to determine the potential of coconut shell liquid smoke in controlling white root fungus in *In vitro* assay.

METHODOLOGY

Pyrolysis of coconut shell liquid smoke

Production of coconut shell liquid smoke was carried out using a simple distillation system following the modified method of Sutin [8]. The first step was to prepare the coconut shell which has been cut into pieces measuring around 6-8 cm and then put it in a burning tool. The material was heated for 5 hours, until a liquid was formed which was flowed by an iron pipe from the combustion device into a glass bottle prepared at the end of the pipe. The next process was filtering the liquid smoke using filter paper.

Observation of cases of white root disease in community nutmeg plantations

Observations of cases of white root disease were carried out on community-owned nutmeg plantations with a land area of 5 hectares. This plantation is located in Lhok Bengkuang, Tapak Tuan District, South Aceh Regency. The location determination was carried out based on information from the South Aceh Regency agricultural service. Observations were made by tracing nutmeg plants that showed symptoms of white root fungus attack. Next, the roots are dug up to be used as samples for fungal isolation.

Sampling of infected nutmeg roots

Root samples of infected nutmeg plant were taken for isolation of pathogenic fungi from community plantations in South Aceh district. The sample was placed in a closed container and then taken to the laboratory for the isolation process.

Isolation of White Root Fungus

White root fungus was isolated from nutmeg root samples that showed symptoms of white root disease. The Root samples were cleaned from soil, then placed on the surface of sterile potato dextrose agar (PDA) media. Next, they were incubated for 5 days. The fungi colonies that appeared were then purified using new sterile PDA media.

Confirmation test for the pathogenicity of white root fungus isolates on nutmeg seedlings

Pathogenicity tests were carried out to ensure that the isolated fungus is a pathogenic agent that causes disease symptoms. The test was carried out by inoculating fungal isolates on the roots of 10 month old nutmeg seedlings. Plant roots were injured with a sterile needle. Then smeared with white root fungus isolate and covered with cotton moistened with distilled water. Seedlings are watered every day in a screen house. Observations began from inoculation of the pathogen until the onset of symptoms.

Preparation of bioassay medium

Coconut shell liquid smoke was diluted to obtain a concentration according to the treatment; 0%, 0.5%, 1%, 2%, 3%, 4%, 6%, 8% dan 10%, then mixed with PDA media which will later become a medium for bioassay white root fungus growth.

Bioassay of liquid smoke on white root fungus growth *in vitro*

Bioassay of liquid smoke was carried out on fungi growth media to which liquid smoke had been added. This treatment has 8 levels, namely 0%, 0.5%, 1%, 2%, 3%, 4%, 6%, 8% and 10%. Each treatment was repeated 3 times. This test was carried out to measure the inhibitory percentage of liquid smoke against white root fungus *in vitro*. The percentage of inhibition was measured according to the formula based on Oramahi *et al.* [9].

$$\text{DHP} = [(K - P)/K] \times 100 (\%)$$

DHP = percentage of fungal growth inhibition

K = control, the growth of white root fungus in treatment 0% (mm)

P = growth of white root fungus in liquid smoke treatments (mm)

Inoculation was carried out by growing white root fungus on PDA media, using 5 mm diameter cork borer in the center of the media circle, then incubating at 25 °C for 7 days [10].

RESULTS AND DISCUSSION

Case of White Root Fungus Attack in South Aceh

Samples of infected nutmeg roots were taken from community plantations in Lhok Bengkuang Village, Tapak Tuan District, South Aceh Regency. The results of field observations at this location showed that most of them were attacked by white root fungus, with symptoms of wilting, yellowing and drying leaves [11]. Figure 1A



Figure 1. Symptoms of white root disease on nutmeg (A. Nutmeg show symptoms of drying leaves; B. Nutmeg show symptoms of yellowing leaves)



Figure 2. Signs and symptoms of white root disease on nutmeg plants (A. Rhizomorphs on the roots of nutmeg plants; B. Lesions on the transport tissue)

shows one of the nutmeg plants that was attacked by white root fungus with similar symptoms, causing some of the branches to wilt, the leaves to dry and fall. If these symptoms persist, the nutmeg plant will die.

Apart from disease symptoms on the twigs and leaves, signs of disease also appeared in the roots in the form of white rhizomorphs covering the root branches (Figure 2). White root fungus infects through the roots and then enters the transportation system through the vascular tissue. In this tissue, the pathogen absorbs the nutrients in the plant so that the plant becomes deficient in nutrients and water. The spread of white root disease can occur through direct contact with the roots of diseased plants or through the remains of infected plant roots. This causes the spread of white root pathogens in the field to occur quickly. In addition, the virulence ability of white root fungus is influenced by climate and other abiotic components in the surrounding land [12]. Deciduous

disease, which is one of the symptoms of white root disease on nutmeg trees in South Aceh Regency, is thought to have experienced an epidemic since 2003. This disease has spread and is easy to find in South Aceh. The pathogen that causes the symptoms of white root disease after being identified by Noveriza *et al.*, [13] shows high similarities to *Marasmius palmivora* and *Rigidoporus microsporus*. according to research by Susanna *et al.* [14] showed that one of the causes of deciduous disease in nutmeg plants in southern Aceh is the sloping and hilly topography which causes the availability of nutrients for plants to be lower so that plants become more vulnerable and easily infected with disease. Apart from that, this topography also causes the potential for the spread of pathogens due to water flow to be higher. Fungal spores originating from infected plants on slopes can move with water runoff, causing the potential for pathogens to enter lower areas of land.



Figure 3. Nutmeg stump infected by white root disease on nutmeg plantation in South Aceh



Figure 4. White root fungus that infected nutmeg plants (A. Isolate of white root fungus; B. Confirmation test the pathogenicity of white root fungus on nutmeg seedlings, Left: healthy nutmeg seedlings and Right: infected nutmeg seedlings)

In case of white root disease attack in South Aceh district, farmers only realized the plants were infected when the nutmeg plants showed symptoms of yellowing and hanging leaves. About 2 months after these symptoms appear, the nutmeg plant will dry out and die. The action taken at this stage is to cut down the nutmeg plants. However, the lower part of the infected nutmeg plant still remains on the ground (Figure 3). It is thought that these remaining plant stumps could be a route for further infection in other plants. This happens because the pathogens that are still residing in the plant roots have not been destroyed and can still grow. Pathogenic mycelia originating from infected plant root stumps can become the primary inoculum that will attack new plants [15].

White root pathogen isolated from infected root samples showed thin white colonies that filled the petri dish (Figure 4A). According to Susana *et al.* [16], one of the pathogens that causes rotting disease in nutmeg plants is

Rigidoporus sp.. Symptoms that appear as a result of this fungus attack are dry and wilted leaves on several twigs, then spread from one branch to another until all parts of the plant are attacked (Figure 4B). Leaves turn brown, curl, hang, and eventually fall off. However, there are plants that wilt completely, showing yellowing leaves, drying out, falling off, and eventually dying completely.

Potential for inhibiting white root fungus in liquid smoke media

Based on the results of bioassay for the potential to inhibit the growth of white root fungus on PDA media with a combination of liquid smoke, it can be seen that the diameter of white root fungus colonies in the 2% treatment was smaller than the control, the treatment 3% to 10% showed no colonies growing (Table 1). This shows that liquid smoke has an effect on inhibiting the

Table 1. Colony diameter (mm) of white root fungus in media treatment with the addition of liquid smoke

Day	Treatment								
	Control	0,50%	1%	2%	3%	4%	6%	8%	10%
1	34,08 d	26,27 c	12,93 b	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a
2	73,60 d	56,09 c	36,30 b	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a
3	90,05 d	83,43 d	65,73 c	14,37 b	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a
4	90,05 c	90,90 c	90,57 c	23,27 c	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a
5	90,05 c	90,90 c	90,57 c	41,47 b	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a
6	90,05 c	90,90 c	90,57 c	54,87 b	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a
7	90,05 c	90,90 c	90,57 c	65,92 b	0,00 a	0,00 a	0,00 a	0,00 a	0,00 a

*Values with the same letters in the same raw denote no significant difference at $P<0.05$ according to Tukey's honest significant difference (HSD) test.

Table 2. Percentage of relative inhibition of white root fungus colonies in liquid smoke treatment

Day	Treatment							
	0,50%	1%	2%	3%	4%	6%	8%	10%
1	22,93 c	62,05 b	0,00 a	100,00 a	100,00 a	100,00 a	100,00 a	100,00 a
2	23,79 c	50,68 b	0,00 a	100,00 a	100,00 a	100,00 a	100,00 a	100,00 a
3	7,35 d	27,00 c	14,37 b	100,00 a	100,00 a	100,00 a	100,00 a	100,00 a
4	0,00 c	0,00 c	23,27 c	100,00 a	100,00 a	100,00 a	100,00 a	100,00 a
5	0,00 c	0,00 c	41,47 b	100,00 a	100,00 a	100,00 a	100,00 a	100,00 a
6	0,00 c	0,00 c	54,87 b	100,00 a	100,00 a	100,00 a	100,00 a	100,00 a
7	0,00 c	0,00 c	65,92 b	100,00 a	100,00 a	100,00 a	100,00 a	100,00 a

*Values with the same letters in the same raw denote no significant difference at $P<0.05$ according to Tukey's honest significant difference (HSD) test.

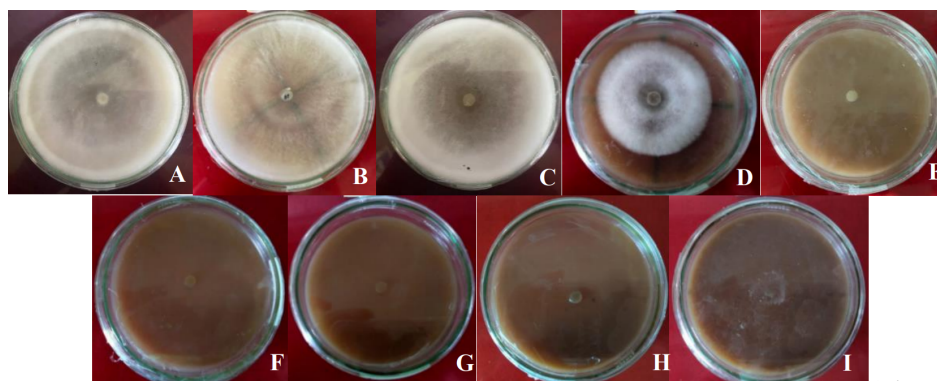


Figure 5. Bioassay of the potential for JAP inhibition in growth media containing liquid smoke (A. Control; B. 0.5% liquid smoke; C. 1% liquid smoke; D. 2% liquid smoke; E. 3% liquid smoke; F. Liquid smoke 4%; G. Liquid smoke 6%; H. Liquid smoke 8%; I. Liquid smoke 10%)

growth of white root fungus. Liquid smoke given to the white root fungus growing medium at a concentration of 2% began to show significant differences in the growth of white root fungus colonies compared to the control. Meanwhile, liquid smoke treatment with a concentration of up to 1% had no effect on fungal growth on day 7, but showed slower growth than the control treatment on days 1 - 3 post-inoculation.

Table 2 shows that the percentage of total inhibition occurred starting from treatment 3% up to treatment 10%. In the 2% treatment, the inhibitory percentage on day 7 of incubation was 65.92%. The control mechanism by liquid smoke can occur due to the high phenol content [17][18]. The combination of functional phenol components and high organic acid content works synergistically to prevent and control microbial growth. High acid levels can inhibit the growth of microbes because microbes can only grow at low acid levels. The antimicrobial properties of acetic acid are related to pH conditions. This is because undissociated acetic acid penetrates cells more quickly. Meanwhile, propionic acid is able to inhibit microbes by blocking the cell

metabolic system through inhibiting enzyme activity. Nineteen volatile compounds were detected by GC-MS in the coconut shell liquid smoke, based on their function group's composition percentage: phenol (90.75%) such as 2-hydroxymethylphenol, 2-methoxyphenol, 2,6-dimethoxyphenol, carbonyl (3.71%) i.e., 3-methyl-2-cyclopenten-1-one, 2-hydroxy-3-methyl-2-, alcohol (1.81%) 2-furanmethanol, and benzene (3.73%) as 4-ethyl-benzene-1,3-diol, and 1,2,3-trimethoxybenzene [19]. Phenol acts as a disinfectant which may inhibit enzyme activity in pathogens, Carbonyl is effective in destroying the NH₂ group in pathogenic chitin. Alcohol functions as a protein denatured, which able to damage pathogen cell membranes. Benzene plays a role in reducing the phytotoxic properties of pathogens [19].

Figure 5 shows the growth of white root fungus on media with a combination of liquid smoke in various treatments on day 7 post-inoculation. In Figure 3A-3C, fungal growth filled the petri dish and there were no indicators of inhibition, while in the treatment in Figure 3E-3I no fungus was seen growing until day 7 or there was 100% inhibition of the growth of white root fungus.

From several studies, coconut shell liquid smoke has been proven to be a botanical pesticide because it has anti-microbial capabilities. Research by Dalimunthe and Tistama (2018) shows that liquid smoke at a dose of 50 ml per liter is able to control white root fungus disease > 75% which infects rubber plants [20]. Not only against white root fungi, the use of liquid smoke has also been proven to be able to control several other pathogenic fungi. Liquid smoke at concentrations of 5% and 10% is able to inhibit the activity of *Phytophthora palmivora* which causes fruit rot in cocoa [4] and the fungus *Elsinoe fawcetti* which causes scab in orange fruit [23]. The research results of Nurfadilah *et al.* [22] showed that liquid smoke with a concentration of 2% could suppress the development of *Bulkholderia glumae* in-vitro and did not cause phytotoxicity in rice seeds. Liquid smoke with a concentration of 2% can also reduce the severity of disease in the seedling phase with a relative inhibition level of 43.8%. Other research also states that the application of liquid smoke can reduce the percentage of *Fusarium oxysporum* attacks on rubber plants with a better percentage of attack reduction compared to chemical fungicides [23]. The difference in effective concentration that can inhibit fungal growth can vary according to the type. This is because different species of fungi have different stress tolerance ranges for liquid smoke content. Different types of pathogens have different sensitivities to fungicide exposure depending on their ability to respond to fungicide chemical stress [24].

CONCLUSION

In vitro test results showed that liquid smoke treatment at a concentration of 2% was able to inhibit the growth of white root fungus by 65.92% on day 7 post inoculation. Liquid smoke treatment 3% was the best treatment in the in vitro test because it was the lowest concentration causing total inhibition of the growth of white root fungus isolated from the roots of infected nutmeg plants in South Aceh district.

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