INHIBITORY EFFECT OF *ULIN* WOOD LIQUID SMOKE AND *GOGO* RICE ENDOPHYTIC FUNGI AGAINST PATHOGEN *Pyricularia oryzae*

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Received 22 March 2021 / Accepted 4 January 2022

ABSTRACT

Diseases in rice plants (paddy) caused by microorganisms such as *Pyricularia oryzae* lead to a decrease in rice production. Therefore, it is essential to find out biological agents for protecting paddy and plants in general, against plant diseases. Liquid smoke and endophytic fungi have been known as biological agents to enhance the protection of plants against disease. The purpose of this study was to determine the ability of liquid smoke, endophytic fungi and the concentrations combinations to suppress the growth of *P. oryzae*. The results showed that liquid smoke concentrations of 0.17% to 1.75% and endophytic fungi filtrate of 2% to 10% showed significant ability against pathogen *P. oryzae*. However, the combination of liquid smoke and endophytic fungi filtrate at selected concentrations (0.17% liquid smoke combined with 2% endophytic fungi filtrate and 0.34% liquid smoke combined with 2% endophytic fungi filtrate) showed no significant inhibition percentage against *P. oryzae* compared to control. In conclusion, this study showed that the respective applications of liquid smoke and endophytic fungi filtrate inhibit the growth of *P. oryzae*.

Keywords: endophytic fungi, inhibition ability, liquid smoke, *P. oryzae*

INTRODUCTION

Rice plants are the most dominating food crop commodity in Indonesia (Andriani 2008). As many as 95% of Indonesians choose rice as a staple food (Norsalis 2011). One of the rice plants cultivated in Indonesia is Gogo rice, which is grown on moorland (a type of habitat with the characteristic of low growing vegetation on acidic soil) or on dry land (Hairmansis *et al.* 2016).

"Gogo" rice Maninjau variety (*Oryza sativa* L. var. *maninjau*) is one of the Indonesian native varieties of brown rice that comes from the area of Lake Maninjau located in the Sumatera Barat Province of Indonesia. This variety is able to live on the dry land of Central Kalimantan, is resistant to leaf blight disease and able to survive in soil with high iron content (BPPP Jateng 2014).

Among microorganisms attacking Gogo rice plants are pathogenic fungi such as *P. oryzae*, *Rhizoctonia solani*, *Helmithosporium sigoideum* and *Cercospora janseana*. *P. oryzae* is one of the main diseases of rice crops due to its impact on reducing rice productivity (Wang *et al.* 2014; Suganda *et al.* 2016). This pathogen causes blast disease and serious damage to panicles (panicle blast) and leaves (leaf blast) of rice plants, where damage to panicles greatly affects rice productivity (Hayashi *et al.* 2019).

Plant resistance to pathogens can be improved by utilizing the interaction of microorganisms with endophytic microbes.

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Endophytic fungi are a symbiotic between fungi and plants, which has a role to protect plants from pathogens by using the compounds produced by the symbiosis. Symbiosis mutualism of endophytic fungi with plants produces secondary metabolites, such as phytohormones, nutrients and colony formations. Some studies state that secondary metabolites produced by endophytic fungi inhibit pathogenic microbes (Lalngaihawmi *et al.* 2019). Therefore, endophytic fungi are potential biological agents to inhibit pathogenic fungi.

Liquid smoke bioactive materials can also be used to prevent disease caused by microorganisms. Liquid smoke is a mixture of solution and colloidal dispersion of wood smoke vapor in water obtained from the process of wood pyrolysis or made from a mixture of pure compounds (Darmadji 2002; Soldera *et al.* 2008; Lee *et al.* 2011). Liquid smoke has good antimicrobial properties because it can inhibit the growth of microbes.

This study used liquid smoke from "Ulin wood" (*Eusideroxylon zwageri* Teijsm. & Binn). Ulin wood has density characteristics and a tight structure of hardwood, containing complex constituent compounds. Several studies reported that one type of hardwood, "alaban" wood (*Vitex pubescens* VAHL.), contains phenol, carboxylic acid and carbonyl that are antimicrobial (Oramahi & Yoshimura 2013). Based on literature studies, "Ulin" wood liquid smoke may have the ability to inhibit *P. oryzae*.

Both endophytic fungi and liquid smoke of "Ulin" wood have the ability to inhibit pathogenic microorganisms. In this study, both were tested both independently and by combining the two. As an initial effort to find one of the solutions to overcome diseases that often occur in rice plants "gogo" Maninjau varieties. This study was aimed to determine the ability of "Ulin" wood liquid smoke, endophytic fungi and their combinations to suppress the growth of *P. oryzae*.

MATERIALS AND METHODS

Sampling

Samples of "Gogo" rice (*O. sativa* L var. *maninjau*) were taken from the same stretch of land in Jaar Village, East Dusun Subdistrict, East Barito District, Central Kalimantan Province. 2°06'53.9" North-South Latitudes 115°15'22.1" West-East Longitudes. Samples consisting of all parts of the Gogo rice plants were taken along with the soil around the roots of the Gogo rice plants, by using a machete. The samples were stored in polybags and transported to the Microbiology Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Lambung Mangkurat.

Isolation, Purification and Identification of Endophytic Fungi

Endophytic fungi were isolated from the roots of the Gogo rice plants, by using surface sterilization method. The roots were washed using running water, then cut to a size of 10 cm. The root cuts were surface-sterilized by using 0.05% bleach for 60 sec and rinsed twice using sterile distilled water. Each part of the root tip was then slightly cut to totally drain the root tip. The root tip was then planted in a sterile Potato Dextrose Agar (PDA) medium and incubated at a temperature of 28 °C for 3-5 days and observed daily. The grown fungi were then purified (Manurung *et al.* 2014; Nurzannah *et al.* 2014). Fungi identification was carried out by using morphological observations, consisting of macroscopic and microscopic observations with reference to the identification book titled "Illustrated Genera of Imperfect Fungi 4th edition" (Barnett & Hunter 1998).

Screening of Endophytic Fungi

Pathogenicity and antagonism tests were used for the screening of endophytic fungi. Pathogenicity test were carried out by using rice seed refering to Waruwu *et al.* (2016). Prior to being used in the pathogenicity test, the surface of the rice seeds (20 grains) were sterilized by soaking the rice seeds in 70% ethanol for 30 sec, followed by soaking in 1% NaOCl for 60 sec. Subsequently, the rice seeds were flushed 3 times in sterile distilled water. After that, the rice seeds were inoculated in PDA medium that had been previously overgrown by 7-day pure isolates of endophytic fungi and then incubated for 2 weeks at room temperature (27-29 °C). Observation on the growing rice sprouts was carried out at the end of incubation. Isolates of endophytic fungi that did not interfere with rice

germination were used for further testing. Seed germination rate was calculated using the formula (Talukdar 2011):

$$Germination\ (\%) = \frac{Number\ of\ germinated\ seed}{total\ number\ seed} \times 100\%$$

A confirmation test for endophytic fungi infection in the roots of rice plants was conducted for germination by using a method of Luqman *et al.* (2015) that has been modified. Prior to being used in the test, the roots of rice plants were washed thoroughly in running water, drained, then soaked in 5.25% NaClO solution for 5 min, then rinsed using distilled water. Subsequently, the roots were soaked in 1% KOH solution for 30 min and then rinsed using distilled water. After that, the roots were then pre-soaked in 1% H_2O_2 solution for 5 min. The coloring stage was started by soaking the roots in 0.5% vinegar solution, followed by being soaked in ink with a ratio of 1:5 for 30 min. Then, the roots were rinsed with distilled water. Finally, the roots of the rice plants were placed on an object glass and covered by a cover glass, and then observed under a microscope with 40x and 100x magnifications.

The antagonism test of endophytic fungi isolates against *P. oryzae* was conducted by using dual culture method (Tomah *et al.* 2020), which put isolates of pathogenic fungi and endophytic fungi on PDA medium in a Petri dish that has been divided into two quadrants. Each isolate was placed at a distance of 3 cm from the edge of the Petri dish and incubated at a temperature of 28 °C for 5-7 days. After incubation, the inhibition percentage of the pathogens was measured using the formula developed by Rabha *et al.* (2014):

$$Inhibition\ (\%) = \frac{diameter\ of\ pathogen\ control\ colony}{diameter\ of\ pathogen\ treatment\ colony} \times 100\%$$

Filtrate Harvesting of Endophytic Fungi

Filtrate production methods used in this study were modified based on Elita *et al.* (2013) and Malinda *et al.* (2015). Endophytic fungi with the highest inhibitory ability obtained in previous tests were inoculated on PDA slant media then incubated for 7 days at 28 °C. After being incubated, the filtrate was harvested by adding 9 mL of sterile distilled water. The fungi surface was then gently wiped with a fine brush. Subsequently, the water suspension and fungi were transferred to a new test tube, then centrifuged at 3,500 rpm for 20 min. Finally, the supernatant was filtered by using a syringe filter with a pore size of 0.45 µm. The filtrate was then used in testing the inhibitory activity of endophytic fungi against pathogens.

Inhibition Test of Liquid Smoke-Endophytic Fungi to *P. oryzae*

Inhibition test of liquid smoke was conducted at various liquid smoke concentrations, i.e., 0.085%, 0.17%, 0.34%, 0.68%, 1.36% and 1.75%, based on a method of Malinda *et al.* (2015) that have been modified. Liquid smoke was obtained from condensation during the production process of "Ulin" wood charcoal by Talasiana Charcoal Production Group, located at Tanah Laut Regency. The liquid smoke was mixed into the Potato Dextrose Agar (PDA) medium. The pathogen isolates were then grown on the mixture of liquid smoke and PDA for 5-7 days with daily observation. The inhibition percentage was calculated by the formula developed by Rabha *et al.* (2014):

$$Inhibition\ (\%) = \frac{diameter\ of\ pathogen\ control\ colony}{diameter\ of\ pathogen\ treatment\ colony} \times 100\%$$

The inhibition test for endophytic fungi was carried out by using the serial dilution method. Endophytic filtrate with concentrations of 2, 4, 6, 8, and 10% was mixed with PDA medium to be used for growing pathogen, and then incubated at 28 °C for 7 days and observed daily. The inhibition percentage was calculated with the same formula as the one used for calculating the inhibition percentage for the liquid smoke.

Liquid smoke and the endophytic filtrate (Ketoconazole) with various concentrations were then combined to be tested for their inhibitory ability against pathogens. Testing methods and measurements of inhibition percentage were conducted by using the same method as for the previous tests.

RESULTS AND DISCUSSION

Endophytic Fungi of *O. sativa* L var. *maninjau* Root

Endophytic fungi obtained from the roots of "Gogo" rice var *maninjau* were coded AP.2,

AP.3, AP.4, AP.7, AP.8, and AP.9. Microscopic observation of the endophytic fungi isolates showed that there are morphological differences. Based on Barnet & Hunter (1998), the six isolates found refers to several species. AP2 is *Curvularia* sp., AP3 and AP7 are *Penicillium* sp., AP 8 is *Geotrichum* sp., AP9 is *Aspergillus* sp., while AP4 has not yet been able to be identified (Fig. 1). The four fungi species were also reported by Lalngaihawmi *et al.* (2018) as endophytic fungi in rice.

The presence of endophytic fungi in the roots of the Gogo rice plants varies based on the various tissues in which they grow. A study conducted by Naik *et al.* (2009) reported that the colonization of endophytic fungi in rice plants happens more dominantly at the roots of rice plants.

Pathogenicity of Endophytic Fungi isolated from *O. sativa* L. var Maninjau Seeds

Our study showed that the germination percentage of Gogo rice seeds varied (Table 1), while the invasions of endophytic fungi against the seed germination were shown in Figure 2. Each endophytic fungi showed different levels of pathogenicity, both at 7 days after inoculation and 14 days after inoculation. The level of pathogenicity is useful for determining the best isolates to be used for subsequent tests. Based on the results of pathogenicity levels of rice sprouts that grew normally, abnormally and did not grow, the best pathogenicity value was provided by *Geotrichum* sp. AP8, followed by *Penicillium* sp. AP7 and *Curvularia* sp. AP 2.

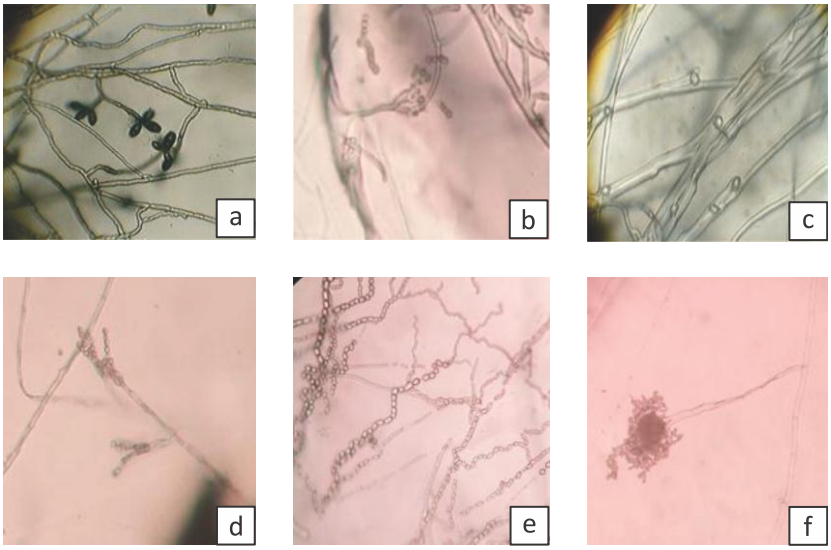

Figure 1 Microscopic characteristics of endophytic fungi isolates obtained from the roots of *O. sativa* L var. *maninjau*
Notes: a. *Curvularia* sp. AP2 (40x); b. *Penicillium* sp. AP3 (40x); c. AP4 (100x); d. *Penicillium* sp. AP7 (40x); e. *Geotrichum* sp. AP8 (40x); f. *Aspergillus* sp. AP9 (40x).

Table 1 Germination rate of the *O. sativa* L. var. *maninjau* for the 7 and 14 Days After Incubation (DAI)

Endophytic fungi	Germination (%)*					
	7-DAI			14-DAI		
	Normal	Abnormal	No growth	Normal	Abnormal	No growth
Without adding endophytic fungi	60.00 ± 8.16b	0.00 ± 0.00a	36.67 ± 4.71a	63.33 ± 9.43b	0.00 ± 0.00a	33.33 ± 4.71a
Curvularia sp. AP2	40.00±14.14a	10.00 ± 8.16a	40.00 ±8.16a	20.00 ± 8.16a	36.67 ± 12.47a	33.33 ± 4.71a
Penicillium sp. AP3	43.33 ± 4.71ab	13.33 ± 4.71a	43.33 ± 9.43a	56.67 ± 4.71ab	16.67 ± 4.71a	26.67 ± 4.71a
AP4	0.00 ± 0.00ab	0.00 ± 0.00a	100.00 ± 0.00b	6.67 ± 9.43ab	13.33 ± 12.47a	80.00 ± 14.14b
Penicillium sp. AP7	43.33 ± 9.43ab	13.33 ± 12.47a	50.00 ± 8.16a	56.67 ± 12.47ab	16.67 ± 9.43a	26.67±4.71a
Geotrichum sp. AP8	50.00 ± 8.16b	0.00 ± 0.00a	50.00 ± 8.16a	70.00 ± 8.16b	16.67 ± 4.71a	16.67±4.71a
Aspergillus sp. AP9	43.33 ± 4.71ab	6.67 ± 4.71a	50.00 ± 8.16a	46.67 ± 4.71ab	20.00 ± 0.00a	33.33±4.71a

Note : * = numbers followed by the same letter are not significantly different based on Duncan test at $P < 0.05$.

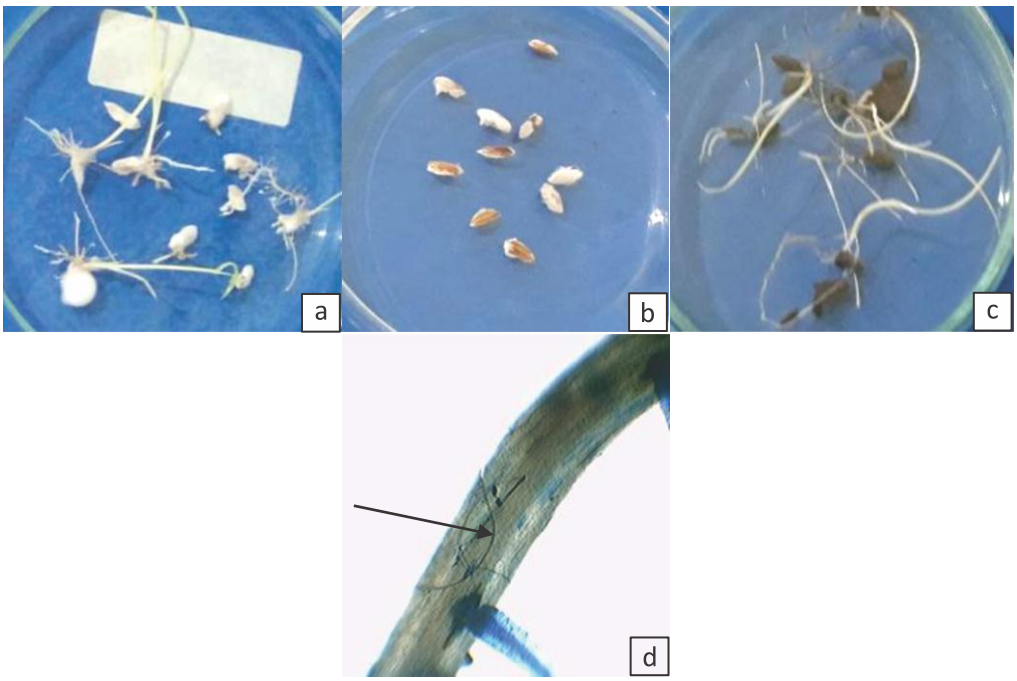

Figure 2 Rice germination with various treatments of endophytic fungi
Notes: a. rice germination without the addition of endophytic fungi; b. rice seeds with the addition of AP4 endophyte fungi (not germinated); c. rice germination with the addition of endophytic fungi *Geotrichum* sp. AP8; d. roots of rice seed undergoing treatment with endophytic fungi *Geotrichum* sp. AP8 (the arrow shows endophytic fungi invading the rice root tissue).

The results of the endophytic fungi antagonism test against *P. oryzae* showed no significant difference between the three selected fungi ($P > 0.05$) (Table 2).

Although the antagonism test did not show significant differences, the selection of the best isolate for subsequent tests was determined based on the best inhibition percentage and the diameter of the pathogen successfully inhibited. Thus, *Geotrichum* sp. AP8 was the chosen isolate. Endophytic fungi can inhibit pathogens having metabolite compounds by inhibiting the permeability of the pathogenic cells (Ting *et al.* 2011). White *et al.* (2019) added that endophytic fungi can use the mechanisms of space and nutrient competition for suppressing pathogen growth.

The Ability of Liquid Smoke and Endophytic Fungi in Inhibiting the Growth of *P. oryzae*

The results of our study indicated that liquid smoke at all tested concentrations was significantly able to inhibit the growth of pathogens compared to control (Fig 3). At liquid smoke concentrations of 0.17% to 1.75%, the inhibition percentages differed significantly ($P < 0.05$). The selection of the right liquid smoke concentration for subsequent tests is indispensable, given that liquid smoke contains several antimicrobial components that may affect not only the growth of pathogen, but also the growth of endophytic fungi.

Table 2 Diameter of *P. oryzae* colony and inhibition percentage of endophytic fungi

Endophytic fungi	Diameter of *P. oryzae* (mm)*	Inhibition percentage (%)*
Curvularia sp, AP2	41.47±2.92	21.98±5.54
Penicillium sp. AP7	32.03±7.84	41.87±14.32
Geotrichum sp. AP8	30.21±6.31	41.87±11.71

Note: * = not significantly different based on Duncan test ($P < 0.05$).

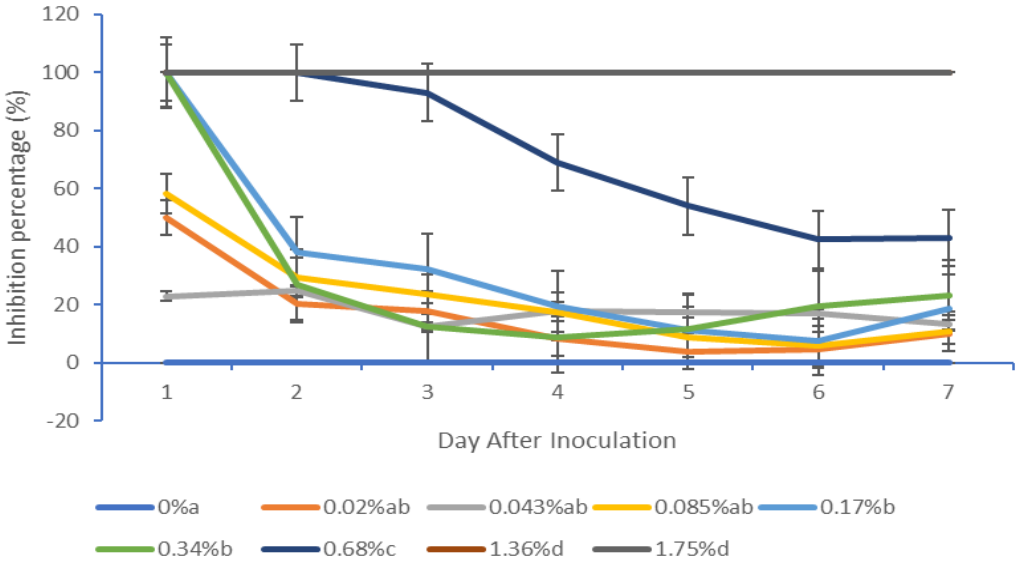

Figure 3 Inhibition percentage of different concentrations of liquid smoke against *P. oryzae* on 1 DAI until 7 DAI
Notes: The bar indicates the standard deviation. Numbers followed by the same letter are not significantly different based on Duncan test ($P < 0.05$).

The results also showed that the smallest liquid smoke concentration (0.02%) was able to inhibit the growth of pathogens despite the daily decrease in ability, while the largest liquid smoke concentration (1.75%) was able to inhibit pathogens at an inhibition percentage of 100%. Our study also indicated that the 0.17% and 0.34% liquid smoke concentrations were considered the best concentration for inhibiting pathogens compared to other concentrations. The inhibitory curve of those two concentrations had a tendency of increase after passing 4 days of inoculation, in contrast to other concentrations that had a tendency to decrease. The two liquid smoke concentrations also showed inhibiting capabilities against the tested pathogen despite the small concentrations.

The inhibiting capabilities of liquid smoke against the growth of microorganisms may have been due to the contents of active compounds originating from the pyrolysis of wood constituents (cellulose, hemicellulose, and lignin). Cellulose and hemicellulose produce organic acid compounds such as acetic acid, while lignin produces phenol compounds. The higher the content of the wood constituents, the more complex liquid smoke obtained (Pszczola 1995). Contents of active compounds in "Ulin" liquid smoke are acids, phenolics, alcohol, ketones, ethers and esters, with acetic acid as the main active compounds (71.57% of the total active compounds) (Junaidi *et al.* 2020). Liquid smoke of "Ulin" wood also contains a total acid of up to 8.88% (Junaidi *et al.* 2019), which has antimicrobial properties.

Various concentrations of selected isolate endophyte fungi filtrate (*Geotrichum* sp.) significantly inhibited the growth of *P. oryzae* compared with the control. However, there were no significant differences in inhibition percentages among concentrations (2-10%) ($P < 0.05$) (Fig. 4).

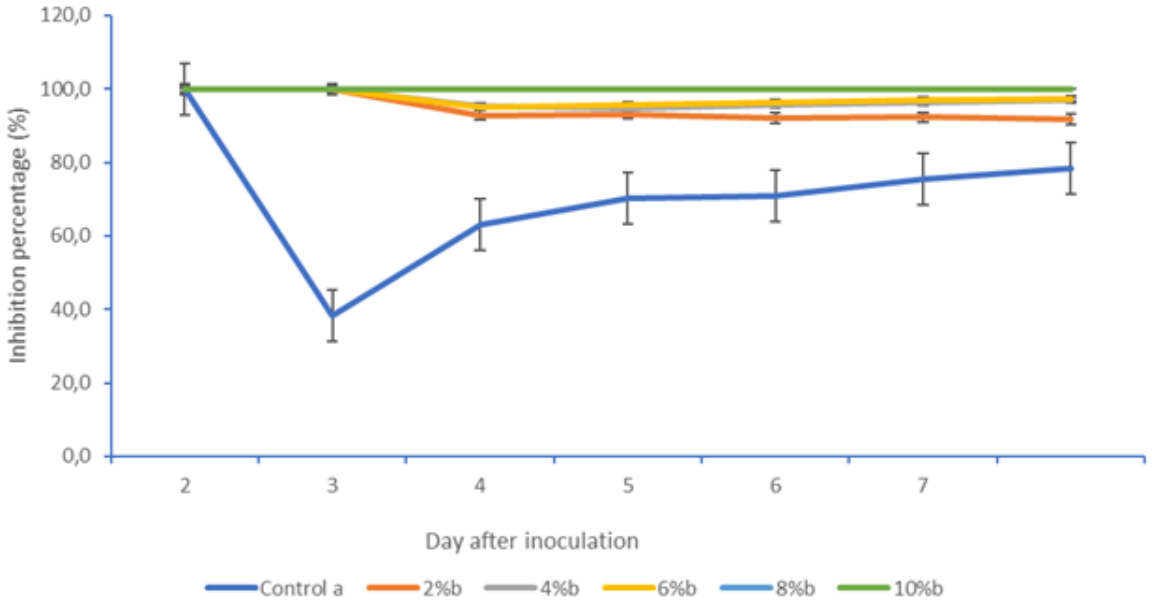

Figure 4 Inhibition percentage of *Geotrichum* sp. AP8 filtrate concentrations against *P. oryzae* on 1 DAI until 7 DAI
Notes: The bar indicates the standard deviation. Numbers followed by the same letter are not significantly different based on the Duncan test ($P < 0.05$).

Geotrichum sp. AP8 filtrate concentration of 2% was able to inhibit the growth of *P. oryzae* grown at 1 DAI up to 7 DAI with inhibition percentages ranging from 91.8 up to 100%. Therefore, the 2% concentration of *Geotrichum* sp. AP8 filtrate was chosen for testing the inhibitory synergism with liquid smoke. This result of *Geotrichum* sp. AP8 filtrate is similar to the results of previous research conducted by Imaningsih *et al.* (2021), which used endophytic fungi filtrate of "Hiyung" cayenne pepper with a concentration of 2% for inhibiting pathogen *Colletotrichum capsici* at almost 100% inhibition percentage. Our study also provides better inhibitory results for the genus *Geotrichum* compared to the study of Lalngaihawmi *et al.* (2019), which tested *Geotrichum candidum* for inhibiting *P. oryzae* with a 68% inhibition percentage at 7 DAI.

Endophytic fungi inhibit pathogen growth through anti-microbial compounds (Schulz & Boyle 2005; Singh *et al.* 2021). In our study, the filtrate of endophytic fungi was tested directly to inhibit the growth of *P. oryzae* and successfully showed high inhibition percentage. Results of our study showed that the presence of bioactive substances produced by endophytic fungi has anti-microbial properties against the tested pathogen. Singh *et al.* (2021) stated that bioactive compounds of endophytic fungi can be alkaloid, flavonoid, lignan, saponin, quinone, xanthone and miscellaneous compounds.

Synergism of Liquid Smoke and Endophytic Fungi Inhibit *P. oryzae* Growth

The growth of pathogenic *P. oryzae* was inhibited by the combination between liquid smoke concentrations of 0.17% and 0.34% and *Geotrichum* sp. AP.8 endophytic filtrate concentration of 2% with a range of inhibition percentages from 42% up to 100% on 1 DAI until 7 DAI. However, the inhibition percentages of the combination did not differ significantly among treatment combinations. The inhibition percentages of the treatments were not significantly different when compared to the control (Fig. 5). A previous study conducted by Imaningsih *et al.* (2021) showed that the concentrations combinations between liquid smoke of "Ulin" wood and endophytic fungi filtrate of "Hiyung" chili significantly inhibit the growth of pathogen *C. capsici* compared to the control.

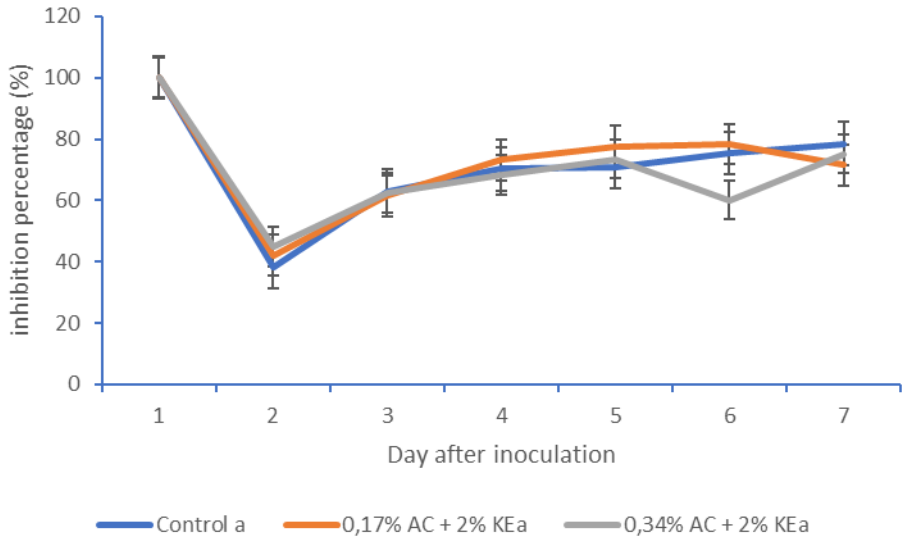

Figure 5 Inhibition percentages of several concentrations combinations between *Geotrichum* sp. AP8 filtrate (KE) and liquid smoke (AC) against *P. oryzae* on 1 DAI until 7 DAI
Notes: The bar indicates the standard deviation. Numbers followed by the same letter are not significantly different based on Duncan test ($P < 0.05$).

Based on the results of our study, the inhibition percentage achieved when combining liquid smoke and endophytic fungi filtrate was higher compared to the inhibition percentage of only using liquid smoke at the same concentration. The inhibition percentage of combining liquid smoke and endophytic fungi filtrate was lower when compared to the inhibition percentage of only using endophytic filtrate at the same concentration. During the filtration process, there might still be fungi cells carried away, due to the pore size of the filter membrane of 0.45 microns. Hyphae fragments and spores of *Geotrichum* sp. AP8 possibly penetrates the filter pores and grows during the inhibition process against the pathogens. Meanwhile, Sayer *et al.* (1969) grouped *Geotrichum* sp. into fungi with intermediate spore size, which size is smaller than the usual fungi spores size. It is suspected that the ability of endophytic fungi filtrate decreased due to the presence of the carried-away fungi cells during the filtration process. However, those carried-away fungi cells died in the presence of the liquid smoke. This was confirmed by a study conducted by Oramahi *et al.* (2011) as well as Oramahi and Yoshimura (2013) which showed that liquid smoke possesses antifungal properties because it contains phenol, carbonyl and acid compounds. In addition, acetic acid and propionate components are able to neutralize fungi cells and inhibit enzyme activity (Karseno *et al.* 2001). Therefore, in addition to inhibiting the growth of pathogenic fungi, liquid smoke is also suspected to inhibit endophytic fungi.

CONCLUSION

Liquid smoke of "Ulin" wood and endophytic fungi of "Gogo" rice var. *maninjau* has the ability to inhibit the growth of pathogen *P. oryzae*. Concentrations of 0.17% to 1.75% liquid smoke and 2% to 10% endophytic fungi filtrate showed high inhibition percentage against pathogen *P. oryzae*. The test of synergism, however, did not show an increase in inhibition percentage. Further research on the inhibitory ability and the best concentrations of liquid smoke and endophytic fungi filtrate should be conducted to provide more protection against plant pathogens, especially for rice plants.

ACKNOWLEDGMENTS

The authors sincerely thank the Ministry of Research, Technology and Higher Education of the Republic of Indonesia who has funded this research (2018-2019), as well as to the Talasiana Business group, Ranggang Village, Tanah Laut Regency. Our sincere thanks go to Mrs. Tambuy

as an elder of Dayak Maanyan Community, Jaar Village area, East Dusun District, East Barito Regency, Central Kalimantan Province.

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