THE PROSPECT OF ROT FUNGI IN CONTROLLING OF *TRICHODERMA* SPP. IN MUSHROOM CULTIVATION

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ABSTRACT

Trichoderma spp. are often encountered in mushroom cultivation. They could contaminate substrates at the time of spawning or after. The heavy infections could reduce the yield considerably. The JPA isolate is a rot fungus that was found in oil palm plantations. Based on the initial detection by paper disc method, the methanolic extract of JPA mycelia cultivated in 1.5% of malt extract could inhibit the growth of *Bacillus subtilis*. Based on the bioautography test, the crude extract gave the inhibition zone as well with the R_f in a range of 0.7-0.8. The antagonistic test of JPA isolate with *Trichoderma* sp. S2-2 was conducted in the baglogs containing 500 g of oil palm empty fruit bunches (OPEFB) supplemented with 15% bran, 1.5% lime and 1.5% gypsum. Both isolates were inoculated at both sides of the baglogs simultaneously. Based on this test, JPA isolate could inhibit the growth of *Trichoderma* sp. S2-2 and covered the colony of *Trichoderma* sp. S2-2 and the OPEFB substrates after 8 weeks of incubation. Furthermore, the methanolic extract of the OPEFB substrates inoculated only with JPA isolate were tested against *Trichoderma* sp. S2-2 by paper disc method. The results showed that the extracts could inhibit the growth of *Trichoderma* sp. S2-2 with formation of very thin *Trichoderma* colony zones or clear zones around the paper discs containing the extracts. Based on ITS1 and ITS4 squences data, JPA isolate is *Lentinus* sp. but the data do not match closely with any species registered in GenBank. Further research is the development of the extract formula and test it in mushroom cultivation. The mycelial extracts isolated from other tropical *Lentinus*, especially *Lentinus cladopus* will also be tested.

Keywords: control, JPA isolate, rot fungi, *Trichoderma*

INTRODUCTION

Indonesian society has known wild mushrooms as food since ancient times. Straw mushroom is the first wild mushrooms cultivated in Indonesia. At present many mushrooms have been cultivated in Indonesia, among others, button mushroom, ear mushroom, shiitake mushroom, etc. But white oyster mushrooms are widely cultivated and accepted by society in Indonesia since 20 years.

Generally mushroom cultivation in Indonesia is still done traditionally or moderately. Only a few large companies that cultivate mushrooms, especially button mushroom, using the latest equipment and methods. Consequently, mushroom cultivations in Indonesia have often pest and disease problems. In the pocket book of Indonesian Ministry of Agriculture [1] for the farmers, they suggested applying of prevention, physical and chemical controls using such as lime, dichloros, dicofol, malathion, diflubenzuron for control of undesirable insects while 0.5% formalin solution, benomyl, carbendazim for control of fungal diseases although over time, the mushroom insects and pathogens have developed resistance to the pesticides.

Trichoderma spp. is the cause of green mould disease in mushroom cultivation in many countries and led to losses of up to tens of millions of dollars. Likewise, *Trichoderma* is often complained by farmers in Indonesia. Generally these fungi are not controlled by the farmers and losses are never counted. Farmers leave the contaminated substrates at growing house and near the farm, consequently the fungal spores spread around the house and reinfection occurred easily on new bags and cultivation equipment.

Trichoderma can be prevented by applying strict sanitation, but if heavy infection has already occurred then the proper fungicide should be applied to the spawn, casing or compost. A number of fungicides are recommended for control of *Trichoderma* such as Environ [2], Prochloraz, mixture of Prochloraz and Carbendazim [3], Thiabendazol [4] and Imidazole

[5]. Many chemical compounds are no longer effective and there is emergence of resistant strains. Therefore, growers have to improve disease prevention and research is to be carried out for alternative control methods.

Many researchers have conducted studies on *Trichoderma* in order to control these fungi in mushroom cultivation. Antagonistic bacteria against *Trichoderma* spp. have been found [6, 7]. The brown strains of *Agaricus bisporus* were highly resistant to green mold caused by TA4 [8]. Effort was made to obtain metabolites that can inhibit the growth or induce resistance to *Trichoderm*a but the Lysing Enzymes of *Trichoderma harzianum* could not increase the resistance of *A. bisporus* against *T. aggressivum* [9].

The purpose of this research is to determine the ability of the colony and secondary metabolites of JPA isolate in inhibiting the growth of *Trichoderma*. JPA isolate is a wood rot fungus that was found in oil palm plantation. In addition, the position of JPA isolate was investigated based on phylogenetic analyses of ITS sequences.

MATERIALS AND METHODS

Isolates

JPA isolate and *Trichoderma* sp. S2-2 were isolated from fruiting body grown on root and soil of oil palm plantation, respectively. Both isolates were stored on agar media of PSA (200 g potatoes, 20 g sucrose, 15 g agar and 1 l distilled water) at room temperature.

Direct confrontation between colonies

Confrontations between JPA isolate and *Trichoderma* sp. S2-2 were investigated in 35 x 20 cm sized polyethylene bags (baglogs) containing 500 g of oil palm empty fruit bunches (OPEFB) supplemented with 15% bran, 1.5% lime and 1.5% gypsum. Initially, OPEFB was chopped, water soaked for 24 hours and drained in a closed plastic bags which were hung for 6 hours to gain 75% moisture content. Afterwards all bags were sterilized, spawned with grain spawns with four different treatments of inoculations, i.e. the substrates were inoculated with JPA isolate (JPA); with *Trichoderma* sp. S2-2 (T); with both isolates (JPA+T) and with *Trichoderma* sp. S2-2 but the sterile substrates were previously fully colonized by JPA isolate [(JPA)+T]. All substrates were incubated at room temperature (in a range of 28-30 ^{o}C) for 8 weeks. Ten replications were used for each growing trial.

Extraction of mycelia and substrates of JPA isolate

Mycelia were obtained from ten liquid cultures of JPA isolate. Each culture consisted of 1.5% of malt extract. One of 7 mm inoculum was inoculated on the surface of each culture containing 100 ml of media in 250 ml of Erlenmeyer flask. Cultures were incubated at room temperature at static condition for 4 weeks. Substrate samples were obtained from ten substrates, each containing of 500 g OPEFB supplemented with 15% bran, 1.5% lime, 1.5% gypsum and inoculated with JPA isolate, then incubated at room temperature for 8 weeks. Substrate samples were obtained as well from mixture substrate containing *Paraserianthes falcataria* sawdust and OPEFB with proportion 1:1, respectively. Mycelia extraction is done as follows: mycelia were separated from the culture filtrates with filter paper. Mycelia of each culture was ground with mortar and then extracted twice with methanol at a concentration of 5%, respectively and agitated overnight on a rotary shaker for each extraction. The mycelial methanolic extract was separated from residues with fritted glass filter no. 3 and then dried under vacuum, with a 30 ^{o}C water bath and a rotary evaporator, then redissolved in methanol. The mycelial ethanolic extract was obtained as well with the same procedure. Substrate extraction is done as follows: substrates were ground by the hammer mill and extracted twice with methanol at a concentration of 10%, respectively and left overnight for each extraction. The further procedure was similar to those described in the section on mycelial extraction.

Activity test

The extract activities were tested by paper disc method against *Bacillus subtilis* and *Trichoderma* sp. S2-2. The test media were tryptone glucose yeast (TGY) (tryptone 5 g, yeast extract 5 g, glucose 1 g, K_2HPO_4 1 g, agar 7.5 g, distilled water 1 l) for *B. subtilis* and (PSA for) *Trichoderma* sp. S2-2. The cell suspensions of *B. subtilis* were obtained by growing 100 µl of stock suspension on 100 ml of TGY medium which was then 25 ml poured on Petri dish and incubated at room temperature for 15 days. Each Petri dish was then added with 3 ml of sterile distilled water, the suspension was

then scrapped with glass rod, filtered with sterile cotton and pasteurized three times at 60°C for 30 min.The cell suspensions of *Trichoderma* sp. S2-2 were obtained by growing one of 7 mm inoculum on PSA and incubated at room temperature for 5 days. Each Petri dish was then added with 10 ml of sterile distilled water, the suspension was then scrapped with glass rod, filtered with sterile cotton. One percent of each extract were poured on 12 mm paper discs. The controls were the discs with similar solvent without the extract. The solutions were dried by air in order to remove solvent. The discs were sterilized with UV ray (254 nm) for 30 min and then placed on the surface of test media containing 1-3 x 10^5 cells of target microbes per ml. These test media were then incubated at 10°C for 3-4 hours in order to diffuse of extracts into agar media. After incubation for 24 hours at 35°C for *B. subtilis* and for 2 days at room temperature for *Trichoderma* sp. S2-2, activities of extract were estimated by measuring the diameters of inhibition zones which had been reduced by the diameters of disc.

Detection of active compounds

Detection was done by bioautography method using analytic thin layer chromatography (TLC) and *B. subtilis* [10]. The medium for *B. subtilis* was TGY. One hundred microliter of this cell suspension were added to 100 ml of TGY medium and then medium was poured on TLC plate. TLC was carried out with silica gel plates (Merck 60 F254, 0.1 mm thick, 20x5 cm). The mycelial methanolic extracts were deposited as spots and developed in a butanol-acetic acid-water mixture (3:1:1, vol:vol:vol). Dry chromatograms were covered with 15 ml TGY medium containing *B. subtilis* and then incubated at 10 °C for 3-4 hours in order to diffuse of extracts into agar media. The locations of inhibition zones were estimated by measuring the R_f values of inhibition spots.

Molecular character of JPA isolate

Two-week-old JPA isolate colony cultured in 100 ml of 1.5% malt extract and 0.5% peptone in 250 ml Erlenmeyer flask at room temperature and static conditions was used to extract genomic DNA. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) and performed according to the instructions of the manufacturer with slight modifications. DNA concentrations were estimated visually in 1.2% agarose gel. The PCR reactions were performed in a 25 ì l volume (8.8 µl ddH_2O; 5 µl 5x Q5 buffer; 5 µl 5x Q5 Enhancer; 1µl dNTPs; 1 µl 10 pmol ITS 1–F; 1 µlpmol ITS4-R 10; 3 µl DNA extract; 0.2 µl Tag HF). The thermal cycles consisted of 95 °C for 2 min, followed by 35 cycles at 95 °C for 45 s, 58 °C for 1 min, 72 °C for 1 min, with a final extension step of 72 °C for 7 min and storage 15 °C for 20 min. The PCR products were verified by staining with ethidium bromide on 1.5% agarose electrophoresis gels stained with ethidium bromide in 1x Tris-boric acid EDTA buffer. ITS 1 and ITS 4 were used to sequence both strands of DNA molecules at PT. Genetika Science Indonesia. Phylogenetic analysis was performed using Mega 5.5.

RESULTS AND DISCUSSION

Direct confrontation between colonies

The results showed that the antagonistic interaction had occurred between *Trichoderma* sp. S2-2 and JPA isolate or vice versa when both isolates were simultaneously inoculated on each side of polybags (JPA+T treatment) (Fig. 1). It depended on the period of colonization, at 30 days of interaction, *Trichoderma* sp. S2-2 colonized 75% of baglog volumes while JPA isolate colonized only 25% of bag log volumes. It seems *Trichoderma* sp. S2-2 inhibited the growth of JPA isolate, because if only JPA isolate was grown on the substrates (JPA treatment), it took 35 days for JPA isolate to fully colonize the substrates. At 45 days of interaction, JPA isolate started to give antagonistic interaction against *Trichoderma* sp. S2-2 because JPA isolate colonized 50% of baglog volumes and covered of *Trichoderma* sp. S2-2 colonies which were previously occupied 75% of baglog volumes. Furthermore, at 60 days of interaction, JPA isolate colonized 75% of baglog volumes, whereas *Trichoderma* sp. S2-2 colonized only 25% of baglog volumes. So, JPA isolate could inhibit the growth of *Trichoderma* sp. S2-2 and covered the colonies of *Trichoderma* sp. S2-2 and the OPEFB substrates. The sterilization of the colonized substrates [(JPA)+T treatment] could damage the active metabolites which were produced probably by JPA isolate. Therefore *Trichoderma* sp. S2-2 could grow on such sterile substrates which were previously full colonized by JPA isolate and it took 22 days for 100% substrate colonization. It also took 26 days for 100% substrate colonization for only *Trichoderma* sp. S2-2 grown on the substrates (T treatment).

Extract Activities of mycelia and OPEFB substrates of JPA isolate against *Bacillus subtilis* and *Trichoderma* sp. S2-2

The previous results showed that JPA isolate could inhibit the growth of *Trichoderma* sp. S2-2 on OPEFB substrates. So there is a possibility that JPA isolate had the ability to produce active compounds that inhibit the growth of *Trichoderma* sp. S2-2. Tests showed that the extract of OPEFB and mixture substrates could inhibit the growth of *B. subtilis* which was used as a sensitive test microbe (Fig. 2, 3). The same results were shown by methanolic and ethanolic extract of mycelia, even the activities were higher. Further tests were conducted against *Trichoderma* sp. S2-2. The OPEFB substrates inoculated only with JPA isolate were tested against *Trichoderma* sp. S2-2 and the results showed that the OPEFB substrate extracts could inhibit the growth of *Trichoderma* sp. S2-2 with formation of very thin *Trichoderma* sp. S2-2 colony zones (Fig. 3B) or clear zones around the paper discs containing the extracts (Fig. 3C). The clear zones were not rounded with diameter of zones in a range of 20-38 mm. In the next test the methanolic extract of mycelia will be tried against *Trichoderma* sp. S2-2. In addition the methanolic extract of JPA mycelia could inhibit the growth of *Ganoderma boninense* as well (unpublished).

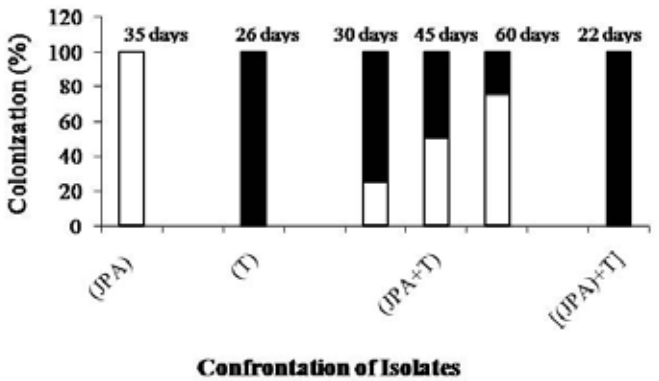


Figure 1. The oil palm empty fruit bunches substrates were colonized by JPA isolate (JPA) (blank bars); *Trichoderma* sp. S2-2 (T) (black bars); both isolates (JPA+T) and *Trichoderma* sp. S2-2 but the sterile substrates were previously full colonized by JPA isolate [(JPA)+T].

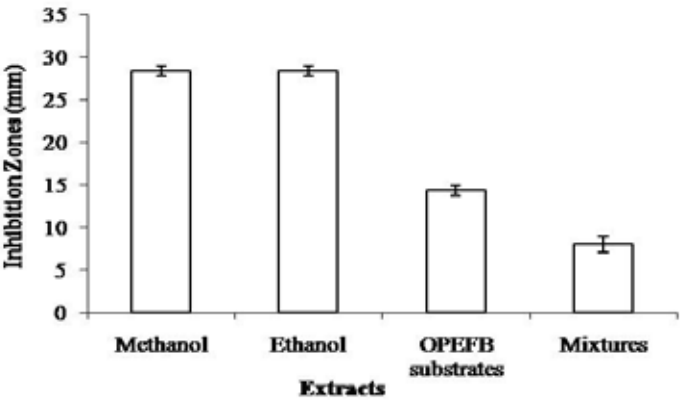
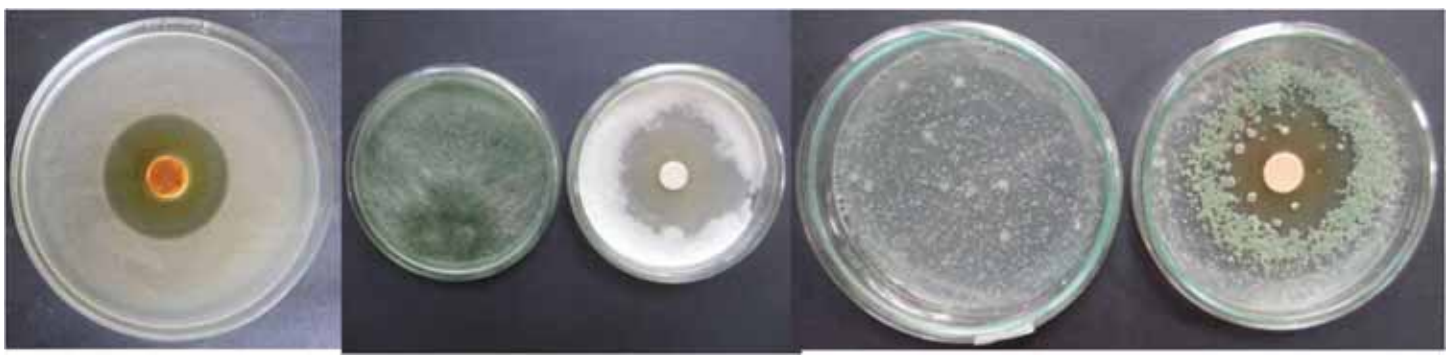


Figure 2. Mycelial extract, OPEFB substrate extractand mixture extract activities of JPA isolate against *Bacillus subtilis*



A **B** **C**

Figure 3. Mycelialextract activity of JPA isolate against *Bacillus subtilis* (A), OPEFB substrate extract activities of JPA isolate against *Trichoderma* sp. S2-2(B and C)

Detection of active compounds

Based on the bioautography test, the mycelial crude extract of JPA isolate gave the inhibition zone against *B. subtilis* as well with the R_f in a range of 0.7-0.8 (Fig. 4). This active compound should be separated and tested against *Trichoderma* sp. S2-2. The characterization of this compound is in progress.

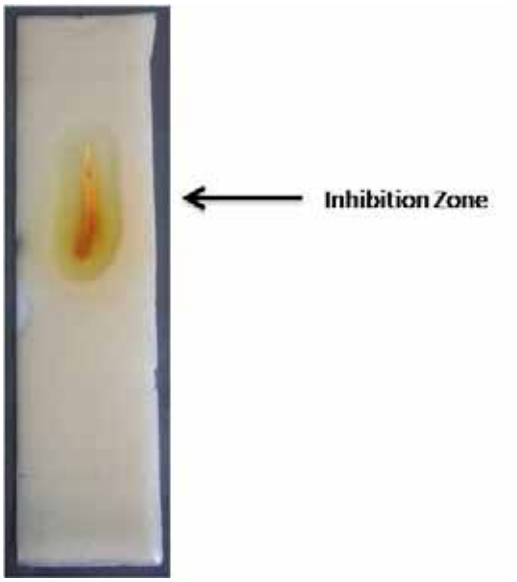


Figure 4. Detection of active compound from mycelial crude extract of JPA isolate on thin layer chromatogram. The activity was tested against *B. subtilis*

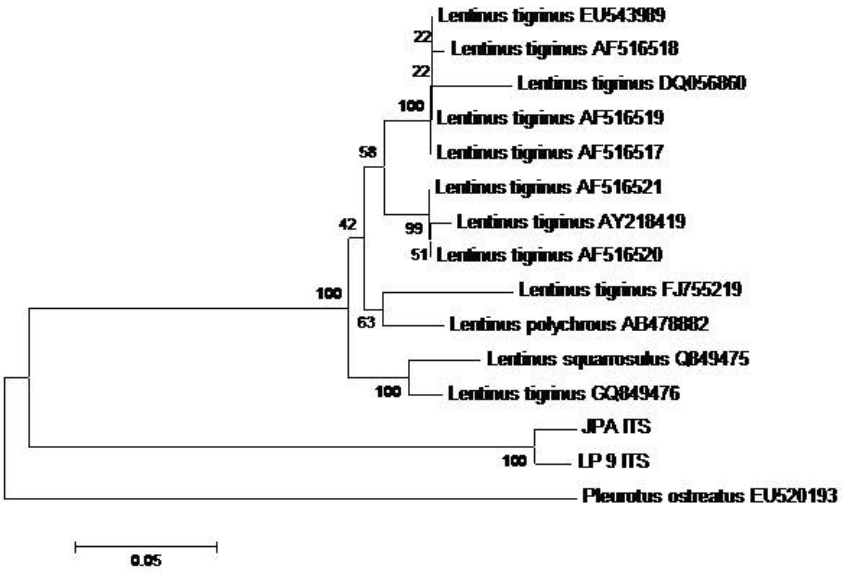


Figure 5. Phylogenetic relationships among JPA isolate and some *Lentinus* and *Pleurotus ostreatus* EU520193

Molecular character of JPA isolate

JPA isolate was grown on root of oil palm trees. Based on morphological characters of fruiting body, JPA isolate was similar to *Lentinus* or *Pleurotus* characters with white pileus, short and off-centre stipe (unpublished). Therefore we investigated the position of JPA isolate based on phylogenetic analyses of the nrITSsquences. Based on ITS sequences data of JPA isolate, they did not match closely with any species presently lodged in GenBank but there were phylogenetic relationships among JPA isolate with some selected species of *Lentinus*. JPA isolate was positioned in the same clade as some selected species of *Lentinus* and the tree is rooted with *Pleurotus ostreatus* EU520193 (Fig. 5). JPA isolate is in need of further research including morphological and cultural identification. For this purpose, JPA isolate will be attempted to cultivate to obtain its fruiting body.

Future research is to test JPA isolates as inducers of resistance in white oyster mushrooms by determining of laccase enzyme activity after confrontation between the two fungi. Increased laccase activity is a way to overcome another antagonist by *Pleurotus*. The laccases activity increase in *Pleurotus ostreatus,* induced by antagonistic bacteria *Bacillus* spp such as *Paenibacillus polymyxa* [11].

CONCLUSION

JPA isolate was potential as source of antimicrobial compounds and their active compounds could probably be used as biopesticides in control of mushroom diseases.

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