Isolation and identification of Endophytic Fungus *Fusarium sp* from Agarwood (*Aquilaria sp*) population originated from the forest of Aceh Tamiang district, Indonesia

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Abstract. *Aquilaria sp* is one of higher plant types that can produce agarwood. Agarwood is commonly stimulated by the biological response of endophytic fungal infection of *Fusarium sp*. This research aims to isolate and identify endophytic fungus *Fusarium sp* from *Aquilaria sp* populations originated from the forest of Aceh Tamiang District. The pure isolates of *Fusarium sp* derived from *Aquilaria sp* that reinfected on *Aquilaria sp* populations to stimulate agarwood formation. The endophytic fungal isolates of *Fusarium sp* were growth on a selective media of Sabouraud Dextrose Agar (SDA) containing antibiotics Streptomycin 10 µg/dl and Chloramphenicol 25µg/dl. Ten percent of infected *Aquilaria sp* from the population was taken aseptically. Isolation and identification of endophytic fungus *Fusarium sp* were conducted from June to August 2016. The results showed that within 10 infected *Aquilaria sp*, several microbe species were found such as three *Fusarium sp*, six *Aspergillus sp*, two *Rhizopus sp*, two *Penicillium sp*, one *Bacillus sp*, one *Actinomyces sp* and one *Streptomyces sp* species. The result of identification towards three species of endophytic fungus *Fusarium sp* that has been purified were strongly suspected to be *Fusarium solani*, *Fusarium oxysporum*, and *Fusarium moniliformae*.

1. Introduction

*Aquilaria sp* is one of the *Thymelaeaceae* family plant. This plant is also known as aloe plant because its ability to stimulate the formation of resin or aloe resin (agarwood). *Aquilaria* has several species, but species that grow a lot in Sumatra, Borneo, and Malaysia are *Aquilaria microcarpa*, *Aquilaria ardesiaca*, *Aquilaria hirta*, *Aquilaria beccariana* and *Aquilaria ardesiaca* [1]. The province of Aceh, particularly in Aceh Tamiang Regency, has a large biodiversity of *Aquilaria sp*. This plant has high non-wood forest economic value. Up to this point, this potential did not get much attention from both

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educational and government institutions because of the lack of knowledge and methods of aloe
cultivation, especially in Aceh Tamiang district.

_Fusarium sp_ is one of stimulant (probiotic) for agarwood formation on _Aquilaria spp_ stem [2].
several injection and infusion methods of _Fusarium sp_ has been commercialized to stimulate
agarwood formation on _Aquilaria sp_ plant, but this method has not showed satisfying results. It may
be caused by several factors like biological factor. For that reason, it is important to conduct _in situ_
endophytic fungal isolation of _Fusarium sp_ from _Aquilaria sp_ stem from the forest area population for
reinfection using _Fusarium sp_. Reinfection is essential to stimulate the in situ formation of endophytic
fungus _Fusarium sp_ so that the secondary metabolites produced by endophytic fungus is effective
against the pathogen bacteria, thus the quality of the agarwood is improved. Some endophytic fungal
species which had been commercialized as probiotics for reinoculum in _Aquilaria sp_ cultivation are
_Torula sp., Cladosporium sp., Aspergillus tamarii, Pencillium citrinum, Botryodiplodia theobromae,
Cytosphaera mangiferae, Epicoccum granulatum, Fusarium cylindrosporum, Fusarium oxysporum,
Fusarium solani_ and _Chaetonium globosum_.

Agarwood can be found in _xylem_ stem of _Aquilaria sp_ (Figure 1), this is formed by biological
response to _Fusarium sp_ either naturally or artificially with endophytic fungal injection method.
Agarwood is an export commodity which has a high economic value as a raw material for perfume
and pharmaceutical industries or medicines. Agarwood produces fragrant aroma when burned because
it contains _agarospiral_ and _jinkohol-eramol_.

![Figure 1. A; resin or aloe's resin (Agarwood) in xylem stem of _Aquilaria sp_ occurred naturally, B; resin or aloe's resin (Agarwood) formed by the endophytic fungal injection method, C; resin or aloe's resin (Agarwood) of Kemedang type.](image)

Agarwood that formed, is a product from fungal infection process that is opportunistic on xylem
infected _Aquilaria sp_. It suspected that the formed resin is the resistance of phytoalexins in cambium
producing aromatic oils. For a long time, the resin will be formed in endophytic infected xylem. The resin
generally is light brown and later turns black. Besides containing _agarospiral_ and _jinkohol-eramol_, the resin also contains other chemical compounds such as _ethyl methoxyphenyl chromate_ (27%) and _phenylethyl chromate_ (15 %).

Endophytic fungus on agarwood is opportunistic and symbiotic microorganisms that live in the
plant cell and stimulate secondary metabolite formation helping plant as a self defense against the
predator or other pathogen bacteria. Endophytic microorganisms can be either bacteria or fungus in
_xylem_ or _floem_ tissue. Sometimes their presence does not cause a disturbance on plants[3]. Endophytic
fungus _Fusarium sp_ is the most predominant fungi found on the _Aquilaria malaccensis Lamk_. stem.
Agarwood is generally formed on the top, middle and bottom parts of the stem [4]. Some studies
indicate that the endophytic fungus _Fusarium sp_ has an important role as a stimulant (probiotics) for
_Aquilaria sp_ in the agarwood formation from response to fungal infection (endophyte) in xylem tissue.
This research aims to map the plant population of _Aquilaria sp_. in Aceh, particularly in Aceh Tamiang
Regency, followed by isolation and identification of endophytic fungus _Fusarium sp_. This study also
aims to find out the type of endophytic fungus that presents in _Aquilaria sp_. There are four selected
populations of *Aquilaria* sp such as a population area of forest Sekrak, Seruwai, Kampung Pipa and Selele, Aceh Tamiang regency. In this research, isolation and identification methods of endophytic fungus *Fusarium* sp were performed on selective media *Sabouraud Dekstrosa Agar* (SDA) containing *Streptomycin* [10µg/dl] and *Chloramphenicol* [25µg/dl]. To prevent the occurrence of contamination and to ensure the purity of endophytic fungal isolates of *Fusarium* sp, macroscopic and microscopic inspection were conducted to see the fungus morphology.

2. Experimental

2.1 Materials

'Sabouraud Dekstrosa Agar' (SDA) media, *Nutrient Agar* (NA) media (Oxoid), *chloramphenicol*, *Nystatin*, aquadest, and sodium hypochlorite 5.3%. The materials and Tools Used for sterilization are alcohol 70%, sodium hiphoklorit (klorox) 0.5%, the sterile water and sterile wipes to dry samples. The media used is MA 2% (Malt Agar) containing 20 g of extract malt, 20 g of agar, 1000 mL of tap water and 100 mg of Streptomycin to suppress the growth of bacteria. The equipment used were a test tube, , measuring cup, thermometer. Erlenmeyer (size 1000 ml, 500 ml and 250 ml), beaker glass, aluminium foil, laminar air flow cabinet, erlenmeyer, scissors, Petri dish, bag, cork borer, Oose needle and measuring cup [5]. All glassware is sterilized with the dry heat sterilization techniques using oven at 170 °C for 40 minutes.

2.2 Methods

2.2.1 Sampling of Endophytic Fungal *Fusarium* sp on *Aquilaria* sp. Population. The survey of *Aquilaria* sp population was done in Aceh Tamiang forest area starting at early June 2017 until June 2018. The *Aquilaria* sp population selected on four locations of forest area which is the natural habitat of *Aquilaria* sp i.e. Sekrak, Seuwai, and Selele and Kampung Pipa. Sampling of endophytic fungus *Fusarium* sp is done by observing the wounded limb, stem or branch that have colour range from brown to black, which are allegedly contained resin. The wounded bark was cleaned, slashed with knives, and tested the aroma with burned test. For the endophytic fungal isolation of *Fusarium* sp on plant parts containing resin was done with sterile sculpture of xylem 10 times. Samples are put into adhesive plastic aseptically and taken to the laboratory to do the isolation and identification of fungal species.

2.2.2 Preparation of Media *Sabouraud Dekstrosa Agar* (SDA). 6.5 g SDA powder was dissolved in 975 mL aquadest in an Erlenmeyer, the flask was then heated until SDA dissolves homogeneously. The solution was sterilized by autoclave at 121 °C for 15 minutes. After that, the solution was cooled at room temperature until the temperature dropped to 40-50 °C. 25 mL of the SDA media solutions were taken and put in the erlenmeyer 100 mL followed by added 100 mg antibiotic of Streptomycin and then was dissolved homogeneously (Solution A). 25 mL SDA media solution was taken and put in other erlenmeyer followed by the addition of 250 mg antibiotic of chloramphenicol and then was dissolved homogeneously (Solution B). Solution A and Solution B were mixed homogeneously until the temperature dropped to 40 °C. The mixture was poured into petridish (9 mm diameter), each of petridish was 18-20 mL. The mixture was cooled until it was solidified. This SDA media was used to isolate endophyte fungung *Fusarium* sp. For isolate culture rejuvenation media of *Fusarium* sp, 5-8 mL SDA solutions were taken and put into sterile tubes and then was placed on the 30-45 °slope position until it was solidified [6].

2.2.3 Preparation of Media *Nutrient Agar* (NA). 28 g of NA powders were dissolved with 1000 mL sterile aquadest in an erlenmeyer 1 L. The solution was heated and dissolved homogeneously then sterilized by autoclave at 121 °C for 15 minutes. The NA solution was cooled at room temperature until the temperature dropped to 40-60 °C. 20 mL NA solution were introduced into an erlenmeyer...
100 mL followed by the addition of 100 mg antibiotic of Nystatin, dissolved homogeneously and cooled until the temperature dropped to 40 °C. The solution was poured into petridish (9 mm diameter), each of petridish was 18-20 mL. The solution was cooled until it was solidified. This media was a selective media for bacterial growth [6].

2.2.4. Inoculation of endophytic fungal isolate. Inoculation was done by cutting the wood fiber containing agarwood into 0.5-1.0 cm long bars. The wood fiber was sterilized using sodium hypochlorite 5.3% for 1 minute followed by several times rinsing with sterile aquadest and alcohol 70% for 15 seconds, respectively. The wood fiber was drained above sterile filter paper for several minutes at 30 °C in an incubator. In aseptic circumstances, the pieces of wood fiber were placed above the petridish containing SDA media and antibiotic. The petridish was incubated at room temperature in the septic cabinet for 48-72 hours. Fungus that growth was identification both in macroscopic and microscopic. The identification of edophytic fungal isolate was performed using NA contained media with the same previous method.

3. Results
Aquilaria sp population selected on four different locations in the forest that were the natural habitat of Aquilaria sp namely, Sekrak, Seuwai, Selele and KampungPipa can be observed in Table 1.

Table 1. The endophyte fungal sampling location on some Aquilaria sp populations on four different locations in Aceh Tamiang Regency.

| No | Sampling Location | Number stem samples | Number endophytic fungal samples |
|----|-------------------|---------------------|----------------------------------|
| 1. | Sekrak            | 3                   | 3                                |
| 2. | Selele            | 2                   | 2                                |
| 3. | Kampung Pipa      | 2                   | 2                                |
| 4. | Seruwai           | 3                   | 3                                |
| Total |             | 10                  | 10                               |

Sampling of wood fiber containing agarwood for isolation of the endophytic fungus in ten different sampling locations can be observed in Figure 2.

Figure 2. The sampling of endophytic fungus Aquilaria sp. a; sampling of endophytic fungus with three month incubation commercial inoculation, b; sampling of endophytic fungus from xylem tissue of aquilaria sp., c; sampling of endophytic fungus from natural infected fungus
Table 2. Endophytic fungal and bacteria isolates isolated from ten stems of *Aquilaria* sp from four different forest in AcehTamiang.

| No. | Sample Code | endophytic fungal isolates | endophytic bacteria isolates |
|-----|-------------|-----------------------------|------------------------------|
| 1   | 01          | *Aspergillus* sp and *Fusarium oxysporum*, *Fusarium oxysporum*, *Penicillium* sp. and *Rhizopus* sp. | Gram positive rods branch (*Actinomices/Streptomyces*), dan Bacillus sp |
| 2   | 02          | *Aspergillus* sp., *Curvularia* sp., *Fusarium solani*, and *Fusarium oxysporum* | Gram positive stem bark (*Actinomices/Streptomyces*), and Bacillus sp |
| 3   | 03          | *Aspergillus* sp., *Fusarium moniliformae* | Gram positive stem bark (*Actinomices/Streptomyces*), and Bacillus sp |
| 4   | 04          | *Fusarium solani*, *Fusarium oxysporum*, and *Trichoderma* sp. (kapang) | Gram positive stem bark (*Actinomices/Streptomyces*), and Bacillus sp |
| 5   | 05          | *Fusarium oxysporum*, and *Penicillium* sp., *Aspergillus* sp., and *Fusarium solani*, *Fusarium oxysporum*, *Trichoderma* sp. (kapang), and *Fusarium solani*. | Gram positive stem bark (*Actinomices/Streptomyces*), and Bacillus sp |
| 6   | 06          | *Fusarium oxysporum*, and *Penicillium* sp., *Aspergillus* sp., and *Fusarium solani*, *Fusarium oxysporum*, *Trichoderma* sp. (kapang), and *Fusarium solani*. | Gram positive stem bark (*Actinomices/Streptomyces*), and Bacillus sp |
| 7   | 07          | *Aspergillus* sp., *Rhizopus nigricans*, *Trematosroma* sp., (kapang), and *Trichoderma* (kapang). | Gram positive stem bark (*Actinomices/Streptomyces*), and Bacillus sp |
| 8   | 08          | *Aspergillus* sp., *Fusarium moniliformae*, *Fusarium oxysporum*, and *Trichoderma* (kapang). | Gram positive stem bark (*Actinomices/Streptomyces*), and Bacillus sp |
| 9   | 09          | *Aspergillus* sp., *Rhizopus nigricans*, *Trematosroma* sp., (kapang), and *Trichoderma* (kapang). | Gram positive stem bark (*Actinomices/Streptomyces*), and Bacillus sp |
| 10  | 10          | *Aspergillus* sp., *Fusarium moniliformae*, *Fusarium oxysporum*, and *Trichoderma* (kapang). | Gram positive stem bark (*Actinomices/Streptomyces*), and Bacillus sp |

Sample of wood fiber was put into sterile adhesive plastic aseptically (can be seen in Figure 3) and taken to laboratory for isolation and identification of fungal and bacterial species that produced from agarwood. *Lacto phenol cotton blue (LPCB)* was used to determine the morphology of endophytic fungus and bacteria such as conidiospore, conidia, hifa and septa that can be seen in Figure 4.

The observation of conidium structures of fungus that grows on the media was conducted under the microscope with 400x magnification. Based on the surface structure of the colony, texture, color, aerial hyphae vegetative, and the morphology of pseudohypha, septahypha,conidia form (microconidia and macroconidia), conidiospore and the mycellium of endopyhtic fungus *Fusarium sp*, we concluded that the endophytic fungal isolates of *Fusarium sp* were *Fusarium solani*, *Fusarium oxyforum* and *Fusarium monyliformae* (Figure 4).
Figure 3. The wood fibre containing agarwood from sampling techniques, a; sample of wood fibre containing agarwood in sterile adhesive plastic, b; endophytic fungus from xylem, c; endophytic fungus from wood fibre of *Aquilaria* sp.

Figure 4. The endophytic fungal isolates of *Fusarium solani*, *Fusarium oxyformum* and *Fusarium moniliformae* grown on SDA media, A; *Fusarium oxyformum* grown on SDA media, incubation at room temperature, day 5 of incubation, B; *Fusarium oxyformum* grown on SDA media, incubation at room temperature, on day 7 of incubation.

To determine the type of bacteria, Gram-positive or Gram-negative, from endophytic bacterial isolates of agarwood, resin staining test was conducted using a solution of Gram Staining microscopically at 1000x magnifications followed by biochemistry fermentation test. This observation showed that the bacterial isolates commonly in basil form. The bacterial are Gram-positive (+) if the color of the bacterial cells change from violet to purple and Gram-negatif (-) if the cells were red in color.

Research results of *Aquilaria* sp population from the four different locations in Aceh Tamiang with macroscopic, microscopic and biochemistry test, we found thirteen (13) endophytic fungal isolates and three (3) endophytic bacteria isolates. The thirteen endophytic fungal isolates consisted of three (3) *Fusarium* sp, six (6) *Aspergillus* sp, two (2) *Rhizopus* sp and two (2) 1s *Penicillium* sp species. The
three (3) species of endophytic bacterial isolates was Gram-positive bacteria, namely Bacillus sp, Actinomyces sp and Streptomyces sp. Results identification of endophytic fungal and endophytic bacterial isolates can be seen in Table 2. The results of the identification of three (3) endophytic fungal isolates of Fusarium sp, we found three species namely Fusarium solani, the Fusarium oxyforum and Fusarium monyliformae.

4. Discussion
The population of Aquilaria sp from four isolation locations in Aceh Tamiang Regency mostly found in Sekrak and Seuawai areas reached 0.6-1 stem per hectare while the endophytic sample containing agarwood mostly found in Kampong Pipa behind the palm plantation border of PTP Aceh Tamiang. This area can be used as a location of agarwood agro-industry reinfection research. In addition, the forest is still natural and the local people ever get natural agarwood.

Fusarium sp generally breed with conidiophore which produces macroconidia and microconidia on each hypha area of mycelium, conidia is a black- that are proliferated spores antigen for tissue fitoaleskin compounds in cambium of Aquilaria sp. Microconidia is considered as foreign matter by plants. The fitoaleskin compound can be a resin mixed with black conidia, so the resin can be brown and fragrant. The resin will be accumulated on the xylem and floem. This vessels prevent the wound to spread to other tissue.

From the three species found from the isolation, only Fusarium solani and Fusarium oxyforum that were reported produced the natural agarwood. Both of this Fusarium sp fungal spesies allegedly act in the agarwood formation or in trials of infected birds on the stem of Aquilaria sp. This is an opportunity to develop agarwood agro-industry reininfected in Aceh Tamiang. Beside on infecting, Fusarium oxysporum caused withered deeces on potato and banana [7]. Both of this fungus will produce fermentation of secondary products [8]. The secondary product will be further analyzed in order to determine the content of metabolites contained in both species of Fusarium sp [9].

5. Conclusion
The population of Aquilaria sp in Aceh Tamiang mostly found in Sekrak, Seuruwai, Selele, and Kampung Pipa. In this study, we found that endophytic fungus containing agarwood producing an aromatic fragrant are commonly derived from Selele and Kampung Pipa. Based on the identification towards the endophytic fungus and bacteria that isolated from the infected Aquilaria sp, we have found thirteen (13) endophytic fungus and bacteria isolates. In this study, we concluded that the endophytic fungal isolates of Fusarium sp are Fusarium solani, Fusarium oxyforum and Fusarium monyliformae. These two spesies of Fusarium solani and Fusarium oxyforum are expected to be responsible in resin and secondary metabolite products formation in Aquilaria sp. Both of these possible Fusarium sp will produce secondary fermentation products [8], secondary product will be further analyzed to determine the content of metabolites in both species of Fusarium sp [9].

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