

Isolation and Characterization of Arbuscular Mycorrhiza Fungi from Gaharu Wood (*Aquilaria* spp.) Rhizosphere

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Abstract

In the last 30 years there have been increasing demand of agarwood. Unlimited hunting of agarwood causing it become threatened species. *Aquilaria* spp. is the main genus producing agarwood that considered as threatened species. Conserving *Aquilaria* spp. as germ plasm needs is essential against destruction. Arbuscular mycorrhizal fungi (AMF) have been used as inoculant to increase the growth of *Aquilaria* spp., but information about the existence of AMF in *Aquilaria* spp. rhizosphere has not been reported. This research was carried out with objective to identify AMF from *Aquilaria* spp. rhizosphere. Soil and root samples from rhizosphere of *Aquilaria* spp. taken from four location that is: Carita (*A. malaccensis*), Ciapus (*A. filaria*), Cifor (*A. crasna* & *A. malaccensis*), and Bantar Kemang (*A. beccariana*, *A. crasna*, & *A. malaccensis*). Roots staining was conducted with modified Phillips and Hayman procedure (1970). Soils sample was run dry wed and placed in pot culture with *Pueraria javanica* as the host plant. Spore was separated from pot culture using wet sieving and decanting method continued with centrifugation. The result showed the occurrences of AMF in *Aquilaria* spp. roots i.e. external hypha, internal hypha, coiled hypha, arbuscules, and vesicles. All AMF colony structure found in *Aquilaria* spp. roots show asosiation between the plant and AMF. Four species of AMF were found i.e. *Glomus* sp.1, *Glomus* sp.2, *Gigaspora albida*, and *Dentiscutata* sp.

Key Words: *Isolation, Arbuscule Mycorrhizae Fungi, Rhizosfer, Agarwood*

INTRODUCTION

Arbuscular mycorrhizal fungus (AMF) are fungus that has mutualistic association with plant roots. Estimated around 80% of AMF live in symbiosis with plant roots (Goss *et al.*, 2017). AMF is grouped under the phylum Glomeromycota (Schüßler *et al.*, 2001; Schüßler & Walker 2010). Glomeromycota comprise of 11 families : *Diversisporaceae*, *Acaulosporaceae*, *Sacculosporaceae*, *Pacisporaceae*, *Dentiscutataceae*, *Gigasporaceae*, *Intraomatosporaceae*, *Dentiscutataceae*, *Racocetraceae*, *Enthrophoraceae*, *Glomeraceae*,

Ambisporaceae, *Geosiphonaceae*, *Archaeosporaceae*, and *Paraglomeraceae* (Redecker *et al.*, 2013). A total number of 334 species have been identified to date (SYMPLANTA, 2020).

Gaharu wood known as agarwood, eaglewood, and aloewood in trading. Gaharu wood naturally formed special aromatic resinous product, therefore it is often used as insence, perfume and traditional medicine (Sumarna, 2013). These gaharu wood product are formed by several Thymelaceae family member. *Aquilaria* spp. Is one of the gaharu wood main species. There are six

Aquilaria species in Indonesia : *A. beccariana*, *A. cumingiana*, *A. filaria*, *A. hirta*, *A. malaccensis*, and *A. Microcarpa*. *Aquilaria* spp. distributed in western Indonesia, Sumatra, Kalimantan, papua barat and papua new guinea (Lee & Mohamed, 2016).

In the last 30 years gaharu wood demand is sharply increasing. These lead to higher intensity of gaharu wood hunting. Thus result in *Aquilaria* spp. scarceness. *International Union for Conservation of Nature* (IUCN) and *Covention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES) have reported all gaharu wood species including *Aquilaria* spp. in the preserved list (IUCN, 2018; CITES, 2019). Therefore preserving *Aquilaria* spp. is an obligatory to prevent extinction.

AMF plays an important role for the plants such as in water and mineral absorption, also in increasing plant immunity against plants pathogen. The main benefit for AMF symbiotic relation is wider soil area explored by AMF extracellular mycelium. AMF mycellia is longer and thinner than root hair, making its able of reaching wider soil pore. In addition AMF inoculum has ben reported to increase plant seeds growth (Goss *et al.*, 2017).

Despite the fact that AMF has been often used as inoculum to increase gaharu wood plant growth (Susilowati *et al.* 2019), information about indigenous AMF associated with *Aquilaria* spp. in nature is rarely observed. This paper aim to study indigenous AMF from *Aquilaria* spp. rizosphere.

MATERIAL AND METHODS

This study conducted at Forestry Microbiology Laboratory and Green House of forest and nature conservation research and development center, Forestry Department in Bogor. And also at Micology laboratory Bogor Agricultural University.

Soil and Roots Samples

Soil and roots samples were collected from four different sites, Carita-Serang (*A. malaccensis*), Ciapus-Bogor (*A. filaria*), Cifor-Bogor (*A. crasna* and *A. malaccensis*), and Bantar Kemang-Bogor (*A. beccariana*, *A. crasna*, and *A. malaccensis*). One Kg soil sample were collected in arround 0-20 cm depth using plastic bag. Soil sample were immediately air dried for culture. Roots sample were cleaned and soaked in formalin acetic acid alcohol (FAA) for fixation. FAA was made by mixing 13 ml formalin, 5 ml acetic acid (glacial) and 200 ml ethyl alcohol.

Root Staining

After fixation *Aquilaria* spp. roots then cut in to twenty small pieces one cm each. The roots are stained using modified Phillips and Hayman staining method (1970). Roots were subjected to bleaching in a solution of KOH 10% for 5-7 days. Every day KOH solution is replaced with the new one until roots are cleared. Browning of the solution is an indication of the clearing process. Cleared roots then washed with water and soaked in HCl 2% overnight. To minimize over colored roots, Roots were stained with 0.05% trypan blue overnight then transferred in to glycerol acid solution. Stained roots were observed for AMF internal structure under light microscope with 100-400 magnification. Infected root by AMF are known by the structure of AMF: vesicles, arbuscules, and hyphae on roots.

Pot Cultures

Rhizosphere soil sample were applied for three pot cultures. Pots were filled with sterilized zeolite and air dried rhizosphere soil sample, arranged in layer inside 200 g pots. The first layer was 50 g zeolite, following 100 g rhizosphere soil sample, and 50 g zeolite as the last layer. Once culture media ready, pots were planted with two weeks old *Pueraria javanica* as host plants.

Pot cultures grown in a greenhouse for three months to initiate AMF spore production. Three months old pot cultures were left to dry undisturbed within three weeks. After three weeks AMF spores were isolated and identified.

Spore Isolation

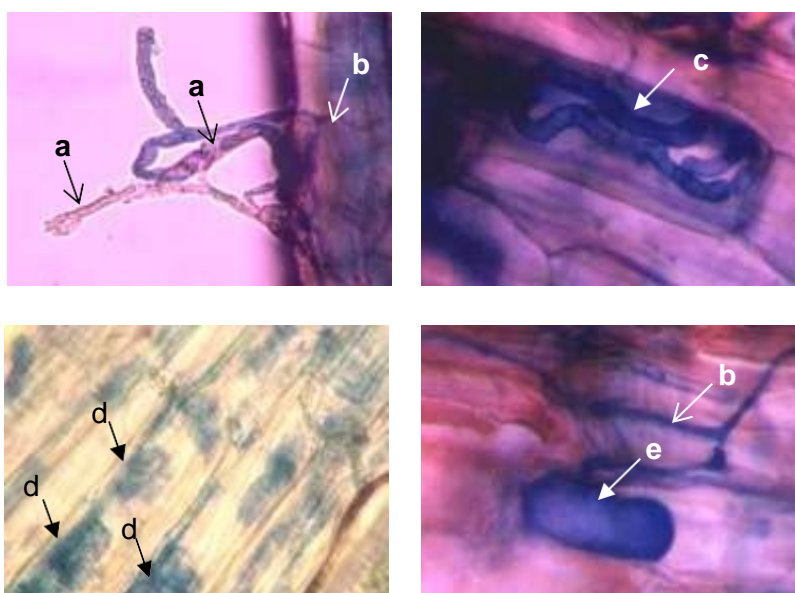
AMF spore were isolated by wet sieving technique followed by centrifugation method (Brundrett *et al.*, 1996). Culture media mixed thoroughly until homogenous. Afterwards 100 g of culture media mixed in 1000 ml water, leave it still for a few second, and then sieved with layered sieving from 425 μm , 250 μm , and 38 μm . Soil left on 250 μm and 38 μm pore washed with water, mixed together and centrifuged at 2000 rpm for three minutes. Pellet then added with 50% sucrose solution and mixed. Subsequently, pellet-sucrose solution centrifuged at 1000 rpm for 60 seconds. Supernatant sieved with 38 μm pore and washed with water. Spores were analyzed under compound microscope and light microscope.

Spore Identification

Spore identification was done manually by making permanent slides. Slides were made by using mounting media polyvinyl alcohol-lactic acid-glycerol (PVLG) and some of the slides were added with Melzer's reagent. Identification was done by sporulation structure and subcellular spore structure. Spore wall colour was described based on percentage of cyan blue, red magenta, yellow and black colour, known as C-M-Y-K colour chart (INVAM 2017a).

RESULTS AND DISCUSSION

Stained roots showed AMF infection on *Aquilaria* spp. These roots are from Bantar Kemang, Carita, Ciapus, and Cifor. AMF infection structures were external hyphae, internal hyphae, coiled hyphae, arbuscules and vesicle (Picture 1). All of these AMF infection structure were present in all *Aquilaria* spp. observed roots.



Picture 1. AMF infection on roots : a. external hyphae, b. internal hyphae, c. coiled hyphae, d. arbuscule hyphae, e. vesicles

All of *Aquilaria* spp. observed roots showed AMF infection structure on root cells. This means there are symbiotic association of AMF and

Aquilaria spp. plants. AMF roots infection structure observed were: external hyphae, internal hyphae, coiled hyphae, arbuscules, and vesicles.

External and internal hyphae are present in all AMF genus. While coiled hyphae is present in *Acaulospora*, *Archaeospora*, *Entrophospora*, *Gigaspora*, & *Scutellospora*. Moreover, in *Glomus* coiled hyphae is rarely present except in hyphae entry point on root. Arbuscules present in all AMF genus. Vesicles present in all AMF genus, except in *Gigaspora* and *Scutellospora* (Souza 2015).

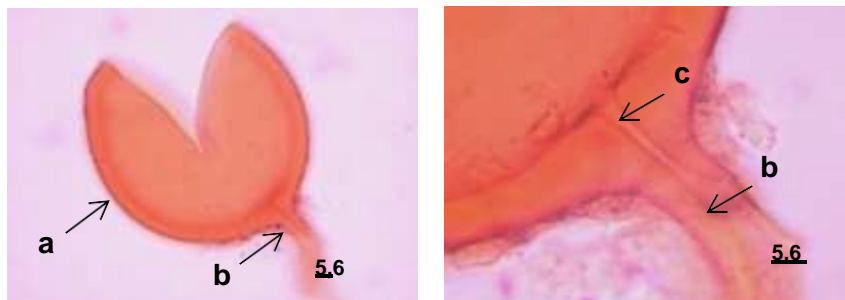
Spore collected and characterized from *Aquilaria* spp. rhizosphere were three AMF genus: *Glomus*, *Gigaspora* and *Dentiscutata*. Further identification showed *Glomus* sp.1, *Glomus* sp.2 and *Gigaspora albida*.

1. *Glomus* sp.1

Spore colour is red brown (0-20-80-0) to dark brown (20-80-100-0), subglobose to oval, 80,5-(77,62)-94.35 μm in diameter. Spore wall produce red to brown colour in PVLG and darker

colour in Melze's reagent. The outer layer has a smooth surface with no ornamentation. The spore wall consists only one layer (Picture 2a), All layers in cell wall originated from subtending hypal wall (Picture 2b), 3.33-(5,43)-7.77 μm thick. Flexible germinall wall is absent. Spore wall ended at the same point where subtending hypha attached (Picture 2c). Subtending hypha is 2.22-(3,60)-5.55 μm thick and 5.55-(10,22)-23.31 μm in diameter. It consists of two wall layers that are a continuation of the spore wall. It has the same colour as the spore wall and becomes thicker when continuously forming the spore wall.

Glomus sp.1 spore isolated from various soil sample and host plants It is originated from Bantar kemang (*A. malaccensis*), Carita (*A. malaccensis*), Ciapus (*A. filaria*), & Cifor (*A. crasna* and *A. malaccensis*).



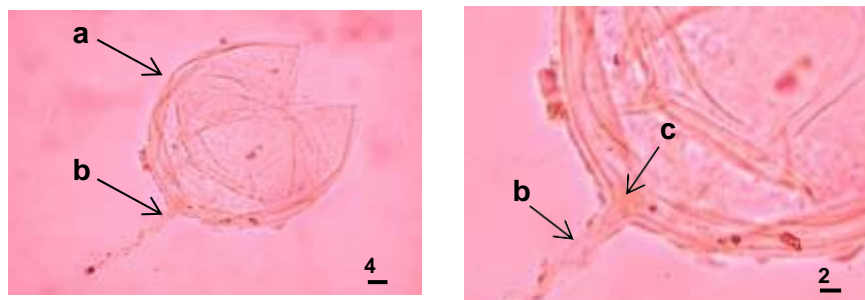
Picture 2 *Glomus* sp. 1 spore from *A. Malaccensis* rhizosphere, Carita: a. broken spore, b. subtending hyphae, c. pore

2. *Glomus* sp. 2

Spore is hyalin (0-0-5-0) to yellow 0-10-60-0), globose, subglobose to oblong shape, 51.06-(76,59)-115 μm in diameter. Spore cell wall produce hyalin to yellow colour in PVLG and darker yellow in Melzer's reagent (Picture 3a). Spore cell wall surface is smooth with no ornamentation. Spore consists of one cell wall, all layers in cell wall originated from subtending hypal wall (Picture 3b), spore cell wall is 1.11-(2,56)-4.44 μm in diameter. Flexible germinal wall

is absent. Spore cell wall ended with a pore (Picture 3c) in subtending hyphae attachment point. Subtending hyphae has the same colour as the spore wall. It is 1.11-(4,92)-6.66 μm , in diameter and 1.11-(1,51)-3.33 μm thick, it gets thicker towards the spore cell wall.

Glomus sp.2 spore isolated from various soil sample and host plants. It is originated from dari Bantar kemang (*A. beccariana*, *A. crasna*, and *A. malaccensis*), Carita (*A. malaccensis*), and Cifor (*A. crasna* & *A. malaccensis*).

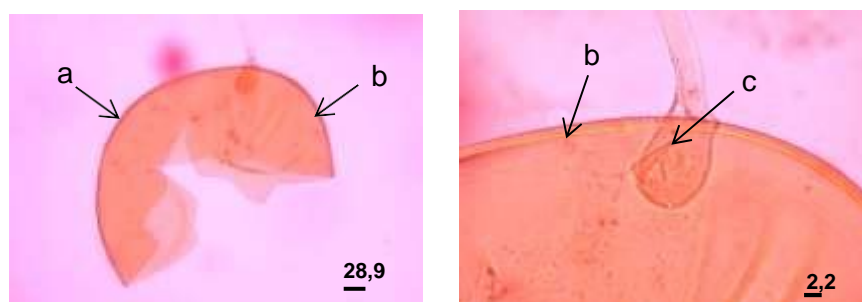


Picture 3. *Glomus* sp. 2 spore from *A. Malaccensis* rhizosphere, Bantar kemang: a. broken spore, b. subtending hyphae, c. pore

The *Glomus* genus collected has the same description with the previous studies (Souza 2015; Gerdemann and Trappe 1974). Spore has only one type of cell wall that is spore wall. Spore wall produce red to brown colour in PVLG and produce darker colour in Melzer's reagent. Spore wall surface is smooth without ornamentation. There is only one spore wall, generally undergo thickening, all layers in the spore wall originated from subtending hyphae wall. Spore wall ended at the pore where subtending hyphae attached.

3. *Gigaspora albida*

Spore produce yellow colour (0-10-60-0) in PVLG and light brown (0-30-60-0) in Melzer's reagent, globose to subglobose in shape, 260-(265)-270 μm in diameter. Spore has two cell walls. The outer cell wall varied in thicknes, colour, and it attached to the second layer, spore cell wall is 3.33-(3,89)-5.55 μm thick. Flexible germinall wall is absent (Picture 4a). Both of the spore wall have a smooth surface (Picture 4b). Suspensor is attached on the surface of outer spore wall (Picture 4c), has the same colourwith spore wall, 22.2-(30,53)-38.85 μm in diameter, 1.11 μm thick. Spore isolated from Ciapus (*A. filaria*).



Picture 4. *Gigaspora albida* spore from *A. filaria* rhizosphere, Ciapus: a. broken spore, b. spore wall, c. Suspensor

The isolated *Gigaspora albida* spore has simillar description which reported by INVAM (2017b). Spore size is 260-270 μm while INVAM (2017b) reported 200-280 μm spore size. Spore wall produce the same colour yellow (0-10-60-0) in PVLG as reported by INVAM (2017b). It

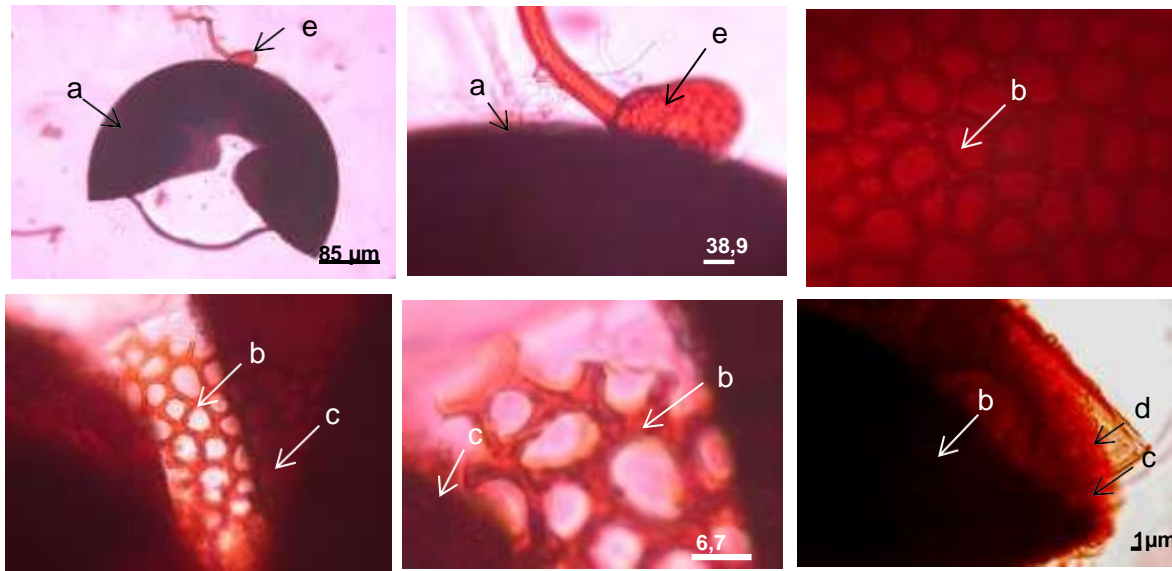
produce darker colour in Melzer's reagent. Other simillar descriptions are there are no flexibel geminall walls formed, suspensor attaches on the outer spore wall and it has the same colour as the spore (0-10-60-0), hyphal wall consists of two wall layers resembling spore wall and has the same

colour as the spore. Suspensor is 22.2-(30,53)-38.85 μm in diameter, similar to INVAM (2017b) report where diameter is around 41-32 μm .

4. *Dentiscutata* sp.1

Spore has dark brown colour (40-80-80-10) to darker brown (80-80-70-0), globose to subglobose in shape, 340 μm in diameter. Spore wall produce dark brown colour in PVLG and doesn't change its colour in Melzer's reagent. Spore wall surface has ornamentation. Spore has spore wall (Picture 5a) consists of two types of wall. The

first wall varies in colour, has ornamentation and some times attaches with the second layer (Picture 5c), overall 3.33 μm thick, ornament size 6.66-(13,48)-29.97 μm . Spore has one to two hyalin germinal flexible wall (Picture 5d) that is absent in *Gigaspora* and *Glomus*. Two germinal flexible wall may attached as one wall. Germinal flexible walls surfaces are smooth. Suspensor attaches on the outer spore wall surface (Picture 5e), it has brown colour (0-60-70-10), 38.85 μm in diameter, 1.11-2.22 μm thick and it's thicker towards spore wall. Spore isolated from Carita (*A. malaccensis*).



Picture 5 *Dentiscutata* sp.1 spore from *A. malaccensis* rhizosphere, Carita:
a. broken spore, b. the first spore wall with ornamentation,
c. the second spore wall, d. flexible germinal wall, e. suspensor

Dentiscutata spore observed is similar with genus *Dentiscutata* described by Oehl *et al.* (2008). Spore has two spore walls, the outer spore wall and the second spore wall that might attached as one spore wall. Spore wall has ornamentation similar to *Dentiscutata* sp. spore reported by Khade (2010) and INVAM (2017c; 2017d). Spore has one to two hyalin flexible germinal walls. It has suspensor that attached on the outer surface spore wall (Khade, 2010; INVAM, 2017c; INVAM, 2017d).

Spore distribution varies for each characterized spore. *Glomus* sp. 1 present in almost all sampling area except in *A. beccariana* and *A. crasna* rhizosphere from Bantar Kemang. *Glomus* sp. 2 isolated from all sampling area, except in *A. filaria* rhizosphere from Ciapus. *Dentiscutata* sp. only present in *A. malaccensis* rhizosphere from Carita. AMF diversity is related with plant species diversity. As reported by earlier studies that plant species diversity, types of soil, and environmental condition difference may result in different AMF

species diversity (Muryati *et al.*, 2016; Stürmer *et al.*, 2018; Kurnia *et al.*, 2019).

CONCLUSION

Indigenous AMF structures observed were external hyphae, internal hyphae, coiled hyphae, arbuscules and vesicles. AMF spore isolated, characterized and identified from *Aquilaria* spp. rhizosphere were *Glomus sp. 1*, *Glomus sp. 2*, *Gigaspora albida* and *Dentiscutata sp.*

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