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# IDENTIFICATION OF ANATOMICAL CHARACTERISTICS OF *Aquilaria microcarpa* IN ITS INTERACTION WITH *Fusarium solani*

RIMA HS SIBURIAN[1*], ULFAH J SIREGAR[2], ISKANDAR Z SIREGAR[2], ERDY SANTOSO[3], and IMAM WAHYUDI[2]

[1] *Post Graduate Student - Faculty of Agriculture, Bogor Agricultural University, Bogor, Indonesia*
[2] *Faculty of Forestry, Bogor Agricultural University, Bogor, Indonesia*
[3] *Center for Research and Development - Conservation and Rehabilitation Ministry of Forestry, Bogor, Indonesia*

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## ABSTRACT

*Aquilaria microcarpa* is one of agarwood-producing plants. Interaction of pathogenic fungi may cause physiological changes that have an impact to cells, tissues or organs of plants. This study aimed to determine the differences in anatomical characteristics of wood between inoculated and uninoculated *Aquilaria microcarpa* with *Fusarium solani*. Result showed that traits of inoculated and uninoculated *Aquilaria microcarpa* wood in general were partly different. The differences were found in wood color, odor, deposit inside the lumen vessel and the frequency of included phloem. Chemical compounds in fusarium-inoculated wood were elemol, baimuxinal, 3-phenyl-2-butanone, and chromen-4-one.

**Key words:** *Aquilaria microcarpa,* anatomical characteristics, pathogenic fungi, *Fusarium solani*.

## INTRODUCTION

*A. microcarpa* is one of gaharu-producing plants, contains a distinctive color (brown-black) and levels of mastic (Dewan Standard Nasional 1999). Agarwood is formed as a reaction of plant to the disruption due to biotic or abiotic factors. The most widely biotic disturbances reported are the role of fungus in the formation of agarwood impaired by *Fusarium* sp. (Gong & Shun 2008; Siregar 2009; Isnaini *et al.* 2009; Mohamed *et al.* 2010).

In the interaction process between the fungus with its host, the fungus pathogenicity greatly affect the response given by the plant (Mendgen & Deising 1993). The response is a plant defense mechanism that serves as a physical biochemistry of plant barriers in cells and tissues that would kill or inhibit the growth of pathogens (Groenewald 2005).

* Corresponding author : rhsiburian@yahoo.com

Biochemical resilience is a series of biochemical reactions that occur in cells and tissues of plants in order to produce toxic substances to pathogens or create conditions that inhibit the growth of pathogens in the plant (Agrios 1996). Biochemical changes that may occur including the synthesis and accumulation of salicylic acid (Wobbe & Klessig 1996) or fitoaleksin (Beynon 1997), the compound result of secondary metabolites toxic to viruses, bacteria, and fungus-like fatty acid (Lowton *et al.* 1992), and issuance of oligosaccharide elicitor by the plant (Nothnagel *et al.* 1983). These compounds may not only protect plants against pathogens comprehensively but can also suppress the development of pathogens without reducing production. In addition, the plant can defend itself without producing metabolites that are required by the pathogen in order to inhibit the developmental process of pathogen.

Research on the interaction of *A. microcarpa* plant with *Fusarium* sp. has been conducted by examining young plants/seedlings (Rahayu 2009; Putri 2009) but the extent of the difference between the anatomical characteristics and its content at inoculated and uninoculated plants have not been studied and reported. Therefore, this study aimed to identify differences in the anatomical characteristics and content of chemical compounds between fusarium-inoculated and uninoculated of *A. microcarpa* plants.

## MATERIALS AND METHOD

Plant material used was *A. microcarpa* that had been inoculated three years prior to the research initiation and formed tree aloes compound at tree ID# 5. Samples were also selected from un-inoculated plants at the same approximate age and grown in location close to inoculated plants with ID# 22. Sampling was carried out by drilling trunk of wood at four cardinal directions (east, west, north and south).

Chemicals used were technical alcohol, technical glycerine, FAA solution (37% formaldehyde: glacial acetic acid: 70% alcohol = 5:5:90), a solution of n-Butanol, Gifford solution (glacial acetic acid: 60% ethanol: technical glycerin = 20:80:5), 4% formaldehyde, 100 mM $K_2HPO_4$, 2% safranin, 1% $I_2KI$, and a solution of copper acetate [Cu ($CH_3COO$) 2 $H_2O$] 50%. All of these chemicals were used to make preparative slides. The tools used were foto-microscope (Nikon Obtiphot 2), microscope (Nikon AFX-DX Labophot -2), rotary microtome (Yamato RV-240), sliding microtome (HM 400 microM R) and frozen microtome (Yamato RV-240).

Observations were conducted through the microtome specimens after being stained by safranin to identify the differences in anatomical characteristics between inoculated and un-inoculated trees. Wood samples were then evaluated by gas chromatography using Gas Chromatography Mass Spectrometry (GCMS).

### Microtome Specimen Preparation

Wood samples from inoculated and uninoculated plants were used for anatomical observation. The samples were soaked into 70% ethanol immediately, infiltrated with 20% polyethylene glycol 2000 in ethanol, dried for 3-4 days in the oven of 60ºC, and

then sliced to a thickness of 12-20 μm in transverse, radial and tangential directions. The thin specimen obtained was stained using 2% safranin for 1-2 hours, washed and then dehydrated with ethanol 30%, 50%, 70%, 90%, and absolute consecutively for 10 minutes each. Furthermore, the specimens were immersed in xylol for about 1 minute and then mounted on the object glass. After mounting, the specimen was put on a slide warmer at 40-50ºC for 1 day and ready to be observed. The procedure was based on Richter (1990) after modification.

**Observation**

Characteristics of both inoculated and un-inoculated wood were observed by using photomicroscope (Nikon Obtiphot 2) and microscope (Nikon Labophot AFX-DX -2). The observed characteristics consist of wood color and odor, growth ring, vessel elements (shape, distribution, diameter, length, arrangement and type of perforation plate), ray parenchyma (type, width, height and frequency), axial parenchyma, intercellular canals, sediment or tyloses and mineral inclusions as well as the frequency of included phloem. Results obtained in each sample were described qualitatively.

Encountered exudates were further tested by using GCMS to determine the chemical compounds. Analysis was only performed on wood sample containing sediment, using pyrolysis GCMS of Shimadzu GCMS-QP2010. Helium (0.8 ml/min.) was used as a carrier, equipped with capillary column DB-5 MS (60 mm x 0.25 mm, film thickness 0.25 μm), operated by electron impact (EI) mode at 70 eV and ion source temperature of 2000ºC. Identification of chemical compounds was conducted on retention time and MS analysis.

## RESULTS AND DISCUSSION

It was observed that anatomical characters of wood from the inoculated and un-inoculated of *A. microcarpa* were partly different. The difference was found in wood color, odor, deposits in vessel-element and the frequency of included phloem. In case of inoculated plants, the color of wood especially around the injection area is slightly darker than that of inoculated plants. Wood odor was also somewhat different: the inoculated wood has a little bit distinct odor compared to that of un-inoculated wood. It was also observed that inoculated wood has a gold-plated resin inside their lumen. Such kind of resin was not observed in the uninoculated wood. Some similarities and differences in anatomical features of inoculated and un-inoculated of *A. microcarpa* wood were presented in Table 1.

Resin produced by *A. microcarpa* tree was not exudated out of wood, but deposited and infiltrated inside the tissues. It then contributes to the changes in tissue color from white to dark brown in general. According to Rao *et al.* (1992), a large amount of resin in case of *A. agalocha* was accumulated in included phloem, but in small amount in other parts such as trachea xylem elements, fiber cells and ray parenchyma. Included phloem is a secondary phloem located in the secondary xylem (Mauseth 1988). Network in which the *Aquilaria* resin accumulated was well known, but the networking

for its secretion was still unknown (Mandang & Wiyono 2002). It was assumed that secreting tissue of parenchyma phloem was composed by the living cells capable to store starch, fat, organic compounds, and some secondary metabolite materials such as tannins and resins (Fahn 1991).

Table 1. Observation of anatomical characters of *A. microcarpa* wood

| Characters Observed | *A. microcarpa* | |
|---|---|---|
| | Inoculated | Uninoculated |
| Wood color | Light to dark brown | White (light) |
| Wood odor | Distinct odor | Regular |
| Growth ring | Unclear | Unclear |
| Vessel elements distribution | Mostly in radial multiple and partly solitaire | Mostly in radial multiple and partly solitaire |
| | Simple | Simple |
| Perforation plate | Present | None |
| Deposit in pores | Higher | Lower |
| Frequency of included phloem | Scented | Unscented |
| Wood scent | | |

Included phloem was produced during secondary growth. According to Blanchette (2005), included phloem is a dedicated networking capable to secrete *Aquilaria* resin. In addition to the *Aquilaria*, the similar structure was also found in Nyctaginaceae and Amaranthaceae. Included phloem on *Aquilaria* has the same function as the resin glands on *Pinus* sp. It was referred as a place to resin being accumulated (Nagy *et al.* 2000). Included phloem that located in the secondary xylem (Mauseth 1988) is a complex structure formed by the cambium to the inside, made up of filter elements, companion cells, parenchyma tissues, and fibers (Rao *et al.* 1992). Cell wall of included phloem components is very thin, less of lignin content so it was not stained by the safranin, and has a plate-type sieve foraminate (Rao *et al.* 1992; Nobuchi & Siripatanadilok 1991). If close to or surrounded by the included phloem, parenchyma cells will die and function as the resin storage (Fahn 1991).

Response of inoculated plant to the infection of *Fusarium* sp. occurred at cellular level, tissues or organs. It was observed that in case of inoculated plant there was a large number of included phloem in which the terpenoid compound and others were accumulated as the deposit but has no plate-gold resin inside the lumen of vessel elements (Figs. 1 & 2).

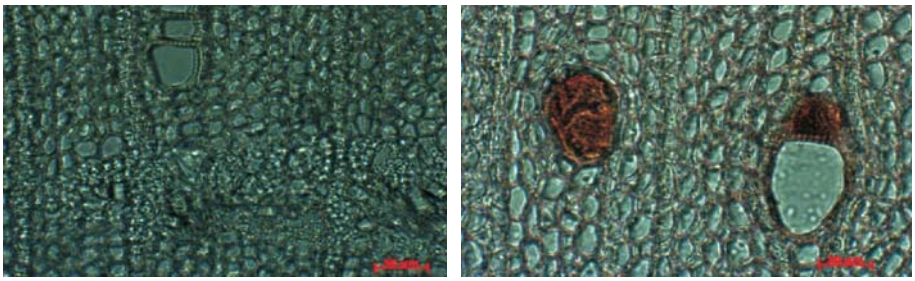

Figure 1. Cross section of *A. microcarpa* (a) uninoculated and (b) inoculated

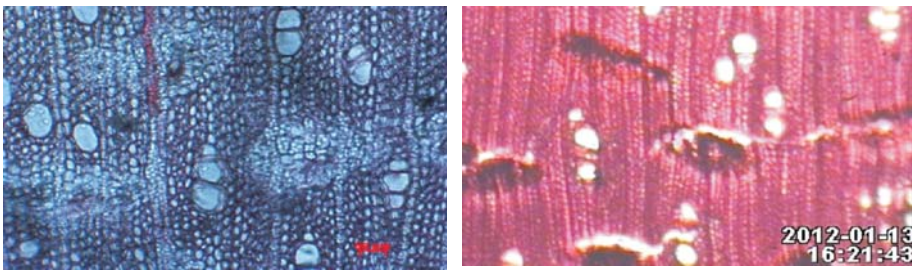


Figure 2. Included phloem islands in *A. microcarpa* (a) uninoculated (b) inoculated

**Chemical Components of *A. microcarpa***

The chemical compounds detected from *A. microcarpa* by using GCMS analysis are listed on Table 2. Table 3 shows similarity and also variation in the chemical composition of several agarwood oil samples.

Generally, agarwood oils are mixtures of sesquiterpenes, sesquiterpene alcohols, oxgyenated compounds, chromone derivatives and resins. Some important compounds are agarospirol, jinkohol-eremol, jinkohol and kesenol that may contribute to aroma of agarwood (Nakanishi 1984; Ishihara 1993). Yuan (1995) found differences in chemical components between high and low quality of gaharu. Agarol was the first sesquiterpene compound isolated from aloe (Yuan 1995), and oxo-agarospirol, an aloe fragrance components resulting from infection of *A. sinensis* fungus on the wood. The oxoagarolspirol content in *A. sinensis* increased two months after inoculation. According to Ishihara *et al.* (1991), kusunol, dihidrokanon, karanon, and oxo-agarospirol were a sesquiterpene compound isolated from low-quality gaharu. Benzilaseton is a constituent of aloes identified by Yang and Cheng in *A. sinensis* (Burfield 2005). These compounds and the compounds of kromone (also known as a constituent of aloes (Yagura *et al.* 2005; Konishi *et al.* 2002) start to be detected since the third day of inoculation. Agarwood is a type of tree defense fitoaleksin compounds triggered its formation after the attack, then the formation of kromone benzilaseton compounds. This prompts the induction of defense responses against *F. bulbigenum* tree.

Table 2 Agarwood components from inoculated *A. microcarpa*

| Peak | Range Time | Area | Concentration (%) | Name |
|---|---|---|---|---|
| 1 | 3.946 | 43336763 | 0.93 | Wolfram, Tricarbonyl- (2,5-Norbornadien) (E-Cycloocten) |
| 2 | 4.030 | 21481234 | 0.46 | 2-PROPYNOIC ACID |
| 3 | 4.521 | 581871973 | 12.54 | (3a.alpha.,4.alpha.,6a.alpha.)-Hexahydro-4-methylene-1(2H)-penta |
| 4 | 6.019 | 24235257 | 0.52 | 1-Propanol, 2-methyl- (CAS) Isobutyl alcohol |
| 5 | 8.809 | 14749584 | 0.32 | 2-Pentanone, 4-methyl-(CAS) 4-Methyl-2-pentanone |
| 6 | 12.122 | 66907808 | 1.44 | 2-Pentanone, 4-hydroxy-4-methyl-(CAS) Diacetone alcohol |
| 7 | 14.075 | 416783661 | 8.98 | Ethanol, 2-butoxy- (CAS) 2-butoxyethanol |
| 8 | 15.679 | 23859825 | 0.51 | Benzaldehyde (CAS) Phenylmethanal |
| 9 | 17.099 | 16151567 | 0.35 | Benzene, 1-methoxy-4-methyl-(CAS) p-Methylnisole |
| 10 | 18.694 | 21427548 | 0.46 | Phenol, 4-methoxy- (CAS) Hqmme |
| 11 | 20.623 | 983694261 | 21.19 | CYCLOBUTANE, 1-DEUTERO-2-ETHYL-1-(PENTADEUTEROETHYL) |
| 12 | 21.643 | 153913534 | 3 32 | 2-Butanone,4-phenyl-(CAS) Benzylacetone |
| 13 | 23.226 | 62806288 | 1.35 | Benzenepropanoic acid (CAS)PHENYLPROPIONIC ACID |
| 14 | 23.563 | 17828687 | 0.38 | 1-(2-VINYL-PHENYL)-ETHANONE |
| 15 | 25.567 | 44916967 | 0.97 | 2-Butanone, 4-(4-methoxyphenyl)-(CAS) ENT 20,279 |
| 16 | 26.441 | 30587272 | 0.66 | Methyl ortho-methoxybenzyl acetate |
| 17 | 26.694 | 27159246 | 0.59 | 2-(2'-3"-isopropenyl-1"-cyclopropenyl-2'-methylpropyl) cyclopropyl methyl |
| 18 | 27.034 | 24215834 | 0.52 | Beta,-Ionol |
| 19 | 27.996 | 78236288 | 1.69 | ETHANONE, 1-(1-HYDROXY-2,6,6-TRIMETHYL-2,4-CYCLOHEXADIEN |
| 20 | 28.550 | 23934213 | 0.52 | 1-(3-ISOPROPYL-4-METHYL-PENT-3-EN-1YNYL)-1-METHYL-CYCLOPRO |
| 21 | 28.971 | 80905192 | 1.74 | Gamma, 1-cadinene aldehyde |
| 22 | 29.476 | 85011925 | 1.83 | 2-Furancarboxaldehyde, 5-(5-methyl-2furanyl) methyl-(CAS) 5-FORMYL |
| 23 | 29.875 | 109066979 | 2.35 | "KW3 AUS EPIGLOBULOL" |
| 24 | 30.275 | 193368835 | 4.17 | Baimuxinal |
| 25 | 30.875 | 105313915 | 2.27 | 3,4-Dihydro-alpha-ionone |
| 26 | 31.132 | 61314943 | 1.32 | 1,3-CHYCLOPENTADIEN, 5-5 DIMETHYL-1,2-DIPROPHYL |
| 27 | 31.338 | 140581115 | 3.03 | LEDENOXID-(II) |
| 28 | 32.006 | 225615566 | 4.86 | Velleral |
| 29 | 32.163 | 65290314 | 1.41 | 3-Cyclohexene-1-methanol,.alpha.,.alpha.,4-trimetyl-(CAS) CYCLOHEKSENE |
| 30 | 32.625 | 69816367 | 1.50 | Octanal,7-hydroxy-3,7-dimethyl-(CAS) Hydroxycitronella |
| 31 | 32.838 | 34881132 | 0.75 | Elemol |
| 32 | 33.013 | 52133006 | 1.12 | 9-Heptadecene-4,6-dyn-8-ol. (Z)-(CAS) |
| 33 | 37.340 | 148108946 | 3.19 | 8-NAPHTHOL, 1-(BENZYLOXY) |
| 34 | 39.843 | 20042318 | 0.43 | 1,2-Benzenedicarboxylic Acid, bis(2-ethylhexyl) ester (CAS) Bis (2-ethylhexyl) |
| 35 | 41.873 | 44232320 | 0.95 | PENTANAL, 2[BIS(PHENYLMETHYL)AMINO]-4-METHYL |
| 36 | 43.126 | 247582648 | 5.33 | 8-METHOXY-2-92PHENYLETHYL)CROMEN-4-ONE |
| 37 | 44.957 | 112603924 | 2.43 | 7-(Benzyloxy)-5-hydroxy-2-methyl-4H-1-benzopyran-4-one |
| 38 | 4.421 | 44320723 | 0.95 | PENTANAL, 2-[ BIS(PHENYLMETHYL)AMINO]-4-METHYL |
| 39 | 51.114 | 58536492 | 1.26 | NORFLUOROCURINE |
| 40 | 52.638 | 64578087 | 1.39 | Benzene, (1-methoxypropyl)-(CAS) 1-Methoxy-1-phenylpropane |

Table 3 Similarity and variation in chemical compounds of several *Aquilaria* spp.

| Compounds | Authors |
|---|---|
| Elemol | Chen H. *et al.* 2011 (*Aquilaria sinensis* Lour.) |
| Baimuxinal | Chen H. *et al.* 2011 (*Aquilaria sinensis* Lour.) |
| 3-phenyl-2-butanone | Faridah S. 2009 (*Aquilaria maleccencis*) |
| Chromen-4-one | Faridah S. 2009 (*Aquilaria maleccencis*), Dai H. *et al.* 2009 (*Aquilaria sinensis* Lour.) |

## CONCLUSIONS

The difference in anatomical features of plants that interacted with *Fusarium* sp., was distinctively found in wood color, deposited material deposition in pore, the smell/aroma of wood and sedimentation in included phloem. Further, examination of deposited material in pores by using GCMS in inoculated plants interacting with *Fusarium* sp., its aloe contains several compounds that forming the basic structure of seskuiterpenoid such as baimuxinal, elemol, 6-Methoxy-2-(2-phenylethyl)-4H-cromen-4-one and 3-phenyl-2-butanone.

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