Journal of Tropical Forest Science 32(1): 97–103 (2020)
https://doi.org/10.26525/jtfs32.1.97

ISOLATION OF ENDOPHYTIC FUNGI FROM JUVENILE *AQUILARIA MALACCENSIS* AND THEIR ANTIMICROBIAL PROPERTIES

Mochahari D, Kharnaior S, Sen S & Thomas SC*

Assam Don Bosco University, Tapesia Campus, Sonapur, Assam, 782402, India

**shinyct@gmail.com*

Received February 2019; accepted July 2019

Aquilaria malaccensis is a precious tropical plant reputed for its agarwood resin, which is the outcome of biochemical interaction of the plant with fungus. The present study was aimed at investigating the process of agarwood production by isolating endophytic fungus from juvenile plant, effect of stem pieces of juvenile plant on the growth of fungus, and antibacterial effect of isolated fungus and leaves. Four endophytic fungi (*Alternaria*, *Curvularia*, *Rhizopus* and *Sterilia* species) were isolated from juvenile (1-year-old) *A. malaccensis* plant and three fungal colonies (*Penicillium*, *Fusarium* and one putative *Cladosporium* species) were isolated from agarwood chips. The addition of stem pieces of juvenile *A. malaccensis* to the culture media (PDA) exhibited profuse and faster growth of six different fungi. The methanolic leaf extract (concentration 200 µL) showed clear inhibitory zone against *Streptococcus pneumonia* (15.66 mm), *Escherichia coli* (12.80 mm), and *Shigella dysenteriae* (13.66 mm). However, 100 µL of methanolic leaves extract showed inhibition of 12.66, 12.30 and 11.41 mm on *S. pneumonia, E. coli* and *S. dysenteriae* plates respectively. The study concludes that agarwood is the product of persistent interaction between endogenous fungi and the plant, which triggers at early stage of its growth and the resin production probably is visible at onset of maturity.

Keywords: Agarwood, endophytes, biotic and abiotic stress, biochemical process, heartwood, oleoresin, bioactive compounds

INTRODUCTION

Agarwood is a scented product obtained after a pathological infection in the wood of the standing trees of the family Thymelaeaceae (most commonly the *Aquilaria* genus). Endophytes are microorganisms that live inside plants (inter- or intracellular in nature) without causing any plant disease. Although its true functions in the plants are poorly understood, reports suggest that bacterial endophytes can produce bioactive compounds in their host (Mehanni & Safwat 2010). These compounds have potential in providing new drugs (Strobel & Daisy 2003), plant hormones (Bhore et al. 2010) and novel natural products (Guo et al. 2008).

In order to meet the demand for agarwood and protect the wild *Aquilaria* trees, many countries with *Aquilaria* plantations also include research on agarwood cultivation and inoculation techniques in their strategy. Agarwood-producing *Aquilaria* species harbours 18 different types of culturable endophytic bacteria (Bhore et al. 2013). Agarwood formation takes place in infected woody organs such as stem, branch and roots. Agarwood bioinduction technology by means of endophytic fungi is proven to be a promising technique for agarwood formation (Turjaman et al. 2016).

Due to the importance of infection in inducing agarwood, several induction techniques have been developed. In general, there are three approaches used to develop agarwood inoculation techniques: physical–mechanical, chemical, and biological. Nonetheless, the quantity and quality of agarwood produced through these techniques are still without certainty.

The main compound in agarwood essential oil is sesquiterpenoid (Liu et al. 2017). Although the plant produces aromatic resin from the heartwood at onset of maturity (25–30 years), wounding trees to inoculate fungal assemblages in order to amplify resin production artificially is a well-known practice among the farmers. The resinous product is called the oleoresin (Nagajothi et al. 2016). In one of our recent studies, juvenile *Aquilaria* plants inoculated

with a *Fusarium* isolate from agarwood showed characteristic discoloration similar to resinous wood and a GC–MS profile of its hexane extract detected substrates of terpenoids among other unique metabolites (Sen et al. 2017). The actual mechanism that takes place behind the resin production is still unclear.

Three hypotheses exist regarding agarwood formation, namely, that it is the result of pathological, wounding/pathological and/or non-pathological processes (Ng et al. 1997). Studies have not provided conclusive evidence for any of these hypotheses. It has also been suggested that resin production is a kind of response to fungal infection (Oldfield et al. 1998). One reason is that there are several environmental factors that influence agarwood formation. They include soil fertility, temperature, humidity, light intensity, and pests and diseases (Pratiwi et al. 2011, Purnomo & Turjaman 2011). Agarwood plant has developed many defense strategies to survive these harsh environmental factors (Wong et al. 2013). Plant and endophytes association develops after overcoming many physical and chemical barriers and particular fungi established as endophytes in a particular niche or localised in the tissue in a systemic manner (Hyde & Soytong 2008). Subsequently, pathogenicity is also affected by the surrounding environment and fungus endophytic to host plant in one ecosystem can be pathogenic in another ecological niche. The endophytic and pathogenic association of fungus with host plant is inter-convertible due to environmental, chemical and molecular elicitors (Schulz et al. 2002, Eaton et al. 2011). With lacking information about fungal diversity in immature plants and their role in fragrant resin formation in *Aquilaria* spp., the present investigation focused to isolate endophytic fungus from juvenile *A. malaccensis* plant to understand whether their association starts right from the beginning of their growth and development. The study was also aimed at studying the bioactivity of the isolates such as antimicrobial efficacy. The effect of juvenile *Aquilaria* twigs (crushed to dust form) on the growth of endophytic fungal mycelia was also investigated.

MATERIALS AND METHODS

Isolation of endophytic fungi from juvenile *Aquilaria malccensis* plants

Aquilaria malaccensis (1-year-old) plants were obtained from Harisinga in the district of Udalguri, Assam, India. The plants were kept under observation till they acclimatised inside the greenhouse of the institute. Stem cuttings were collected from the plants, washed, surface sterilised and ground in distilled water. The filtrate was collected and used as fungal source for the isolation of pure fungal culture.

Figure 1 (a) dried infected agarwood chips, (b) fresh infected agarwood chips and (c) fresh uninfected agarwood chips

Isolation of endophytic fungi from *A. malaccensis* wood chips

Three types of agarwood chips (dried infected chips 1.22 g, fresh infected chips 5.88 g and fresh uninfected chips 4.12 g) were collected from Harisinga on 11th February 2017 (Figures 1a, b & c). The wood chips were surface sterilised three times using 70% ethanol, ground and filtered separately. The filtrate obtained was used as the fungal source for the isolation of pure fungal culture.

Morphological identification of the fungal isolates and the endophytic fungus

The fungi isolated from the samples were identified by a standard protocol of morphological identification using lactophenol cotton blue staining. The prepared slide was observed under a compound microscope for morphological identification at 10×, 45× and 100× magnifications.

Effect of *A. malaccensis* stems pieces on fungal growth

The effect of stem pieces of juvenile *A. malaccensis* on the growth of different fungi were determined by adding the stem pieces (1–3 cm) to PDA fungal culture plates. Autoclaved agar chips (200 and 300 mg) were added to the PDA plates while the media was poured on the plates. The mycelia mat of each fungus (*Sterilia* spp. *Rhizopus* spp. *Curvularia* spp. and putative *Cladosporium* spp. isolated from the juvenile plants and chips and two non-endophytic fungal species—*Ustilago maydis* and *Cladosporium herbarum*—obtained from the Microbial Culture Collection and Gene Bank, Chandigarh) was inoculated on the plate which was supplemented with stem pieces of juvenile agar plant. The plates were incubated at room temperature for 5–6 days until fungal growth was observed.

Antibacterial activity of leaves and fungal isolates

The leaves of 1-year-old *A. malaccensis* were collected, washed, dried and ground into coarse powder. The dried powder (27 g) was extracted in methanol solvent for 24 hours for crude extracts. The antibacterial activity of crude extract (200 mg mL^{-1}) was tested against five bacterial strains (*Streptococcus pneumonia*, *Escherichia coli*, *Shigella dysenteriae, Klebsiella pneumonia* and *Bacillus subtilis*) by disc diffusion method. Antibacterial activity of the seven isolated fungi was carried out against *E. coli* and *B. subtilis*. The bacterial strains were cultured by spread plate method on Luria agar (LA) plates. A strip of the mycelia mat from each isolated fungi was inoculated at three positions on the plates at equal distance.

RESULTS

Isolation of endophytic fungi from juvenile *A. malaccensis* and wood chips

Four endophytic fungi were isolated from the stem of juvenile *A. malaccensis* plants and three different fungi were isolated from the infected agarwood chips.

Identification of the fungal isolates by lactophenol cotton blue staining

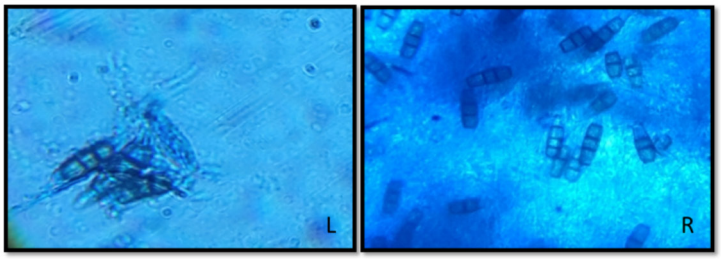
The four endophytic isolates were identified to be the species of *Alternaria*, *Curvularia*, *Rhizopus*, and *Sterilia* by analysing their hyphal and spores structures through microscope (Figure 2). Three different fungi (*Penicillium*, *Fusarium* and putative *Cladosporium*) were isolated from three different agarwood chips (Figure 3).

Effect of stem pieces on fungal growth

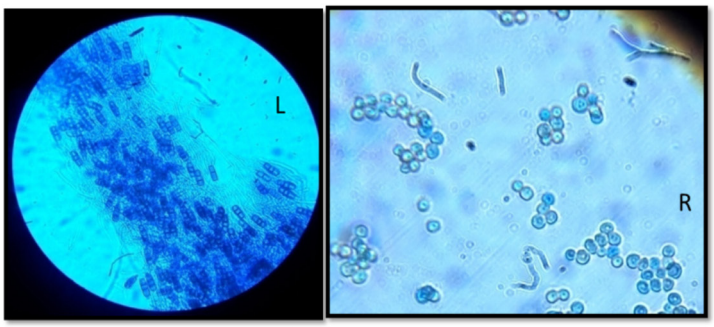
The addition of stem pieces from juvenile *A. malaccensis* to the PDA culture media showed good response (Figure 4). All six different fungi exhibited profuse and faster growth than control plates without stem pieces.

Antibacterial activity of the leaf extract of *A. malaccensis*, fungal isolates and endophytic fungi

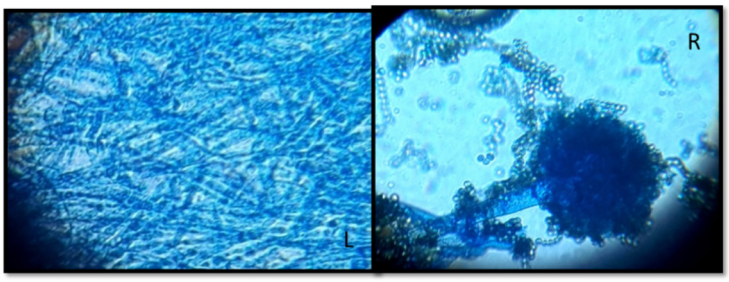
The methanolic extract of juvenile *A. malaccensis* leaves showed clear inhibitory action (15.66, 12.8 and 13.66 mm respectively) against *S. pneumonia*, *E. coli* and *S. dysenteriae* at 200 µL and the inhibition zones for 100 µL extract were 12.66, 12.30 and 11.41 mm respectively. Meanwhile *K. pneumonia* and *B. subtilis* did not form distinct clear zones (Table 1). Antimicrobial potential of the extract was evaluated by measuring the diameter



Spores of *Alternaria* (L) and *Curvularia* (R) under, 45×



Spores of *Curvularia* (L) and *Fusarium* (R) under, 100×



Hyphae of *Sterilia* (L) and spores of *Rhizopus* (R), 45×

Figure 2 Morphological identification of isolated fungal species from juvenile *Aquilaria malaccensis* plants

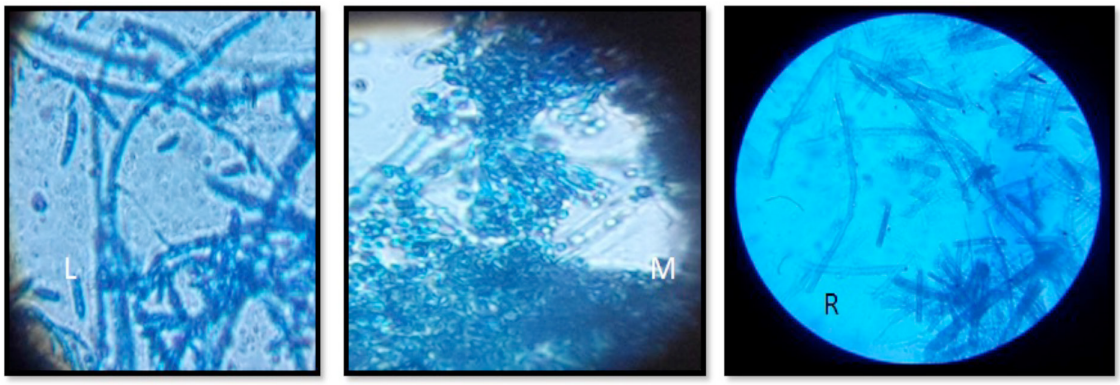


Figure 3 Morphological identification of isolated fungal species from agarwood chips; L–R: *Penicillium*, *Fusarium* and putative *Cladosporium* species (45×)

inhibition zones (mm) in the LA plates as well as minimum inhibitory concentration. Of the seven fungi (three from agarwood chips and four endophytic fungi), only one (putative *Cladosporium*) showed antibacterial effect on *E. coli* and *B. subtilis*. The rest showed negative results.

DISCUSSION

Four different endophytic fungi (*Alternaria*, *Curvularia*, *Rhizopus* and *Sterilia*) were isolated from 1-year-old *A. malaccensis* plant. Thus, it can be assumed that agarwood formation or oleoresin production does not occur immediately

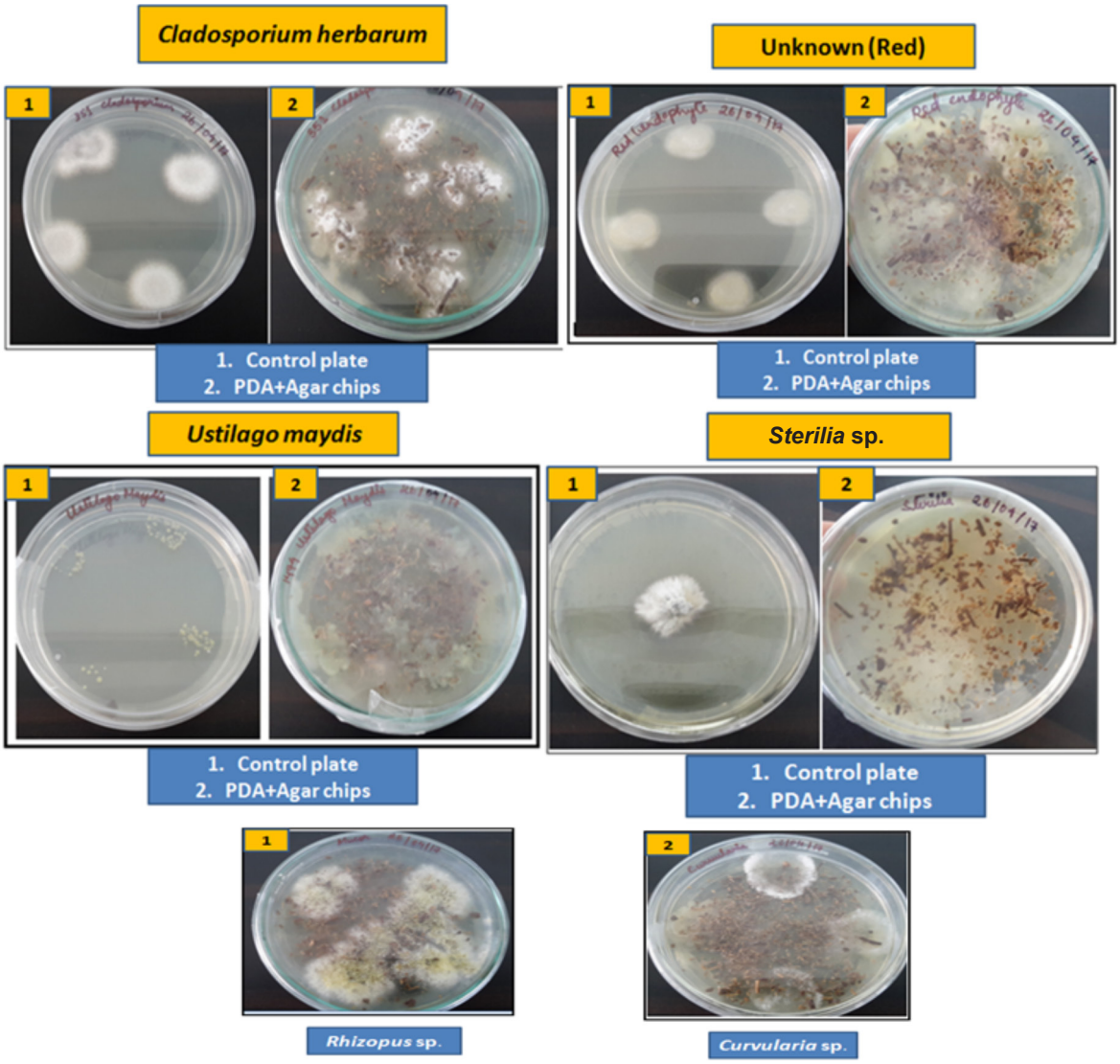


Figure 4 Effect of juvenile *Aquilaria malaccensis* stem pieces on fungal growth in PDA plates

Table 1 Antibacterial activity of *Aquilaria malaccensis* leaves on *S. pneumonia*, *E. coli*, *S. dysenteriae*, *K. pneumonia* and *B. subtillis*

Bacterial strain	Control (150 µL)	Leaf 100 µL methanol extract (mm)	Leaf 200 µL methanol extract (mm)
Streptococcus pneumonia	-	12.66	15.66
Escherichia coli	-	12.30	12.80
Shigella dysenteriae	-	11.41	13.66
Klebsiella pneumonia	-	-	-
Bacillus subtilis	-	-	-

due to infection of pathogenic fungi on the wood either naturally or artificially at onset of maturity. Instead, agarwood is the product of persistent interaction between endogenous fungi and the plant, which triggers at the early stage of its growth.

The source of fungus in agarwood–fungus interaction is also not well understood. From this study, indications of interaction of endophytic fungi with juvenile plants offers a new direction to their origin and prevalence. We suggest that agarwood production in *Aquiliaria* is steered

through a continuous rather than a sudden process of fungal infection and the fungi show latent pathogenicity, which created the conditions for resin formation. In response to abiotic or biotic stresses faced by the plant at different stages of their life cycles, these organisms modify their nature of association with the host. Similar to *Fusarium, Alternaria* found extensively in mature *Aquilaria* trees, and also implicated in resin formation, is by nature opportunistic pathogens and can be quiescent for long periods until favourable conditions for its infection.

Endophytes play an important role in plant physiology. Most of the endophytes are helpful in host plant growth, stress tolerance, and explained as the microorganisms that are not detrimental to the plant (Oses et al. 2008, Nimnoi et al. 2010). However, some had no beneficial impact and showed latent pathogenicity in plant species (Pojanagaroon & Kaewrak 2005). Latent pathogenicity of endophytes could be an economical source of local community and *Aquilaria* is an example in this regard, which produces oleoresin after infection and enhances the value of tree in market (Barden et al. 2000, Pojanagaroon & Kaewrak 2005).

This is the first report of the presence of agarwood associated fungi from juvenile plants which indicates that the association is more dormant and symbiotic rather pathogenic. To adapt and overcome different factors of stress, the associated fungus change their mode of association from dormant to pathogenic. This induces the plant to produce secondary metabolite (resin) as their defensive mechanism. Future research needs to be focused on this suggestion to confirm and comprehend the role of fungi and their continuous association with *Aquilaria* plant that leads to resin production.

Since the plant–microbe interaction in agarwood production is still not well understood, it is assumed that the addition of juvenile agar plant stem pieces in the fungal culture medium (PDA) may give a pilot clue about their pathophysiology. Interestingly, the plates which were added with agar plant twig pieces exhibited profuse growth of mycelia compared with control (Figure 4). The growth of fungus is enhanced by the addition of the agarwood pieces. The study suggests that association of fungus initiates at juvenile stage. Therefore, we can presume that the resin production in *A. malaccensis* is due to various biological processes. The initiation and regulation of agar resin production is the combined biochemical processes beginning at juvenile stage of the plant and the resin production probably is visible at onset of maturity.

Although the plant is known for its agarwood production, the methanol extract of the leaves showed visible inhibition zones against *S. pneumonia, E. coli* and *S. dysenteriae* in the antimicrobial assay (Table 1). Although rigorous research is needed to confirm this finding, it would be an additional benefit for *A. malaccensis*. Interestingly the putative *Cladosporium* fungus isolated from wood chips exhibited promising antibacterial effect against *E. coli* and *B. subtilis*, while the rest of the six isolated fungi from wood chips and juvenile plant did not show antibacterial effect. This is the first report of the study of the endophytic fungus from juvenile plant of *A. malaccensis* and the antimicrobial property of its methanolic leaf extract.

ACKNOWLEDGEMENT

We sincerely acknowledge the facility provided by Department of Life Science, Assam Don Bosco University.

REFERENCES

Barden A, Awang A, Mulliken T & Song M. 2000. *Heart of the Matter Agarwood Use and Trade and CITES Implementation for Aquilaria malaccensis*. Traffic International, Cambridge.

Bhore SJ, Jagadesan P & Kandasamy KI. 2013. Isolation and identification of bacterial endophytes from pharmaceutical agarwood-producing *Aquilaria* species. *Pharmacognosy Research* 5: 134–137.

Bhore SJ, Ravichantar N & Loh CY. 2010. Screening of endophytic bacteria isolated from leaves of Sambung Nyawa [Gynura procumbens (Lour.) Merr.] for cytokinin-like compounds. *Bioinformation* 5: 191–197.

Chowdhury M, Hussain D, Chung S, Kabir E & Rahman A. 2016. Agarwood manufacturing: a multidisciplinary opportunity for economy of Bangladesh—a review. *Agricultural Engineering International: The CIGR Journal* 18: 171–178.

Eaton CJ, Cox MP & Scott B. 2011. What triggers grass endophytes to switch from mutualism to pathogenism? *Plant Sci*ence 180: 190–195. doi: 10.1016/j.plantsci.2010.10.002.

Guo B, Wang Y, Sun X & Tang K. 2008. Bioactive natural products from endophytes: A review. *Prikl Biokhim Mikrobiol* 44: 153–158.

Hyde KD & Soytong K. 2008. The fungal endophyte dilemma. *Fungal Diversity* 33: 163–173.

Liu Y, Wei J, Gao Z, Zhang Z & Lyu J. 2017. A review of quality assessment and grading for agarwood. *Chinese Herbal Medicines* 9: 22–30. doi: 10.1016/S1674-6384(17)60072-8.

Mehanni MM & Safwat MSA. 2010. Endophytes of medicinal plants. *Acta Horticulturae* 854: 31–39.

Nagajothi MS, Parthiban KT, Kanna SU, Karthiba L & Saravanakumar D. 2016. Fungal Microbes Associated with Agarwood Formation. *American Journal of Plant Sciences* 7: 1445–1452.

Ng LT, Chang YS & Kadir AA. 1997. A Review on agar (gaharu) producing *Aquilaria* species. *Journal of Tropical Forest Products* 2: 272–285.

Nimnoi P, Pongsilp N & Lumyong S. 2010. Endophytic actinomycetes isolated from *Aquilaria crassna* Pierre ex Lec and screening of plant growth promoters production. *World Journal of Microbiology and Biotechnology* 26: 193–203. doi: 10.1007/s11274-009-0159-3.

Oldfield S, Lusty C & Mackinven A. 1998. *The Word List of Threatened Trees*. World Conservation Press, Cambridge.

Oses R, Valenzuela S, Freer J, Sanfuentes E & Rodriguez J. 2008. Fungal endophytes in xylem of healthy Chilean trees and their possible role in early wood decay. *Fungal Diver*sity 33: 77–86.

Pratiwi P, Santoso E & Turjaman M. 2011. Soil physical and chemistry of the gaharu (*Aquilaria* spp.) stands habitat in West Java. Pp 105–120 in Turjaman M (ed) *Proceedings of Gaharu Workshop: Development of Gaharu Production Technology—A Forest Community Based Empowerment*. 1 July 2011, Bogor.

Purnomo E & Turjaman M. 2011. The environmental characteristics of Kandangan site for gaharu plantation project. Pp 95–104 in Turjaman M (ed) *Proceedings of Gaharu Workshop: Development of Gaharu Production Technology—A Forest Community Based Empowerment*. 1 July 2011, Bogor.

Pojanagaroon S & Kaewrak C. 2005. Mechanical methods to stimulate aloes wood formation in Aquilaria crassna Pierre ex H.Lec. (Kritsana) trees. Pp 161–166 in Jatisatienr A et al. (eds) *ISHS Acta Horticulturae 676: III WOCMAP Congress on Medicinal and Aromatic Plants. Volume 2: Conservation, Cultivation and Sustainable Use of Medicinal*. International Society for Horticultural Science, Chiang Mai.

Schulz B, Boyle C, Draeger S, Römmert Ak & Krohn K. 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research* 106: 996–1004. doi: 10.1017/S0953756202006342.

Sen S, Dehingia M, Talukdar NC & Khan MR. 2017. Chemometric analysis reveals links in the formation of fragrant bio-molecules during agarwood (*Aquilaria malaccensis*) and fungal interactions. *Scientfic Reports* 7: 44406. doi: 10.1038/srep44406.

Strobel G & Daisy B. 2003. Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews*. 67: 491–502.

Turjaman M, Hidayat A & Santoso E. 2016. Development of agarwood induction technology using endophytic fungi. Pp 57–71 in Mohamed R (ed) *Agarwood: Science Behind the Fragrance*. Springer, Basel.

Wong MT, Siah CH, Faridah QZ & Mohamed R. 2013. Characterization of wound responsive genes in *Aquilaria malaccensis*. *Journal of Plant Biochemistry and Biotechnology* 22: 168–75.