MINISTRY OF FORESTRY OF INDONESIA
IN COOPERATION WITH
INTERNATIONAL TROPICAL TIMBER ORGANIZATION

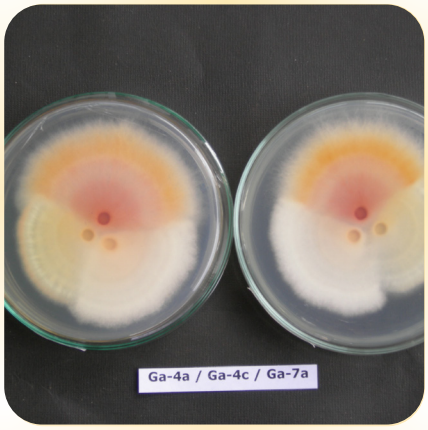
ITTO PD425/06 Rev. 1 (I)

Production and Utilization Technology
for Sustainable Development of Eaglewood (Gaharu)
in Indonesia

TECHNICAL REPORT NO. 2

Better Inoculation Engineering Techniques

by :
Erdy Santoso, Ragil S.B. Irianto, Irnayuli R. Sitepu and Maman Turjaman



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FORESTRY RESEARCH AND DEVELOPMENT AGENCY (FORDA)
MINISTRY OF FORESTRY
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PREFACE

This report signifies as part of research results from the output entitled “Better Inoculation-Engineering Techniques”, which comprised three activities, namely (1) Selecting Suitable Inoculums; (2) Developing Several Prospective Inoculants in Large Scale; and (3) Implementing Several Prospecting Inoculums for Artificial Inducement. The technical report of this output reflects the very substantial spirits as accomplished from the development of gaharu-inoculation technology funded by the ITTO PD425/06 Rev.1 (I) Project, entitled “Production and Utilization Technology for Sustainable Development of Eaglewood (Gaharu) in Indonesia”.

Those three above-mentioned activities presented the research which was very closely related and conducted by the expert researchers who were very proficient in their field. The isolates of gaharu-developing fungi were procured from the Indonesia’s tropical forest, then selected and tested in adequate sample-amount, thereby greatly assisting their selection process based on multi-locations and differing-conditions.

This report provides information about the selection process and testing on isolates that developed gaharu at different species of indicatively gaharu-yielding trees, and the realization process was measured and evaluated thoroughly and properly.

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SUMMARY

Gaharu is formed as an gaharu producing-tree responsed to particular factors which are the plant physiology and fungal infection. Fungi isolates which are potential to induce gaharu-forming have been isolated from various regions. This activity was carried in order to provide information about the diversity of isolates that have been collected. Natural infected wood samples were taken from several locations, from cultivated plants as well as nature (Java, Sumatera, Kalimantan, Sulawesi, and Maluku). Isolation, purification, and cultivation were done with adding standard medium, while qualification was carried with observing *Aquilaria mallacensis* and *A. microcarpa* characteristics. Cultured isolates on Potato Dextrose Agar (PDA) medium were incubated in room temperature for seven days. Isolates that have been collected include *Fusarium solani* (Mart), Appell and Walenw, *F. sambunicum*, and *F. tricinctum*. Inoculation of four isolates of *Fusarium* to *Aquilaria microcarpa* was carried in KHDTK Carita, Banten. Inoculation of Gorontalo-originated *Fusarium* to *Aquilaria microcarpa* stems caused the largest and fastest infection compared to *Fusarium* originated from West Sumatera, West Kalimantan, or Jambi in 2-6 months.

From the molecular identification as inflicted by 36 gaharu-yielding fungi strains, could be acquired the species of the so-called *Fusarium solani* which became the most dominant of the other strains. *Fusarium solani* species presented as the best inoculant that developed gaharu at the four gaharu-yielding tree species, and this species comes from consecutively Gorontalo and Jambi. Morphologically, the *Fusarium* spp. isolates were dominated by while-colored mycelia, but there existed colonies with weak red, yellow, and violet colors. Almost all the isolates exhibited the aerial-mycelium characteristics. Histologically, the *Fusarium* spp. isolates afforded the macroconidia characteristics dominated by elliptical shape. The fungi originated from Gorontalo exhibited viability and virulence which was very excellent compared to those of other fungi from Jambi, West Kalimantan, as well as West Sumatera. At the different research locations, the *Fusarium* spp. fungi could induce the gaharu trees more excellently compared to other fungi from Jambi, West Kalimantan, as well as Padang (West Sumatera), due to among others the fungi suitability, fungi violence, and the resistance of the trees themselves.

The uses of *Fusarium solani* fungi isolates from Gorontalo seems the most recommendable, since this isolate affords high viability and virulence, and hence can be implemented to various gaharu-yielding tree species, which grow on several regions in Indonesia. This is so by considering and following inoculation protocols as enacted by the FORDA (Forestry Research and Development Agency, administratively under the Indonesia's Ministry of Forestry). For the isolates recommended as the second-best rank, it is the *Fusarium solani* originated from Jambi, since this fungal isolate exhibits superiority and afford evolving the fragrant aromas which differs from those of isolates with Gorontalo origin.

FORDA developed fermentation technique for large scale production and establish quality control procedure to produce inoculant of high quality. Quality control of inoculant plays an important role in ensuring the effectiveness and virulence of inoculant for certain period of time. For large scale production, one shaker with capacity of 500 liter inoculants per month has been placed in Forest Microbiology Laboratory, FORDA.

It still deserves the further tests on developing *Fusarium* spp. fungi originated from various regions, and have been collected by the FORDA to assess the potency of gaharu development at other gaharu-yielding tree species in the different locations.

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1
INTRODUCTION

Gaharu, which is a comercial product which has a highly economical value, is actually a resin deposit which is accumulated in wood tissue as a reaction toward wounding or pathogen infection. Gaharu has been traded since hundreds years ago. According to Suhartono and Mardiastuti (2002), the trading of this product in Indonesia was first registered in fifth century, and China was reported as the main buyer. In international trading this comodity was known with several names; agarwood, aloeswood, karas, kresna, jinkoh, oudh, and many others. Trading shape varies from chunks, chips, powder, and gaharu oil (Surata and Widyana 2001). Oil-formed comodity was usually achieved by distilation or extraction from low quality chips.

Nowadays, gaharu has a high sale value especially from its fragrant resin which is called 'Scent of God', although the usage of gaharu is not limited to fragrance industry. In principal, gaharu can be used for medicine, incense, and fragrance (Barden *et al.,* 2000). Gaharu incense is used in beliefs rituals and religious ceremonies, as room fragrance, and religious accecories such as rosario and *tasbih* (Barden *et al.,* 2000). Meanwhile, in medical industry, gaharu is used as analgesic and anti-inflammatory agent (Trupti *et al.,* 2007) and is known has benefits to cure various diseases like toothache, kidney pain, reumatics, asthma, diarrhea, tumor, diuretic, liver, hepatitist, cancer, smallpox, malaria, tonic for pregnancy and after-labor, and also has anti-toxic, anti-microbes, and neuron and digestive stimulant characteristics (Heyne, 1987; Barden *et al*., 2000; Adelina, 2004; Suhartono and Mardiastuti 2002).

Researches concerning various aspects related to gaharu have been done for a long time and is still developing. These researches were primely initiated by the nature-dependent gaharu comodity. Due to the high gaharu-collecting activity which was solely dependent to nature, the main genus of gaharu-producing tree, *Gyrinops* and *Aquilaria* were included in Appendix II CITES. Not all gaharu-producing trees contain gaharu which is only synthesized under certain stress conditions. Gaharu forming process requires a long time, in which during the process various levels of quality are formed and at the end of the process, gaharu with highest quality will be achieved (Sumadiwangsa and Harbagung, 2000).

Gaharu-forming is initiated by biotic or abiotic factors. To synthesize gaharu artificially, one of these methods can be used; mechanical wounding on the stem, or chemical inducing methods (methyl jasmonic, oil, or brown sugar). Abiotic gaharu forming as mentioned above did not distribute its mechanism to other regions in the tree which are not directly affected by the abiotic factor. On the contrary, gaharu-forming by biotic factor such as fungi or other microbes let the mechanism spread into other region on the tree. Due to the spreading of gaharu-forming mechanism to other tissues, the quality and quantity of the gaharu product would be more satisfying.

Process of natural gaharu production occurs due to the injury inflicted on its corresponding host trees and then infected by pathogens. Meanwhile, from the results of isolation could be identified the species of gaharu-synthesizing microbes from various

production-center regions. At each of the isolates could be found the pathogenic-fungi genus with the particular species such as *Fusarium* spp., *Phytium* sp. and *Botrydiplodia* sp, *Penicilium* sp., *Rhizoctonia* sp., *Acremonium* sp., *Cystosphaera* sp., *Thielaviopsis* sp., *Libertella* sp., *Trichoderma* sp. and *Scytalidium* sp. (Sumarna and Santoso, 2002). Further, it was reported that results of purification on those gaharu-synthesizing fungi were presumably dominated by the species of *Fusarium* spp. (Santoso *et al*., 2006). Still further, Sumarna and Santoso (2002) also reported that the production of gaharu could be artificially engineered. The presumed gaharu-yielding tree-stem after being injured was further engineered using the tree-boring method on that injured stem by injecting into it the pathogen inoculant. For the pathogen species to be inoculated, those species should be originated from the environment-ecology condition where that trees species themselves grow, and are suitable with the trees themselves.

Novel findings by gaharu team's have revealed important aspects that determine the successful of gaharu formation by artificial induction, i.e. methods of injection, fungal strain type, and growing media for delivering the fungi. These methods are more practice, effective, and efficient.

The objective of this technical report is to give thorough information concerned with selection of pathogens for gaharu (eaglewood) inoculation. This technical report based on three activities of the project, as follows : (i) selecting suitable inoculums; (ii) developing several prospect inoculums in large scale; and (iii) implementing several prospecting inoculum for artificial inducement.

2
APPLIED METHODOLOGY

2.1 Selecting suitable inoculums

Thirty six isolates of inoculums originated from Jambi, Padang, Mentawai, Bohorok, West Kalimantan, Central Kalimantan, Gorontalo, Maluku, and Papua (and other regions not yet mentioned) were grown in PDA (Potato Dextrose Agar) medium. The isolation of DNA was done with Wizard Genomic DNA Purification Kit (Promega, madison, USA). A region of 443 bases were amplified by PCR using primer Fus1 (5'-TGAAATCTGGCTCTCGGG) and Fus2 (5'-CATGCGCGAACCTCAGTC) (Hannequin *et al*. 1999). PCR reaction for 50µL were including 5µL buffer, 1 µL dNTP mix PCR Grade (Qiagen, Germany), 2.5 µL Fus1, 2.5 µL Fus2, 0.5 µL Amplitaq 360 DNA Polymerase Grade (Qiagen, Germany), 2.5 µL DNA template, and 36 µL MilliQ. The PCR condition was started at 95°C for 10 minutes; followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and post extension at 72°C for 1 minute. PCR product purification prior to cycle sequencing was carried out with Wizard SV Gel and PCR Clean-Up Systems (Promega, USA). Purified PCR products were sequenced using ABI 3130 Genetic Analyzer (Applied Biosystem, USA). Sequenced results were analyzed using FinchTV software and aligned with GeneBank database using BLASTN program. Multiple sequence alignments were run using Clustal X 2.0 software (Higgins, Germany). Neighbor-joining method used for trees and matrix distances of the Phylip package. Species names were determined based on matching identity (Figure 1.).

2.2 Developing several prospect inoculums in large scale

2.2.1 *Preparation of the inoculant media*

The solid media inoculant was already developed prior to the start of this project. The media composition consisted of sawdust from legume-tree wood and rice husk, then mixed with PDA solution. All the mixture as such was put into the bottle of 250-cc capacity. Before being used, all the glassware and raw materials were sterilized at 121°C under one-atmosphere pressure for 20 minutes. The mouths of the bottles were each closed with aluminum foil, and further the closed bottles with their contents inside incubated in a dark room at ambient temperature for 21 days. The liquid media for inoculant was prepared using PDA solution, and added with several vitamins for the sake of inoculant growth. The thread pieces (mycelia) of *Fusarium* sp. fungi were put into the sterilized liquid media. Those bottles were shaken vigorously using the shaker device, through the fermentation process. The inoculant was incubated for 14 days, and shaken at 125 rpm speed. The morphology characterization of mycelia growth from as many as 21 fungi isolates that synthesized gaharu was observed during their growth process.

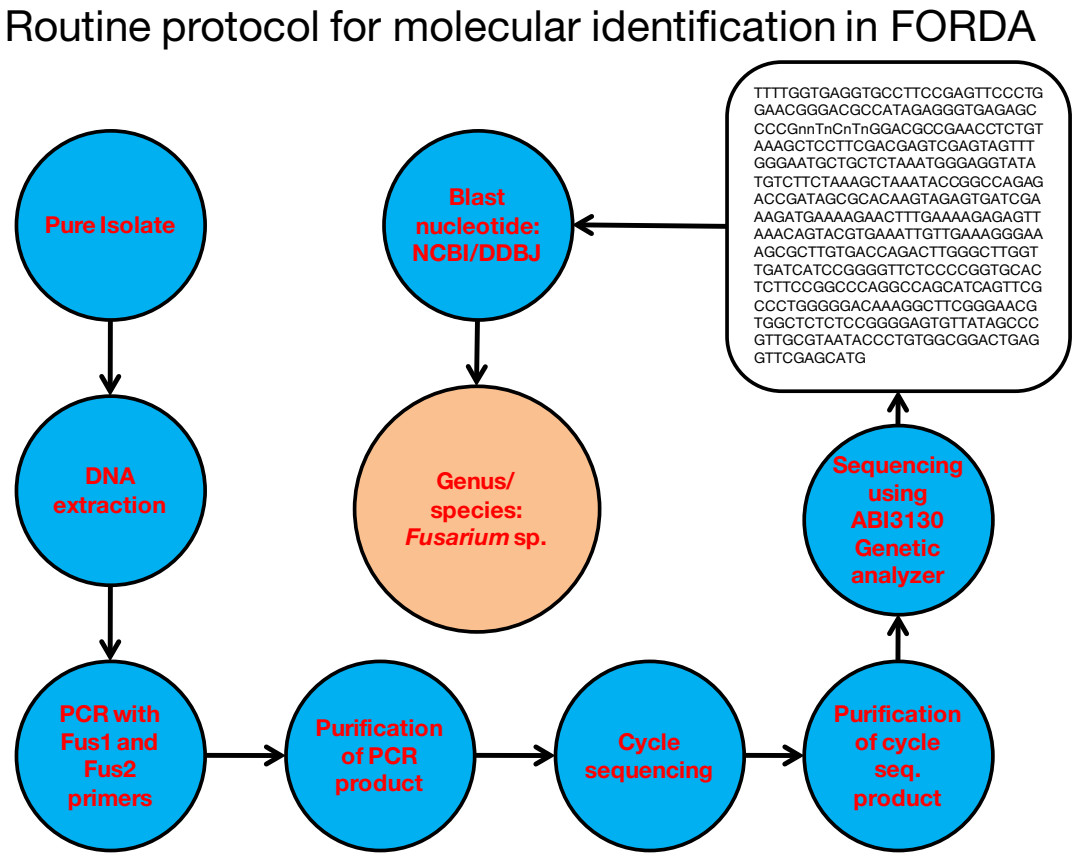


Figure 1. Protocol for molecular identification of *Fusarium* sp.

2.2.2 *Mass production of inoculant*

Trial test on inoculant production was already carried out at the larger-scale work by using large plastic bottles each of l0-litre capacity. By using liquid PDA media, the pieces of *Fusarium* sp. inoculant were incubated for 14 days. Before being used, all the glassware and raw materials were sterilized at 121°C under one-atmosphere pressure for 20 minutes. Each work was done aseptically, and the oxygen consumption was met by using rotor continuously, in order that the development of *Fusarium* sp. biomass could proceed more and more intensively throughout the days. The process of inoculant packaging was done by providing plastic bottles each of 300 ml or 600 ml capacity. This size (capacity) was practical viewed from its purpose for uses, inoculant security, quality stabilization, storage, transportation, as well as for commercialization (Figure 2).

Figure 2. Processing of mass production of gaharu inoculant production in large scale.

2.3 Implementing several prospecting inoculums for artificial inducement

In the realization to find the best inoculums to synthesize gaharu, in the initial stage there were four isolates of *Fusarium* sp. which seemed prospective for the trial test. Those four isolates were originated from consecutively Gorontalo, Padang, Jambi, and West Kalimantan. Further, those four isolates have been identified molecularly, and already tested at laboratory and greenhouse scales. Those four inoculums were entirely produced at liquid media. Several modification methods were already conducted beginning from scrutinizing the distance between injection holes, the injection depth, size of drill bits, until the number of inoculants as injected into each of the holes (Figure 3). Size of drill bits was 3 mm as a tool for inoculating hole, made by radial of motor cyclist (Figure 4). There were four gaharu-yielding trees which have been trial tested, comprising consecutively *Aquilaria microcarpa*, *A. crassna*, *A. beccariana*, and *Gyrinops versteegii.* Automatic injection was used to determine a dosage of liquid inoculum for each hole, i.e. 1 ml per hole, 2 ml per hole, etc. (Figure 5).

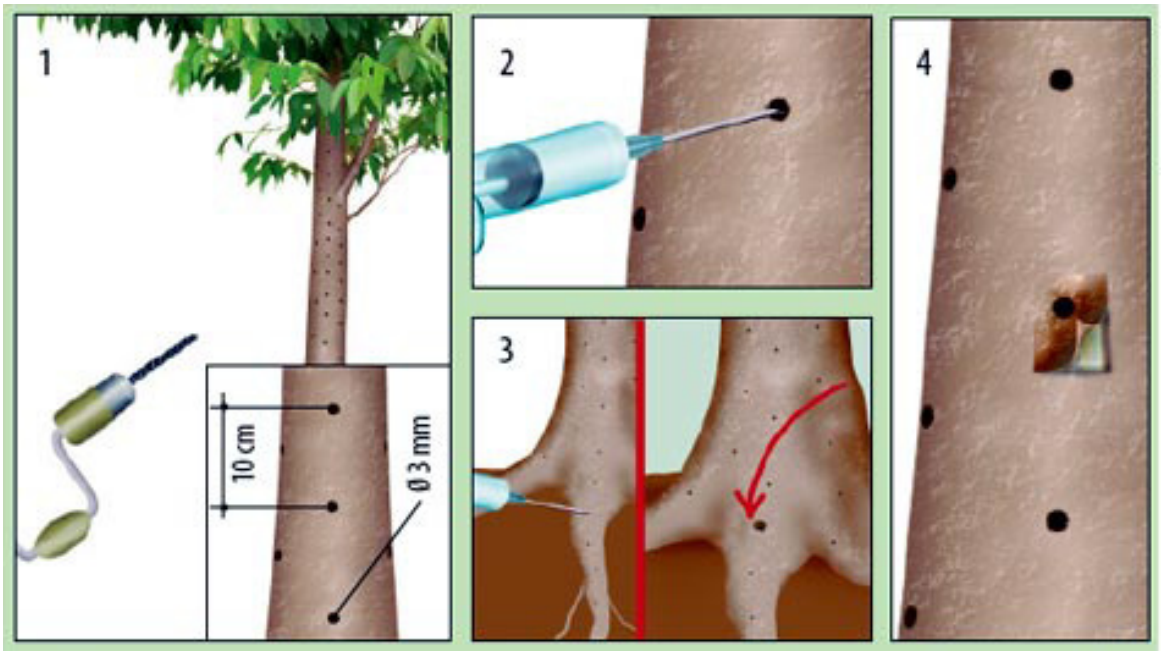


Figure 3. Gaharu inoculation technology by FORDA.

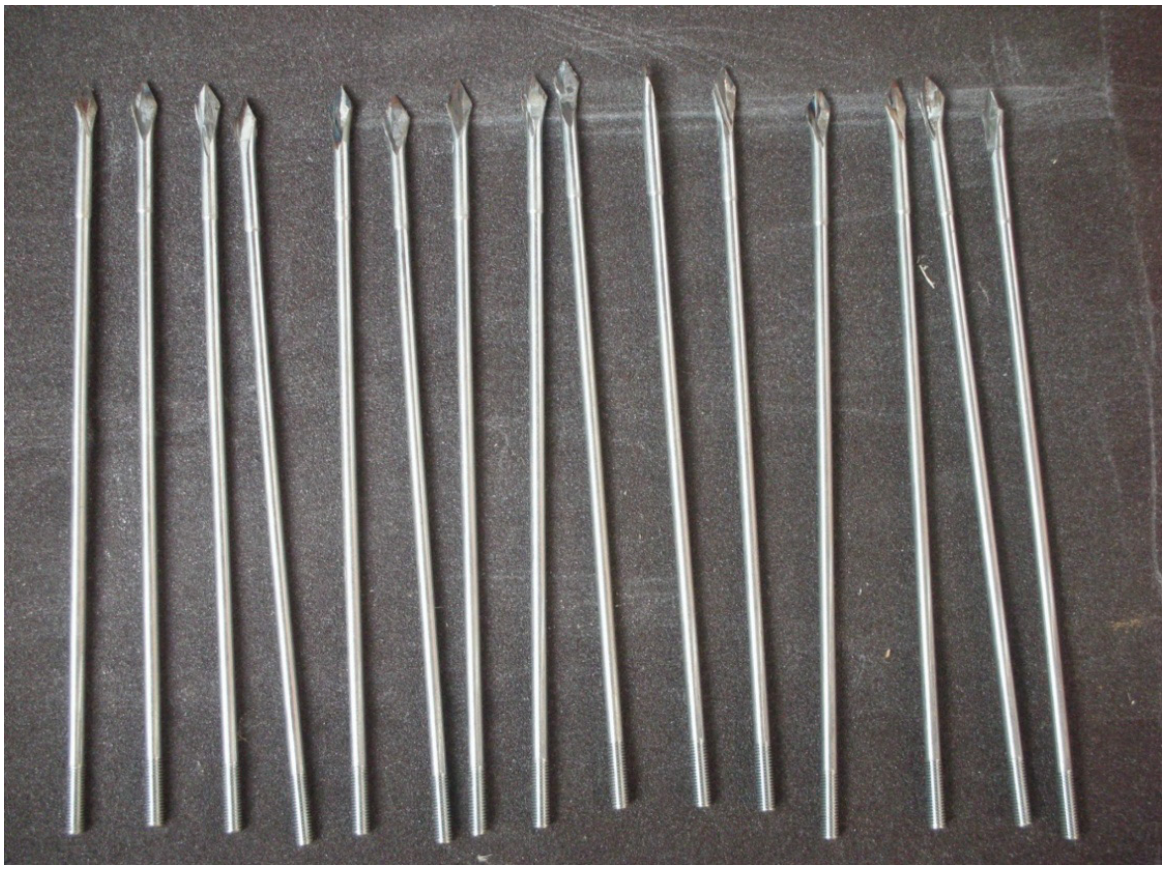


Figure 4. A tool for making an inoculation hole made by radial of motor cyclist.

Figure 5. Automatic injection for liquid inoculum.

3
PRESENTATION OF THE DATA

3.1 Selecting suitable inoculums

Results of molecular identification on 36 gaharu-developing fungi strains as collected from 17 provinces are presented in Table 1. From the 36 strains after being analyzed molecularly, it turned out that 100% of the identified fungi belonged to the genus of *Fusarium*. Specifically, results of such molecular identification strongly indicated that *Fusarium solani* was the most dominant (>80%).

Table 1. Molecular identification of 36 strains of gaharu-inducing fungi collected from 17 provinces in Indonesia

No.	Isolate Number	Origin (Province)	Molecular identification
1	FORDACC506	North Sumatra	*Fusarium solani*
2	FORDACC509	Gorontalo	*Fusarium solani*
3	FORDACC503	West Sumatra	*Fusarium solani*
4	FORDACC512	Papua	*Fusarium solani*
5	FORDACC500	Jambi	*Fusarium solani*
6	FORDACC501	West Sumatra	*Fusarium solani*
7	FORDACC510	Molucca	*Fusarium solani*
8	FORDACC497	Central Kalimantan	*Fusarium solani*
9	FORDACC499	West Kalimantan	*Fusarium solani*
10	FORDACC2372	East Nusa Tenggara	*Fusarium solani*
11	FORDACC504	Riau	*Fusarium solani*
12	FORDACC514	Papua	*Fusarium solani*
13	FORDACC502	West Sumatra	*Fusarium ambrosium*
14	FORDACC515	East Nusa Tenggara	*Fusarium* sp.
15	FORDACC2379	Molucca	*Fusarium solani*
16	FORDACC511	West Nusa Tenggara	*Fusarium solani*
17	FORDACC2370	Bangka Belitung	*Fusarium solani*
18	FORDACC517	Bangka Belitung	*Fusarium solani*
19	FORDACC513	Papua	*Fusarium solani*
20	FORDACC519	West Java	*Fusarium falciforme*
21	FORDACC2375	East Kalimantan	*Fusarium oxysporum*
22	FORDACC520	West Java	*Fusarium solani* f. batatas
23	FORDACC518	Babel	*Fusarium solani* f. batatas
24	FORDACC2371	Babel	*Fusarium solani*
25	FORDACC2377	West Java	*Fusarium solani*

No.	Isolate Number	Origin (Province)	Molecular identification
26	FORDACC507	Lampung	*Fusarium solani* f. batatas
27	FORDACC498	Central Kalimantan	*Fusarium solani*
28	FORDACC2369	West Sumatra	*Fusarium ambrosium*
29	FORDACC495	South Kalimantan	*Fusarium solani*
30	FORDACC2373	West Nusa Tenggara	*Fusarium solani* f. batatas
31	FORDACC2374	East Kalimantan	*Fusarium solani*
32	FORDACC508	Bengkulu	*Fusarium* sp.
33	FORDACC505	North Sumatra	*Fusarium solani*
34	FORDACC496	South Kalimantan	*Fusarium solani* f. batatas
35	FORDACC516	Babel	*Fusarium solani*
36	FORDACC2378	West Java	*Fusarium solani*

Note: FORDA CC: Forestry Research and Development Agency Culture Collection (Source: Sitepu *et al.,* in preparation for publication).

3.2 Developing several prospect inoculum in large scale

From the results of developing from 21 inoculant which were multiplied through the PDA media, could be obtained the data about the morphology characterization of each gaharu-developing fungi colony (Table 2). Such morphology characters as measured covered colony size, the body ability on media surface, and colony color on PDA media (Figure 6).

From the results regarding the production development of gaharu-synthesizing inoculum, could be acquired the technology of mycelia multiplication using a simple shaker and the liquid-PDA growth-media. As such, it could multiply the inoculums, which in volume reached 500 litres a month. For the mass and commercial scale operation, the inoculum packaging has been tried using the bottles each with 300-ml and 600-ml capacity, which were easily transported and practical in the field (Figure 7).

Table 2. **Variety of morphology of *Fusarium* spp. from several location**

No.	Isolate codes	Origins	Morphology characters		
			Colony diameter mm/7 days	Aerial miselium	Color on PDA medium
1	Ga-1	Kalteng	61	Yes,+++	White, bright yelloW
2	Ga-2	Maluku	49	Yes,++	White, bright brown
3	Ga-3	Sukabumi	48	Yes,+	Bright brown
4	Ga-4	Kalsel	50	Yes,++	White
5	Ga-5	Kaltim	45	Yes,++	White
6	Ga-6	Belitung	38	Yes,+	White
7	Ga-7	Riau	59	Yes,++	Cream white
8	Ga-8	Bengkulu	49	Yes,++	White

No.	Isolate codes	Origins	Morphology characters		
			Colony diameter mm/7 days	Aerial miselium	Color on PDA medium
9	Ga-9	Jambi	59	Yes,+++	Cream white, bright brown
10	Ga-10	Padang	61	Yes,+++	White
11	Ga-11	Gorontalo	58	Yes,+++	Brownish white
12	Ga-12	Lampung	58	Yes,+++	Bony white, pink
13	Ga-13	Bangka	59	Yes,+++	White
14	Ga-14	Bogor	61	Yes,++	White
15	Ga-15	Mentawai	56	No	Brown, yellow, white
16	Ga-16	Kaltim LK	57	Yes,+	White, purple
17	Ga-17	Kalbar	59	Yes,+++	Creamy white
18	Ga-18	Yanlapa	58	Yes,++	White, bright yellow
19	Ga-19	Mataram	52	Yes,++	White
20	Ga-20	Kalsel MIC	50	Yes,++	White, bright yellow
21	Ga-21	Kaltel TL	69	Yes,++	White, creamy

Table 3. **Histology Character of *Fusarium* spp. Isolates from different location**

No	isolate code	Characteritic of histology			
		Macroconidia	Microconidia		
		Number of Septa	conidiofor	Relative abundance	shape
1	Ga-1	3	Simple	Many	Elips
2	Ga-2	4	Branch	Many	Elip, oval
3	Ga-3	3	Simple	Many	Elips
4	Ga-4	-)	-)	-)	-)
5	Ga-5	2	Simple	Little	Elips
6	Ga-6	3	Simple	Little	Elips, oval
7	Ga-7	2	Simple	Little	Elips, oval
8	Ga-8	2	Simple	Little	Elips, oval
9	Ga-9	5	Simple	Little	Elips, septa
10	Ga-10	3	Simple	Many	Elips, septa
11	Ga-11	-)	branch	Many	Elips
12	Ga-12	5-6	Simple	Many	Elips
13	Ga-13	3-4	Simple	Many	Elips
14	Ga-14	3	Simple	Little	Elips
15	Ga-15	3-4	Branch	Many	Elips
16	Ga-16	6-7	Simple	Little	Elips, septa 3
17	Ga-17	5	Branch	Little	Elips
18	Ga-18	3	Branch	Many	Elips
19	Ga-19	3-4	Branch	Many	Elips

No	isolate code	Characteritic of histology			
		Macroconidia	Microconidia		
		Number of Septa	conidiofor	Relative abun-dance	shape
20	Ga-20	3	Branch	Little	Elips, oval
21	Ga-21	3	Branch	Many	Elips

-) not observed.

Table 4. Caracteristic different of morphology of Fusarium spp.

No	Isolat Code	location	Morphology	
			Basal cell	Apical cell
1	Ga-1	Kalteng	*papillate*	*hooked*
2	Ga-2	Maluku	*blunt*	*conical*
3	Ga-3	Sukabumi	*blut*	*blunt*
4	Ga-4	Medan	-)	-)
5	Ga-5	Kaltim	*blunt*	*blunt*
6	Ga-6	Belitung	-)	-)
7	Ga-7	Riau	*blunt*	*conical*
8	Ga-8	Bengkulu	-)	-)
9	Ga-9	Jambi	*foot shaped*	*blunt*
10	Ga-10	Padang	*papillate*	*blunt*
11	Ga-11	Gorontalo	-)	-)
12	Ga-12	Lampung	*blunt*	*conical*
13	Ga-13	Bangka	*blunt*	*nipple-like*
14	Ga-14	Bogor	-)	-)
15	Ga-15	Mentawai	-)	-)
16	Ga-16	Kaltim LK	*blunt*	*conical*
17	Ga-17	Kalbar	*foot shaped*	*blunt*
18	Ga-18	Yanlapa	*blunt*	*hooked*
19	Ga-19	Mataram	*blunt*	*nipple-like*
20	Ga-20	Kalsel MC	-)	-)
21	Ga-21	Kalteng TL	-)	-)

-) Not Observed.

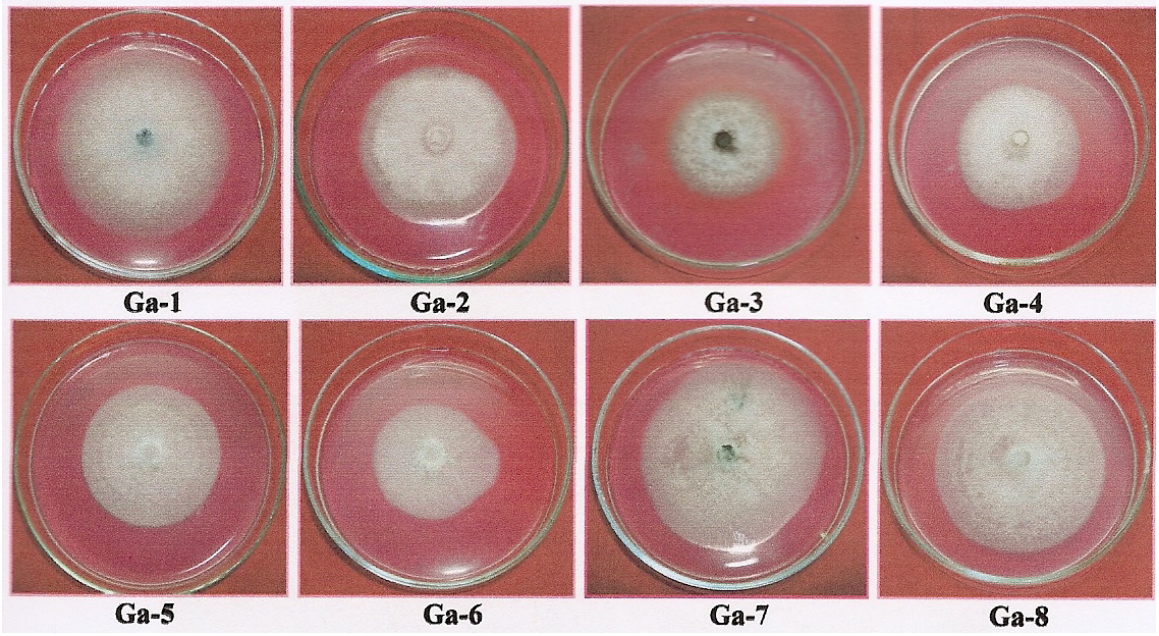


Figure 6. Colony of *Fusarium* spp. from different location in Indonesia

Figure 7. Mass production of prospect inoculums in large scale.

3.3 Implementing several prospecting inoculums for artificial inducement

Prior to conducting inoculum tests on the stem of gaharu-yielding tree at the field stage, those four gaharu-developing isolates, each originated from consecutively

Gorontalo, Jambi, West Kalimantan, and West Sumatera were tested with regard to the levels of their growth ability and viability on the PDA media (Table 5).

Table 5. Viability test for four isolates of *Fusarium* origin from Gorontalo, Jambi, Kalbar, and Sumbar

No	Fungal Isolate															
	Gorontalo				Jambi				West Kalimantan				West Sumatera			
	Fungal growth viability				Fungal growth viability				Fungal growth viability				Fungal growth viability			
	week				week				week				week			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	+++	+++	++	++
2	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	+++	+++	++	++
3	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	+++	+++	++	++
4	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	+++	+++	++	++
5	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	+++	+++	++	++
6	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	++++	+++	++	++
7	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	++++	+++	++	++
8	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	+++	+++	++	++
9	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	++++	+++	++	++
10	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	+++	+++	++	++

Note :
\+ : very weak
++ : weak
+++ : strong
++++ : very strong

After going through the viability test, those four gaharu-developing fungi were tested of their virulence. Such test was conducted on the gaharu-yielding tree-seeds with their age still in the range of 6-12 months old. The virulence tests would detect the level of fungi attack on the stem of gaharu-yielding tree (Figure 8 and Table 6).

Figure 8. The virulence test of *Fusarium* spp. to gaharu seedling in greenhouse conditions

Table 6. Virulence test to *Aquilaria* spp. seedlings in greenhouse condition

Fungal Isolate	Amount of seedling	fungal virulensi level		
		week		
		1	2	3
Gorontalo	1	+++	+++	
	2	+++	+++	
	3	+++	+++	
	4	+++	+++	
	5	+++	++	
Jambi	1	++	++	
	2	++	++	
	3	++	++	
	4	++	++	
	5	++	++	
West Kalimantan	1	-	+	
	2	+	-	
	3	-	+	
	4	+	-	
	5	-	+	
West Sumatra	1	+	-	
	2	-	+	
	3	+	-	
	4	-	+	
	5	+	-	

note :
+ : withered symptoms
++ : languish, withered symptoms and yellowish
+++ : death

In the realization to find the inoculums which were satisfactorily prospective in the gaharu development, inoculation has been experimentally conducted on the species of *Aquiliaria microcarpa* trees that grew at the KHDTK Carita (Forest Area for Special Purpose), situated in Banten Province. There were four inoculum species already trial tested, and each of those inoculums originated from Jambi. Gorontalo, Padang (West Sumatra), and West Kalimantan. Scrutinizing those four isolates as inoculated on *A. microcarpa*, it turned out that 75 days afterwards, the symptom of gaharu development on *A. microcarpa* stems occurred with 100% chance to all the inoculation holes on those stems. In the control treatment, where the tree stems were holed, but the isolate liquid was not injected into the resulting holes, no gaharu development occurred (Figure 9).

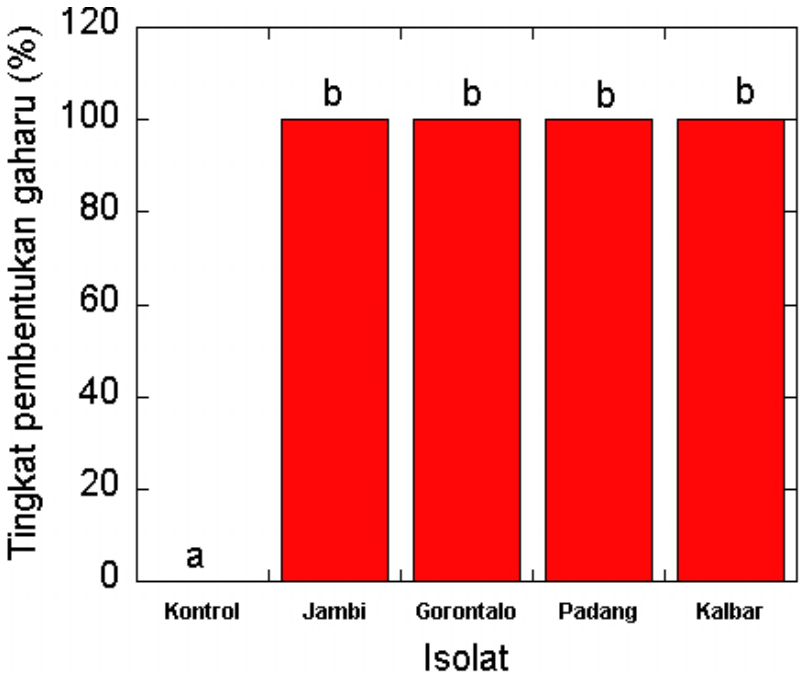


Figure 9. Bargraph *Aquilaria microcarpa* inoculated by four isolates of *Fusarium* spp. after 75 days inoculation at KHDTK Carita, Banten

Further, on *A. microcarpa* stems that revealed the symptom of gaharu development, some gaharu thin-slices were taken randomly from the portion of those stems. Those slices as taken and selected should be blackish brown in color to be further tested qualitatively regarding the fragrant aroma of gaharu as evolved. The results of inoculation that have reached 75-day age could already develop gaharu with satisfactory qualities.

The same treatment was also conducted on the species of *Aquilaria crassna*. The research for conducting such treatment took place in Sukabumi. As such, the *A. crassna* trees have already reached 8-year age, which grew on the former rubber plantation. In number, as many as 80 *A. crassna* trees grew there. It turned that those four isolates which were trial tested, entirely afforded the gaharu development 75 days after their inoculation. Likewise, in the control treatment, no gaharu development occurred (Figure 10).

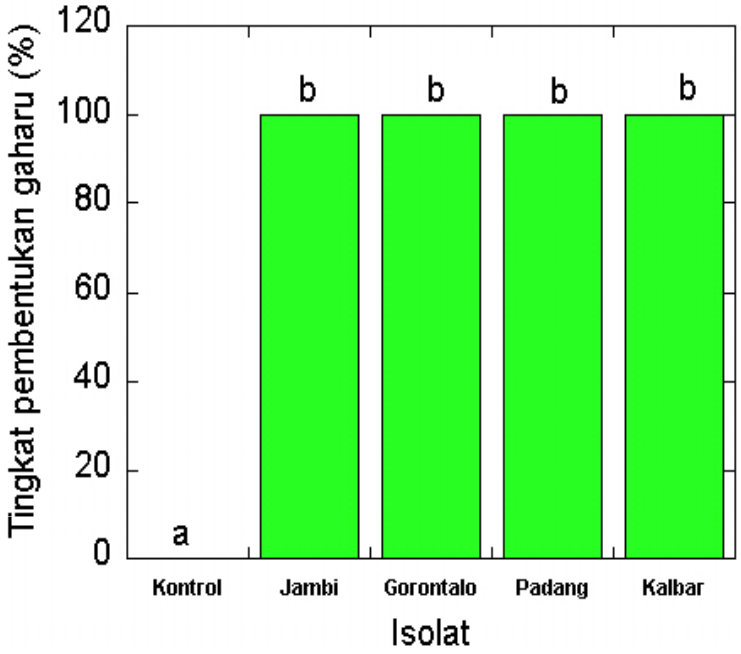


Figure 10. Bargraph *A. crassna* inoculated by four isolates of *Fusarium* spp. after 75 days inoculation in Sukabumi, West Java

In realization, the trial test on gaharu development that seemed prospective at *Aquilaria beccariana* species was already done in Sanggau Regency, West Kalimantan (Figure 11). The trial-test method as implemented was similar to that done on *A. microcarpa* dan *A. crassna*. Those four *Fusarium* spp. isolates were inoculated to *Aquilaria beccariana*-tree stems, and when the inoculation reached 75-day age, all the isolates afforded the symptom of gaharu development.

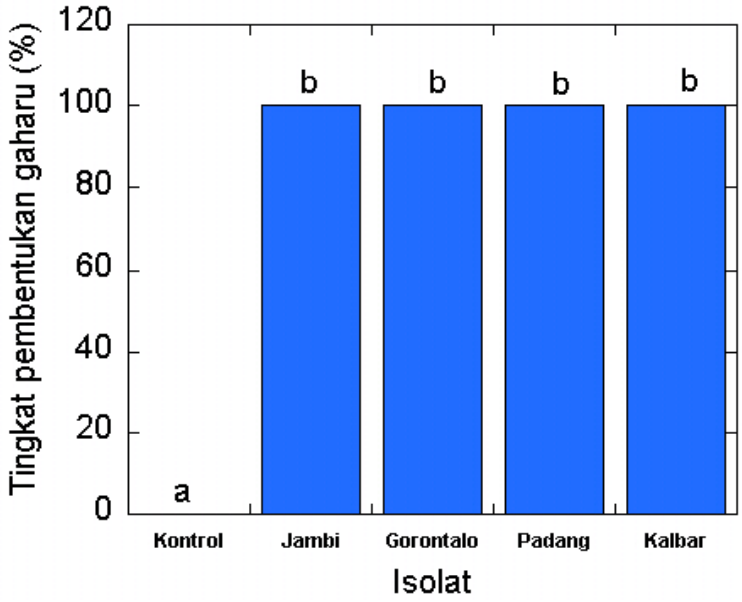


Figure 11. Bargraph *A. beccariana* inoculated by four isolates of *Fusarium* spp. after 75 days inoculation at Sengoret, Sanggau, West Kalimantan

These research results was also added with the data/information as acquired from the trial test on the gaharu-yielding *Gyrinops vergeestii* tree species, which grew on Nagekeo Regency, Flores Island, East Nusa Tenggara (Figure 12). The stem of these gaharu-yielding trees measured over 20 cm in diameter and 8 meters in height. Those four isolates was already inoculated to those *Gyrinops versteegii* trees, and 75 days afterwards there found the symptom of gaharu development at each tree sample.

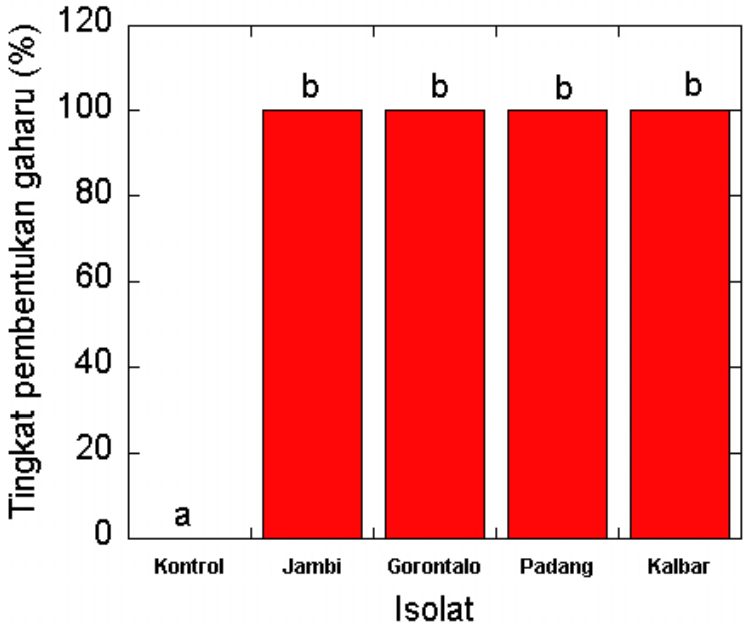


Figure 12. Bargraph *G. versteegii* inoculated by four isolates of *Fusarium* spp. after 75 days inoculation in Nagekeo, Flores Island, West Nusa Tenggara

When the length was measured either horizontally or vertically on the symptom of gaharu development at the stem of *A. beccariana* trees, it turned out that the isolate originated from Gorontalo exhibited the fastest response compared to the other three isolates (Figure 13 and Figure 14). Likewise, for the species of *A. crassna* dan *A.microcarpa* (data are not provided)*.*, the symptom of gaharu development on these two species due to the isolate inoculation revealed similar responses as those occurred to *A. beccariana* species.

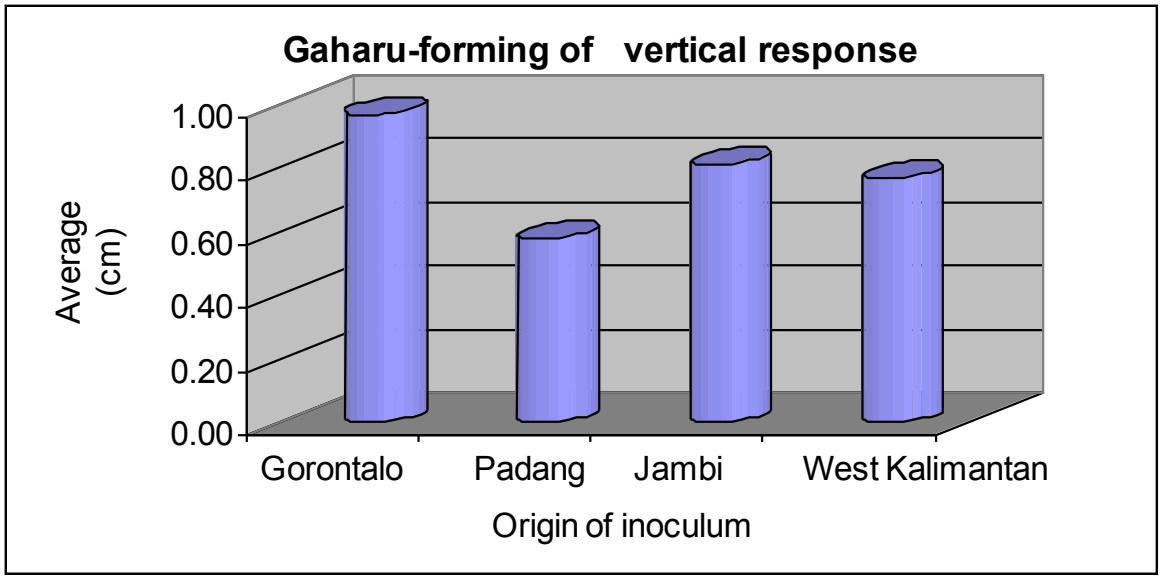


Figure 13. Vertical response of *A. beccariana* after inoculation by four isolates of *Fusarium* spp. in Sanggau, West Kalimantan

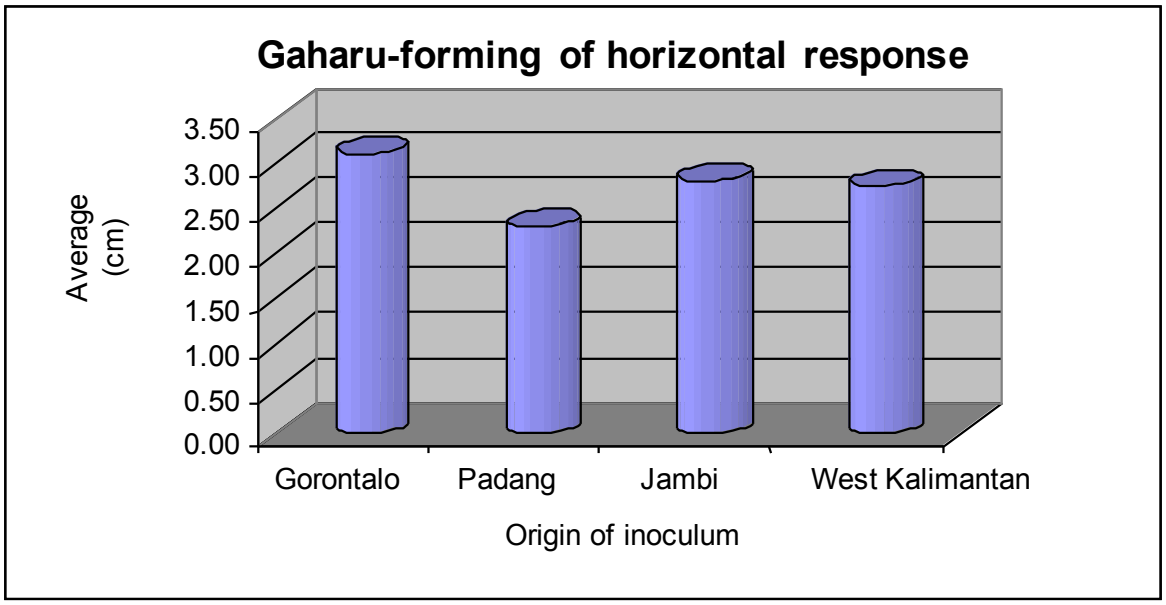


Figure 14. Horizontal response of *A. beccariana* after inoculation by four isolates of *Fusarium* spp. in Sanggau, West Kalimantan

Special for the realization of the inoculation at the gaharu-yielding trees of *Gyrinops versteegii* species, it was already done so at Lombok island, whereby the results of horizontal and vertical response judged as the fastest were inflicted by the *Fusarium* spp. isolates originated from Gorontalo (Figures 15 and 16).

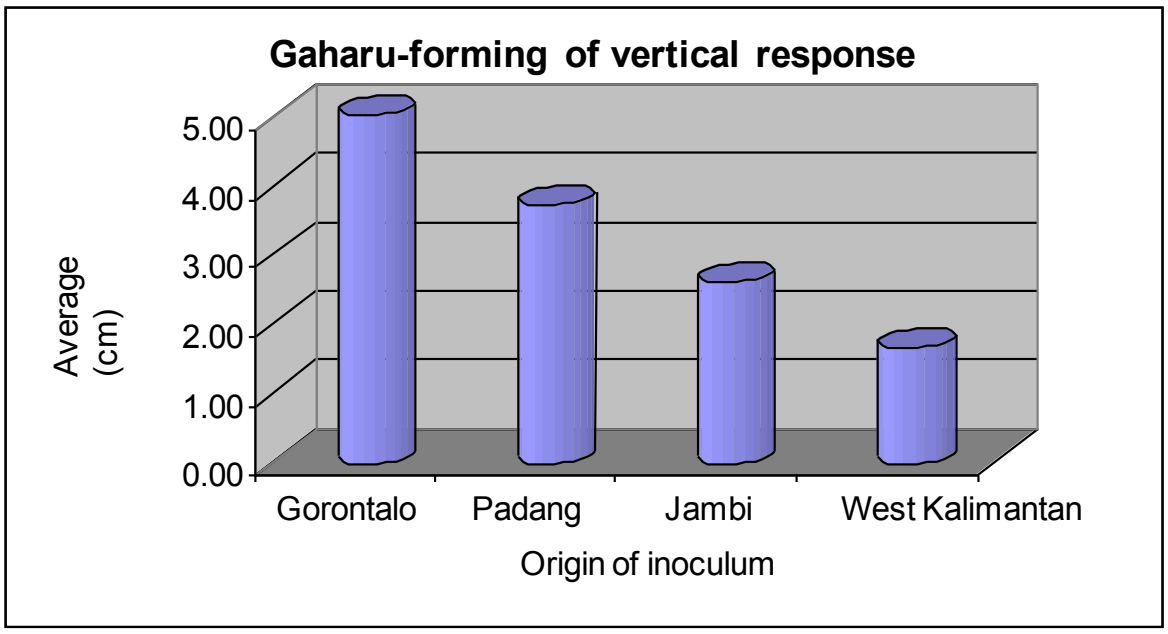


Figure 15. Vertical Response of *G. versteegii* after inoculation by four isolates of *Fusarium* spp., Lombok Island, West Nusa Tenggara (NTB)

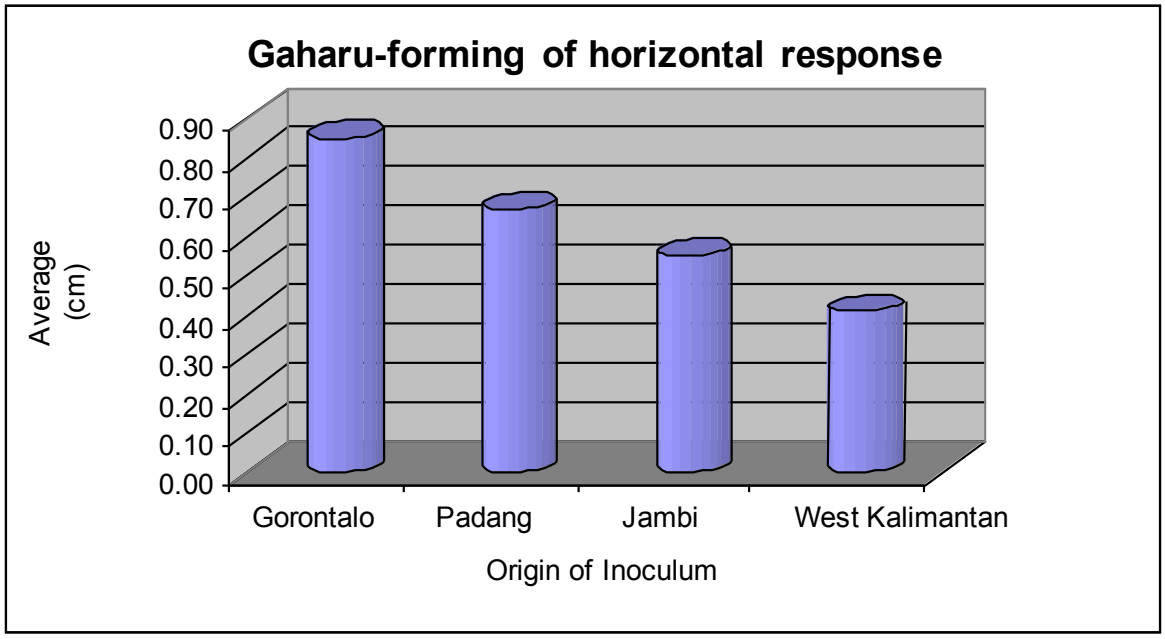


Figure 16. Horizontal Response of *G. versteegii* after inoculation by four isolates of *Fusarium* spp, Lombok Island, West Nusa Tenggara (NTB)

Several gaharu samples that resulted from the inoculation work were analyzed of their chemical-compound content. This intended to determine which chemical compounds inside those samples that either qualitatively or quantitatively brought about the fragrant

aroma/smell as evolved from the gaharu yielded by the species of *Aquilaria* spp. and *Gyrinops* spp. (Tabel 7).

Table 7. Chemical compound of gaharu in *Aquilaria* sp. dan *Gyrinops* sp.

Aromatic compounds			
No	**Name of compounds**	***Aquilaria* sp.**	***Gyrinops* sp.**
1	Cyclopropyl carbinol	3.87	4.76
2	Benzene, 1, 2, 3, D-trimethoxy, 5-methyl-	0.85	0.82
	(CAS) Tolerene, 3, 4, D-trimethoxy		
3	2. Butanone (CAS) Methylethyl ketone	3.26	-
4	Benzene : 1, 4 - dimethoxy-(CAS) DMB	-	2.52
5	1, 2. Cyclopentanedione	3.45	5.17
6	Cyclopentanone (CAS) Dumasin	1.2	1.36
7	2, 2 - Binapthalene, 5, 5', 6, 6', 7, 7', 8, 8'-	0.38	-
	octahydro		

4

ANALYSIS AND INTERPRETATION OF THE DATA AND RESULTS

4.1 Selecting suitable inoculums

Dr. Erdy Santoso and Ir. Ragil SB. Irianto, MSc. and their team R&D for Forest Conservation and Rehabilitation, FORDA have started exploration for potential gaharu-inducing fungi before the project. The pure cultures of isolates are maintained in the laboratory and have been screened for their efficiency and effectiveness in inducing the formation of gaharu (Annex 1-17). In addition, They have been conducting concurrent activities including searching for potential fungal isolates from gaharu in their natural habitats and gaharu plantation forests, and *in-vitro* and *in-vivo* screenings for their efficiency and effectiveness, and mass formulation of product for artificial inductions. Selection of potent gaharu-inducing fungi has been conducted in *in vitro* (laboratory) and in the field. Four potent fungi that showed high ability to induce gaharu were selected: Isolate-G-06, isolate-J-06, isolate WK-06 and isolate WS-06 isolated from natural gaharu-producing tree in Gorontalo-Sulawesi, Jambi-Sumatra, Sanggau-West Kalimantan, and Padang-Sumatera. These isolates were maintained in both solid and liquid media in order to keep the virulence. Their efficiency in inducing gaharu is ongoing on *Aquilaria microcarpa* in Forest area with specific purpose (KHDTK) Carita, Java. Selection of potent inoculant for gaharu induction has been 100% completed. Four isolates designated as Gt, Kb, Jb, and Pd were selected as the most efficient gaharu-inducing isolates. The second fiscal year may further screen other isolates collected from many localities in Indonesia to select more potent isolates in inducing gaharu. These isolates are collections of FORDA-CC microbe bank of Forest Microbiology Research Group.

Molecular identification of gaharu-inducing fungi by means of 28S rRNA partial gene sequencing. We believe that the formation of gaharu follows a pathological process initiated with an infection of fungi on stem/branch tissues of certain tree species. We have isolated 36 fungi from infected gaharu trees from 17 provinces in Indonesia. Some of these fungi have been identified as *Fusarium* spp. conventionally by observing their morphological characteristics, however this identification needs to be confirmed with molecular identification by means of 28S rRNA partial gene sequencing. Identification was conducted in the Laboratory of Forest Microbiology, FORDA. FUS1 and FUS2 primers were used that enabled amplification of up to 460-bp fragment. Most isolates identified were members of *Fusarium solani* species complex (Table 1). Only one isolate, FORDACC-02375, originated from East Kalimantan showed high similarity to *Fusarium oxysporum*. This study highlighted a rapid molecular identification protocol for gaharu-inducing fungi over the conventional measure.

4.2 Developing several prospect inoculum in large scale

This activity has focused on formulating inoculant for large scale production. Inoculant can be formulated as solid, pellet, alginate bead or liquid inoculant. For large

scale production, this activity has focused on producing liquid inoculants of the four potent isolates: Gorontalo, West Kalimantan, Jambi, and Padang following protocols developed by FORDA researchers. We developed fermentation technique for large scale production and establish quality control procedure to produce inoculant of high quality. Quality control of inoculant plays an important role in ensuring the effectiveness and virulence of inoculant for certain period of time. For large scale production, one shaker with capacity of 500 liter inoculants per month has been purchased and placed in Forest Microbiology Laboratory.

The development of inoculant in more practical shapes kept inside the bottles each with 10-15 litres capacity should be done, in order that the farmer group are able to produce the gaharu themselves. Meanwhile, the sources of core isolates are kept being held by the research institutions in order that the qualities of the gaharu-developing fungi could be maintained. Other methods which seem possible to be done are among others the isolate extract should be packaged using the so-called swelling with 20-30 ml capacity, and in this way that system would be more practical to be carried away in the field for its application. The farmers are just to dissolve the isolate extract into the sterile water as much as 2 liters, afterwards the resulting solution is vigorously agitated to render the resulting solution evenly distributed, and further can be applied by the users.

4.3 Implementing several prospecting inoculums for artificial inducement

By paying attention thoroughly to Table 5 and Table 6, results of viability and virulence tests indicated that the fungi originated from Gorontalo, which developed the disease symptom, worked out better compared to other fungi from Jambi, West Kalimantan, and West Sumatera, since the latter fungi (i.e. from Jambi, West Kalimantan, and West Sumatera) caused the death to the inoculated trees. For example, the trees inoculated with the fungi from Jambi sooner or later suffered from unhealthy growth with their leaves withering and in color turning yellow. Meanwhile, likewise, the trees inoculated with the fungi originated from West Kalimantan and Padang (West Sumatera) only showed the sign that their leaves withered.

From the results of molecular identification as inflicted by 36 gaharu-yielding fungi strains, could be acquired the species of the so-called *Fusarium solani* which became the most dominant of the other strains. *Fusarium solani* species presented as the best inoculant that developed gaharu at the four gaharu-yielding tree species, and this species comes from consecutively Gorontalo and Jambi. Morphologically, the *Fusarium solani* isolates were dominated by while-colored mycelia, but there existed colonies with weak red, yellow, and violet colors. Almost all the isolates exhibited the aerial-mycelium characteristics.

According to histologically, the *Fusarium solani* isolates afforded the macroconidia characteristics dominated by elliptical shape. The fungi originated from Gorontalo exhibited viability and virulence which was very excellent compared to those of other fungi from Jambi, West Kalimantan, as well as West Sumatera. At the different research locations, the *Fusarium* spp. fungi could induce the gaharu trees more excellently compared to other fungi from Jambi, West Kalimantan, as well as Padang (West Sumatera), due to among others the fungi suitability, fungi violence, and the resistance of the trees themselves.

From the results of chemical analysis, it indicated that the wood portion (particularly sapwood) in *Aquilaria* sp. and *Gyrinops* sp. afforded material characteristics or released specific fragrant smell. Particular for *Aquilaria* sp., its wood portion contained 2-butanone compounds as commonly encountered inside the gaharu, in high concentration (3.26%). From the chemical analysis on both samples as described above (*Aquilaria* sp. and *Gyrinops* sp.), it turned out that each isolate species or each gaharu-yielding tree species exhibited or released different fragrant-smell characteristics. Further, regarding the active contents, were also analyzed the gaharu as yielded by those two tree species (i.e. *Aquilaria* sp. and *Gyrinops* sp.).

Secondary metabolites in plants defense system, like phytoantisipin or phytoalexin, play a big role (Verpoorte *et al.,* 2000). Phytoantisipin is an active compound with anti-microbe activity which present in plant, but sometimes its activity is stimulated by wounds. Phytoalexin is an anti-microbial active compound which is produced *de novo* after wounding or infection. The biosynthesis of both compound are stimulated in gene level (Verpoorte *et al.,* 2000; Vidhyasekaran, 2000).

Plants secondary metabolites which are derivated from terpenoid have various functions in plants; like as an anti-microbial agent (sesqui-, di-, and triterpena). Based on the various functions, the expression of the related biosynthesis pathways would be different. There are biosynthesis pathways that are stimulated in gene level after wounding or infection and there are others that occur in compounds level, where the already present compounds are to change enzimatically into active compounds when there is a wound. For instance, certain sesquiterpena biosynthesis in solanaceae is stimulated when there is microbe infection, whereas in other plants, sequiterpenoid biosynthesis is a common expression. In *Morinda citrifolia,* anthraquinone can be found in all area of the plant (Verpoorte, 2000).

The secondary metabolite concentration varies between species, inter-tissues (the highest is in the derm, teras wood, roots, branch base, and wounding tissues), between trees in the same species, inter-species, and is also season-dependent. Tropical and sub-tropical species usually contain higher extractive amount than temperate trees (Forestry Comission GIFNFC, 2007).

Under the condition facing the infection by fungi, the gaharu-yielding trees will exhibit their responses to defend and restore themselves. The resistance of trees will determine which will be winner between the infected trees and the diseases inflicted by such gaharu-developing fungi. In the aspects of gaharu development, certainly the pathogen fungi are expected to be the winner, thereby the gaharu products could be yielded as desired. The chemical compounds owned by the tree body serves as one attempt to defend those trees themselves against the pathogen attacks. Meanwhile, the chemical compounds in the gaharu have been identified as among others sesquiterpenes, acting as defending compound of phytoalexin type. The vulnerability of trees in facing the pathogen infection will be related to the gaharu as developed, and these are each reflected by the extent of infection and the content of other compounds.

Some modification of inoculation engineering technique have been tried in Lombok (West Nusa Tenggara). There was a problem about environmental condition in mix plantation between *Gyrinops* spp. and cacao trees. The local condition is very high humidity and high rainy intensity. Some of Gyrinops tree were decay condition after inoculation. They used some nails with pores and also plastic pipe to ensure the liquid of inoculum inside in stem of gaharu tree (Annex 18 and Annex 19).

Novel findings by gaharu team's have revealed important aspects that determine the successful of gaharu formation by artificial induction, i.e. methods of injection, fungal strain type, and growing media for delivering the fungi. Intensive studies for several years have confirmed efficient gaharu inducing methods, as follows: (i) Injection hole is of small size of about 3 mm in diameter. The holes will be closed naturally by the plant, not long after inoculants injection. This closing process of the injection hole is important in stimulating the formation of gaharu; (ii) Inoculant is delivered in the form of liquid by injection with a syringe of about 1 ml per hole; (iii) Type of fungal strain determines the gaharu formed, so screening of efficient strain using few samples in several locations to confirm its efficiency is essential prior to establishing large demonstration plots; (iv) Spaces in between holes should be wide enough (about 25 cm apart) to prevent from overlapping of vertical disease development from each other's hole; (v) The quality of gaharu formed becomes higher with longer incubation time. the Gaharu product harvested after three years of induction using this method was classified as *tanggung* a grade higher than kemedangan, while gaharu harvested from shorter incubation period was considered as *kemedangan* grade A-B.

5
CONCLUSION

From the molecular identification as inflicted by 36 gaharu-yielding fungi strains, could be acquired the species of the so-called *Fusarium solani* which became the most dominant of the other strains. *Fusarium solani* species presented as the best inoculant that developed gaharu at the four gaharu-yielding tree species, and this species comes from consecutively Gorontalo (coded as 0509) and Jambi (coded as 0500). Morphologically, the *Fusarium* spp. isolates were dominated by while-colored mycelia, but there existed colonies with weak read, yellow, and violet colors. Almost all the isolates exhibited the aerial-mycelium characteristics. Histologically, the *Fusarium* spp. isolates afforded the macroconidia characteristics dominated by elliptical shape. The fungi originated from Gorontalo exhibited viability and virulence which were very excellent compared to those of other fungi from Jambi, West Kalimantan, as well as West Sumatera. At the different research locations, the *Fusarium* spp. fungi could induce the gaharu trees more excellently compared to other fungi from Jambi, West Kalimantan, as well as Padang (West Sumatera), due to among others the fungi suitability, fungi violence, and the resistance of the trees themselves.

FORDA developed fermentation technique for large scale production and establish quality control procedure to produce inoculant of high quality. Quality control of inoculant plays an important role in ensuring the effectiveness and virulence of inoculant for certain period of time. For large scale production, one shaker with capacity of 500 litres inoculants per month has been placed in Forest Microbiology Laboratory, FORDA.

6
RECOMMENDATION

The uses of *Fusarium* spp. fungi isolates from Gorontalo seems the most recommendable, since this isolate affords high viability and virulence, and hence can be implemented to various gaharu-yielding tree species, which grow on several regions in Indonesia. This is so by considering and following inoculation protocols as enacted by the FORDA (Forestry Research and Development Agency, administratively under the Indonesia's Ministry of Forestry). For the isolates recommended as the second-best rank, it is the *Fusarium* spp. fungi originated from Jambi, since this fungi isolate exhibits superiority and afford evolving the fragrant aromas which differs from those of isolates with Gorontalo origin.

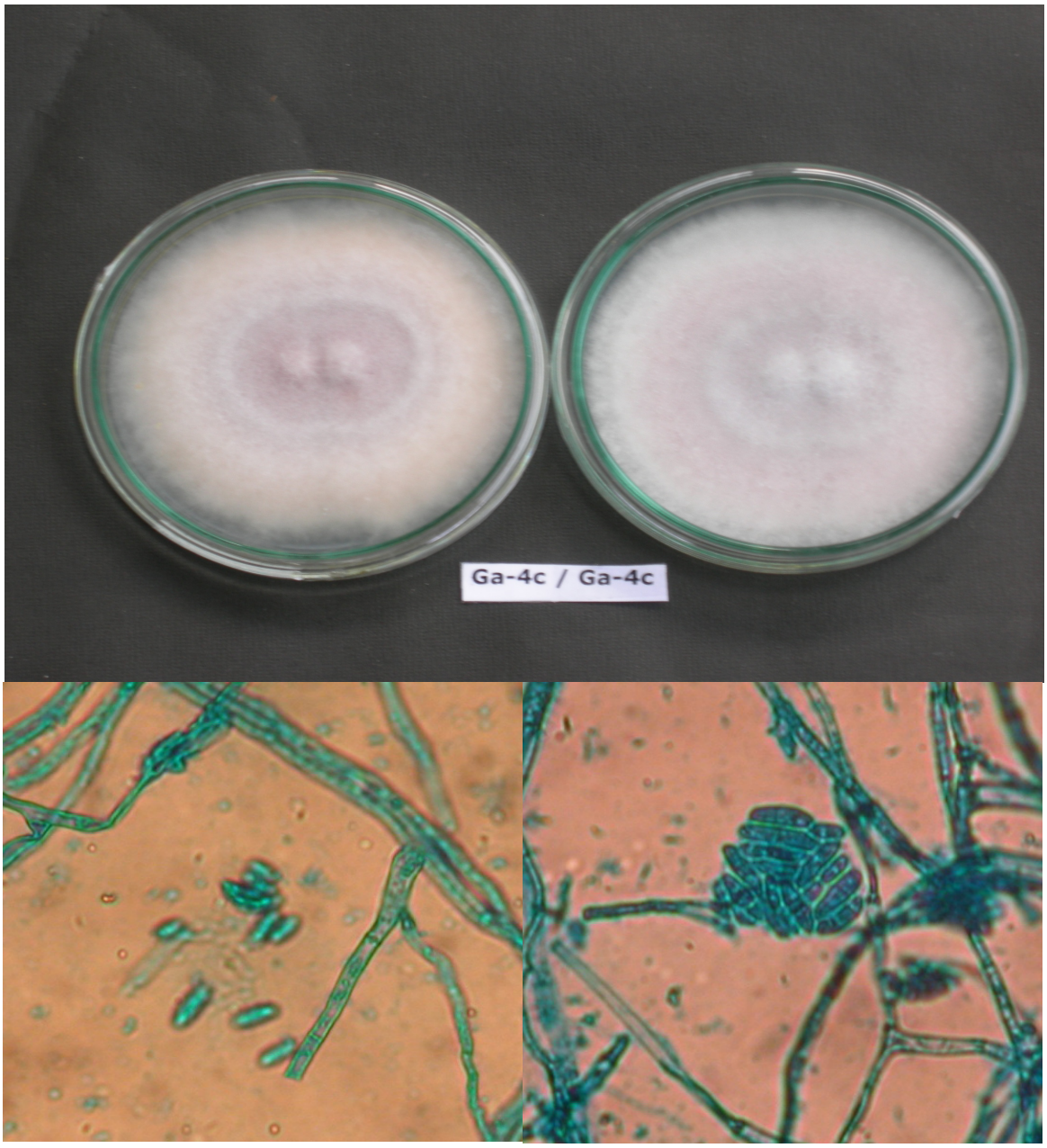
It still deserves the further tests on developing *Fusarium* spp. fungi originated from various regions, and have been collected by the FORDA to assess the potency of gaharu development at other gaharu-yielding tree species in the different locations.

7
IMPLICATION FOR PRACTICE

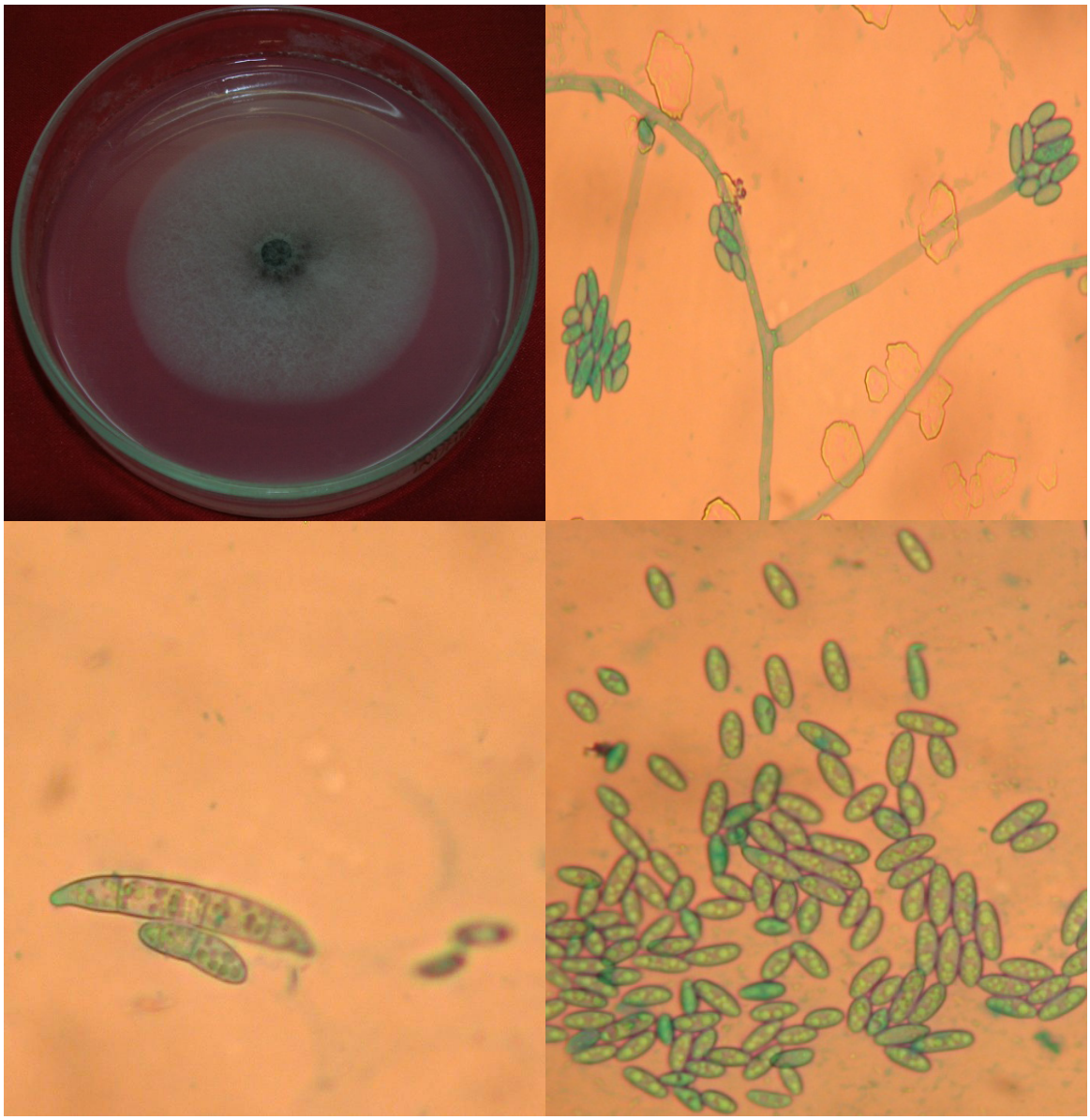
Novel findings by gaharu team's have revealed important aspects that determine the successful of gaharu formation by artificial induction, i.e. methods of injection, fungal strain type, and growing media for delivering the fungi. These methods are more practice, effective, and efficient.

ANNEX

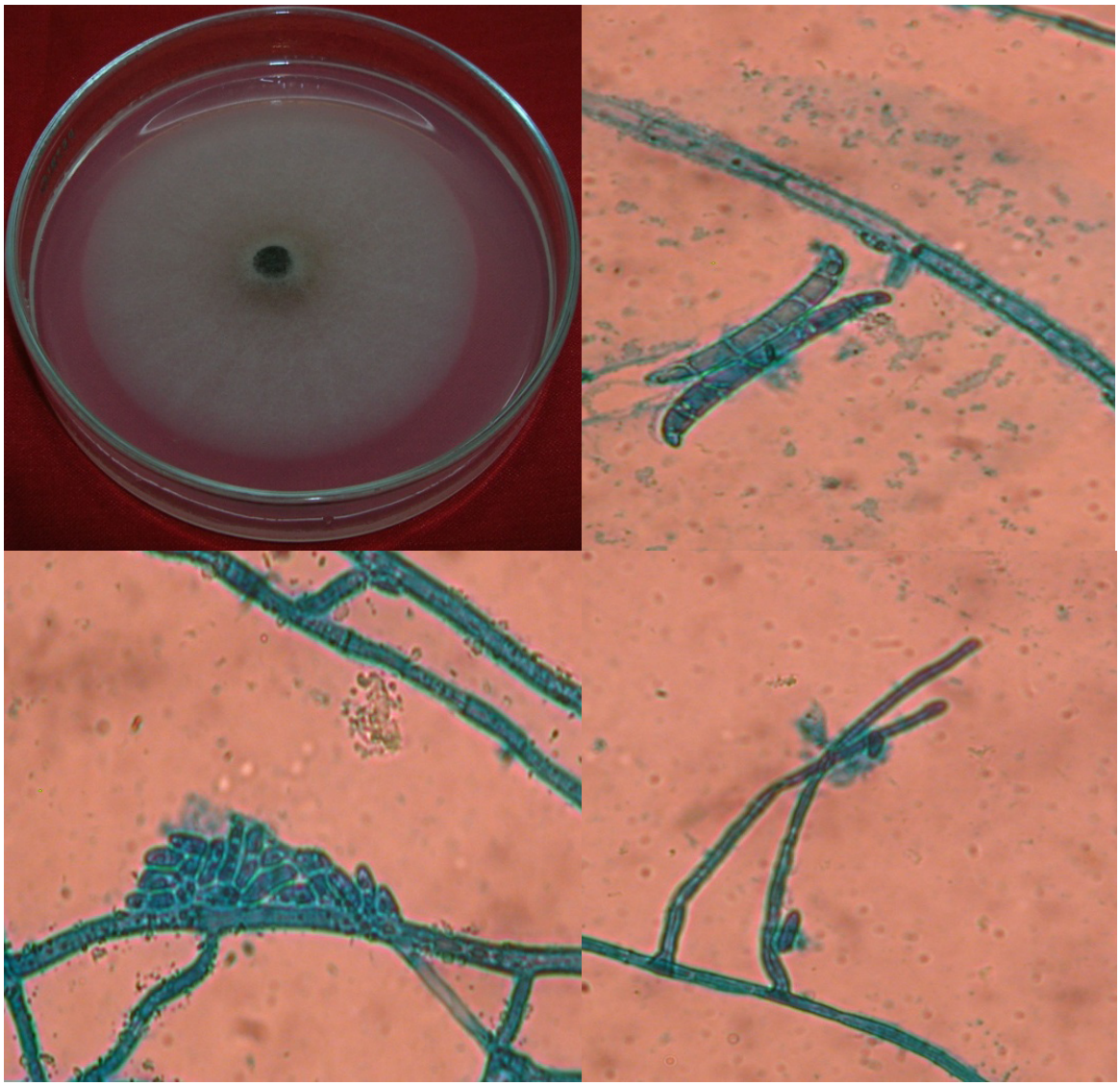
Annex 1. Pure culture of *Fusarium solani* from Gorontalo (Sulawesi Island)



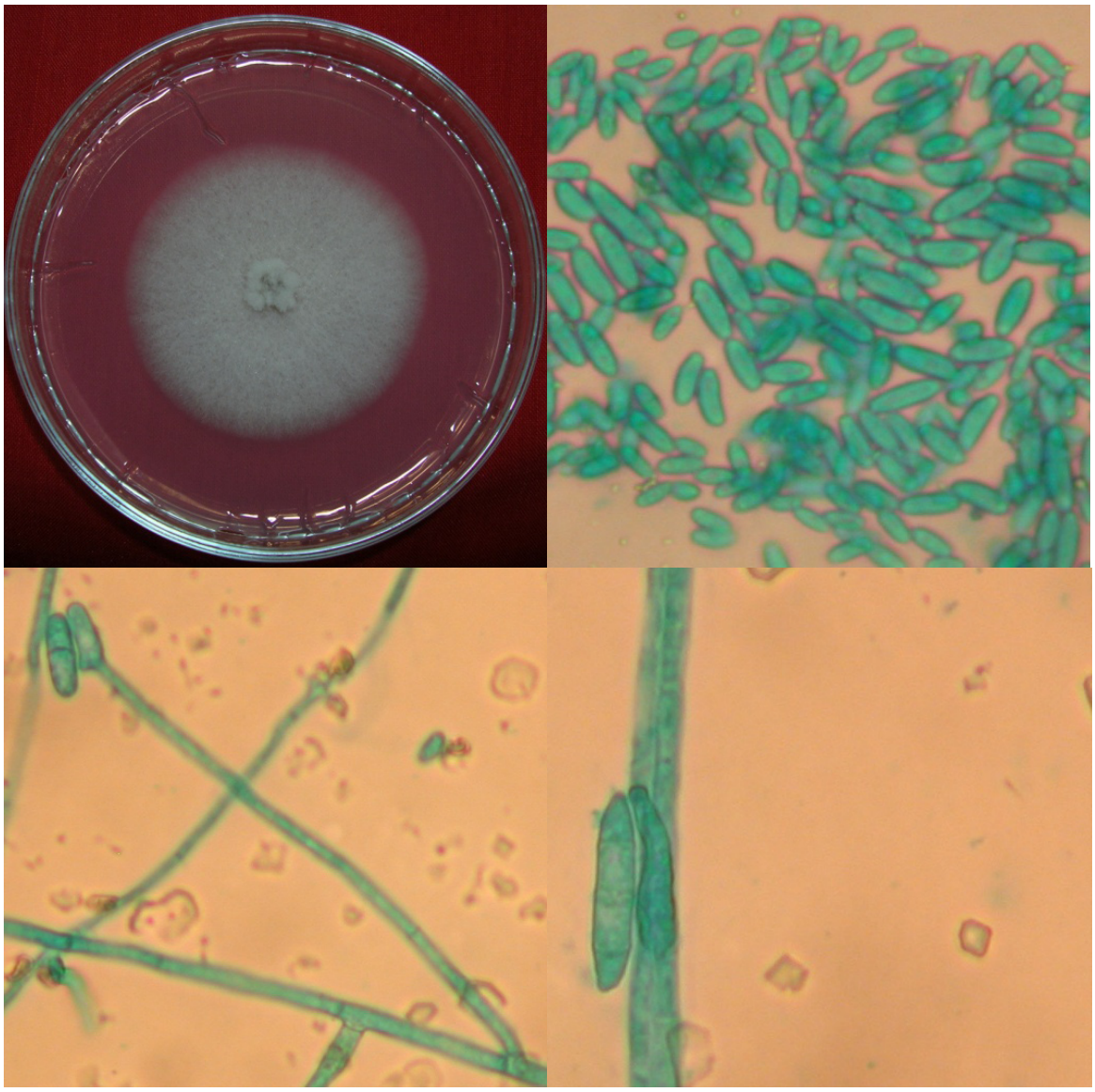
Annex 2. Pure culture of *Fusarium solani* from Jambi



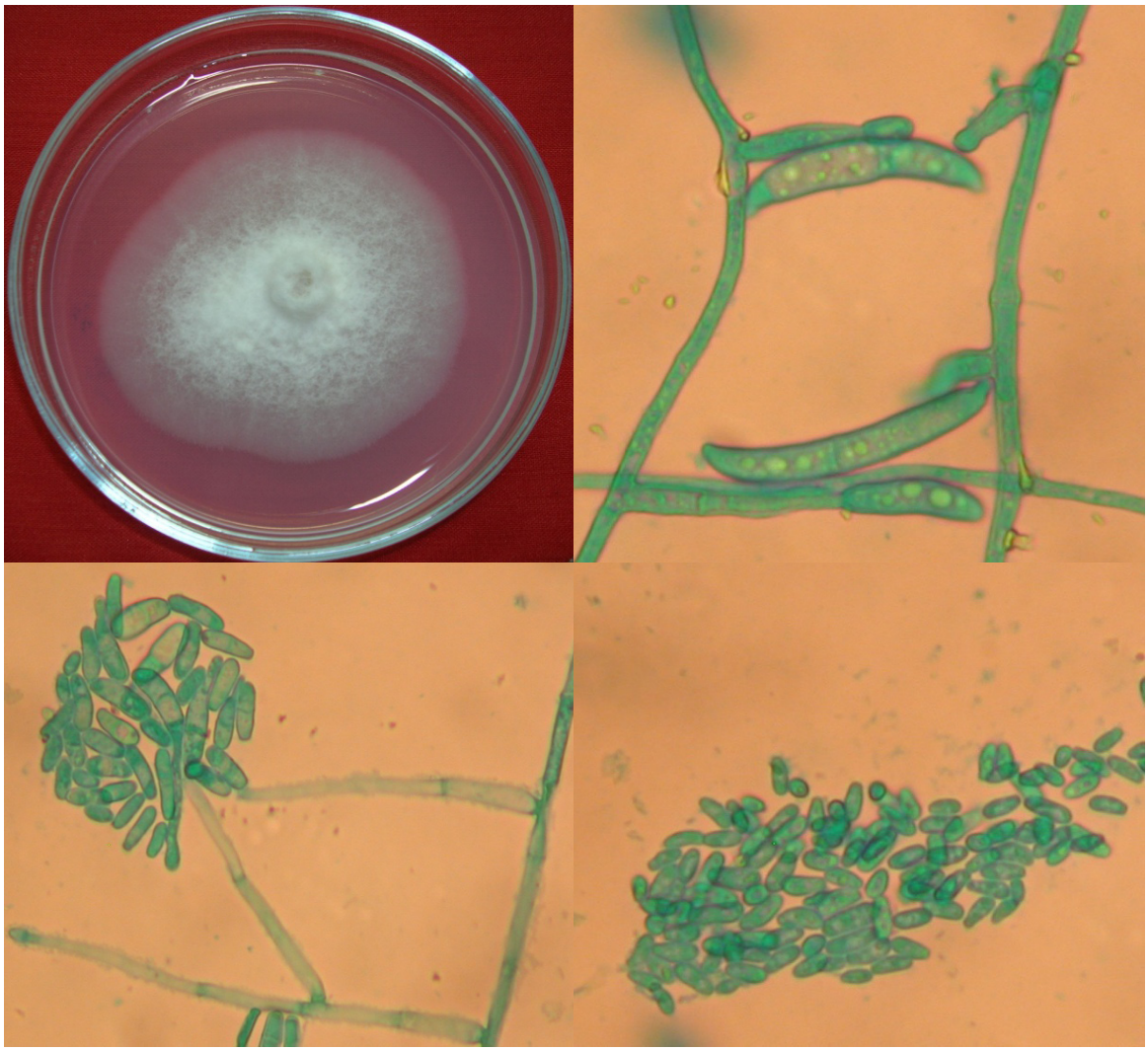
Annex 3. Pure culture of *Fusarium solani* from West Kalimantan



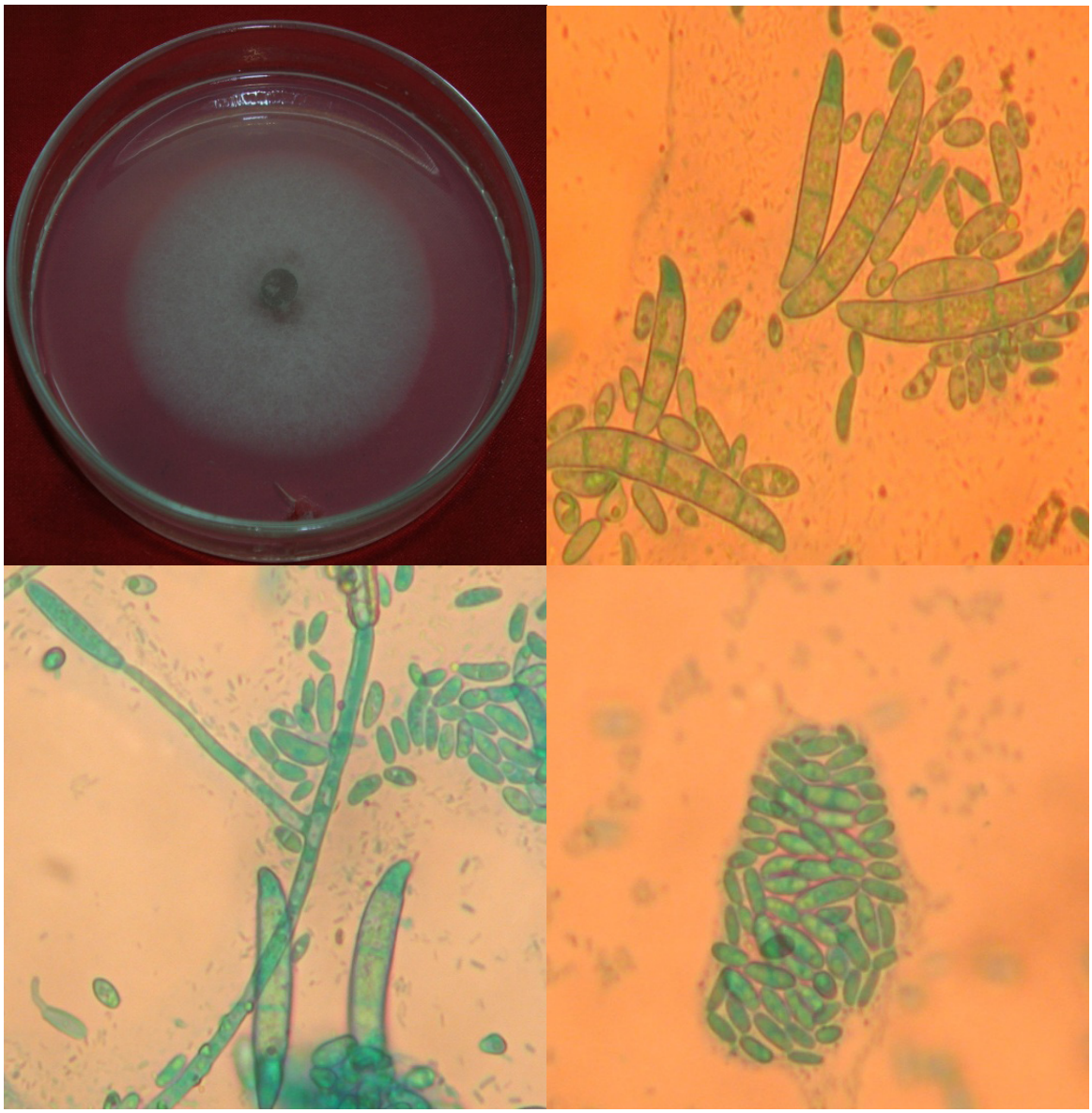
Annex 4. Pure culture of *Fusarium solani* from Padang (West Sumatra)



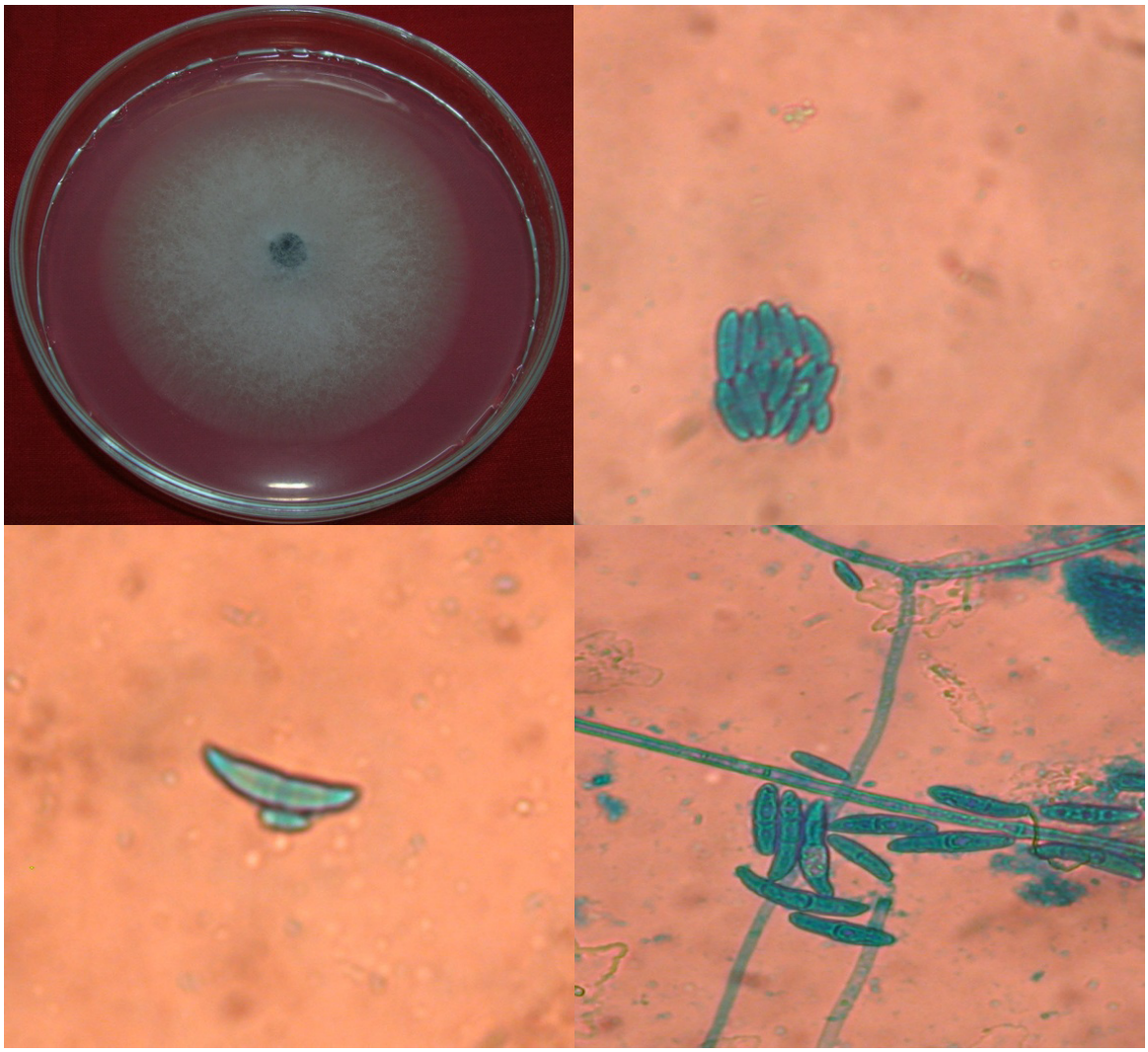
Annex 5. Pure culture of *Fusarium solani* from West Nusa Tenggara



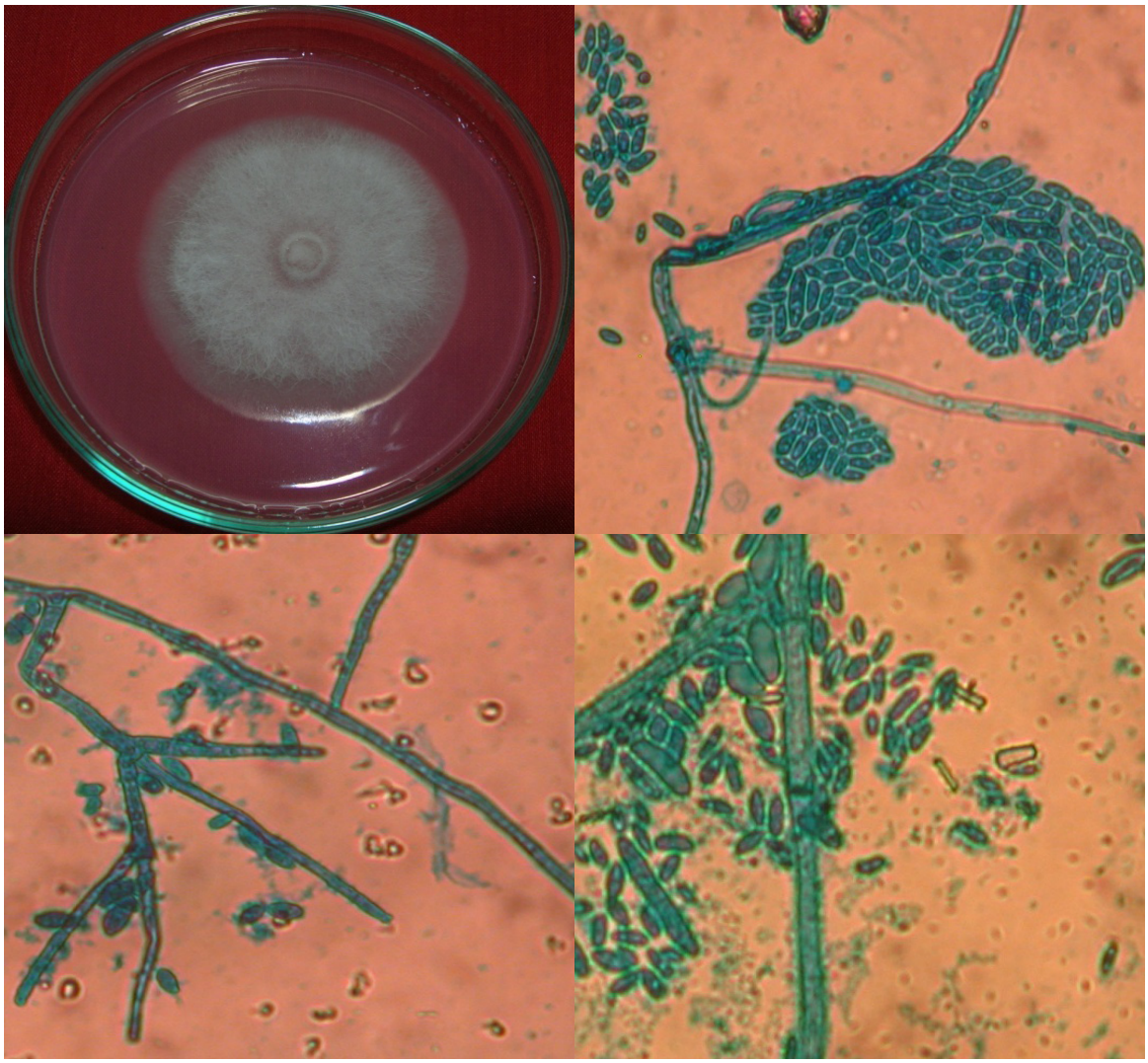
Annex 6. Pure culture of *Fusarium solani* from Lampung



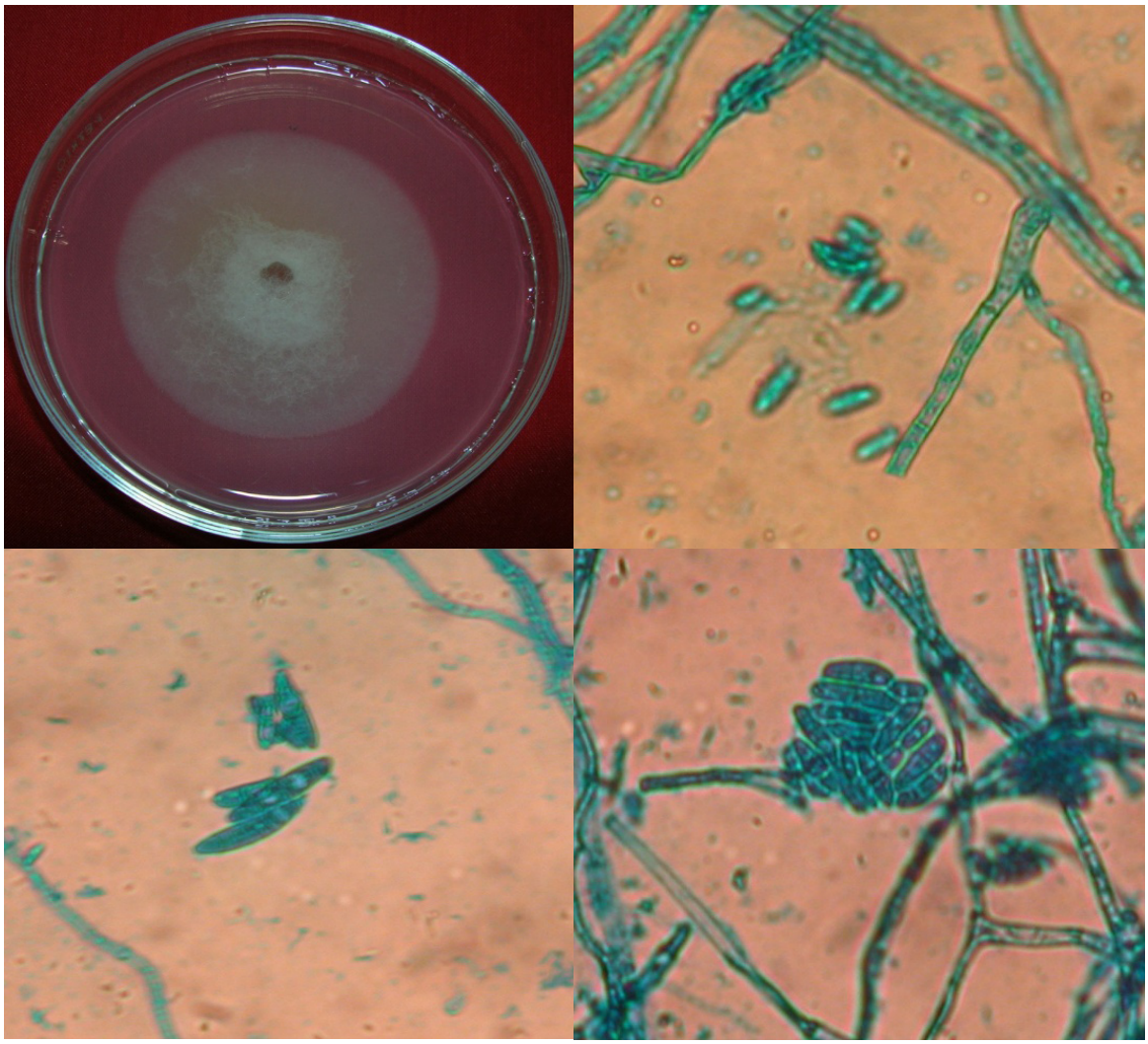
Annex 7. Pure culture of *Fusarium solani* from Central Kalimantan



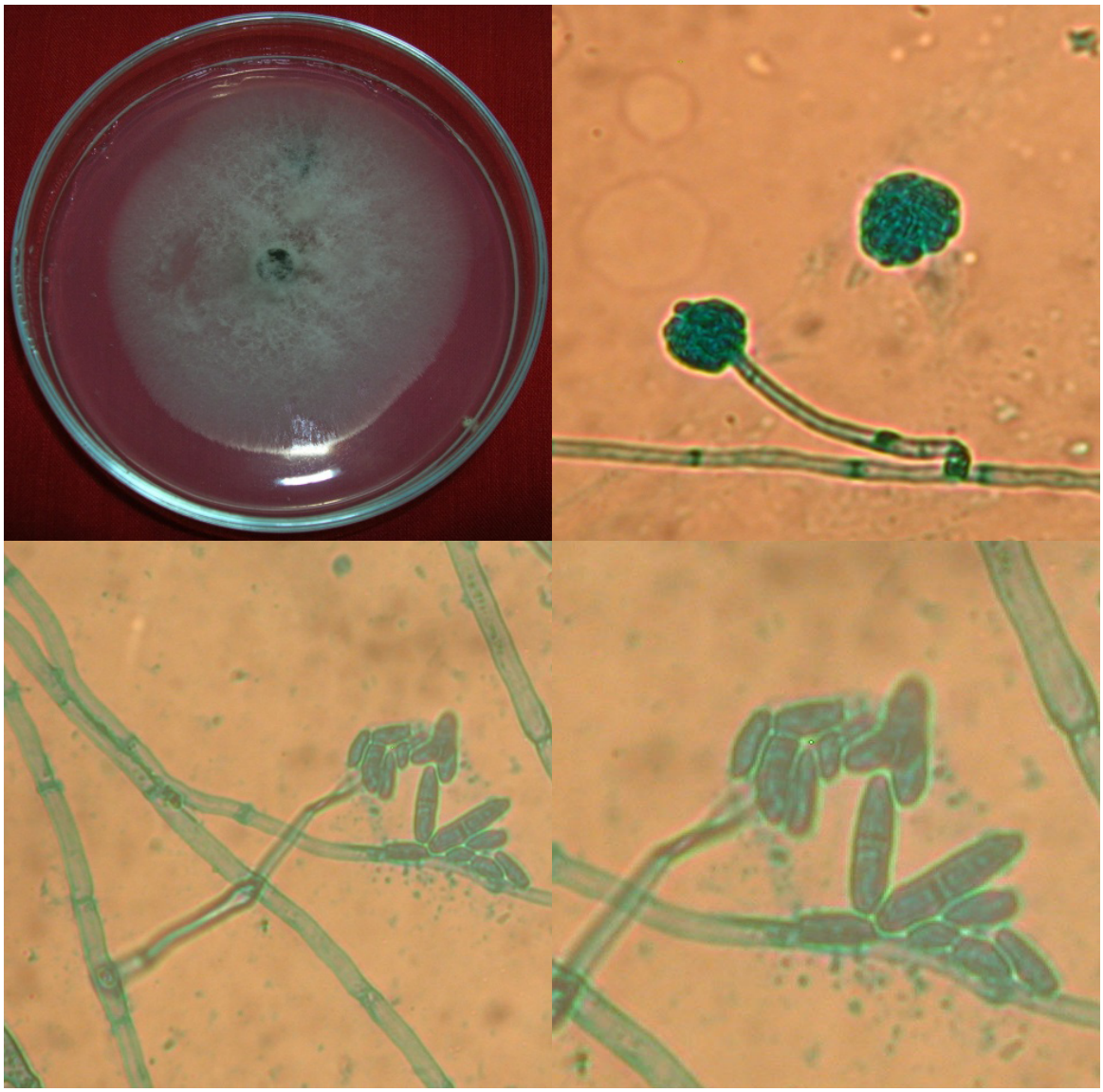
Annex 8. Pure culture of *Fusarium solani* from Mollucas.



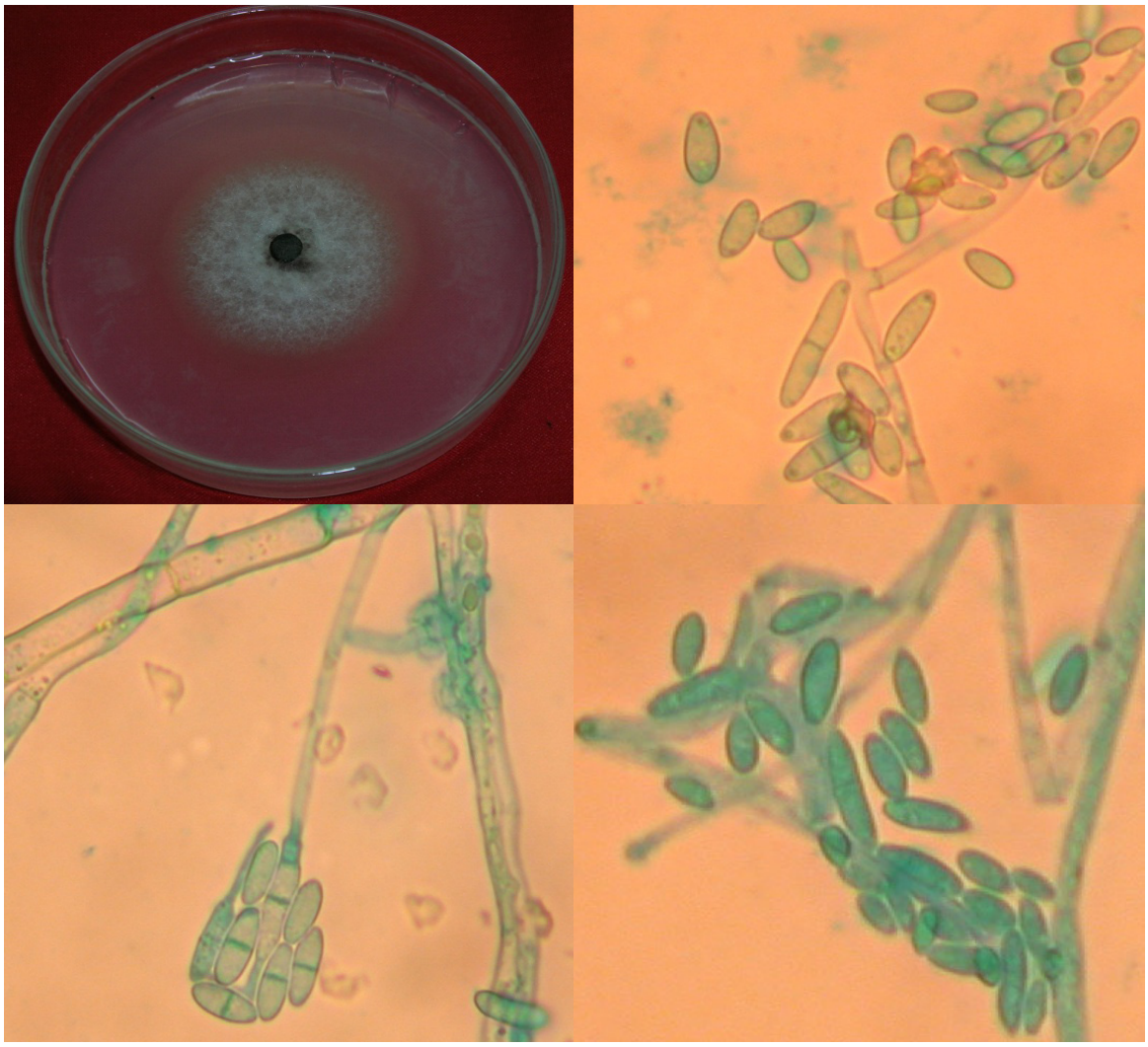
Annex 9. Pure culture of *Fusarium solani* from South Kalimantan



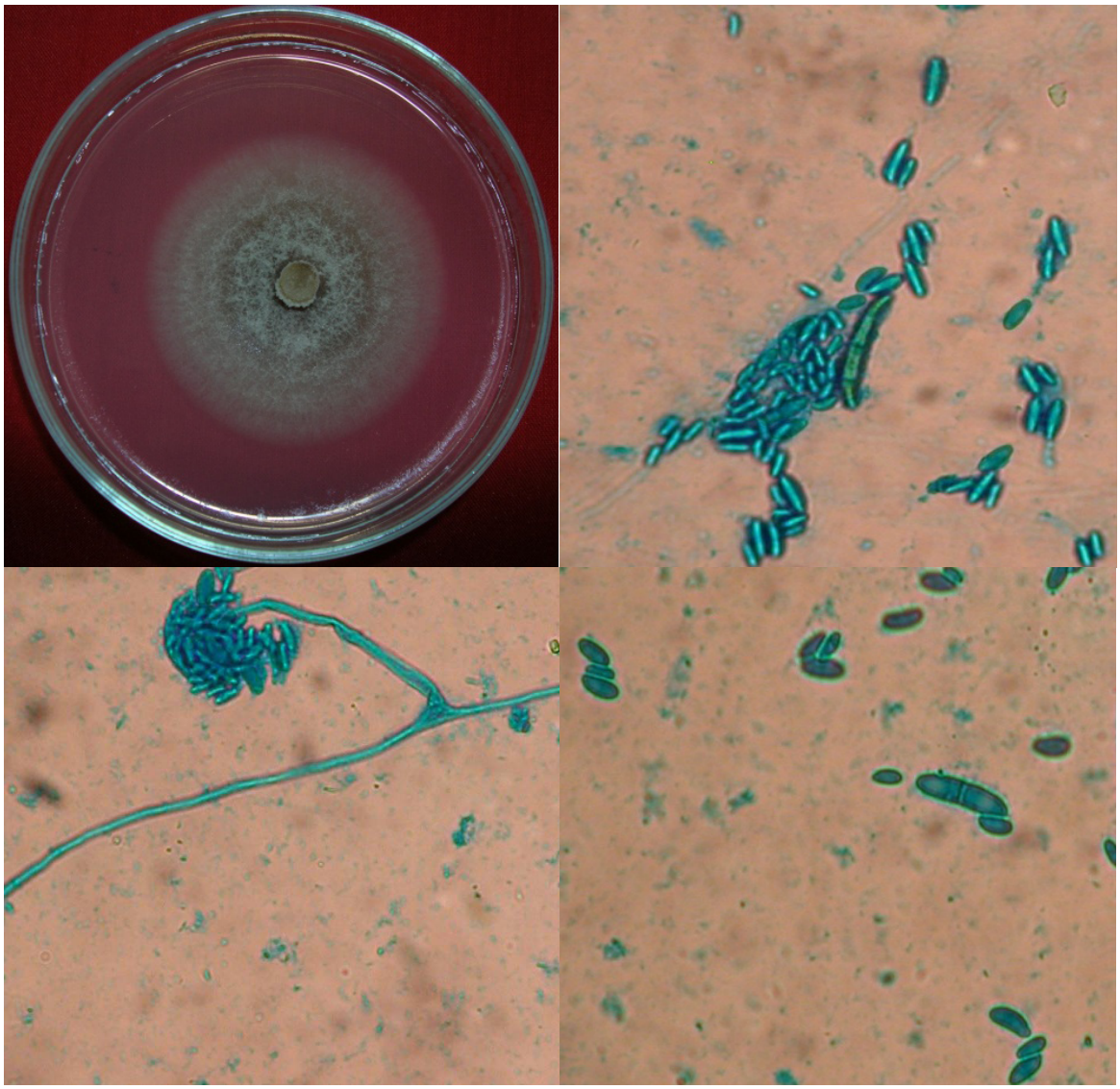
Annex 10. Pure culture of *Fusarium solani* from Riau



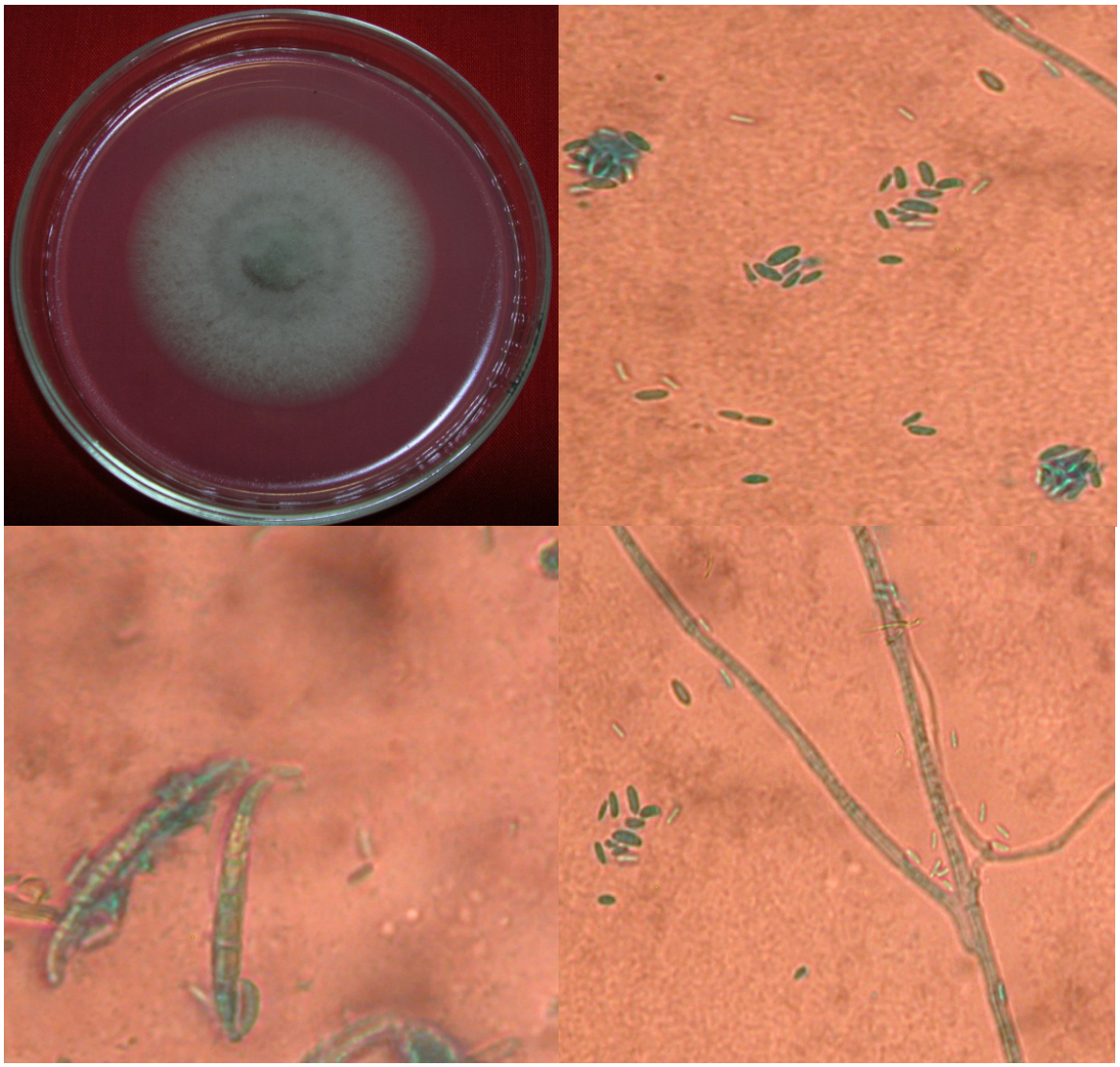
Annex 11. Pure culture of *Fusarium solani* from Sukabumi (West Java)



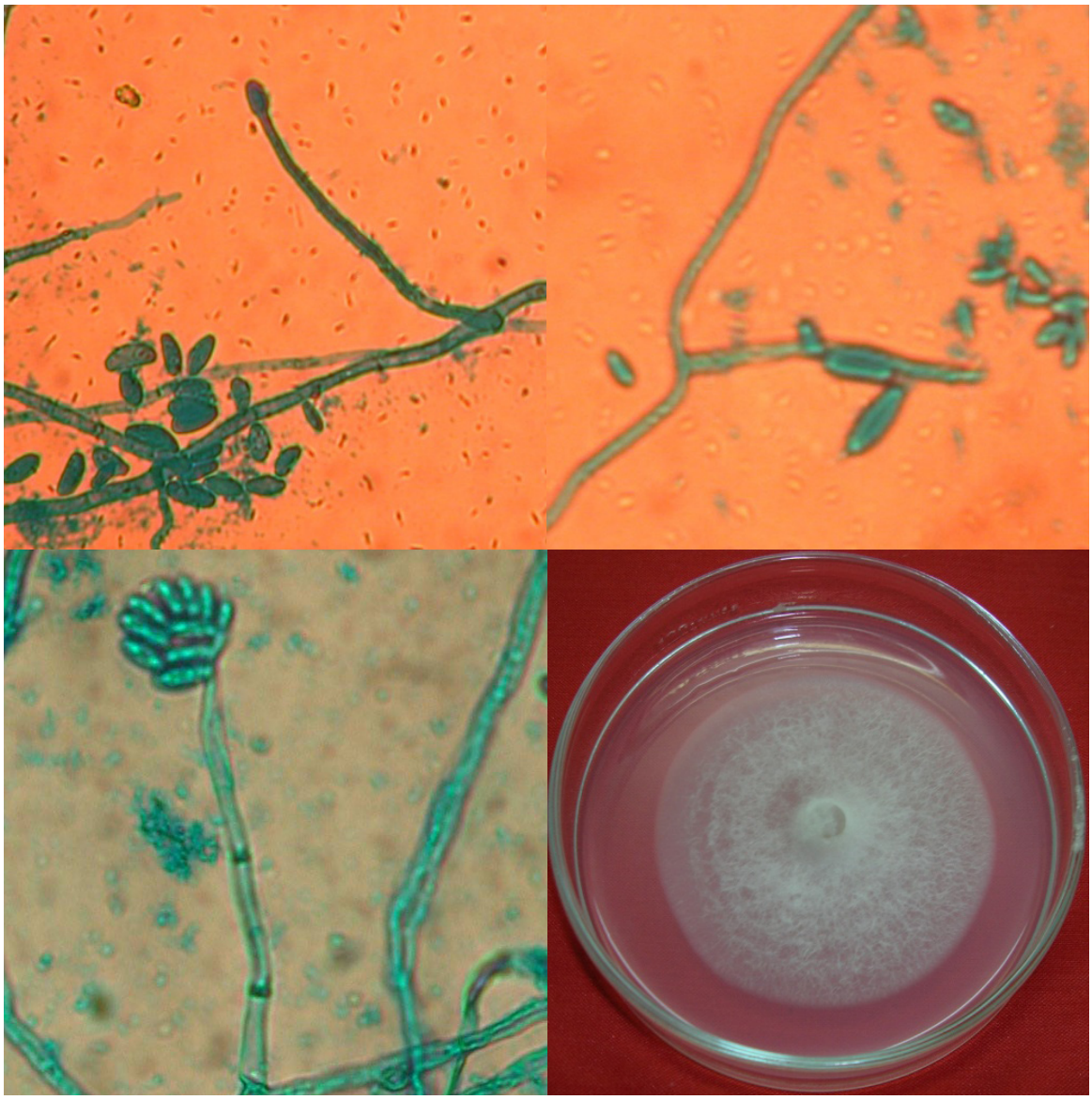
Annex 12. Pure culture of *Fusarium solani* from Yanlapa (West Java)



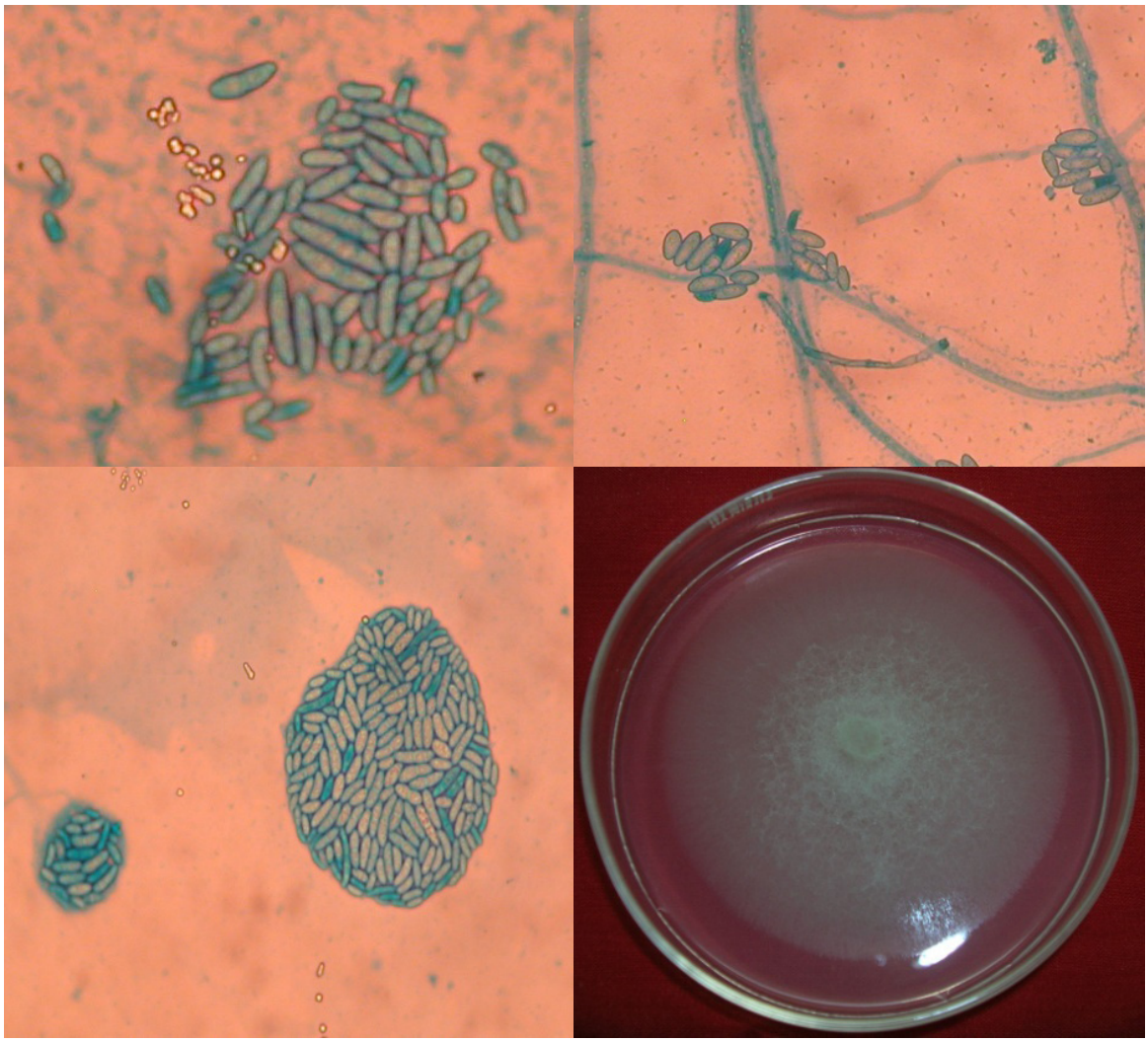
Annex 13. Pure culture of *Fusarium solani* from Bahorok (Nort Sumatra)



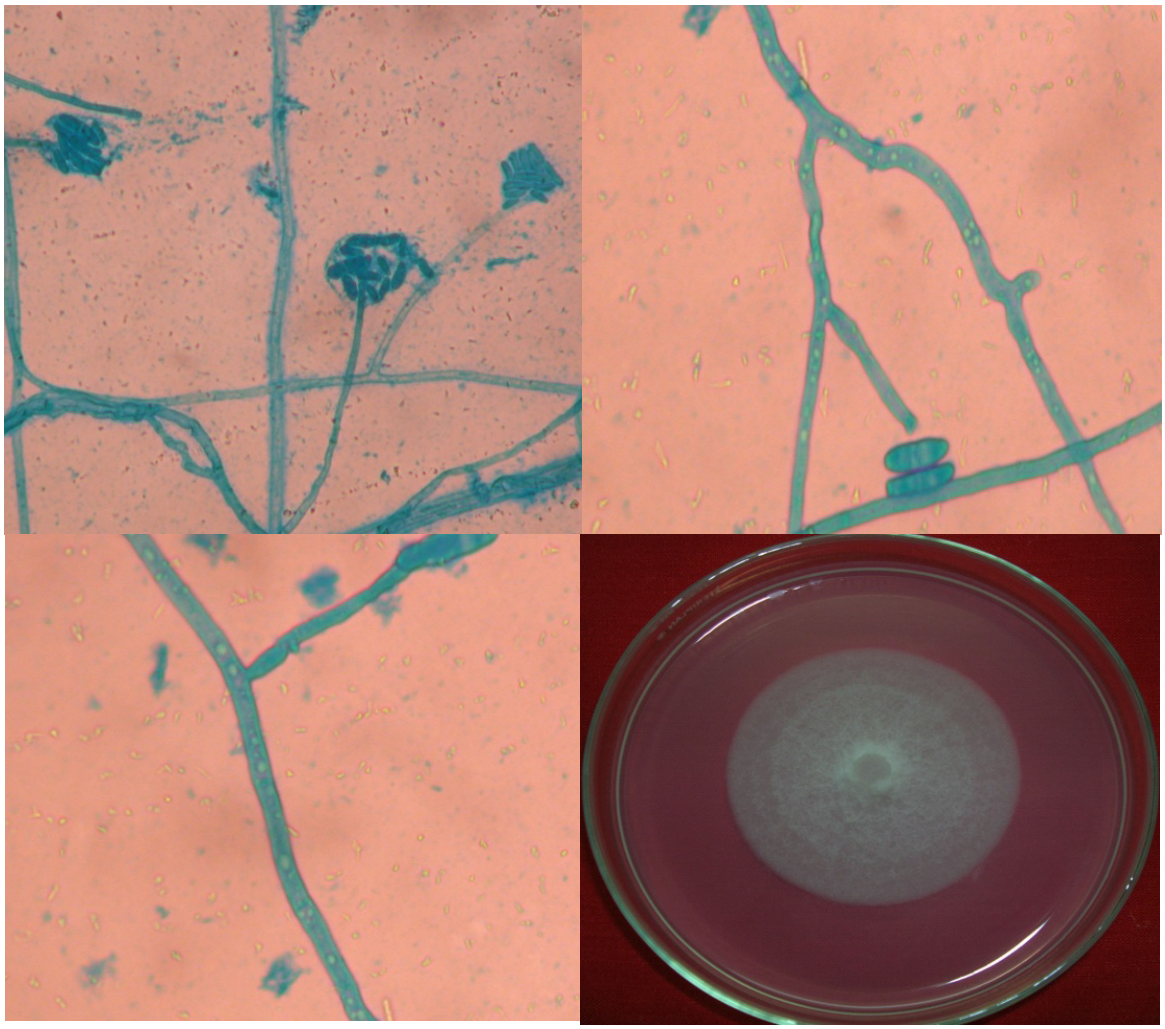
Annex 14. Pure culture of *Fusarium solani* from Bengkulu



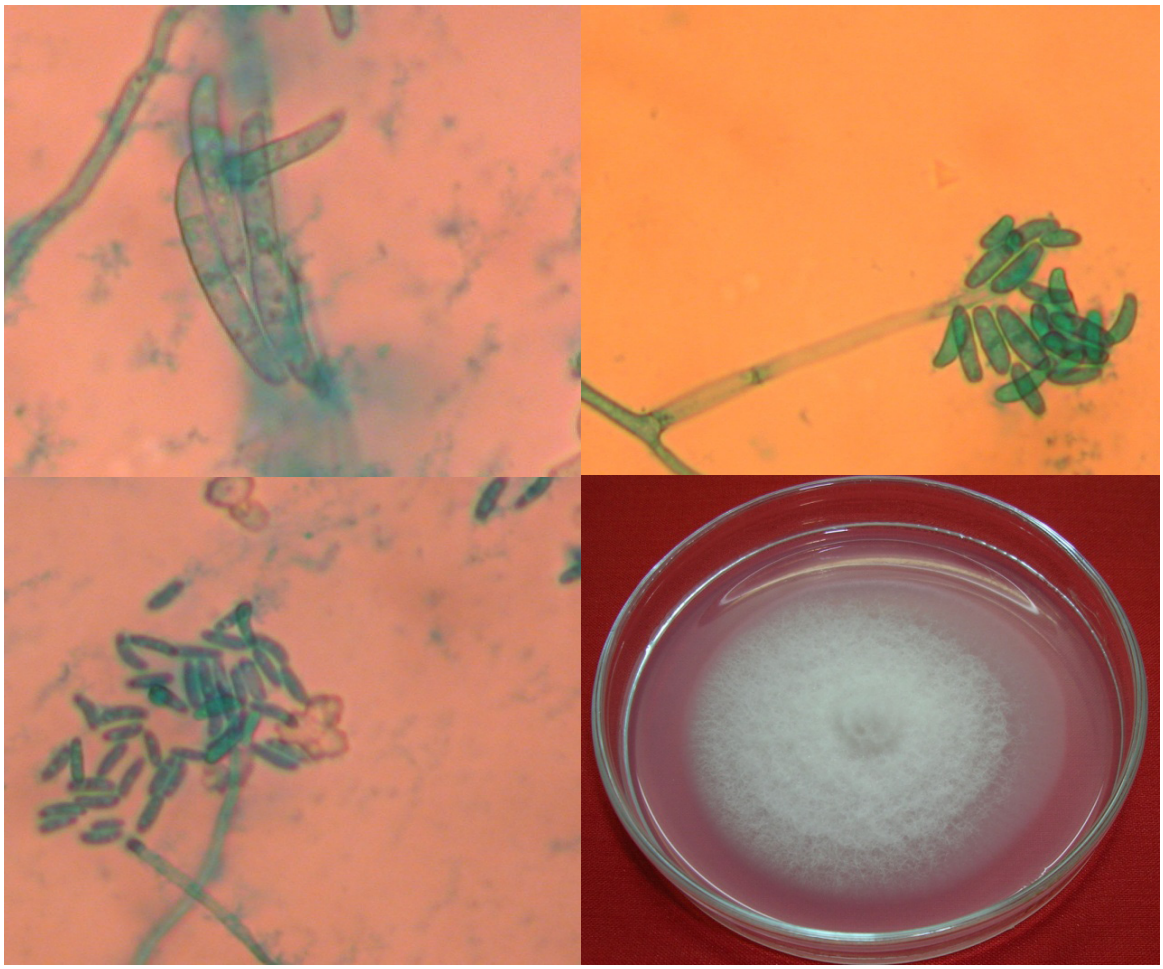
Annex 15. Pure culture of *Fusarium solani* from Bogor (West Java)



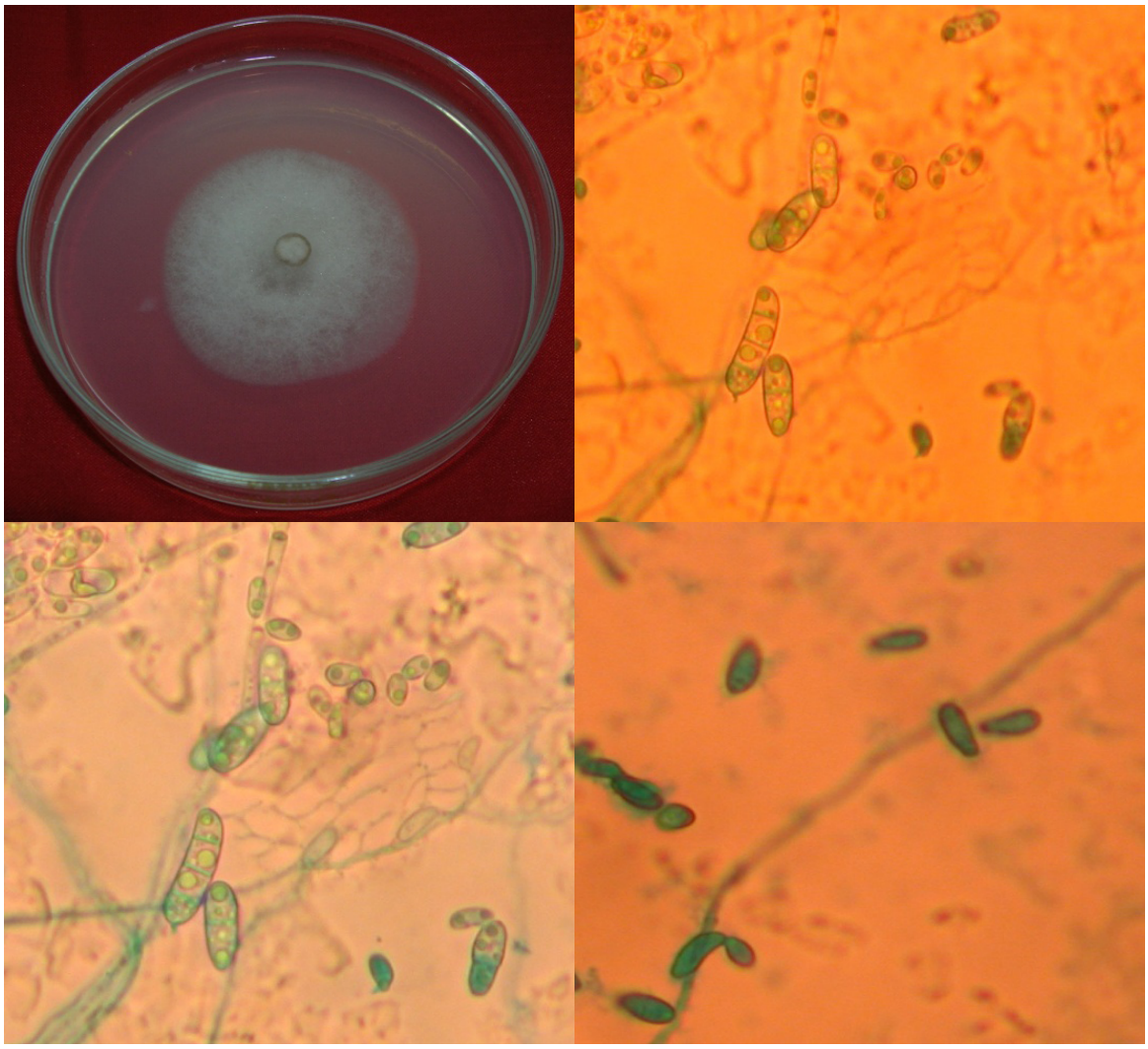
Annex 16. Pure culture of *Fusarium solani* from East Kalimantan



Annex 17. Pure culture of *Fusarium solani* from Bangka Island.



Annex 18. Pure culture of *Fusarium solani* from Medan (North Sumatra)



Annex 19. Nail with pores designed by Forest Research Institute (FRI) Mataram, Lombok (West Nusa Tenggara).

Annex 20. Plastic pipe to keep liquid inoculum inside of gaharu stem in West Nusa Tenggara.

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