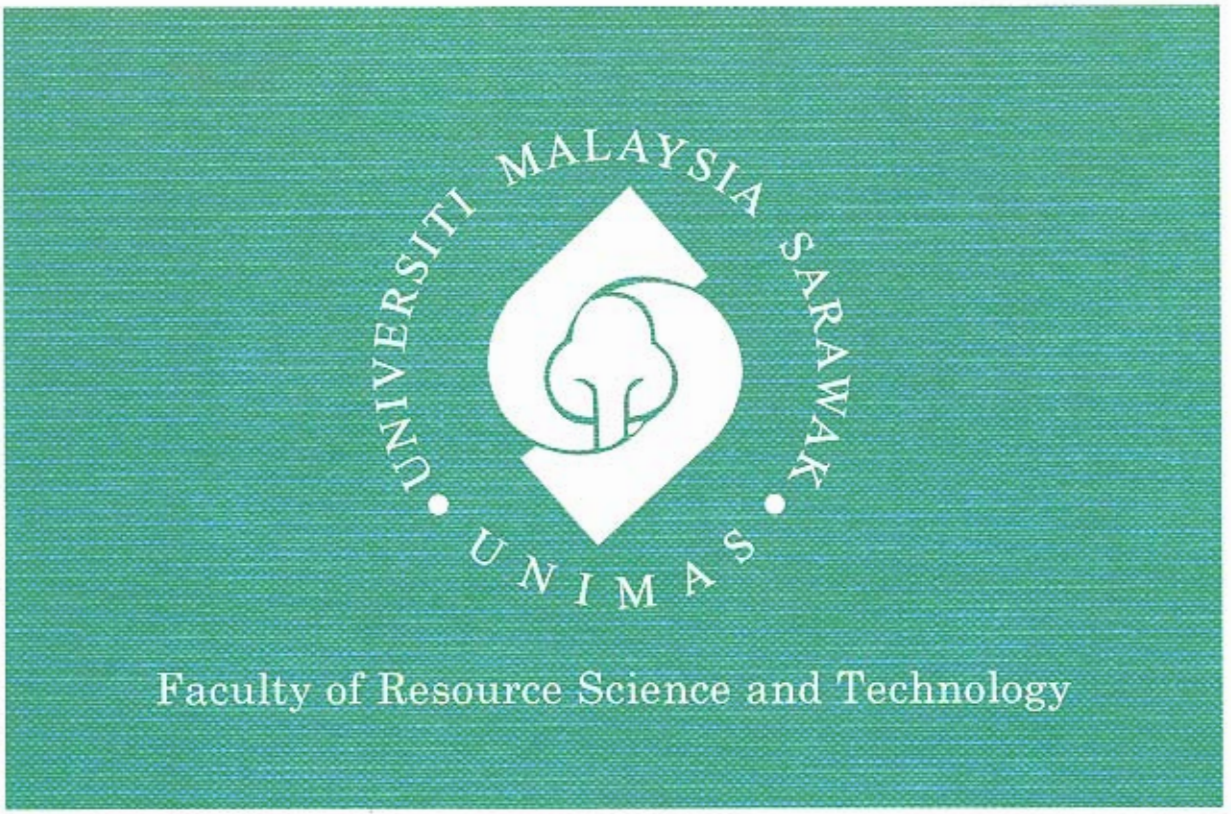

ISOLATION AND CHARACTERIZATION OF ENDOPHYTES ISOLATED FROM *AKAR GAHARU*

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Bachelor of Science with Honours
(Resource Biotechnology)
2013

Isolation and Characterization of Endophytes Isolated from *Akar Gaharu*

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A final project report submitted in partial fulfilment for the degree of Bachelor of Science with honours in Resource Biotechnology

Resource Biotechnology
Department of Molecular Biology
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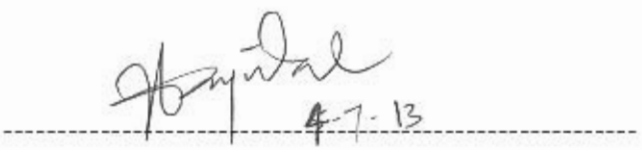
Universiti Malaysia Sarawak

2013

Declaration

I hereby declare that all the writing in this dissertation is from my own work except for some quotes which I have stated their source of origins.



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List of Abbreviations

Akar gaharu	Local name for subject study plant
Cyclosporine	A drug with immunosuppressive properties used to prevent the rejection of grafts and transplants
DNA	Deoxyribonucleic acid
et al.	*et alia* (and others)
griseofluvin	An antifungal drug
ISTH	International Subcommission on *Trichoderma* and *Hypocrea*
Lovastatin	An oral drug to reduce blood cholesterol levels; used when dietary changes have proved inadequate
MJ	Methyl jasmonate
n.d	not dated
PDA	Potato Dextrose Agar
PP	polyphenolic parenchyma
SMARD	Special Management of Agarwood Research & Development
sp. (spp.)	species (more than one species)
^{o}C	degree Celsius

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ABSTRACT

A study was carried out to isolate the endophytes from a wood-tendril plant known as *akar gaharu* that can be found in the jungle near Kota Samarahan, Sarawak. A total of 48 isolates were grown on the PDA and left for around three to five days, but only 16 isolates (AG1-4A, AG2-4A, AG3-1B, AG3-2A, AG3-6, AG4-1B, AG4-4A, AG4-6, AG4-7, AG5-1, AG6-5, AG7-2, AG7-3, AG7-4, AG7-6 and AG7-7) were successfully grown and chosen for further identification. Identification through colony growth morphology and microscopic observation was done after a week subculture of isolates. The morphology of the endophytes isolates was observed and classified which were based on their colour, shape and mycelia for the colony observation on PDA, while observation on structure of hyphae, spore, and conidia were for the microscopic examination. Among the 16 endophytes which were subjected to characterization, nine species of endophytes have been identified. Two isolates (AG1-4A and AG7-3) were identified as *Mucor* spp., three isolates (AG2-4A, AG4-6, and AG4-7) were identified as *Beauveria bassiana,* three isolates (AG3-1B, AG4-4A, and AG7-2) as *Trichoderma* spp., two isolates (AG3-6 and AG5-1) as *Microsporium* spp., two isolates (AG6-5 and AG7-7) as *Aspergillus flavus,* and the other rest isolates (AG3-2A, AG4-1B, AG7-4, and AG7-6) were identified as *Phoma tracheiphila, Penicillium* sp., *Fusarium oxysporum,* and *Colletotrichum gloeosporioides,* respectively. Further test will be needed in order to confirm the identity of these isolates.

Key words: Isolation, endophytes, *akar gaharu,* morphological characterization, identification

ABSTRAK

Satu kajian telah dijalankan untuk memencilkan endofit daripada sejenis pokok kayu menjalar yang dikenali sebagai akar gaharu yang boleh didapati di kawasan hutan berdekatan Kota Samarahan, Sarawak. Sejumlah 48 pencilan telah ditumbuhkan di atas PDA dan dibiarkan selama lebih kurang tiga hingga lima hari, tetapi hanya 16 pencilan (AG1-4A, AG2-4A, AG3-1B, AG3-2A, AG3-6, AG4-1B, AG4-4A, AG4-6, AG4-7, AG5-1, AG6-5, AG7-2, AG7-3, AG7-4, AG7-6 dan AG7-7) telah berjaya ditumbuhkan dan dipilih untuk pengenalpastian yang lebih lanjut. Pengenalpastian melalui pertumbuhan morfologi koloni dan pemerhatian mikroskop telah dijalankan selepas satu minggu pencilan disubkultur. Morfologi pencilan endofit diperhati dan dikelaskan iaitu mengikut warna, bentuk dan mycelia untuk pemerhatian koloni di atas PDA, manakala pemerhatian pada struktur hyphae, spora, dan konidia adalah untuk ujian mikroskop. Dalam antara 16 pencilan yang dihalakan untuk pencirian sembilan spesies endofit telah dikenalpasti. Dua pencilan (AG1-4A dan AG7-3) telah dikenalpasti sebagai Mucor sp., tiga pencilan (AG2-4A, AG4-6, dan AG4-7) telah dikenalpasti sebagai Beauveria bassiana, tiga pencilan (AG3-1B, AG4-4A, dan AG7-2) sebagai Trichoderma spp., dua pencilan (AG3-6 dan Ag5-1) sebagai Microsporium spp., dua pencilan (AG6-5 dan AG7-7) sebagai Aspergillus flavus, dan selebihnya (AG3-2A, AG4-1B, AG7-4, dan AG7-6) masing-masing telah dikenalpasti sebagai Phoma tracheiphila, Penicillium sp., Fusarium oxysporum, dan Colletotrichum gloeosporioides. Ujian lanjutan akan diperlukan bagi kepastian identiti pencilan-pencilan ini.

Kata kunci: Pemencilan, endofit, akar gaharu, pencirian morfologi, pengenalpastian

1.0 Introduction

Akar gaharu is a type of plant that lives in the tropical forest that is suspected to have the novel endophytic fungi which can induce the formation of resin in *Aquilaria* sp. supported by the ability of this plant to produce the high density of black substances on its stem which can be used to fake the super grade of agarwood of *Aquilaria*. *Aquilaria* sp. from the family Thymelaeaceae (Gratzfeld & Tan, 2008), which is the main source of the agarwood, characterized as a timber plant that can produce the most valuable non-timber products where it can be collected from the tropical forest. The resin of agarwood produced by the *Aquilaria* also visualized as a dark substances that spread on the bark or stem of the plant. Factors that can also contribute to the initiation of resin formation include the insects and pathogens attack, wounding and the abiotic stress (Cheong & Choi, 2003). The resin produced contains highly volatile organic compounds is aimed to retard and suppress the growth of the fungus that may cause the plant susceptible to the disease (Gratzfeld & Tan, 2008). This agarwood resin actually resulted from the methyl jasmonate (MJ), a vital substance of cell regulator in plant that induced the plant defense gene and next following with the production of the sesquiterpenes or other phenolic compound that lastly being expressed in the form of dark substances called resin (Cheong & Choi, 2003). The fungi has the ability to activate the methyl jasmonate inducible genes thus secrete the organic compound to be able to suppress the fungal growth forming the dark aromatic resin which contains many bioactive compounds that are known to be the major source of the antibiotics as the resin produced is the products of defense mechanism from pathogens (Cheong & Choi, 2003).

Other than that, as the agarwood has a high commercial value because of the novel resin, the world demands from customer are keep increasing, thus a higher yield of the products is needed. Harvesting the agarwood is not an easy task as the plant takes a few

years in order to become an adult plant that can produce the resin. According to Gratzfeld and Tan (2008) the only an estimated of 7%-10% of the *Aquilaria* trees are infected by the fungus and being able to form dark brown or black resinous agarwood in nature. Besides, this tree is also classified as the threatened species as it has high value market. Therefore, an alternative to minimize the problem is needed by searching for other alternative source of fungal inoculum from other plant as there are less source of fungal inoculum that have been discovered and have the ability to produce the high super grade agarwood but the formation of the high density of black resin on the *akar gaharu* is suspected to be in other way around. According to SMARD (n.d.), the fungi inoculum that have been infected into the *Aquilaria* through the process of inoculation could form agarwood after two months injection to four years old trees thus reducing the seven years which plants usually took to produce the resinous agarwood. This discovery would be able to shorten the time production of resin through infection of fungi and this should be able to fulfil the demands for the products. Therefore, this study is aimed:

1) to isolate the potential fungi from the *akar gaharu* that are able to induce the resin production in agarwood of *Aquilaria* sp.;
2) to identify the species of fungi isolated from *akar gaharu* using morphological characterization;and
3) to compare the identified fungi species isolated from *akar gaharu* with the common fungi found in agarwood of *Aquilaria* plant.

2.0 Literature Review

2.1 Black resinous substances source of bioactive metabolites

Based on the physical appearance of the *akar gaharu*, it is suspected to be able to produce the black substances on its stem. This observation is much similar with the resin produces in the agarwood of *Aquilaria* plants thus indicate that the *akar gaharu* have high probability to resemble the properties of the agarwood as some of the local people use this plant in traditional medication but no study has reveal the significant potential of this *akar gaharu.* The resin is commonly rich of many organic compounds and secondary metabolites. Many plants that can produce resin for example in *Pseudognaphalium vira vira* has the antibacterial properties that can control the soil bacteria and thus this plant was known to be one of the medicinal plant (Gil *et al.*, 2006). Some of the medicinal plants also produced resin that contains diterpenoids, flavonoids, and phenolic compounds (Gil *et al.*, 2006). According to Calvo *et al.* (2002), the microorganisms known as fungi have contributed a lot in the production of secondary metabolites. The production of more than 20,000 bioactive metabolites originated from the microbial proved that the fungi specifically endophytes acts as a major producer of the biologically active metabolites that are used to develop the medicinal drugs (Selim *et al.*, 2012). For example, this is proven by the successs development of the drugs from microbial origin such as antibiotic penicillin from *Penicillium* sp., the immunosuppressant cyclosporine from *Tolypocladium inflatum* and *Cylindrocarpon lucidum,* the antifungal agent griseofluvin from *Penicillium griseofulvum* fungus and the cholesterol biosynthesis inhibitor lovastatin from *Aspergillus terreus* fungus (Selim *et al.*, 2012).

inducing the resin formation in plants especially *Aquilaria* sp. The use of naturally occurring microorganisms such as endophytic fungi can enhance the production of resin in agarwood and the products produced is more perceive than the agarwood produce through the chemical injection.

2.3 Fungal infection

Most of the plants act as a host or reservoir of many microorganisms either bacteria or fungi. Some plants stored a group of microorganism known as fungi or endophytes that can produce novel bioactive metabolites. Therefore, these endophytes have been used as one of the natural sources for antibiotics because of the properties they exhibit as they can be effective against the plant diseases and pests due the observation with the action in the cellular signal and block the metabolisme pathways (Cheong & Choi, 2003).

By definition, endophytes are the microorganisms that live within the tissues of plant that give neither harm nor infection to the host plant (Selim *et al.*, 2012). This colonization of fungal endophytes on beneath tissue of epidermal cells layer of plants produced the bioactive metabolites for example some important enzymes that are crucial for the living process of the plants (Selim *et al.*, 2012). The plants and the fungal endophytes are lived symbiotically with each other where the host plants give the nutrition and living environment for the endophytes while the endophytes give the protection for the plant from the pathogenic invasion as the endophytes acts parasitic when their hosts are in stress (Selim *et al.*, 2012). Moreover, these novel bioactive metabolites are said to be benefits to man as its high potentiality for medical, agriculture and many others fields (Selim *et al.*, 2012).

The fungal infection for the resin induction takes place when the fungi are introduced into the stems of the plants. The stem of the tree is first drilled before the fungal inoculum is injected into the tissues (SMARD, n.d.). This can happens naturally as the stems may wound or injured by means of environment stress and the fungi from nature deposited into tissues and inhabit the exposed site. The process of resin accumulation is based on the interaction of the tree-fungi activity and the longer the "battle" between these sources indicates the high valuable the resin is (SMARD, n.d.). The fungal infection can be made by isolating the fungi and culture into specific media and thus the injected fungal can start producing agarwood after two months into the four-year-old plant (SMARD, n.d.). There are some common fungi that can be used as inoculums for the resin induction in plants. According to the Mohamed *et al.* (2010), common fungi that can be found in the agarwood of the *Aquilaria* are from the genus *Fusarium, Trichoderma, Curvularia and Cunninghamella.* These fungi should give some hints of the fungi that can induce the formation of the resin in agarwood although it is still not absolutely clear which are important or even necessary for the resin formation induction.

2.4 Drugs resistant pathogenic microorganisms

One of the major concerns in world health problem is related with the multi-drug resistant pathogenic microorganisms where it keeps increasing from time to time (Elufisan *et al.*, 2012). This phenomenon visualized as where the pathogenic bacteria are able to resist and defend themselves from being inhibit or destroy by the antibiotics or drugs which is later cause the disease to the infected organisms (Elufisan *et al.*, 2012). Bacteria which are resistant to the existing antibiotics are capable organisms that may have undergone gene mutation or gene resistance through the pathway known as horizontal transmission

(Elufisan *et al.*, 2012). The synthetic drugs may contribute to high probability of increasing the drug-resistant bacteria as it does not occur naturally but through several chemicals that may create resistance to the pathogenic microbes. Isolation of endophytes by screening for antimicrobial properties to develop natural drugs as the replacement of synthetic drugs promising the best way to overcome this multi-drug resistant bacteria problem (Selim *et al.*, 2012).

According to CDC (n.d.), *Staphylococcus aureus, Streptococcus pneumonia, Shigella* sp. and *Klebsiella* sp. are bacteria that have been reported to be able to rapidly developed resistance to antibiotics. For example, the Methicillin resistant strains of *Staphylococcus aureus* have been discovered and the worse issue is these resistant strains can also be transferred to other bacteria species through vertical transmission by means of genetic transfer and mutation and the horizontal transmission process such as conjugation, transformation and transduction (Elufisan *et al.*, 2012).

Thus, some researches have been conducted continuously for example the development of new drugs from the natural sources such as endophytic fungi in order to solve this problem (Elufisan *et al.*, 2012).

3.0 Materials and Methods

3.1 Sources of Materials

3.1.1 Wood sample

A total of seven wood samples with the infected region were obtained from the Samarahan jungle. Each sample was cut into small wood portions and collected from different sites of the tree's bark. The samples were labeled specifically and kept at 4°C in Virology Laboratory before further analysis.

3.1.2 Media and solutions

In preparation of fungal isolates, double distilled water, 70% ethanol and 0.5% of bleach were used and Potato Dextrose Agar (PDA) (Oxoid) was used in this study in order to cultivate the fungal colonies. For microscopic examination, the lactophenol blue was used to stain the endophytes.

3.2 Preparation of wood samples and plating

3.2.1 Surface sterilization of the wood samples

The preparation of the sample was started by washing off all the dirt on the wood samples using tap water. The wood or stem samples were cut into small pieces about 0.5 cm × 0.5 cm before they were surface sterilized. The surface sterilization procedure was conducted by placing the samples in the 0.5% sodium hypochlorite for two minutes followed by 70% ethanol for another two minutes and lastly several rinses in sterile distilled water.

3.2.2 Endophytes cultivation

Isolation of endophytes was accomplished by placing the stems' pieces on the PDA (PDA, Becton Dickinson, USA) and was left for another three to seven days in room temperature. Each colony that grown from the stem was subculture on the Potato Dextrose Agar (PDA, Becton Dickinson, USA), and it was continued until a pure isolate was obtained. For each sample type, two plates were prepared to grow seven to eight stems (replicates) to maximize the growth of possible different endophytes and the isolates were incubated again at room temperature (26°C) for five to seven days before they were subjected to morphological examination.

3.3 Colony growth observation and microscopic examination

The grown endophytes were subjected to morphological examination. Observation of the growth of the each endophyte colony was carried out after from around three to five days or a week of subculture of endophytes isolates on PDA. Following the colony growth observation was the microscopic examination. The slide for each isolate was prepared and labeled specifically. The fungi mycelia was scrapped slowly and carefully to avoid the damage on the hyphae's structure of endophytes. The mycelia were placed slowly on the slide and the lactophenol blue was used to stain the sample. The cover slip then was placed carefully on top of the sample to avoid the presence of bubbles that will affect the observation. Compound microscope was used to observe the structure of endophytes between resolution of 20x and 40x.

3.4 Characterization of endophytes isolates

The morphological characteristics such as spore, mycelia, colour and shape of the growth enophytic fungi on the PDA can help in identification of the types of the fungi (Mohamed *et al.*, 2010). The morphological characteristics of endophytes under compound microscope stained with lactophenol blue that were observed and identified are the hyphae and the shape of the conidia. The species of endophytic fungi have been compared and the same species of fungi found on *akar gaharu* and agarwood tree may contributed to the same properties function on these plants. One of the functions is the ability to induce the formation of resin and other secondary metabolites.

The pictures of the isolated endophytes were captured using digital camera for record and further identification. Reference literatures and some trusted mycology webs were used to aid in the identification of the species.

4.0 Results

4.1 Endophytes grown on PDA

The small pieces of wood samples with infected region were cultivated on the PDA in order to grow the endophytes. After a week incubation in the room temperature, the appearance of endophytes was observed on the plate growing from the cultivated wood sample of *akar gaharu.*

Figure 1: The wood samples of infected parts of *akar gaharu*

Figure 2: The wood samples cultivated on PDA after the surface sterilization preparation

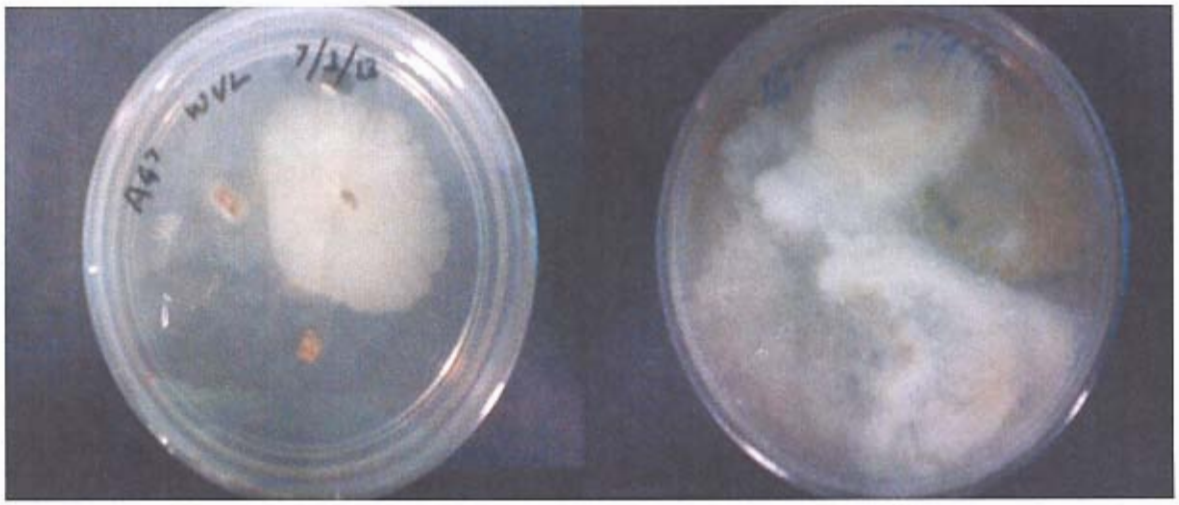


Figure 3: The endophytes grown from the cultivated wood sample

4.2 Species identification

After a week of wood sample cultivation, the endophytes grown were subcultured into other new plates and were left for five days. The endophytes were continuously grown until pure isolates were obtained. The endophytes that did not grow or contaminated were discarded and 16 isolates out of 48 samples were subjected to macroscopic examination by observing the growth of endophytes on the PDA and microscopic examination to identify the species. Table 1 shows the identified species of 16 endophyte isolates from *akar gaharu*.

Table 1: Endophytes isolated and their respective identified species

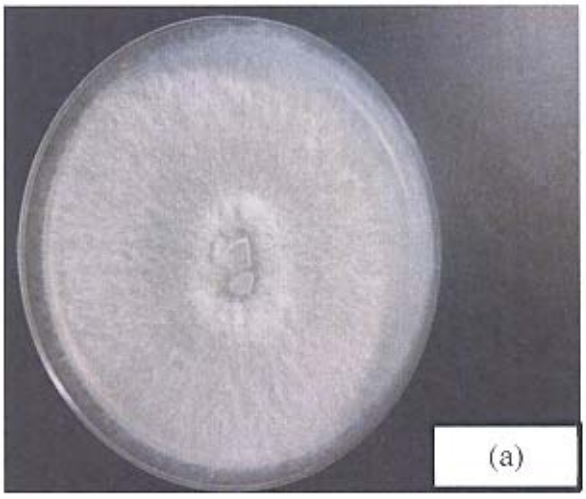
Endophytes isolated	Species identified
AG1-4A	*Mucor* sp.
AG2-4A	*Beauveria bassiana*
AG3-1B	*Trichoderma* sp.
AG3-2A	*Phoma tracheiphila*

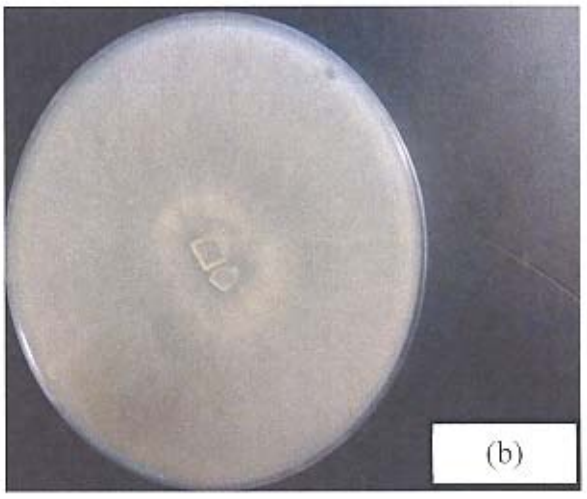
AG3-6	*Microsporium* sp.
AG4-1B	*Penicillium* sp.
AG4-4A	*Trichoderma* sp.
AG4-6	*Beauveria bassiana*
AG4-7	*Beauveria bassiana*
AG5-1	*Microsporium* sp.
AG6-5	*Aspergillus flavus*
AG7-2	*Trichoderma* sp.
AG7-3	*Mucor* sp.
AG7-4	*Fusarium* sp.
AG7-6	*Colletotrichum gloeosporoides*
AG7-7	*Aspergillus flavus*

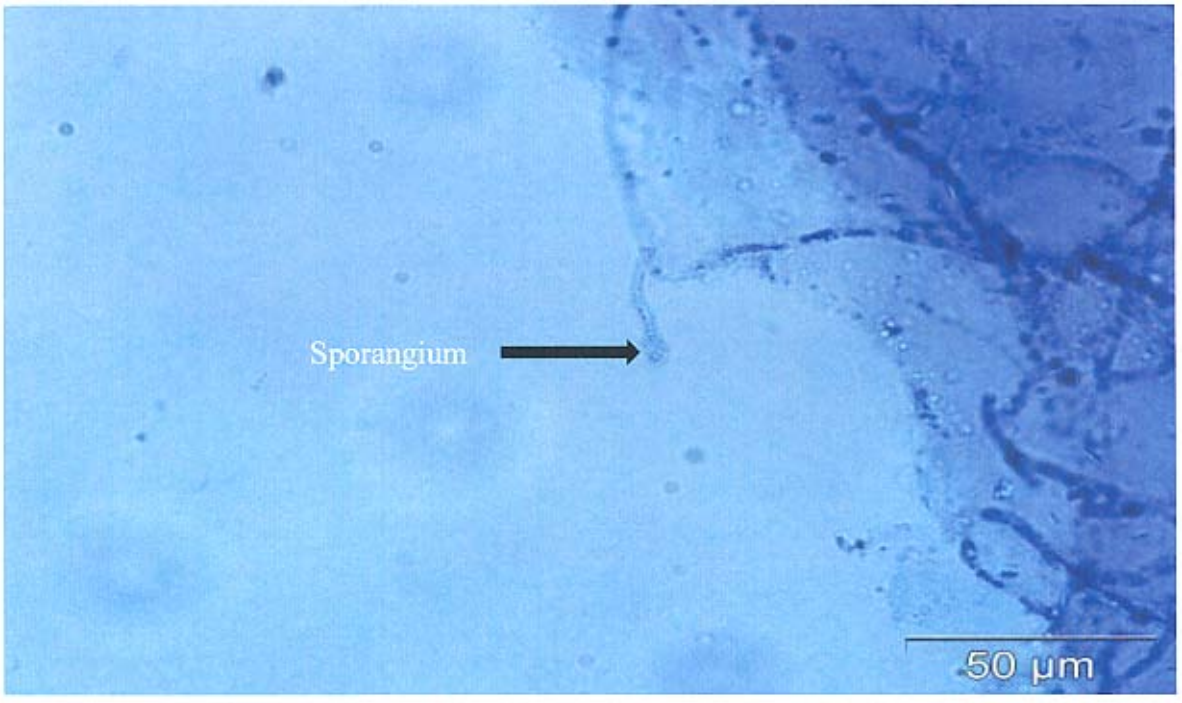
4.3 Morphological description of the endophytes isolated

Mucor sp.

According to the Richa *et al.* (2013), the colonies of *Mucor* sp. were very fast growing and produce white fine threads shape that spread on the plate and become fluffier as the colonies grow older. It produced plain reverse colour and the conidia could be observed under microscope. *Mucor* sp. has both septate and non septate hyphae and conidia attached to the sporangia. The pictures of *Mucor* sp. on the PDA and its conidia were shown in Figure 4 and 5.

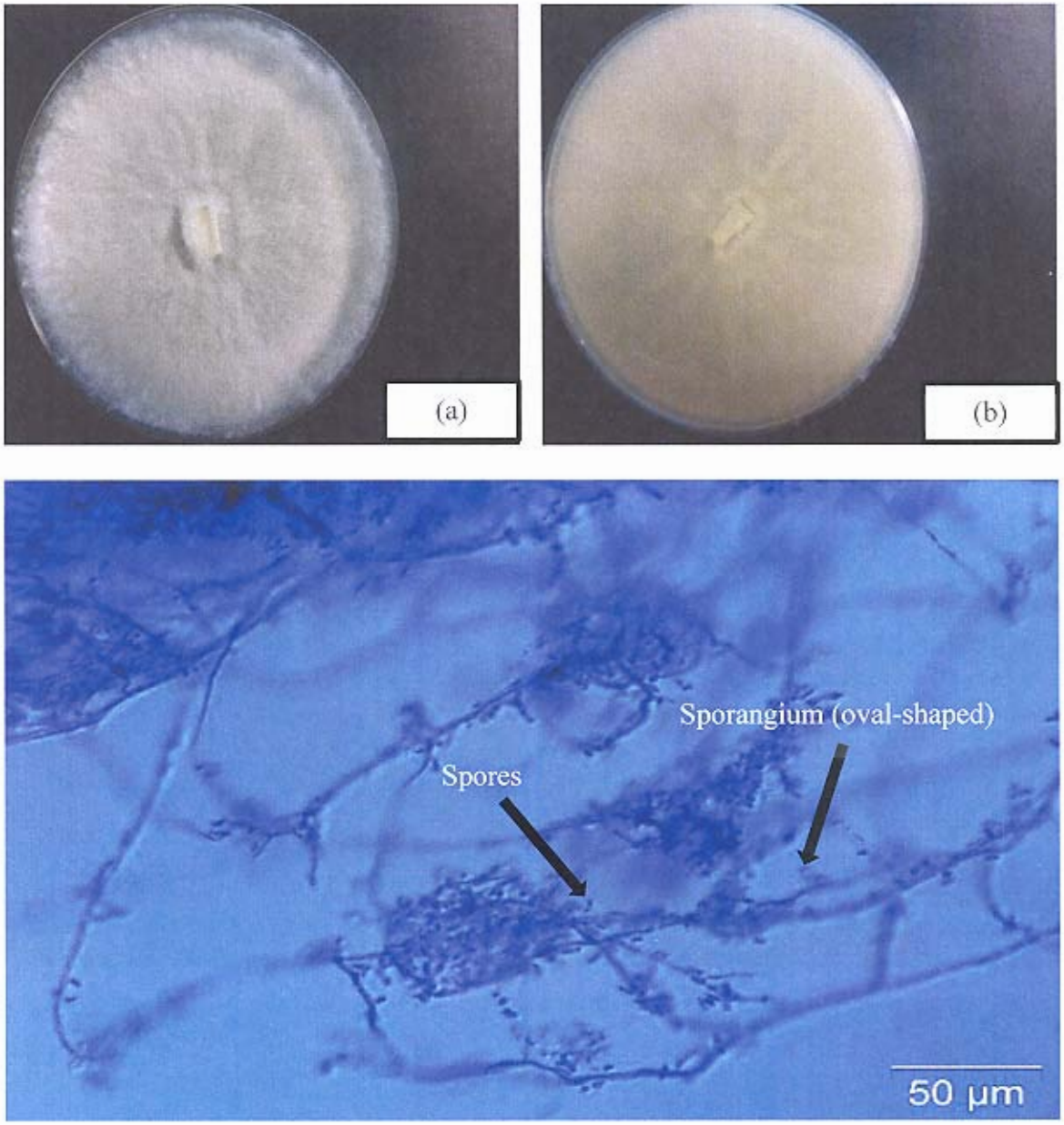






(c)

Figure 4: *Mucor* sp. (a) AG1-4A colony on PDA (b) Reverse plate (c) Conidia



(c)

Figure 5: *Mucor* sp. (a) AG7-3 colony on PDA (b) Reverse plate (c) Conidia