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ISOLATION OF PEAT SWAMP FOREST FOLIAR ENDOPHYTE FUNGI AS BIOFERTILIZER

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ABSTRACT

Peatland restoration activity is facing many obstacles, particularly in planting techniques and poor nutrient in peat soil. Naturally, endophytic fungi are abundant and have great potential as biofertilizer. This research investigates the potential endophytic fungi isolated from leaves of peat swamp tree species for biofertilizer. Research activities include: exploration, in vitro test to examine the phosphate solubilization and identification. Result showed that there were 360 leave segments collected from 4 sampling locations. The colonization percentage of 222 isolates ranged from 52.17% - 60.17%. Fifty seven morphospecies were selected from 222 isolates. Twelve isolates demonstrated ability to produce clear zones and ten isolates were selected for identification. It is concluded that twelve isolated demonstrated potential ability to produce clear zone and *Penicillum citrinum* isolate P3.10 was identified as an isolate that show the highest potential ability as a biofertilizer.

Keywords: biofertilizer, microbe, peat swamp forest, phosphate solubilizing fungi

INTRODUCTION

Fungi are abundant in our environment, and it's recorded that there were almost 1.5 million of species fungus were found on the earth (Hawksworth, 2004). Tropical area tends to have higher biodiversity of fungi compared to temperate area or others (Arnold *et al.*, 2000; Porras-alfaro and Bayman, 2011). Based on their niche, there are various type of fungi such as: saprobe, parasite and also endophyte fungi.

Endophytic fungi is fungi which live within plant tissues, including in leaves tissues which known as foliar endophyte fungi. Fungal endophyte was a fungus that lives within plant leaves tissues. Foliar endophyte known as fungal endophyte was a fungi that lives within plant leaves tissues and considered as the most heterogenic fungi with potential as a latent pathogen, enthomo-pathogen, saprobe and unknown ecological roles (Unterseher and Schnittler, 2015). In addition, fungal endophyte has a significant role in fungal diversity (Arnold *et al.*, 2001) and peat ecosystem. Like others fungi which have ability to transform inorganic phosphate to organic phosphate that can help plant growth (Pradhan dan Sukla, 2005; Barrow dan Osuna, 2002), foliar endophyte fungi also known to have the same ability (Kauppinen *et al*., 2016; Cheplick, Clay and Marks, 1989). Regarding these theories, it is assumed that endophytic fungi which abundant in our environment could be used as biofertilizer.

Kalimantan island is which also known as Borneo island covered by peatland (4.78 bio ha). Nowadays, peat in Kalimantan island degraded due to several human activities, i.e. land degradation, conversion into other land use, intense burning for agriculture leads to poor soil condition and bad drainage (Page *et al*., 2009; Osaki *et al.*, 2016). To overcome this problem, restorations were carried out. Unlike the restoration activity in mineral soils, restoration in peat land facing many obstacles such as: acid soil, low pH, and poor nutrient soil, particularly in Phosphor availability.

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Researches on the utilization of arbuscular mycorrhiza fungi to enhance peat swamp forest plant's growth in the nursery have been carried out (Turjaman *et al*., 2005, 2006, 2008, 2011; Yuwati, 2008; Yuwati *et al*. 2007). Arbuscular mycorrhiza fungi gave significant effect on the height, biomass, leave production and nutrient assimilation at the nursery level (Turjaman *et al*., 2005, 2006, 2008, 2011). Moreover, arbuscular mycorrhiza also reported to give significant effect on the height and diameter growth of *Alstonia pneumatophora* and *Gonystylus bancanus* up to 24 weeks in the nursery (Yuwati, 2008; Yuwati *et al*., 2007). However, the effect of arbuscular mycorrhiza in enhancing plant's growth in the field has not yet understood. Graham *et al*., (2013) inoculated *Glomus clarum* and *Gigaspora decipiens* on *Dyera polyphylla* as well as *Scleroderma columnare* on *Shorea balangeran*. The result showed that inoculation of mycorrhiza on *S. balangeran* and *D. polyphylla* was recommended based on the level of mycorrhiza colonization on seedlings and increasing of nutrient absorption after mycorrhiza innoculation (Graham *et al*., 2013).

Therefore, we assumed that endophytic fungi which isolated from peat land could also be used as an alternative bio-resource to assist the reforestation in peat swamp forest. The objectives of this research are to isolate foliar endophytic fungi from peatland and also to investigate its potential ability to enhance plant growth by *in vitro* method.

MATERIALS AND METHODS

Sampling Location

This research was conducted on April to November 2015. Sample collection was done in two different sites: (a) designated forest for special purpose (KHDTK) of Tumbang Nusa, Central Kalimantan (02°18'34"S and 114°02' 48"E), and (b) Sanitra Sebangau Indah (SSI) Camp, Sebangau National Park, Central Kalimantan (02°35'23"S and 114°01'5"E). In each location, sampling were done in two different vegetation types, namely (a) post fire vegetation dominated by pioneer tree and (b) post fire vegetation dominated by climax tree.

Host Tree.

Foliar endophytic fungi were isolated from four species. Gerunggang (*Cratoxylon glaucum*) and merapat (*Combretocarpus rotundatus*) represent pioneer species, while ramin (*Gonystylus bancanus*) dan punak (*Tetramerista glabra*) represent the climax vegetation.

Sample Collection and Isolation of Foliar Endophytic Fungi.

Healthy leaf sample was taken on July 2015 during dry season and on September 2015 during wet season. For each species, five trees were randomly picked and in each tree five leaves were taken. In total, there were 25 leaves collected for each species. Leaf samples were kept in a cool box and processed in the laboratory for isolation within 48 hours. Isolation was carried out in Microbiology Laboratory Banjarbaru Forestry Research Institute.

First, leaves were rinsed with tap water and cut in segments (0.5 cm x 0.5 cm) from leaf lamina by clean scissor. Surface sterilization method was adopted from (Arnold, Maynard, and Gilbert, 2001). Sterilization were done by soaking the leaf segments in following solutions: Ethanol 70% (2 min); 0.5 % NaOCl (2 min), and rinsed in sterile destile water twice, respectively. Leaves were air dried and transferred to Petridish containing Potato Dextrose Agar (Difco^{TM}Potato Dextrose Agar). To ensure the sterilization process, 1 ml water from surface sterilization solution transferred into PDA Agar. Ninety leaf segments of each species randomly picked and transferred to PDA medium (15 segments/dish). In total, 360 segments were incubated in room temperature and observed every three days over 8 weeks incubation. Fungi that growth from the leaves segments edge were transferred to new PDA plates to obtain single culture isolates. Colonization percentage were determined by dividing the number of leaf segments

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colonized by endophyte by the total segments used during this research times 100.

Soil Water Content Measurement

Five soil samples from the upper soil horizon (0-30 cm) of the each host tree of fungal endophyte were taken from four different sites. Samples from each site were mixed. Samples were analyzed for soil water content parameter by Indonesian Swampland Agriculture Research Institute, Banjarbaru, South Kalimantan.

Phosphate Solubilizing Activity of Foliar Endophyte

We collected fungal morpho-species based on colony morphology. Each fungal morpho-species was tested for its ability to solubilize phosphate using in vitro assay on PVK medium. Fungal plug (0.5 cm x 0.5 cm) of each fungal endophyte isolate was placed in the center of plate containing Pikovskaya Agar (Himedia®) which containing ingredient (g/L): glucose 10.0; Yeast extract 0.5; $(NH4)_2SO_4$ 0.5; $MgSO_4.7H_2O$ 0.1; $Ca3(PO4)_2$ 5; NaCl 0.2; KCl 0.2; $MnSO_4.H_2O$ 0.002; $FeSO_4.7H_2O$ 0.002; and Agar 15. The experiment for each isolate was performed in triplicates. Observation was done after 72 hours. Fungal ability in phosphate solubilizing was indicated by clear zone Solubilization Index (SI) which determined by the ratio of total diameter (colony and clear zone) and colony diameter (Yasser *et al*., 2014; Vitorino *et al*., 2016).

Fungal identification.

Fungi which shown ability to produce clear zone in PVK agar and representing the sampling locations were identified by molecular detection (Polymerase Chain Reaction technique) using primer 18S RNA. Fungal identification was conducted in Institute Pertanian Bogor Culture Collection (IPBCC). Nucleotide sequences were analyzed using BLAST at the National Centre for Biotechnology Information (NCBI) website(www.ncbi.nlm.nih.gov). Furthermore phylogenetic analysis was analyzed using MEGA 6.06 software by *Neighbour-Joining Tree* method (bootsrap = 1000x).

RESULT AND DISCUSSION

Exploration of Foliar Endophyte Fungi

Fungal endophytes were obtained in all tree species observed in this study. The average of colonization percentage of fungal endophyte ranged from 52.17 to 60.17% (Figure 1). Fungal endophyte from secondary forest of Sebangau National Park has highest colonization percentage in each vegetation type. There were 222 fungal colonies observed during this study and 57 difference morpho-species obtained during this research.

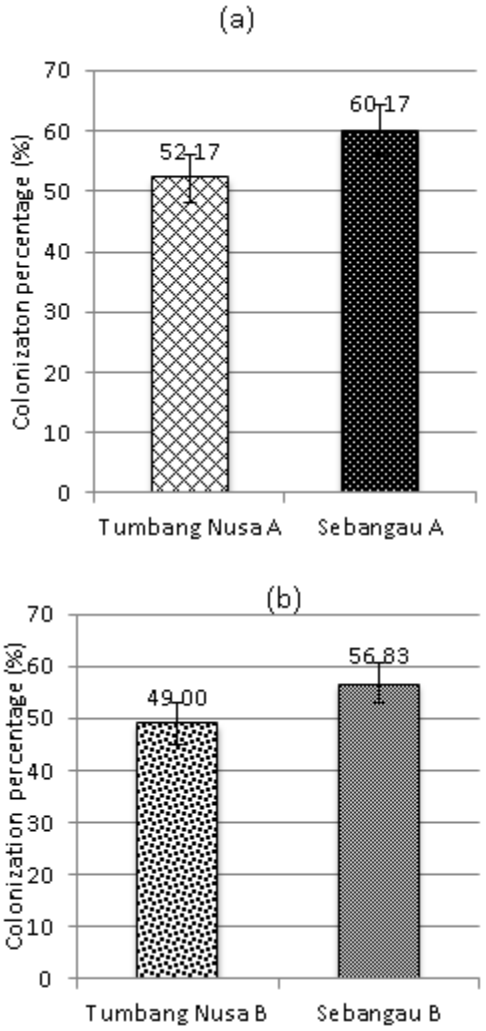

Figure 1 Foliar endophyte colonization in peat swamp forest species in Tumbang Nusa and Sebangau in following vegetation type: (a) Post Fire vegetation dominated pioneer tree) and (b) climax tree.

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Soil Water Content

The soil analysis showed that soil in Sebangau has higher water content compared to Tumbang Nusa (Figure 2).

Phosphate Solubilizing Activity of Foliar Endophyte

A total 57 morpho-species isolated from leaves of four tree species of peat swamp forest, only twelve isolates showed low to strong ability to produce clear zone in PVK Agar. Summary of screening results are presented in Table 1. Isolates code P3.10 isolated from tree species Tetrameristaglabra showed the best ability among others isolates (IS = 1.97) followed by isolate R3.8 (IS =1.26) and isolate P2.11 (IS =1.22). Another isolates showed low ability which produce clear zone approximately only ±1 mm in outer location of fungal mycelia. No quantitative observation of phosphate solubilization measured during this research.

Table 1. Solubilization Index of foliar endophyte

No	Isolate	Solubilization Index (SI)	Host Tree
1	M1.8	1,01±0,01	Merapat
2	G1.4	1,02±0,00	Gerunggang
3	G1.7	1,09±0,14	Gerunggang
4	R3.8	1,26±0,05	Ramin
5	P2.11	1,22±0,07	Punak
6	P3.10	1,97±0,67	Punak
7	G1.12	1,14±0,04	Gerunggang
8	M1.9	1,06±0,02	Merapat
9	M2.6	1,11±0,05	Merapat
10	P1.15	1,03±0,00	Punak
11	P4.6	1,21±0,05	Punak
12	R1.11	1,13±0,05	Ramin

Molecular Identification of Foliar Endophyte Fungi.

Eight of 10 isolates which identified using molecular technique showed similarity with six different species, most of them are from family of Xylariaceae followed by Botrophaeriaceae. Sequencing result showed that endophyte fungi which produce clear zone, obtained from this research had highest similarity to fungal member of Sordario mycetes, Eurotiomycetes, Leotiomycetes, and Dothideomycetes (Figure 3). Two isolates, G3.14 TN and K1.4 TN were unidentified due to bad sequencing result. Seven different species of 8 isolates showed that foliar endophyte fungi have high diversity as shown in Table 2.

Endophyte fungi are abundant and can be found in plant tissues including leaf, wood, bark, stem, etc. (Bayman, 2007; Schulz and Boyle, 2006) . Furthermore, fungal endophyte could be isolated from various habitats, from lower to higher altitude habitat (Schulz and Boyle, 2005). These findings, supporting our theory that fungal endophyte, also known as foliar endophyte could also diverse in peat swamp forest habitat. Some data recorded the existence of fungal endophyte in peat habitat (Dickinson and Dooley, 1967; Pinnoi *et al*., 2006; Pinruan *et al*., 2007; Thormann and Rice, 2007; Yabuki *et al*., 2013)

Foliar endophytes were successfully isolated during this research. Colonization percentages of foliar endophyte are ranging from 49.00-60.17%. This result was lower compared to the result of Arnold *et al*. (2001) which has colonization percentage about 98.77%±0.77% in neo-tropical region. However, this result was comparable to previous study by Orachaipunlap, Roengsumran, and Sihanonth (2009) in tropical area, which stated the colonization percentage of foliar endophyte of dipterocarp tree in tropic area are 75.5% in wet season and 59.3 % in dry season. This present study result is higher compared to the previous study in riparrian habitat with 28.54% of colonization percentage (Lau *et al*., 2013).

Soil analysis also showed that soil in Sebangau has higher soil water content compared to Tumbang Nusa. Our data showed no difference in colonization percentage of foliar endophyte isolated from Tumbang Nusa and Sebangau. Foliar endophyte obtained from Sebangau tended to have higher colonization percentage. We assumed, soil water content play role in foliar endophyte colonization.

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This assumption was linearly with study result by Saikkonen (2007), which shown that colonization percentage increasing linear with soil water content. Colonization percentage may affected by some factor, which were leaf chemical content and leaf water content (Lau, Arnold, and Johnson, 2013). In addition, Saikkonen (2007) also mentioned that the habitat fragmentation significantly reduced the foliar endophyte colonization. This result may reflected that foliar endophyte have strong relationship with many factor that related to forest sustainability. The differences of endophyte colonization result also may be varied based on the differences in leaf segments size, sampling time, and also incubation treatment Arnold (2007). However, there was no patent method to obtain endophyte from leaf tissues.

PVK medium is a low cost yet effective method for screening the Solubilization ability of an organism (Pradan and Suklana, 2005). The ability of fungi to solubilize phosphate related to the ability of fungi to produce inorganic and organic acid.Siva Filho and Vidor (2000) classified Solubilization Index (SI) into three categories : low (SI <2); moderate (2<SI<3); and high (SI>3). According to this theory, isolates obtain during this research categorized in low category. However, based on the observation, the fungal isolate P3.10 was the best isolate with highest Solubilization index.

Molecular analysis also showed that out of 8 isolates from 10 identified isolates were belonged to fungal group of Ascomycota. This result support the theory that fungal endophyte from Angiosperm dominated by Ascomycota fungi (Arnold 2007; *Pinruan et al*., 2007) Identification of isolated endophyte fungi from pioneer tree in peat swamp forest showed that most fungi were belonged to fungal class of Sordariomycetes, Dothideomycetes and Leotiomycetes. This result support the hypothesis that the majority of endophyte fungi are from class Sordariomycetes (Arnold and Lutzoni, 2007).

Fungal isolates P3.10 which identified as *Penicillum* citrinum was also isolated in Frankinchese (*Boswellia* sp.) (Khan *et al*., 2016). In addition, Several studies reveal that *Penicillum* supported plant growth by its phosphate solubilization activity *(Pandey et al*., 2008) in their research, eight species of Penicillum showed to have ability in forming clear zone by *in vitro* screening. Many researches also reported the ability and efficiency of *Penicillum* as fertilizer in field application.

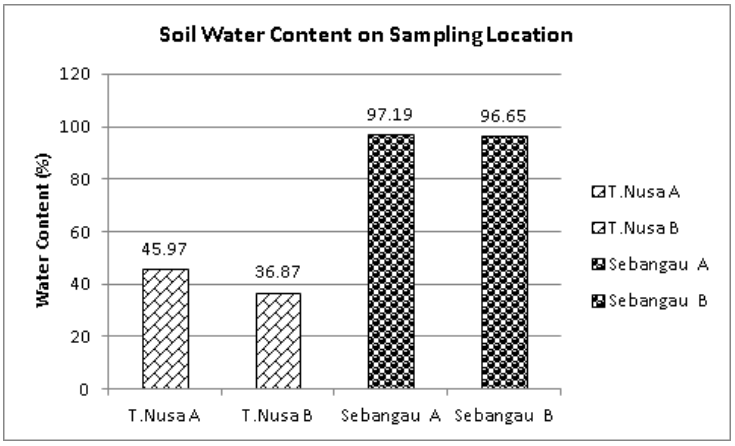

Figure 2. Soil Water Content on each Sampling location; A: Post Fire vegetation and dominated pioneer tree and B: Post Fire vegetation dominated climax tree.

Beside its ability to solubilize phosphate, *Penicillum citrinum* was also known to have ability in producing growth promoting metabolite such as gibberellin and auxin (Khan *et al*., 2008). According to this data, endophyte *Penicillum citrinum* has the potential as biofertilizer. Some fungi identified during this research also recorded

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in other research as fungal endophyte. As example, *Colletotrichum gloeosporioides* also could be isolated from leaf tissues of some *Citrus* sp. (Araújo *et al*., 2001). This result also reveals that there are similarity between endophyte fungi from tropical peat swamp forest and endophyte fungi in temperate region.

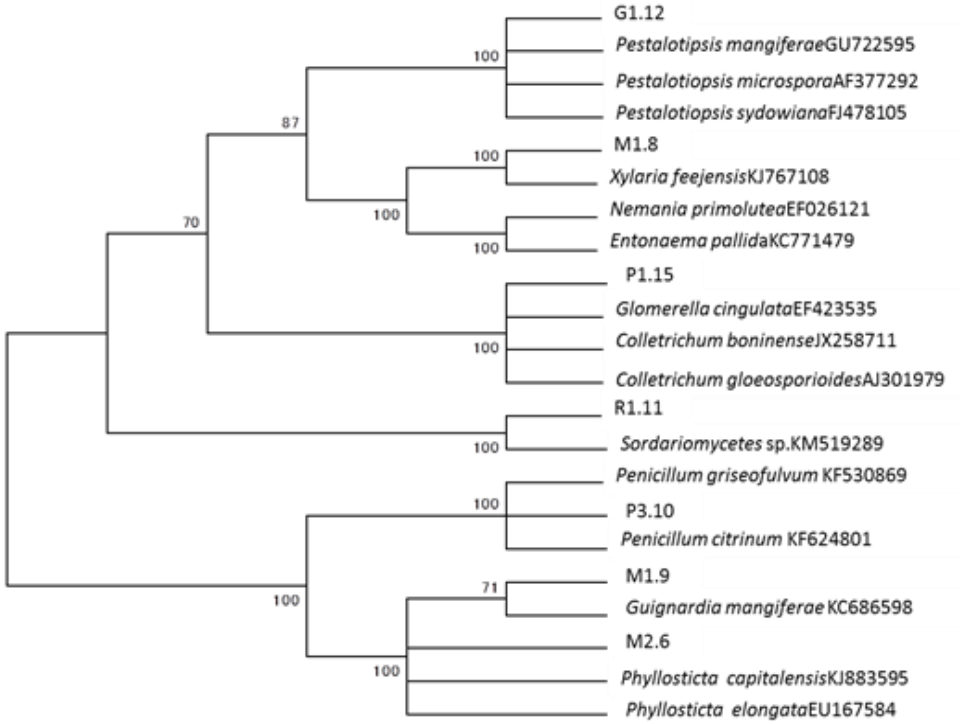

Figure 3. Phylogenetic tree of fungal endophyte isolated from Peat Swamp Forest

Through this research, we generally conclude that peat swamp forest is a harbor of diverse fungi that support global fungal biodiversity. In addition, this research is a preliminary research that supports the bioprospecting of fungi from tropical peatland as novel bioactive for agricultural use and medical use. In the next project, we will use these potential isolates as biofertilizer in field application to examine the effectiveness. Further study of fungal bioactive needs to be done to support the idea of using fungal bioresources from tropical peat land.

Table 2. Molecular identification of Foliar endophyte fungi which produce clear zone from pioneer species of peat swamp forest

No	Isolate Code	Similarity (%)	Most Similar Organism	Accession No.	Family	Class
1	G1.4 TN	97	*Nemania primolutea* 91102001	EF026121.1	Xylariaceae	Sordariomycetes
2	G3.14 TN*	NA	NA	NA	NA	NA
3	M1.8 TN	99	*Xylaria feejensis* HMJAU22039	JX256824.1	Xylariaceae	Sordariomycetes
4	K1.4 TN*	NA	NA	NA	NA	NA
5	R1.11 SB	70	*Geomyces pannorum* Isolat MC13	GU222395.1	Myxotrichaceae	Leotiomycetes
6	G1.12 SB	100	*Pestalopsis mangiferae* Isolat MM102	GU722595.1	Xylariaceae	Sordariomycetes
7	P1.15 SB	100	*Colletrichum gleosporoides* train M91	JX258802.1	Glomerallaceae	Sordariomycetes
8	M1.9 SB	99	*Phyllosticta capitalensis* train M111	KR056283.1	Botrophaeriaceae	Dothideomycetes
9	M2.6 SB	100	*Phyllosticta capitalensis* train M111	KR056283.1	Botrophaeriaceae	Dothideomycetes
10	P3.10	100	*Penicillumcitrinum* train NW-2	KT004401.1	Trichomaceae	Euromycetes

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