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DAFTAR ISI

Identification of Ectomycorrhiza-Associated Fungi and Their Ability in Phosphate Solubilization
(Identifikasi Cendawan Asosiasi Ektomikoriza dan Kemampuannya dalam Pelarutan Fosfat)

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ABSTRACT

The existence of *Pinus* sp. is very dependent on ectomycorrhizal (ECM) fungi. ECM fungi affect the growth of their hosts especially by increasing mineral availability and water intake. However ECM fungi is not the only one that plays a role in the growth of their host. There are many ECM-associated fungi which also have many important roles in the growth of the host. Helotiales which were isolated from the ECM of *Pinus merkusii* are known as the most member of root associated fungi. Three isolated Helotiales identified as *Scytalidium* sp., Helotiales sp., and *Glutinomyces* sp. by morphological and molecular identification based on ITS1, 5.8S rRNA, ITS2 DNAr region. All three isolates have the ability to solubilize phosphate. Compared with *C. geophilum* which already known as P solubilizing fungi, *Glutinomyces* solubilized 16.6 ppm P which is higher than *C. geophilum* which solubilized as much as 13.68 ppm in Pikosvkaya medium with glucose as carbon source and rock phosphate as phosphate source. Then followed by S*cytalidum* sp. and lastly Helotiales sp. Rock phosphate tend to harder to solubilize because its complex chemical form with other minerals.

Keyword: ECM-associated fungi, Helotiales, phosphate solubilizing ability, *Pinus merkusii*

ABSTRAK

Keberadaan *Pinus* sp. sangat bergantung terhadap asosiasi dengan cendawan ektomokoriza (ECM). Cendawan ECM dapat mempengaruhi pertumbuhan terutama dengan meningkatkan ketersediaan hara mineral dan juga serapan air. Akan tetapi cendawan ECM bukanlah satu-satunya yang berperan terhadap pertumbuhan inangnya. Terdapat banyak cendawan yang berasosiasi dengan ECM yang dapat pula berperan terhadap pertumbuhan tanaman inang. Cendawan Helotiales yang telah diisolasi dari ECM *P. merkusii* merupakan cendawan dengan populasi terbanyak ditemukan berasosiasi dengan akar. Tiga isolat Helotiales berhasil diidentifikasi sebagai *Scytalidium* sp., Helotiales sp., dan *Glutinomyces* sp. berdasarkan identifikasi morfologi dan identifikasi molekuler pada daerah ITS1, 5.8S rRNA, ITS2 rDNA. Ketiganya memiliki kemampuan dalam melarutkan fosfat. Dibandingkan dengan *C. geophilum* yang sebelumnya telah diketahui sebagai cendawan pelarut fosfat, *Glutinomyces* sp. dapat melarutkan 16,6 ppm P lebih tinggi dibandingkan *C. geophilum* yang melarutkan 13,68 ppm P dalam media Pikosvkaya cair dengan glukosa sebagai sumber karbon dan *rock phosphate* sebagai sumber P. Selanjutnya diikuti oleh *Scytalidium* sp. dan Helotiales sp. *Rock phosphate* cenderung lebih sulit untuk dilarutkan karena struktur ikatan kimia antar mineralnya yang sangat kompleks.

Kata kunci: Cendawan berasosiasi ECM, Helotiales, kemampuan pelarut fosfat, *Pinus merkusii*

INTRODUCTION

Ectomycorrhiza (ECM) is widely found across the world especially at forest vegetation. Many studies have been conducted to explore ECM communities. The fungi are associated with mostly woody plants including pine. The association between pine species and ECM fungi is obligate. *Pinus merkusii* is one of species from *Pinaceae* family that mostly found in Indonesia. *Suillus* sp., *Rhizopogon* sp., and *Scleroderma* sp. are ECM fungi that commonly associated with *P. merkusii.*

There are many studies discussing the role of fungal ECM regarding promoting plant growth. One of their unique properties is that they are able to solubilize some minerals including phosphate (P). This physiological characteristic is very important to increase soil P availability which tend to very low because they formed in compounds that cannot be absorbed by plants. Many reports have mentioned about their ability to solubilize minerals. *C. geophilum* has been reported to have phosphate solubilizing property with other ECM fungi such as *Hebeloma cylidrosporum, H.*

crustuliniforme, Laccaria laccata, L. bicolor, Paxillus involutus, and *Pisolithus tinctorus* (Lapeyrie *et al.* 1991).

We believe that ECM association in *P. merkusii* are quite complex. Basidiomycota usually is the first fungi that start the succession of ECM community in new roots. They found to be the most fungi colonizing root in upper horizon and Ascomycota are mostly found in the deeper area (Nara *et al.* 2003). For the older roots sometimes there will be community shift from Basidiomycota to Ascomycota. According to Davey *et al.* (2015) root-associated fungi communities which colonize upper area tend to be easily more affected by environmental change. This could mean that the succession in the deeper area is more stable.

These ECM fungi play many big roles to the sustainability of *P. merkusii.* But behind these ECM fungi there are still many fungi which associated with these fungal ECM which also give their contribution to the sustainability of *P. merkusii*. Some studies mentioned about ECM-associated fungi communities and mostly they belong to Helotiales (Wang *et al.* 2006; Tedersoo *et al.* 2009; Nakamura *et al.* 2018). But still there is no studies which covering their physiological ability in P dissolution which has become one of important properties in promoting growth of their hosts.

The objective of this study was to identify fungus associated with the root tips of *P. merkusii* and verify its physiological characters in promoting plant growth.

MATERIALS AND METHODS

Samples were isolated from the root tip of *Pinus merkusii* which have been colonized by ectomycorrhizal fungi. Riddle (1950) method was used for morphological identification. Isolates were cultured on PDA medium at room temperature and observed after 7, 10, and 15 days to get desired reproductive structures. Morphological characteristics were examined with Olympus CX23 compound microscope and compared with Huhtinen (1990) and Seifert *et al.* (2011) then photographed using Beta Industrial Digital Camera.

Isolates were cultured on PDB medium and harvested after 7 days. DNA were isolated using Illustra DNA Extraction kit Nucleon Phytopure. Molecular identification based on ITSr DNA region by using ITS 1 (*forward*) (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (*reverse*) (5'-TCCTCCGCTTATTGATATGC-3') primer amplified by *Polymerase Chain Reaction* (PCR) (White *et al*. 1990). Amplified DNA was sequenced and analyzed using Chromas Pro verion 1.7.7. Fungal identity determined by comparing its homologue sequence using nucleotide BLAST based on http://www.ncbi.nlm.nih.gov and then their sequences were aligned with MEGA X version. Maximum likelihood (ML) phylogenetic trees were inferred from ITS sequences under the Kimura 2-parameter plus gamma distribution (K2+G) model of sequence evolution in MEGA X with 1000 bootstrap replicates. Phylogenetic tree construction then edited using Treegraph 2.

Samples were cultured in 100 ml of Pikosvkaya medium containing 0.5 gr rock phosphate using 500 mL Erlenmeyer flask. Two different carbon source were used for each fungal isolates, i.e., glucose and dextrose, to compare which of the following carbon source gives the best influence in phosphate solubilizing activity. Each culture was incubated in room temperature under agitation condition for 10 days. After 2, 4, 6, 8, and 10 days around 10 mL subculture were withdrawn and centrifuged for 15 minutes at 50.000 rpm. Supernatant were filtered using filter paper (Omar 1998). The pH of sample was measured using glass electrode. The phosphorus content was measured by using ascorbic acid method (Murphy & Riley 1962; Edwards *et al.* 1965).

RESULTS

Morphological and Molecular Identification

To determine some ECM-associated fungi, we presenting three fungal culture which isolated from the root tips of *P. merkusii*. Figure 1 shows the morphology of SM-2. On PDA medium we observed 30 mm in diameter after 7 days of incubation under room temperature with entire margin, white mycelia and brown chlamydospore, and raised floccose colony. Based on its microscopic morphology, SM-2

has two different types of asexual reproduction. In the first type of asexual reproduction, SM-2 formed either terminal or intercalary chlamydospore. As for the second type, SM-2 formed didymo and amero conidia with thalic-arthic conidiogen cell. Pesante (1956) described the genus of *Scytalidium* or the species of *S. lignicola* have conidioma and forms two anamorph: one with thallic-arthric (sub)hyaline or brown conidiogen cells with amero or didymo or phragmo (sub) hyaline or brown in chain conidia and a second is brown intercalary single or chain chlamydospore (Seifert *et al.* 2011). They considered as incertae sedis which taxa that cannot be placed in any of family (inside of Helotiales) accepted in the present classification.

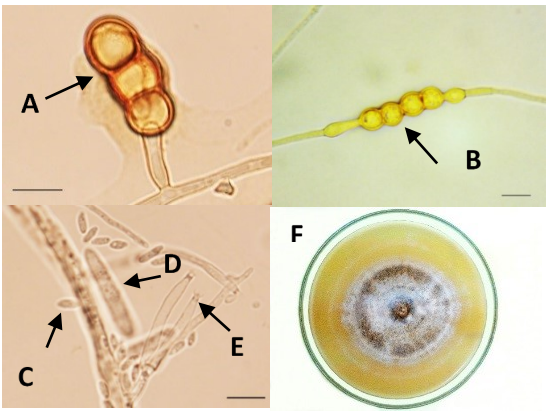

Figure 1. Morphology of SM-2 grown on PDA after 10 days. A) terminal clamydospore, B) intercalar clamydospore, C) amero conidium, D) dydimo conidium, E) conidiogen cell, F) Colony on PDA. Scale: 10 µm

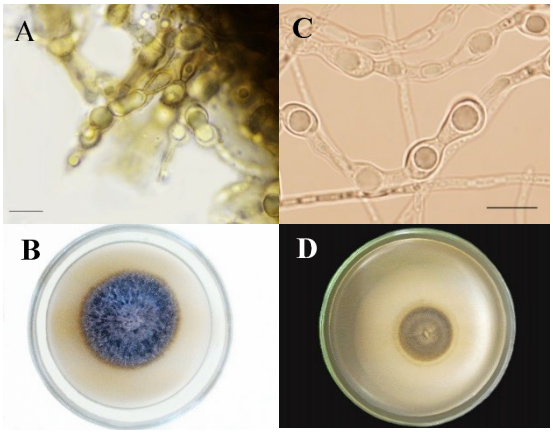

Figure 2. Morphology of SM-5 and Ind-1 grown on PDA after 15 days. A) clamydospore of SM-5, B) SM-5 on PDA medium after 15 days, C) clamydospore of Ind-1, D) Ind-1 on PDA medium after 15 days. Scale: 10 µm

As for SM-5 and Ind-1 (Figure 2), they only formed sterile septate mycelia and formed chlamydospore after 15 days. On PDA medium SM-5 formed 13 mm diameter colony after 7 days of incubation at room temperature with entire margin and dark green raised floccose colony. As for Ind-1, it formed 30 mm diameter colony on PDA medium after 7 days of incubation a room temperature with entire margin and white transparent flat and velvety colony. With the fact that they did not form any distinctive reproductive structure, we couldn't do any morphological identification and could only decide these two as Ascomycota. Based on Huhtinen *et al.* (1990) family of *Hyaloscyphaceae* from Ascomycota are identified based on the morphology of their uncultured ascocarp and not from their cultured morphology. Further identification of SM-5 and Ind-1 will be combined by molecular method.

Molecular identification with BLASTN based on universal fungal genetic marker, ITS region, are shown in Table 1. SM-2 has the closest relation with Scy*talidium* sp. SL3007 with 99% similarity value of 95% covered sequence. *Scytalidium* sp. SL3007 was found to be associated with *C. geophilum* sclerotia based on unpublished article by Obase *et al.* (2014) from NCBI sequence GenBank information. As for the phylogenetic analysis (Figure 3), SM-2 can be confidently placed within the genus of *Scytalidium.* SM-2 is closely related with *Scytalidium* sp. SL3007 with 99% similarity from 1000x bootstrap. These two closest neighbors appeared to be *S. lignicola*. However, we observed a significant branch length difference and only 56% similarity of ITS regions, therefore we are not able to uniquely identify SM-2 as *S. lignicola* and for that SM-2 will be referred as *Scytalidium* sp. The discovery of *Scytalidium* sp. from the root tip of *P. merkusii* in Indonesia is considered as a new record.

BLASTN result for SM-5 showed that Mycorrhizal fungal sp. shylhd09 is the closest homologue sequence. This specimen was isolated from the root tip of *Rhododendron fortunei* in subtropical forests of China (Zhang *et al.* 2009). Compared to its phylogenetic analysis (Figure 4), SM-5 is closely related with

Mycorrhizal fungal sp. shylhd09 with 96% similarity value. The closest neighbor for these two is the clade of Hyaloscypaceae family of the order of Helotiales with only 61% similarity of ITS region. With this we couldn't confidently identify SM-5 as a member of Hyaloscypaceae family, therefore SM-5 will be identified as Helotiales sp.

As for Ind-1, the closest homologue sequence is *Glutinomyces inflatus*. The genus of *Glutinomyces* is considered new and included in the family of Hyaloscyphaceae from the order of Helotiales. This genus is submitted to mycobank in 2017 by Nakamura *et al.* (2018). According to Figure 5, Ind-1 is definitely a member of *Glutinomyces* with the closest relative appeared to be *G. takaragainensis* with only 56% similarity. With this we still couldn't confidently identify its species and therfore Ind-1 will be identified as *Glutinomyces* sp.

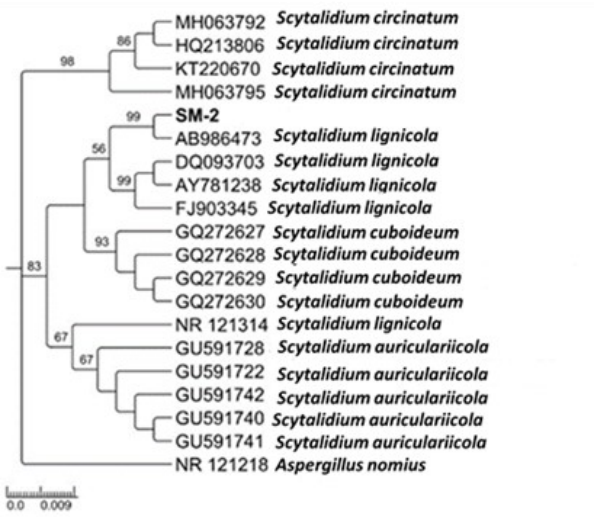

Figure 3. Phylogenetic tree of the ITS region of Scytalidium genus representatives constructed from with ML methods with Kimura 2-parameter subtitution model and Gamma distribution (K2+G). Bootstrap values above 50% are indicated on the left side of the nodes. The studied microorganism (SM-2) is in bold and outgroup species are indicated with asterisks. Scale bar represents the number of nucleotide substitutions per site. There were a total of 388 positions in the final dataset

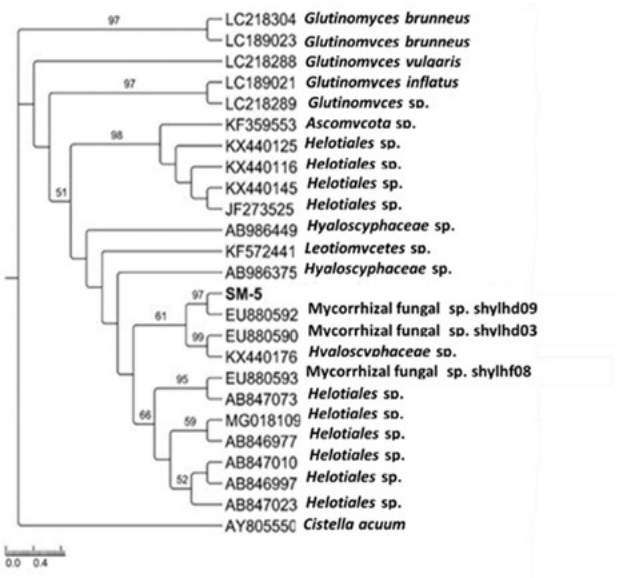

Figure 4. Phylogenetic tree of the ITS region of some Helotiales representatives constructed from with ML methods with Kimura 2-parameter subtitution model and Gamma distribution (K2+G). Bootstrap values above 50% are indicated on the left side of the nodes. The studied microorganism (SM-5) is in bold and outgroup species are indicated with asterisks. Scale bar represents the number of nucleotide substitutions per site. There were a total of 498 positions in the final dataset

Phosphate (P) Solubilizing Ability

Three samples we used were compared with *C. geophilum* as the ECM species which has been known for its ability to solubilize P. Each isolates fermented for 10 days in Pikosvkaya broth medium supplemented with *rock*

Table 1. Molecular identification of fungal isolates obtained from P. merkusii ECM root tips. Isolates were identified using a BLAST nucleotide sequence comparison of the ITS region.

Isolate	Maximum score	Total score	Query cover	E value	Ident	Closest GenBank match	Accession
SM-2	1024	1024	95%	0	99%	*Scytalidium* sp.	AB986473
SM-5	1029	1029	99%	0	99%	Mycorrhizal fungal sp. shylhd09	EU880592
Ind-1	941	941	97%	0	97%	*Glutinomyces inflatus*	LC189021

phosphate (RP) as P source and glucose as carbon (C) source. All medium cultured with each isolates appeared to be underwent acidification after 10 days of incubation except control (Figure 6). Started with neutral slightly acidic pH (6.97), all medium with 4 isolates were slightly become alkaline at the second day and gradually became acidic after 4th day until the last day of observation. At 10th day observation, medium fermented with *Glutinomyces* sp. had the lowest pH followed with *C. geophilum,* Helotiales sp, and *Scytalidum* sp. As for control, its medium acidity remained around neutral. These result will be exactly correlated with their phosphate solubilization activity.

Table 2. Phosphate dissolved after 10 days of incubation in Pikosvkaya liquid medium at room temperature

	Phosphate concentration (ppm)
Scytalidium sp.	12.57 ± 0.22c
Helotiales sp.	7.67 ± 0.22b
Glutinomyces sp.	16.60 ± 0.48e
C. geophilum	13.68 ± 0.22d
Control	0 ± 0a

Notes: The number followed by the same letter in the same column is not significantly different based on Duncan's multiple range test at α = 5%.

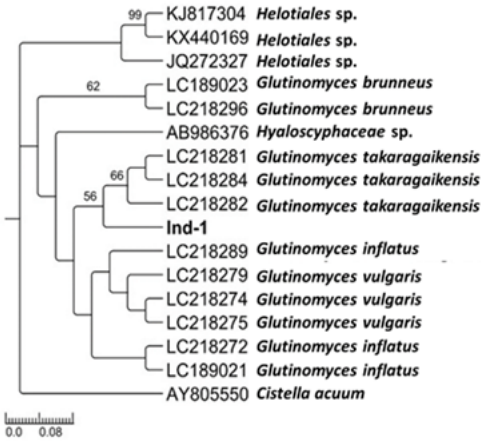

Figure 5. Phylogenetic tree of the ITS region of Glutinomyces genus representatives constructed from with ML methods with Kimura 2-parameter subtitution model and Gamma distribution (K2+G). Bootstrap values above 50% are indicated on the left side of the nodes. The studied microorganism (Ind-1) is in bold and outgroup species are indicated with asterisks. Scale bar represents the number of nucleotide substitutions per site. There were a total of 359 positions in the final dataset

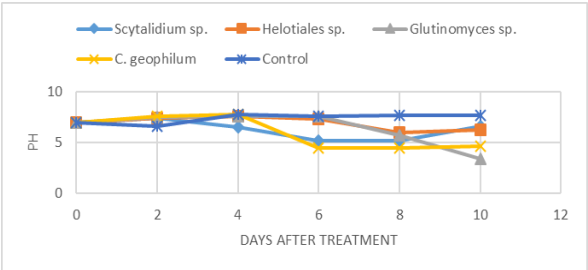

Figure 6. pH level of each fermented medium for 10 days of incubation in Pikosvkaya liquid medium at room temperature

The data from Table 2 shows the average solubilized P concentration in Pikosvkaya medium after 10 days incubation. The average solubilized P level was significantly different for each samples. These results are directly proportional with their medium acidity. The more acidic medium the more P released in the medium. Therefore *Glutinomyces* sp. solubilized most P than the other 3 including *C. geophilum.* On the other hand, control medium released very low P (below 10^{-4} ppm) so we will consider it as 0 ppm. This released of P could be happened naturally by water dissolution without organism interference.

Acidification of medium indicates that each culture produces organic acid metabolites. These acids metabolites are the cause of P solubilizaion (Asea *et al.* 1988). In previous study by Senthilkumar *et al.* (1998) mentioned that *S. lignicola* grown in Pikosvkaya agar medium with tricalcium phosphate (TCP) as P source have the ability to solubilize P by producing clear zone around its colony. In addition Lapeyrie et al. (1991) also conducted a qualitative test of P solubilization by ectomycorrhizal fungi in various forms of non-soluble phosphates and various sources of nitrogen as a treatment. In this *study C. geophilum* grown on a medium containing calcium phytate with ammonium as a nitrogen source has the best phosphate solubilizing activity. The ability of phosphate solubilization in qualitative test is known by the formation of clear zone in the agar medium around the fungal colonies.

Our study this time tried to quantitatively testing P solubilization by some of fungal isolates using RP as P source which tend to be more difficult to dissolve due to their more complex bonds with various other elements compared to TCP which phosphates can be

easily dissolved even with water. With current results their P solubilizing-fungi status will be more convincing that if we were using TCP as P source.

DISCUSSIONS

The status of Helotiales in this study remained unclear. The lack of information from molecular database make it hard to decide. *Helotiales* comprises the largest number of undescribed root-associated fungi such as endophyte, ECM, ericoid, arbutoid, and arbuscular mycorrhiza (Wang *et al.* 2006; Tedersoo *et al.* 2008; Toju & Sato 2018). Some Helotiales also has been known as ECM – Associated Ascomycota (Tedrsoo *et al.* 2009). Because of their many forms of association with plants, Helotiales holds important roles for plant existence. Their existence in plant roots could help increasing mineral absorption by releasing some mineral complex in soil. Phosphate as one of macronutrients for plants tend to have low availability in soil. Acid or alkaline soil tend to have insoluble P that are not ready for plants to absorb. So tons of P fertilizers are applied to increase plant productivity. With the help of soil microorganism they could release some of insoluble P thus increase P availability in soil.

Fungal ability to solubilize P is inseparable from several factors. One of main factors which can affect P solubilization in culture medium is carbon source. Pradhan and Sukla (2015) mentioned in his research that glucose and maltose are the best C source in promoting P dissolution then followed by sucrose, xylose, and galactose. These results may vary depend on fungal species. Other factors that could affect P solubilization are P source and N source.

Mineral dissolution, including P, through a various chemical reaction in soil. Reaction with protons, organic acid ligands, water, and CO_2 are the main mechanisms for mineral dissolution (Sverdrup *et al.* 2002). These mechanism also can be done by plants and soil microorganism including fungi.

Two main mechanisms which are done by fungi in mineral weathering are biophysical and biochemical. By biophysical weathering means that fungal mycelia are spreading throughout mineral containing rocks and broke them out. The second mechanism is done by chemical reactions with fungal metabolites. Four mechanism of biochemical weathering are acidolysis, complexolysis, redoxolysis, and metal accumulation by fungal biomass. Acidolysis is the most common mechanism for mineral dissolution (Burgstaller & Schinner 1993). Correlation between acidity and total of dissolved P due to the presence of organic acids produced from the respiration process of CO_2 which acts as chelating agent by releasing bounded minerals with protons. Arvieu *at al.* (2003) said that one of common produced organic acid is oxalic acid. Most ECM fungi can produce oxalic acid under axenic condition.

CONCLUSIONS

Three ECM-associated Helotiales isolated from ECM of *P. merkusii* identified as *Scytalidium* sp., Helotiales sp., and *Glutinomyces* sp. All three isolates are able to solubilize complex phosphate under axenic culture condition. *Glutinomyces* sp. in this research has the best phosphate solubilizing ability followed by *C. geophilum, Scytalidium* sp., and Helotiales sp.

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PANDUAN PENULIS

Naskah dapat ditulis dalam bahasa Indonesia atau bahasa Inggris. Naskah disusun dengan urutan: JUDUL (bahasa Indonesia dan Inggris), NAMA PENULIS (yang disertai dengan alamat Lembaga/ Instansi), ABSTRAK (bahasa Inggris, dan Indonesia maksimal 250 kata), KATA KUNCI (maksimal 6 kata), PENDAHULUAN, BAHAN DAN CARA KERJA, HASIL, PEMBAHASAN, UCAPAN TERIMA KASIH (jika diperlukan) dan DAFTAR PUSTAKA. Penulisan Tabel dan Gambar ditulis di lembar terpisah dari teks.

Naskah diketik dengan spasi ganda pada kertas HVS A4 maksimum 15 halaman termasuk gambar, foto, dan tabel disertai CD atau dikirim melalui email redaksi/ web JBI. Batas dari tepi kiri 3 cm, kanan, atas, dan bawah masing-masing 2,5 cm dengan program pengolah kata *Microsoft Word* dan tipe huruf *Times New Roman* berukuran 12 point. Setiap halaman diberi nomor halaman secara berurutan. Gambar dalam bentuk grafik/diagram harus asli (bukan fotokopi) dan foto (dicetak di kertas licin atau di scan). Gambar dan Tabel di tulis dan ditempatkan di halaman terpisah di akhir naskah. Penulisan simbol a, b, c, dan lain-lain dimasukkan melalui fasilitas insert, tanpa mengubah jenis huruf. Kata dalam bahasa asing dicetak miring. Naskah dikirimkan ke alamat Redaksi sebanyak 3 eksemplar (2 eksemplar tanpa nama dan lembaga penulis).

Penggunaan nama suatu tumbuhan atau hewan dalam bahasa Indonesia/Daerah harus diikuti nama ilmiahnya (cetak miring) beserta Authornya pada pengungkapan pertama kali.

Pustaka didalam teks ditulis secara abjad.

Contoh penulisan Daftar Pustaka sebagai berikut :

Jurnal :

Achmadi, AS., JA. Esselstyn, KC. Rowe, I. Maryanto & MT. Abdullah. 2013. Phylogeny, divesity , and biogeography of Southeast Asian Spiny rats (*Maxomys*). *Journal of mammalogy* 94 (6):1412-123.**Buku :**

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Gerhart, P. & SW. Drew. 1994. Liquid culture. Dalam : Gerhart, P., R.G.E. Murray, W.A. Wood, & N.R. Krieg (eds.). *Methods for General and Molecular Bacteriology*. ASM., Washington. 248 -277.

Abstrak :

Suryajaya, D. 1982. Perkembangan tanaman polong-polongan utama di Indonesia. Abstrak Pertemuan Ilmiah Mikrobiologi. Jakarta . 15 –18 Oktober 1982. 42.

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Mubarik, NR., A. Suwanto, & MT. Suhartono. 2000. Isolasi dan karakterisasi protease ekstrasellular dari bakteri isolat termofilik ekstrim. Prosiding Seminar nasional Industri Enzim dan Bioteknologi II. Jakarta, 15-16 Februari 2000. 151-158.

Skripsi, Tesis, Disertasi :

Kemala, S. 1987. Pola Pertanian, Industri Perdagangan Kelapa dan Kelapa Sawit di Indonesia. [Disertasi]. Bogor : Institut Pertanian Bogor.

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Schulze, H. 1999. Detection and Identification of Lories and Pottos in The Wild; Information for surveys/Estimated of population density. http//www.species.net/primates/loris/lorCp.1.html.