Hashemite Kingdom of Jordan

Jordan Journal of Biological Sciences

An International Peer-Reviewed Scientific Journal

Financed by the Scientific Research and Innovation Support Fund

This title is indexed in Scopus

http://jjbs.hu.edu.jo/

DAFTAR ISI

2.

Halaman Pengesahan

HALAMAN PENGESAHAN

Judul	: Morphological Identification of Mycorrhizal Fungi Isolated from Native Orchid in Indonesia
Penulis	: **Mahfut**
NIP	: 198109092014041001
Instansi	: Jurusan Biologi, Fakultas MIPA, Universitas Lampung
Publikasi	: Jordan Journal of Biological Sciences, Vol. 14, No. 5, Pp. 1031 – 1034, 2021
Alamat Web (Link)	: https://doi.org/10.54319/jjbs/140523 http://repository.lppm.unila.ac.id/id/eprint/43096
Penerbit	: Hashemite University
ISSN	: 1995-6673
Indeks	: Scopus Q2, SJR 0.29 (2021)
Jenis Publikasi	: Jurnal Internasional Bereputasi

Bandar Lampung, 14 Juli 2022

Mengetahui,
Dekan Fakultas MIPA

Dr. Eng. Suripto Dwi Yuwono, M.T.
NIP. 197407052000031001

Penulis

Dr. Mahfut, M.Sc.
NIP. 198109092014041001

Menyetujui,
Ketua LPPM Universitas Lampung

Dr. Ir. Lusmeilia Afriani, D.E.A
NIP. 196505101993032008

3.

Artikel Final Yang Sudah Dipublikasi

المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)
http://jjbs.hu.edu.jo

Jordan Journal of Biological Sciences (JJBS) (ISSN: 1995–6673 (Print); 2307-7166 (Online)): An International Peer- Reviewed Open Access Research Journal financed by the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research, Jordan and published quarterly by the Deanship of Scientific Research , The Hashemite University, Jordan.

Submission Address
Professor Atoum, Manar F
The Hashemite Universit y
P.O. Box 330127, Zarqa, 13115, Jordan
Phone: +962-5-3903333 ext.4147
E-Mail: jjbs@hu.edu.jo

المجلة الاردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)
http://jjbs.hu.edu.jo

International Advisory Board (Arranged alphabetically)

JJBS

Volume 14, Number 5,December 2021
ISSN 1995-6673

Jordan Journal of Biological Sciences

CONTENTS

Original Articles

JJBS

Volume 14, Number 5,December 2021
ISSN 1995-6673
Pages 1031 – 1034
https://doi.org/10.54319/jjbs/140523

Jordan Journal of Biological Sciences

Morphological Identification of Mycorrhizal Fungi Isolated from Native Orchid in Indonesia

Mahfut*

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Lampung, Indonesia

Received: February 27, 2021; Revised: May 2, 2021; Accepted: November 28, 2021

Abstract

Moth orchid [*Phalaenopsis amabilis* (L.) Blume] is a species of native orchid from Indonesia. The association of this orchid with mycorrhizal fungi in nature is as a biocontrol agent. In a previous study, one *Ceratobasidium* isolate from Yogyakarta, Indonesia was successfully identified based on rDNA-ITS molecular analysis. This study aimed to identify these isolates based on morphological analysis to complement other identification data, namely anatomy and molecular. Verification morphological analysis is carried out by observing macroscopic and microscopic characteristics, as well as observing peloton. The results of showed that the characterization of Yogyakarta isolates had similarities with *Ceratobasidium*. These character equations include colony color, cell length, core number, and cell width. The examination of orchid roots also showed the presence of pelotons in the cortical cells. This study confirms that the fungal isolates of orchid mycorrhiza from Yogyakarta, Indonesia were *Ceratobasidium* based on morphological and molecular analysis. This research is one of the efforts to preserve native orchids in Indonesia using myccorhiza fungi as a biocontrol agent. This study is the first to report regarding *Ceratobasidium* isolated from native orchids in Indonesia based on morphological analysis.

Keywords: *Ceratobasidium*; mycorrhiza; morphological analysis; *Phalaenopsis*; Indonesia

1. Introduction

Orchid is a type of ornamental plant that has a high aesthetic value and is most in demand by the community (Mose et al., 2020). The moth orchid [*Phalaenopsis amabilis* (L.) Blume] is one of Indonesia's national flowers, namely the charm that was set through Presidential Indonesia Decree Number 4/1993. Diseases are still the main obstacle in the cultivation and development of natural orchids (Mahfut et al., 2019; Mahfut, 2020; Mahfut et al., 2020[b]; Mahfut et al., 2021).

Endophytic mycorrhiza is a form of symbiosis between fungi and plant roots during a certain period of their life cycle, forming colonies in plant tissues without endangering their hosts. In general, mycorrhizal fungi help germination of orchid seeds in the presence of ethylene and various vitamins. Another role is to support efforts to provide nutrition for plant growth and development (Haro and Benito, 2019), and to assist in the formation of more buds and flowers. In plant resistance, mycorrhiza fungi serve as a biological agent of control in plant protection against pathogenic infections (Safarini et al., 2020; Song et al., 2020).

In a previous study, Mahfut et al. (2020) reported one *Ceratobasidium* isolate from Yogyakarta, Indonesia based on rDNA-ITS molecular analysis. The results show sequences measuring 600-750 bp DNA products located on the ITS1-5.8S-ITS4 region. Reconstruction of phylogenetic trees resulted in Indonesian isolates having undergone speciation and separated from C*eratobasidium* isolates from other countries. This research is a follow-up study which aims to clarify the identification of these isolates as *Ceratobasidium* based on morphological analysis. Furthermore, this research can be used as a benchmark in developing strategy for conserving moth orchid through protection against plant diseases.

2. Materials and Methods

2.1. Collection of Healthy Orchid Root Samples and Endophytic Mycorrhizal Isolation

Healthy root of *P. amabilis* collected from Yogyakarta, Indonesia on coordinates 8º 30' - 7º 20' LS 109º 40' - 111º 0' BT. The sample was taken to the Biotechnology Laboratory of the Faculty of Biology UGM to isolate endophytic mycorrhiza following the Chand et al. (2020) method. Isolation of endophytic fungi was then carried out by cutting the roots and culturing on PDA medium. The fungal colonies obtained from the isolation were then purified by sub-culturing aseptically and incubating for 7 days at room temperature (20-25℃). It is possible that the isolates obtained are pure and single isolates that are not contaminated by other fungi.

2.2. Macroscopic Characteristic Observation

To observe the macroscopic characteristics of the isolated fungi, small portion of isolates (± 1 mm^2) were placed in the middle of the PDA medium. Furthermore, the growth of the isolates was observed and measured every day for 7 days. Furthermore, observations of colony morphological characteristics such as colony color, colony

* Corresponding author e-mail: mahfut.mipa@fmipa.unila.ac.id.

base color, the surface color of young colonies, the appearance of colonies, and the growth rate of the colony were carried out.

2.3. Making Slide Culture Preparations

Observation of microscopic characteristics was also carried out using the slide culture method (Stoian et al. 2019) with a slight modification. This method was carried out to monitor microscopic characteristics such as the color of hyphae, bulk in hyphae, angle of branching of hyphae, and number of cell nuclei. The number of nuclei was observed after treating with safranin and 3% KOH.

2.4. Observation of Pelotons in roots

Observation of peloton was carried out by making squash preparations on root samples which had been treated with previous endophytic fungal inoculations. Root samples are sliced and soaked in trypan blue dye for 15 minutes. Observations were carried out using a light microscope at 100 x magnification.

3. Results

3.1. Collection of Healthy Orchid Root Samples and Endophytic Mycorrhizal Isolation

One endophytic fungus, namely Yogyakarta was isolated from root samples of *P. amabilis* in Yogyakarta, Indonesia in Figure 1.

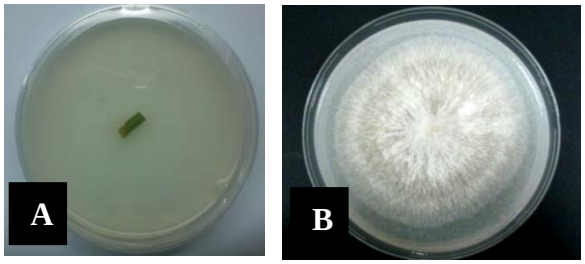


Figure 1. A) Root samples of MP1 cultured on PDA medium, B) Colonies of fungi that grow on 7 days old orchid roots

Identification results based on macroscopic, microscopic, and molecular characteristics showed that the isolate was *Ceratorhiza* which is one of the types of *Rhizoctonia* endophytic mycorrhiza. According to Chand et al. (2020), *Ceratorhiza* is *Rhizoctonia* grouped by cell hyphae and moniloid, and many are found to be associated with orchid roots.

3.2. Macroscopic Characteristics

Special characteristics of Yogyakarta isolate were identified as having branching types that formed elbows or 90º angles and hyphae cells with a cell nucleus 2 (binucleate). These two characteristics are special characteristics of endophytic mycorrhiza. Endophytic fungi have 1-3 nuclei (binucleate) nuclei, whereas pathogenic fungi have more than 3 nuclei (multinucleates). This is in accordance with the opinion of Pannecoucque & Hofie (2009) that in addition to binucleates, the characteristics of endophytic fungi are branching which forms a 90ºC or T-shaped branches.

Overall, the results of identification of Yogyakarta isolates showed similarities with the types of *Ceratorhiza* mycorrhiza. The following characteristics of Yogyakarta isolates compared to supporting references are presented in Tables 1 and 2.

Table 1. Comparison of the characteristics of Yogyakarta isolates and *Cerathoriza* isolates according to Shan et al. (2002)

Characteristics	**Yogyakarta Isolate**	***Cerathoriza* Isolate**
The surface color of young colonies	White to yellow	Yellow to white
The appearance of colonies	Like cotton	Like cotton
Hyphae color	Hyaline	Hyaline
Hyphae diameter (µm)	6.20-6.72	3.8-7.5
Monilioid cell form	Elongated, barrel shaped	Ellipsoidal or elongate barrel shape
Monilioid cell size (µm)	(12.22-14.56) × (2.39-2.91)	(7.5-15.0) × (10.0-25.0)
The growth rate of the colony (mm / hr)	0.72	0.42-0.52
Number of cell nuclei	Binucleate	Binucleate

Table 2. Comparison of the characteristics of Yogyakarta isolate and *Cerathoriza* isolates according to Currah and Zelmer (1992)

Characteristics	**Yogyakarta Isolate**	***Cerathoriza* Isolate**
Colony color	White to yellow	Cream, yellow, or brown
The appearance of colonies	Like cotton	Like cotton
The form of branching hyphae	90 º	90º
Air hyphae diameter	<3 µm	<4 µm
Number of nuclei per cell	Binucleate	Binucleate
Clamp connection	-	-
Surface of the colony	Like cotton	*Flat and waxy*
Monilioid cells	*Elongate barrel shaped*	-
Monilioid cell length	12.22-14.56	-
Monilioid cell width	2.39-2.91	-

Based on observations of hyphae and cells using safranin O-KOH dye **(**Figure 2**)**, it was found that Yogyakarta isolates were endophytic fungi of *Ceratorhiza* with specific characteristics having branching types that form elbows or 90° angles, the color of vegetative hyphae is hyaline, with diameters of 6.20-6.72 µm, fungal colonies form cell monilioids with elongate barrel shape and monilioid size (12,22-14,56) µm x (2.39-2.91) µm.

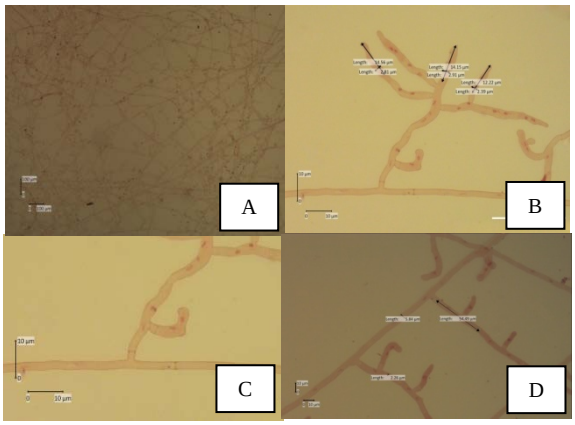


Figure 2. Microscopic observation of hyphae cells and moniliod cells *Ceratorhiza* from Yogyakarta isolates on the 7-day PDA medium using safranin O-KOH staining; (A) Colony, (B) Monilioid cell nucleus, (C) Binucleate, (D) Branching of hyphae 90º. Magnification (A) 10 x, (B, C, D) 40 x. Bar = 10 µm

3.3. *Observation of Endophytic Mycorrhizal Hyphae (Peloton)*

Observation of the cross section of the root sample indicated the presence of pelotons in the root cortex (Figure 3). Currah & Zelmer (1992) explain that the characteristic of endophytic fungal hyphae colonizing plants will form a mass of dense hyphae in cortical cells called pelotons.

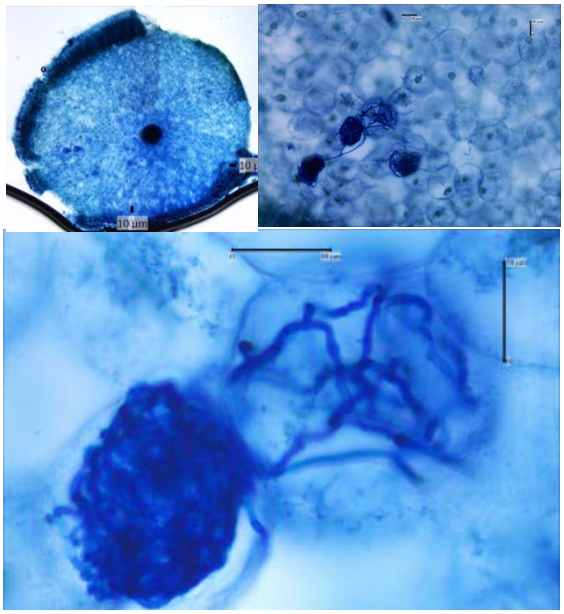


Figure 3. Peloton structure in the anatomy of the root cortex cells of MP1 with Tryphan Blue staining. Magnification (A) 4 x, (B) 10 x, (C) 40 x. Bar = 10 µm

4. Discussion

Characteristics of endophytic mycorrhizal analysis were obtained and compared with the reference characteristics (Shan et al., 2002; Currah and Zelmer, 1992); it was found that Yogyakarta isolates were *Ceratorhiza* isolates. *Ceratorhiza* is an anamorphic phase of *Ceratobasidium*. According to Athipunyakom et al. (2004), the genus *Ceratorhiza* is a mycorrhizal isolate which is commonly found associated with orchids, where endophytic fungi of this type are *Rhizoctonia* which are grouped according to cell hyphae and moniloid. Growth of *Ceratorhiza* reached a diameter of 9 cm after 4 days of incubation by forming a symmetrical zone. The colony turned yellowish after 7 days, and the colony surface appeared as cottony clumps. The lumps are aerial hyphae; this is known from the hyphae that grow on the surface of the agar media. These hyphae are fertile hyphae which play a role for reproduction. The length of moniloid cells is 12.22-14.56 µm, width 2.39-2.91µm and lancet-shaped. If the ratio of length and width is 3.5-4: 1 then it is called the lancet type. The number of nuclei in binucleic cells, according to the characteristics reported by Shan et al. (2002) and Currah and Zelmer (1992).

The peloton structure is evident in the roots of *P. amabilis* which are associated with endophytic fungi (Figure 3). One of the parameters for the mechanism of resistance induction can be the formation of a peloton structure which shows that endophytic fungi penetrate the epidermal finger and into the cortex. In the cortex, the pulp will enter the cell space (intracellular) and form a peloton.

The presence of peloton proves that the endophytic fungi can penetrate into the root tissue of *P. amabilis*. According to Currah and Zelmer (1992), the stage of infection with endophytic fungi begins with the formation of appressorium which is an inflated hyphae. These hyphae originate from spores that germinate or externally hyphae at the root surface of infected host plants. Hyphae will then penetrate the root surface mechanically and enzymatically into the space between the root epidermal cells and into the cortex and beyond. Hyphae develop without destroying the cells of the root cortex. In cortex cells, hyphae form a peloton in the form of dense hyphae. In the platoon, the accumulation of organic materials includes protein, glycogen, fat, and nutrients produced by absorption from the soil.

5. Conclusion

The results showed that the characterization of endophytic mycorrhizae from the roots of *P. amabilis* from Yogyakarta, Indonesia had similarities with the type of endophytic mycorrhizal *Ceratorhiza*. These character equations include colony surface color, colony appearance, hyphae color, hyphae diameter (µm), cell monilioid shape, monilioid cell size (µm), colony growth rate (mm / hour), core number, hyphae branching shape, and clamp connection. The results of mycorrhizal induction also produce a peloton structure in orchid root cortex cells. The peloton structure is evident in the roots of *P. amabilis* which are associated with endophytic fungi. The presence of peloton proves that endophytic fungi can penetrate into the root tissue.

References

Chand K, Shah S, Sharma J, Paudel RM and Pant B. 2020. Isolation, characterization, and plant growth-promoting activities of endophytic fungi from a wild orchid *Vanda cristata*. *Plant Sig Behavior*, **15(5):** 1744294.

Currah RS and Zelmer C. 1992. A Key and Notes for the Genera of Fungi Mycorrhizal with Orchids and A New Species in the Genus Epulorhiza. *Rept Tottori Mycol Institution*, **30:** 43-59.

Haro R and Benito B. 2019. The Role of Soil Fungi in K^+ Plant Nutrition. *Int J mol Sci*, **20(13):** 3169.

Mahfut. 2020. Identification and Detection Odontoglossum ringspot virus on Native Orchids Collection of Nurserys in Java, Indonesia. Proceeding of International Conference on Advanced Information Scientific Development (ICAISD). West Java, Indonesia. **1641:** 1-8.

Mahfut, Anggreiny A, Wahyuningsih S, Handayani TT and Sukimin. 2020[b]. Identification of Disease and Efforts to Protect Native Orchid Plants Against Bacteria Infection in Liwa Botanical Garden. Proceedings of ICAISD Conference. University of Bina Sarana Informatika. West Java, Indonesia.

Mahfut, Daryono BS, Indrianto A and Somowiyarjo S. 2019. Effectiveness Test of Orchid Mycorrhizal Isolate (*Ceratorhiza* and *Trichoderma*) Indonesia and Its Role as a Biofertilizer. *Ann Res Review Biol*, **33(4):** 1-7.

Mahfut, Indrianto A, Somowiyarjo S and Daryono BS. 2020. Molecular phylogeny of orchids mycorrhiza isolated from native tropical orchids in Indonesia. *Malaysian J Microbiol*, **16(1):** 68-72.

Mahfut, Shafira H, Wahyuningsih S, Handayani TT and Sukimin. 2021. Identification of Virus Infection on Native Orchid in Liwa Botanical Garden. Proceedings of ICASMI Conference. University of Lampung. Lampung, Indonesia.

Mose W, Daryono BS, Indrianto A, Purwantoro A and Semiarti E. 2020. Direct Somatic Embryogenesis and Regeneration of an Indonesian orchid Phalaenopsis amabilis (L.) Blume under a Variety of Plant Growth Regulators, Light Regime, and Organic Substances. *Jordan J Biol Sci.* **13(4):** 509-518.

Pannecoucque J and Hofie M. 2009. Interaction Between Cauliflower and *Rhizoctonia* Anastomosis Groups with Different Levels of Aggreeiveness. *BMC Plant Biol*, **9(95):** 1-12.

Safarini M, Rosyidah IH, Mertaniasih NM, Hamzah MNSB, Novianti KA, Primaharinastiti R, Rahmawaty D and Isnaeni I. 2020. In vitro Antibacterial Activity of Cell Free Fermentation Supernatant of *Passiflora edulis* forma flavicarpa Sims. Fruit Fermented by de Man, Rogosa and Sharp Media. *Jordan J Biol Sci.* **13(4):** 469-475.

Shan XC, Liew ECY, Weatherhead MA and Hodgkiss IJ. 2002. Characterization and Taxonomic Placement of *Rhizoctonia*-like Endophytes from Orchids Roots. *Mycologia*, **94(2):** 230-239.

Song Z, Bi Y, Zhang J, Gong Y and Yang H. 2020. Arbuscular mycorrhizal fungi promote the growth of plants in the mining associated clay. *Sci Reports*, **10:** 2663.

Stoian V, Vidican R, Crisan I, Puia C, Sandor M, Stoian VA, Pacurar F and Vaida I. 2019. Sensitive approach and future perspectives in microscopic patterns of mycorrhizal roots. *Sci Reports*, **9:** 10233.

4.

Akreditasi Jurnal

Source details

Jordan Journal of Biological Sciences

Scopus coverage years: from 2014 to Present

Publisher: Hashemite University

ISSN: 1995-6673 E-ISSN: 2307-7166

Subject area: Agricultural and Biological Sciences: General Agricultural and Biological Sciences

Biochemistry, Genetics and Molecular Biology: General Biochemistry, Genetics and Molecular Biology

Source type: Journal

View all documents > Set document alert Save to source list Source Homepage

CiteScore 2021
1.4

SJR 2021
0.290

SNIP 2021
0.778

CiteScore CiteScore rank & trend Scopus content coverage

Improved CiteScore methodology

CiteScore 2021 counts the citations received in 2018-2021 to articles, reviews, conference papers, book chapters and data papers published in 2018-2021, and divides this by the number of publications published in 2018-2021. Learn more >

CiteScore 2021

$$1.4 = \frac{541 \text{ Citations } 2018 - 2021}{380 \text{ Documents } 2018 - 2021}$$

Calculated on 05 May, 2022

CiteScoreTracker 2022

$$1.5 = \frac{531 \text{ Citations to date}}{363 \text{ Documents to date}}$$

Last updated on 05 November, 2022 • Updated monthly

CiteScore rank 2021

Category	Rank	Percentile
Agricultural and Biological Sciences General Agricultural and Biological Sciences	#106/211	50th
Biochemistry, Genetics and Molecular Biology General Biochemistry, Genetics and Molecular Biology	#131/204	36th

View CiteScore methodology > CiteScore FAQ > Add CiteScore to your site

Jordan Journal of Biological Sciences

COUNTRY

Jordan

Universities and research institutions in Jordan

SUBJECT AREA AND CATEGORY

Agricultural and Biological Sciences
- Agricultural and Biological Sciences (miscellaneous)

Biochemistry, Genetics and Molecular Biology
- Biochemistry, Genetics and Molecular Biology (miscellaneous)

PUBLISHER

Hashemite University

Hashemite University in Scimago Institutions Rankings

H-INDEX

15

PUBLICATION TYPE

Journals

ISSN

19956673, 23077166

COVERAGE

2014-2021

INFORMATION

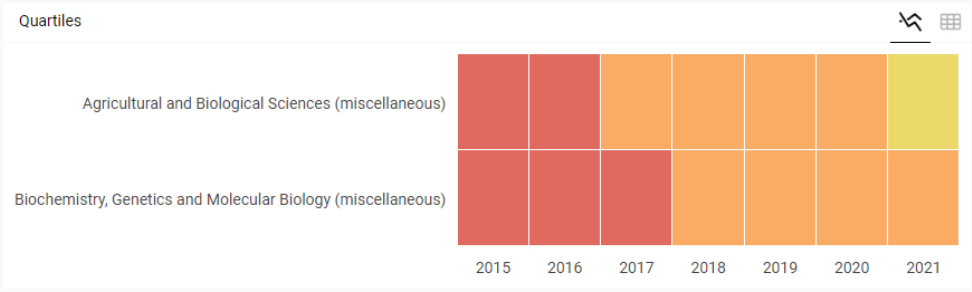
Homepage

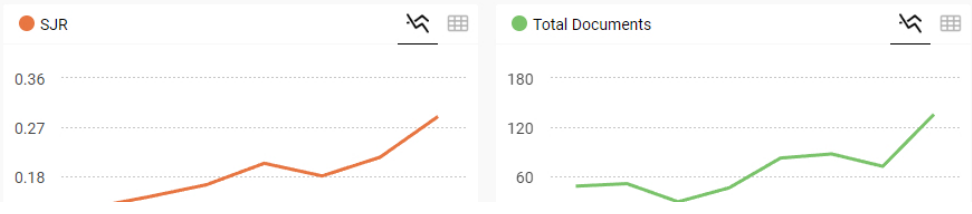
How to publish in this journal

JJBS@hu.edu.jo

SCOPE

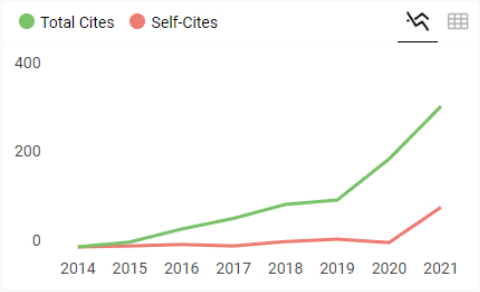
Jordan Journal of Biological Sciences (JJBS) aims to provide a forum for a broad blend of scientific and technical papers to reflect the evolving needs of the biological sciences. Mainly welcome are contributions dealing with cell biology, genomics, microbiology, immunology, molecular biology, biochemistry, embryology, immunogenetics, cell and tissue culture, molecular ecology, genetic engineering and biological engineering, bioremediation and biodegradation, bioinformatics, biotechnology regulations, gene therapy, organismal biology, microbial and environmental biotechnology, marine sciences. The JJBS welcomes the submission of manuscript that meets the general criteria of significance and academic excellence. All articles published in JJBS are peer-reviewed

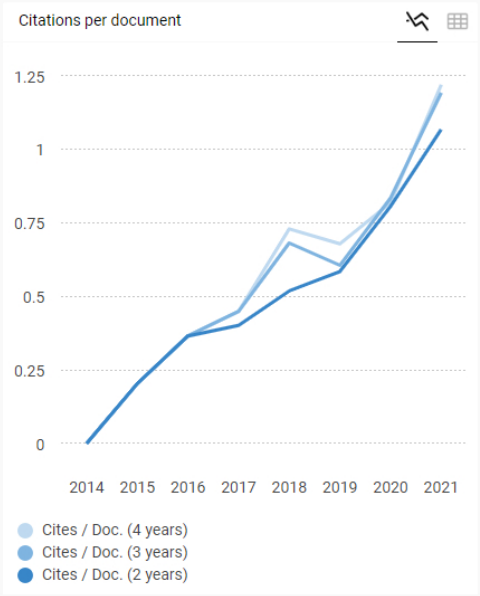


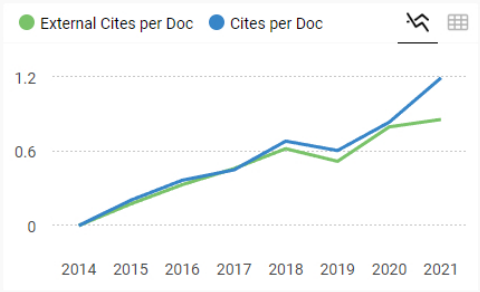


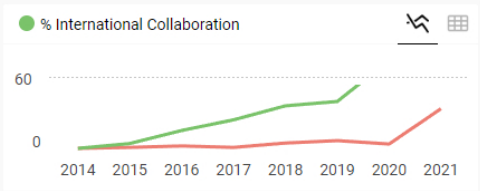

0.09
2015 2016 2017 2018 2019 2020 2021

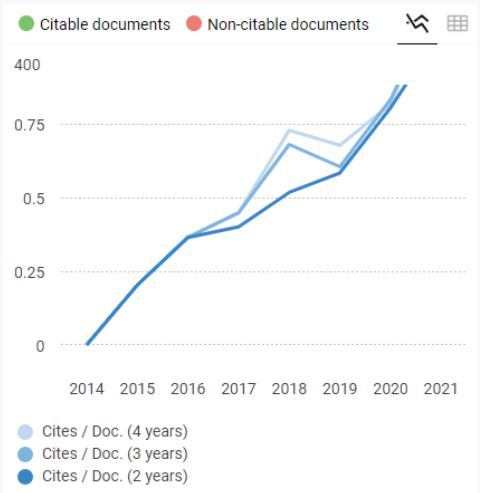

0
2014 2015 2016 2017 2018 2019 2020 2021

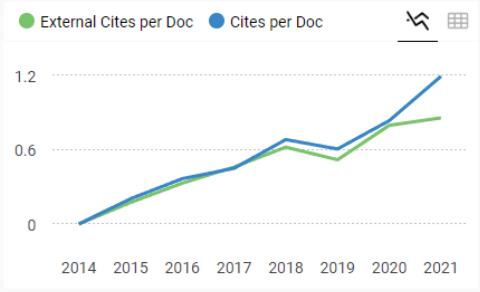

Total Cites
Self-Cites
400
200
0
2014 2015 2016 2017 2018 2019 2020 2021

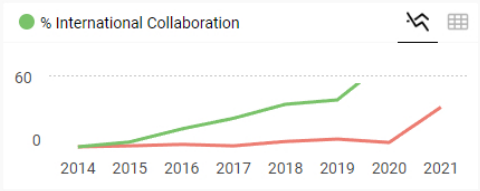

Citations per document
1.25
1
0.75
0.5
0.25
0
2014 2015 2016 2017 2018 2019 2020 2021
Cites / Doc. (4 years)
Cites / Doc. (3 years)
Cites / Doc. (2 years)

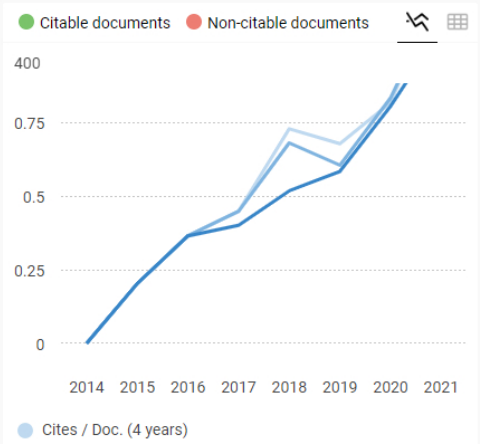

External Cites per Doc
Cites per Doc
1.2
0.6
0
2014 2015 2016 2017 2018 2019 2020 2021

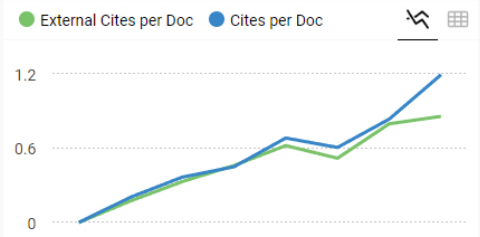

% International Collaboration
60
0
2014 2015 2016 2017 2018 2019 2020 2021


Citable documents
Non-citable documents
400
0.75
0.5
0.25
0
2014 2015 2016 2017 2018 2019 2020 2021
Cites / Doc. (4 years)
Cites / Doc. (3 years)
Cites / Doc. (2 years)


External Cites per Doc
Cites per Doc
1.2
0.6
0
2014 2015 2016 2017 2018 2019 2020 2021


% International Collaboration
60
0
2014 2015 2016 2017 2018 2019 2020 2021


Citable documents
Non-citable documents
400
0.75
0.5
0.25
0
2014 2015 2016 2017 2018 2019 2020 2021
Cites / Doc. (4 years)


External Cites per Doc
Cites per Doc
1.2
0.6
0

5.

Review 1

JJBS 58/21/A8 (evaluation results) Kotak Masuk ×

Jordan Journal Biological
kepada saya

20 Apr 2021 21.10 (2 hari yang lalu)

Jordan Journal of Biological Sciences (JJBS)
ISSN 1995- 6673 (Print), 2307- 7166 (Online)
http://jjbs.hu.edu.jo

Dear Dr. Corresponding author **April 20, 2021**

MORPHOLOGICAL IDENTIFICATION OF MYCORRHIZA FROM NATIVE TROPICAL ORCHIDS IN INDONESIA

Manuscript Number: JJBS 58\21\A8

Thank you for submitting the above mentioned manuscript to Jordan Journal of Biological Sciences (JJBS). It has been considered by an Editor, a Member of the Editorial Board and three independent expert referees. They agree that the paper presents an interesting piece of work, however, before your paper can be accepted for publication, several changes are required; you should also respond satisfactorily to some questions of methodology and interpretation which have been raised by the referees (See attached and Comments Below). **The current decision does not guarantee an automatic accept decision following revision and the handling editor may still reject your manuscript. Please make sure that all References are according to JJBS Format as well as you send me the consent form and the results of plagiarism software (I authenticate or turnitin) with similarity results less than 15%.**

If you decide to submit a revised version of your paper within two-three weeks, please send to me one copy of the revised MS and your response to each Referee at separate sheet. Please be sure to include detailed Response to Reviewers' comments on separate file.

-

Thank you for your interest in our journal. Kindly acknowledge the receipt of this mail.

Sincerely yours,

Professor Manar Atoum

Hashemite University

Zarqa – Jordan

E. mail: jjbs@hu.edu.jo

المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)
ISSN 1995- 6673 (Print), 2307- 7166 (Online)
http://jjbs.hu.edu.jo

Informed Consent

Manuscript Number: JJBS 58/21/A8

MORPHOLOGICAL IDENTIFICATION OF MYCORRHIZA FROM NATIVE TROPICAL ORCHIDS IN INDONESIA

Authorships [Mahfut] and Affiliation:
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Lampung, Indonesia

Conflict of interest/sponsorship (if any):

Ethical committee approval\ Human Research Protections \ or Institutional Review Board (IRB): If Applicable, Please provide a copy of the Approval.

We affirm that the submission represents work that has not been published previously and is not currently being considered by another journal. Also, I confirm that each author has seen and approved the contents of the submitted manuscript.

Signature (on behalf of all co-authors (if any))

Corresponding author
Name: Mahfut
Affiliation: Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Lampung, Indonesia
Tel.: +62 721 702673
Fax: +62 721 702767
E-mail address: mahfut.mipa@fmipa.unila.ac.id
Submission date: February 27, 2021

Auhor,

Mahfut

Jordan Journal of Biological Sciences (JJBS)
ISSN 1995- 6673 (Print), 2307- 7166 (Online)
http://jjbs.hu.edu.jo
Scientific Research and Innovation Support Fund **Hashemite University**
Jordan Journal of Biological Sciences **Deanship of Scientific Research**

Manuscript Evaluation Report- Referee 1

Manuscript ID: JJBS 58/21/A8 **Due date: April 4, 2021**

MS Title: Morphological Identification of Orchid Mycorrhiza Fungi From Native Orchids in Indonesia

Type of Article: ☐ Review Article ☑ Research Paper ☐ Case Report

PART A:

On a scale of 1 – 5 (1 being lowest and 5 being highest), rate the manuscript based on the following criteria;

NO.	Criteria	Score
1	**Is the topic of the manuscript within the scope of the journal?**	3
2	**Does the title clearly and sufficiently reflect its content?**	2.5
3	**Are the keywords and abstract sufficient and informative?**	2.5
4	**What is the scholarly quality of the manuscript?**	2
5	**Is this a new and/ or original contribution?**	2.5
6	**Is the research methodology utilized appropriate and properly administered?**	2
7	**Are the methods of data analysis acceptable?**	2
8	**Are the results and conclusions clear, adequately presented and organized in relation to rest of manuscript?**	2.5
9	**Are the illustrations and tables necessary and in acceptable format?**	2
10	**Are the interpretations/ conclusions sound and justified by the data?**	2
11	**Are the References in a proper format according to JJBS author Instruction?**	2
12	**Is the MS written in correct and satisfactory English?**	1

Please rate the priority for publication of this article (10 is the highest priority, 1 is the lowest priority)

05

PART B: Comments per Section of Manuscript:

Abstract	The abstract needs a thorough revision as some of the sentences are not clear. The fungus studied in the present study was molecularly characterized in a previous study. In such a case, it is not clear why its identity has to be characterized morphologically. Use either *Ceratorhiza* or *Ceratobasidium* which is haphazardly used throughout the text. This study is not the first report on the morphological characterization of *Ceratorhiza* in moon orchid (see Khaterine et al. 2011; Warcup 1981).
Introduction	The introduction should emphasize the need to carrying out the study. It is well known that mycorrhizal fungi are mandatory for orchid seed germination and plant development. The aim of the study should be modified as the protection of orchid from diseases by mycorrhizal fungi was not examined.
Methodology	The methods should explain the procedures used in the study. Although some fungal structures have been measured in the present study it is not clear how many of these structures (n) were measured. See annotated manuscript.
Results	Avoid discussion and reference citations in the results section. It is not necessary to provide magnifications for the microscopic images when scale bars are marked on the images.
Discussion and Conclusion	Many part of the discussion are mere repeat of the results. Moreover, the salient results of the study are not adequately discussed.
References	Some of relevant studies were not consulted. The author should thoroughly check the literature for the mycorrhizal association in *Phalaenopsis*.

PART C: Recommendation (Kindly Mark With An ✓)

Acceptable in its Present Form	
Acceptable with Minor Revision	
Reconsidered after Major Revision	✓
Reject on Ground of (Please be Specific)	

PART D: Additional Comments:

Please add any other additional comments or specific suggestions on the enclosed comments sheet:

This manuscript presents a study on the isolation and characterization of mycorrhizal fungi associated with an epiphytic moon orchid *Phalaenopsis amabilis*. This culture-dependent study resulted in the isolation of one fungus resembling *Ceratobasidium* which was characterized both morphologically and molecularly. Moreover, the presence of fungal pelotons was also observed in the root cortical cells. The study as such lacks novelty as the symbiosis of *Ceratobasidium* and other mycorrhizal fungi with moon orchid is already reported in the literature (see appendix). Further, the presentation is rather poor making reading extremely difficult. The presentation is marred by faulty sentences, spelling errors, etc., spread throughout the manuscript. The fungus is mentioned to control diseases moon orchid. However, this aspect was not examined in the present study. The discussion is unfocused and empty. This should be rectified. I would suggest the author seek the help of a native English speaker while revising the manuscript.

Appendix:

Khaterine, Situmorang, N., & Kasiamdari, R. S. (2011). Isolation and identification of *Rhizoctonia* associated with *Phalaenopsis amabilis* (L.) Blume Roots. Proceeding ICBB (The International Conference on Bioscience and Biotechnology) 1(1): C39-C44.

Mahfut, M., Daryono, BS., Ari, I., & Susamto, S. (2019) Effectiveness test of orchid mycorrhizal isolate (*Ceratorhiza* and *Trichoderma*) Indonesia and its role as a biofertilizer. Annual Research & Review in Biology 33 (4): 1-7.

Warcup JH(1981) The mycorrhizal relationships of Australian orchids. New Phytologist 87(2):371-381.

Morphological Identification of Orchid Mycorrhizal Fungi From Native Orchids in Indonesia

Mahfut

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Lampung, Indonesia

Corresponding author e-mail: mahfut.mipa@fmipa.unila.ac.id

Abstract

Moth orchid ~~([~~*Phalaenopsis amabilis* (L.) Blume~~])~~ is a~~n~~ species native orchid from Indonesia. Its presence in nature to live in symbiosis with endophytic mycorrhiza as biocontrol agent. In a previous study, one *Ceratobasidium* isolate from Yogyakarta, Indonesia was successfully ~~isolated~~ identified based on rDNA-ITS molecular analysis. This study aims to identify these isolates based on morphological analysis. Verification morphological analysis is carried out by observing macroscopic and microscopic characteristics, as well as observing peloton. The results of the analysis showed that the characterization of Yogyakarta isolates had similarities with *Ceratobasidium*. These character equations include colony color, cell length, core number, and cell width. The ~~results~~ examination of ~~the induction of~~ orchid roots ~~mycorrhiza~~ also showed ~~a~~ the presence of peloton~~s~~ ~~structure~~ in the cortical~~ex~~ cells. ~~of orchid root.~~ This study confirms that the fungal isolates of orchid~~s~~ mycorrhiza from Yogyakarta, Indonesia ~~are~~ were *Ceratobasidium* based on morphological and molecular analysis. This research is a conservation and natural protection effort of native orchids in Indonesia using biocontrol agent. This study is the first to report regarding *Ceratobasidium* isolated from native orchids in Indonesia based on morphological analysis.

Keywords: *Ceratobasidium*; mycorrhiza; morphological analysis; *Phalaenopsis*; Indonesia

1. Introduction

Orchid is a type of ornamental plant that has a high aesthetic value and is most in demand by the community (Mose et al., 2020). The moth orchid ~~([~~*Phalaenopsis amabilis* (L.) Blume~~])~~ is one of Indonesia's national flowers, namely the charm that was set through Presidential Indonesia Decree Number 4/1993. Disease~~s~~ ~~infections~~ are still the main obstacle in the cultivation and development of ~~the potential of~~ natural orchids (Mahfut et al., 2019; Mahfut, 2020; Mahfut et al., 2020[b]; Mahfut et al., 2021).

Endophytic mycorrhiza is a form of symbiosis between fungi and plant roots during a certain period of their life cycle, forming colonies in plant tissues without endangering their hosts. In general, ~~endophytic~~ mycorrhizal fungi help germination of orchid seeds in the presence of ethylene and various vitamins. Another role is to support efforts to provide nutrition for plant growth and development (Haro and Benito, 2019), and to assist in the formation of more buds and flowers. In plant resistance, the role of endophytic mycorrhiza can be said to be a biological agent of control in plant protection against pathogenic infections (Safarini et al., 2020; Song et al., 2020).

In ~~the~~ a previous study, Mahfut et al. (2020[a]) ~~was~~ reported one *Ceratobasidium* isolate from Yogyakarta, Indonesia ~~was reported to have been successfully isolated~~ based on rDNA-ITS molecular analysis. The results show ~~bands~~ sequences measuring 600-750 bp DNA products located on the ITS1-5.8S-ITS4 region. Reconstruction of phylogenetic trees resulted in Indonesian isolates having undergone speciation and separated from C*eratobasidium* isolates from other countries. This research is a follow-up study which aims to clarify the identification of these isolates as *Ceratobasidium* based on morphological analysis. Furthermore, this research can be used as a ~~database~~ benchmark in developing strategy for conserving ~~the~~ moth orchid ~~conservation strategy~~ through protection ~~of~~ against plant~~s against infectious~~ diseases.

2. Materials and Methods

2.1. Collection of Healthy Orchid Root Samples and Endophytic Mycorrhizal Isolation

Healthy root of *P. amabilis* ~~(L.) Blume~~ collected from Yogyakarta, Indonesia. The sample was taken to the Biotechnology Laboratory of the Faculty of Biology UGM to isolate endophytic mycorrhiza following the Chand et al. (2020) method. Isolation of endophytic fungi was then carried out by cutting the roots and culturing on PDA medium. The ~~isolates~~ fungal colonies obtained from the isolation were then purified by sub-culturinge aseptically and incubating for ~~ed~~ 7 days at room temperature. It is possible that the isolates obtained are pure isolates that are not contaminated by other fungi.

2.2. Macroscopic Characteristic Observation

To observe the macroscopic characteristics of ~~endophytic~~ the isolated fungi ~~isolated~~, small ~~fragments~~ portion of isolates (± 1 mm^2) were placed ~~± 1 mm^2 inoculated~~ in the middle of the PDA medium. Furthermore, the growth of the isolates were observed

Formatted: Numbering: Continuous

Comment [R1]: Only one was examined in the present study.

Comment [R2]: Not clear. Are you mentioning the association of this orchid with mycorrhizal fungi??

Comment [R3]: What is the need for morphological characterization after a more relevant molecular characterization.

Comment [R4]: Was there any fruiting bodies formed in culture?

Comment [R5]: Not clear.

Comment [R6]: How was this determined?? The genus *Ceratobasidium* also contains plant pathogens.

Comment [R7]: Rewrite for clarity.

Comment [R8]: It is not necessary to cite the authorities at every mention.

Comment [R9]: Provide the coordinates for the sampling site.

Comment [R10]: Specify the room temperature.

Comment [R11]: How was this determined?

and measured ~~the growth of their colonies~~ every day for 7 days. Furthermore, observations of colony morphological characteristics such as colony color, colony base color, and colony characteristics were carried out.

2.3. Making Slide Culture Preparations

Observation of microscopic characteristics was also carried out using the slide culture method ~~as delivered by (~~Stoian et al. ~~(~~2019) with a slight modification. This method ~~is~~ was carried out to monitor microscopic characteristics such as the color of hyphae, bulkhead in hyphae, angle of branching of hyphae, and number of cell nuclei. ~~While for observing t~~The number of nuclei was observed after treating with ~~using~~ safranin and 3% KOH ~~3%~~.

2.4 Observation of ~~Endophytic Mycorrhizal Hyphae (~~Peloton~~s in roots~~)

Observation of peloton was carried out by making squash preparations on root samples which had been treated with previous endophytic fungal inoculations. Root samples are sliced and soaked in tryp~~h~~an blue dye for 15 minutes. Observations were carried out using a light microscope at 100 x magnification. ~~The peloton will appear in the cell portion of the root cortex.~~

3. Results

3.1. Collection of Healthy Orchid Root Samples and Endophytic Mycorrhizal Isolation

~~The results of isolation were obtained by 1 isolate of~~One endophytic fungus~~i~~, namely Yogyakarta was isolated from root samples of *P. amabilis* ~~(L.) Blume~~ in Yogyakarta, Indonesia in Figure 1.

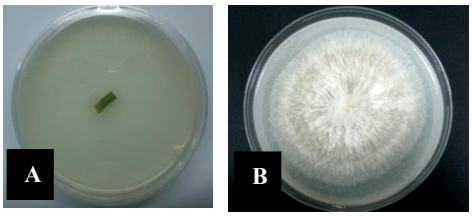


Figure 1. A) Root samples of *P. amabilis* ~~(L.)~~ cultured on PDA medium, B) Colonies of fungi that grow on 7 days old orchid roots

Identification results based on macroscopic, microscopic, and molecular~~–~~ characteristics showed that the isolate was *Ceratorhiza* which is one of the types of *Rhizoctonia* endophytic mycorrhiza. According to Chand et al. (2020), *Ceratorhiza* is *Rhizoctonia* grouped by cell hyphae and moniloid, and many are found to be associated with orchid roots.

3.2. Macroscopic Characteristic~~s~~ ~~Observation~~

Identification of Yogyakarta isolate~~s~~ was carried out by observing colony color, shape of branching hyphae, diameter of hyphae, number of cell nuclei, clamp connection, monoloid cell shape and size.

Special characteristics of Yogyakarta isolate was identified as having branching types that formed elbows or 90º angles and hyphae cells with a cell nucleus 2 (binucleate). These two characteristics are special characteristics of endophytic mycorrhiza. Endophytic fungi have 1-3 nuclei (binucleate) nuclei, whereas pathogenic fungi have more than 3 nuclei (multinucleates). This is in accordance with the opinion of Pannecoucque & Hofie (2009) that in addition to binucleates, the characteristics of endophytic fungi are branching which forms a 90ºC or T-shaped branches.

Overall, the results of identification of Yogyakarta isolates showed similarities with the types of *Ceratorhiza* mycorrhiza. The following characteristics of Yogyakarta isolates compared to supporting references are presented in Table~~s~~ 1 and ~~Table~~ 2.

Table 1. Comparison of the characteristics of Yogyakarta isolates and *Cerathoriza* isolates according to Shan et al. (2002)

Characteristics	Yogyakarta Isolate	*Cerathoriza* Isolate
The surface color of young colonies	White to yellow	Yellow to white
The appearance of colonies	Like cotton	Like cotton
Hyphae color	~~Hialin~~Hyaline	~~Hialin~~Hyaline
Hyphae diameter (µm)	6,20-6,72	3,8-7,5
Monilioid cell form	*Elongated, barrel shaped*	*Ellipsoidal* atau *elongate barrel shape*
Monilioid cell size (µm)	(12,22-14,56) ~~x~~ (2,39-2,91)	(7,5-15,0) x (10,0-25,0)
The growth rate of the colony (mm / hr)	0.72	0,42-0,52
Number of cell nuclei	Binucleate	Binucleate

Table 2. Comparison of the characteristics of Yogyakarta isolate~~s~~ and *Cerathoriza* isolates according to Currah and Zelmer (1992)

Comment [R12]: Specify the characters.

Comment [R13]: What is this? Explain.

Comment [R14]: Figures should be self-explanatory. Do not abbreviate the genus name,

Comment [R15]: This should be in the methods.

Comment [R16]: Change as 6.20-6.72

Comment [R17]: ??

Comment [R18]: Use symbol.

Characteristics	Yogyakarta Isolate	*Cerathoriza* Isolate
Colony color	White to yellow	Cream, yellow, or brown
The appearance of colonies	Like cotton	Like cotton
The form of branching hyphae	90 °	90°
Air hyphae diameter	<3 µm	<4 µm
Number of ~~cell~~ nuclei per cell	Binucleate	Binucleate
Clamp connection	-	-
Surface of the colony	Like cotton	*Flat and waxy*
~~Cell m~~Monilioid ~~form~~cells	*Elongate barrel shaped*	-
~~Length of cell m~~Monilioid cell lengths	12,22-14,56	-
~~Cell m~~Monilioid cell width	2,39-2,91	-

Based on observations of hyphae and cells using safranin O-KOH dye **(**Figure 2) it was found that Yogyakarta isolates were endophytic fungi of *Ceratorhiza* with specific characteristics having branching types that form elbows or 90°~~o~~ angles, the color of vegetative hyphae is hyaline, with diameters of 6.20-6.72 µm, fungal colonies form cell monilioids with elongate barrel shape and monilioid size (12,22-14,56) µm x (2.39-2.91) µm. The number of nuclei in binucleic cells, according to the characteristics reported by Shan et al. (2002) and Currah and Zelmer (1992).

Comment [R19]: Why do you include a discussion when there is a separate section for this?

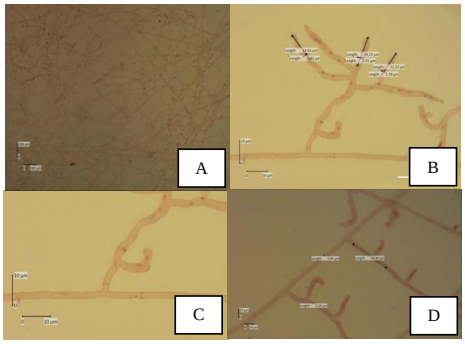


Figure 2. Microscopic observation of hyphae cells and moniliod cells *Ceratorhiza* from Yogyakarta isolates on the 7-day PDA medium using safranin O-KOH staining; (A) Colony, (B) Monilioid cell nucleus, (C) Binucleate, (D) Branching of hyphae 90°. Magnification (A) 10 x, (B, C, D) 40 x. Bar = 10 µm

3.4 Observation of Endophytic Mycorrhizal Hyphae (Peloton)

Observation of the cross section of the root sample ~~found~~ indicated the presence of ~~a~~ peloton~~s~~ in the root cortex (Figure 3). Currah & Zelmer (1992) explain that the characteristic of endophytic fungal hyphae ~~infecting~~ colonizing plants will form a mass of dense hyphae in cortical cells called peloton~~s~~.

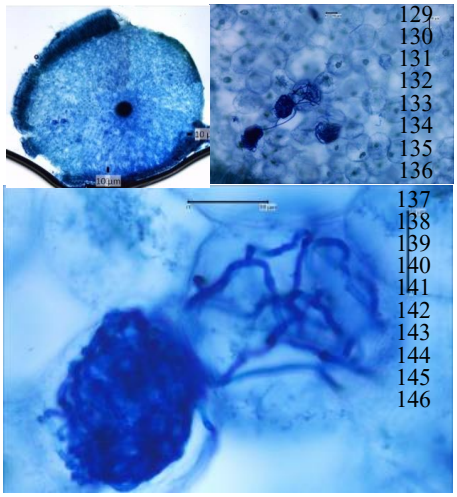


Figure 3. Peloton structure in the anatomy of the root cortex cells of *P. amabilis* ~~(L.) Blume~~ with Tryphan Blue staining. Magnification (A) 4 x, (B) 10 x, (C) 40 x. Bar = 10 µm

4. Discussion

Characteristics of endophytic mycorrhizal analysis were obtained and compared with the reference characteristics (Shan et al., 2002; Currah and Zelmer, 1992), it was found that Yogyakarta isolates were *Ceratorhiza* isolates. *Ceratorhiza* is an anamorphic phase of *Ceratobasidium*. According to Athipunyakom et al., (2004), the genus *Ceratorhiza* is a mycorrhizal isolate which is commonly found associated with orchids, where endophytic fungi of this type are *Rhizoctonia* which are grouped according to cell hyphae and moniloid. Growth of *Ceratorhiza* reached a diameter of 9 cm after 4 days ~~after~~ of incubation by forming a symmetrical zone. The ~~color of the~~ colony ~~will~~ turnned yellowish after 7 days ~~with~~ and the colony surface ~~of the hyphae~~appeared as ~~clumping like~~ cotton~~y~~ clumps. The lumps ~~y hyphae~~ are aerial hyphae, this is known from the hyphae that grow on the surface of the agar media. These hyphae are fertile hyphae which play a role for reproduction. The length of moniloid cells is 12.22-14.56 µm, width 2.39-2.91µm and lancet-shaped. If the ratio of length and width is 3.5-4: 1 then it is called the lancet type.

The peloton structure is evident in the roots of *P. amabilis* ~~(L.) Blume~~ which are associated with endophytic fungi (Figure 3). One of the parameters for the mechanism of resistance induction can be the formation of a peloton structure which shows that endophytic fungi penetrate the epidermal finger and into the cortex. In the cortex, the pulp will enter the cell space (intracellular) and form a peloton.

The presence of peloton proves that the endophytic fungi can penetrate into the root tissue of *P. amabilis* ~~(L.) Blume~~. According to Currah and Zelmer (1992), the stage of infection with endophytic fungi begins with the formation of appressorium which is an ~~thickening~~ inflated ~~of~~ hyphae. These hyphae originate from spores that germinate or externally hyphae at the root surface of infected host plants. Hyphae will then penetrate the root surface mechanically and enzymatically into the space between the root epidermal cells and into the cortex and beyond. Hyphae develop without destroying the cells of the root cortex. In cortex cells, hyphae form a peloton in the form of dense hyphae. In the platoon, the accumulation of organic materials includes protein, glycogen, fat, and nutrients produced by absorption from the soil.

5. Conclusion

The results showed that the characterization of endophytic mycorrhizae from the roots of *P. amabilis* ~~(L.) Blume~~ from Yogyakarta, Indonesia had similarities with the type of endophytic mycorrhizal *Ceratorhiza*. These character equations include colony surface color, colony appearance, hyphae color, hyphae diameter (µm), cell monilioid shape, monilioid cell size (µm), colony growth rate (mm / hour), core number, hyphae branching shape, and clamp connection. The results of mycorrhizal induction also produce a peloton structure in orchid root cortex cells. The peloton structure is evident in the roots of *P. amabilis* ~~(L.) Blume~~ which are associated with endophytic fungi. The presence of peloton proves that endophytic fungi can penetrate into the root tissue.

References

Chand K, Shah S, Sharma J, Paudel RM and Pant B. 2020. Isolation, characterization, and plant growth-promoting activities of endophytic fungi from a wild orchid *Vanda cristata*. *Plant Signaling & Behavior*, **15(5):** 1744294.

Currah RS and Zelmer C. 1992. A Key and Notes for the Genera of Fungi Mycorrhizal with Orchids and A New Species in the Genus Epulorhiza. *Rept. Tottori Mycology Institution*, **30:** 43-59.

Haro R and Benito B. 2019. The Role of Soil Fungi in K^+ Plant Nutrition. *International journal of molecular sciences*, **20(13):** 3169.

Mahfut. 2020. Identification and Detection Odontoglossum ringspot virus on Native Orchids Collection of Nurserys in Java, Indonesia. Proceeding of International Conference on Advanced Information Scientific Development (ICAISD). West Java, Indonesia. **1641:** 1-8.

Mahfut, Anggreiny A, Wahyuningsih S, Handayani TT and Sukimin. 2020[b]. Identification of Disease and Efforts to Protect Native Orchid Plants Against Bacteria Infection in Liwa Botanical Garden. Proceeding of International Conference on Advanced Information Scientific Development (ICAISD). West Java, Indonesia. **1641:** 1-7.

Mahfut, Daryono BS, Indrianto A and Somowiyarjo S. 2019. Effectiveness Test of Orchid Mycorrhizal Isolate (*Ceratorhiza* and *Trichoderma*) Indonesia and Its Role as a Biofertilizer. *Annual Research & Review in Biology*, **33(4):** 1-7.

Mahfut, Indrianto A, Somowiyarjo S and Daryono BS. 2020[a]. Molecular phylogeny of orchids mycorrhiza isolated from native tropical orchids in Indonesia. *Malaysian Journal of Microbiology*, **16(1):** 68-72.

Formatted: Highlight

Formatted: Font: Italic

Formatted: Font: Italic

Mahfut, Shafira H, Wahyuningsih S, Handayani TT and Sukimin. 2021. Identification of Virus Infection on Native Orchid in Liwa Botanical Garden. Proceeding of International Conference on Applied Science, Mathematics, and Informatics (ICASMI). Lampung, Indonesia. **1751:** 1-7.

Mose W, Daryono BS, Indrianto A, Purwantoro A and Semiarti E. 2020. Direct Somatic Embryogenesis and Regeneration of an Indonesian orchid Phalaenopsis amabilis (L.) Blume under a Variety of Plant Growth Regulators, Light Regime, and Organic Substances. *Jordan Journal of Biological Sciences.* **13(4):** 509-518.

Pannecoucque J and Hofie M. 2009. Interaction Between Cauliflower and *Rhizoctonia* Anastomosis Groups with Different Levels of Aggreeiveness. *BMC Plant Biology*, **9(95):** 1-12.

Safarini M, Rosyidah IH, Mertaniasih NM, Hamzah MNSB, Novianti KA, Primaharinastiti R, Rahmawaty D and Isnaeni I. 2020. In vitro Antibacterial Activity of Cell Free Fermentation Supernatant of *Passiflora edulis* forma flavicarpa Sims. Fruit Fermented by de Man, Rogosa and Sharp Media. *Jordan Journal of Biological Sciences.* **13(4):** 469-475.

Shan XC, Liew ECY, Weatherhead MA and Hodgkiss IJ. 2002. Characterization and Taxonomic Placement of *Rhizoctonia*-like Endophytes from Orchids Roots. *Mycologia*, **94(2):** 230-239.

Song Z, Bi Y, Zhang J, Gong Y and Yang H. 2020. Arbuscular mycorrhizal fungi promote the growth of plants in the mining associated clay. *Scientific Reports*, **10:** 2663.

Stoian V, Vidican R, Crisan I, Puia C, Sandor M, Stoian VA, Pacurar F and Vaida I. 2019. Sensitive approach and future perspectives in microscopic patterns of mycorrhizal roots. *Scientific Reports***, 9:** 10233.

Formatted: Font: Italic

Morphological Identification of Orchid Mycorrhizal Fungi From Native Orchids in Indonesia

Mahfut

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Lampung, Indonesia

Corresponding author e-mail: mahfut.mipa@fmipa.unila.ac.id

Abstract

Moth orchid ~~{[~~*Phalaenopsis amabilis* (L.) Blume~~]}~~ is a~~n~~ species native orchid from Indonesia. The association of this orchid with mycorrhizal fungi in nature ~~. Its presence in nature to live in symbiosis with endophytic mycorrhiza~~ as biocontrol agent. In a previous study, one *Ceratobasidium* isolate from Yogyakarta, Indonesia was successfully ~~isolated~~ identified based on rDNA-ITS molecular analysis. ~~This study aims to identify these isolates based on morphological analysis.~~ This study aimed to identify these isolates based on morphological analysis to complement other identification data, namely anatomy and molecular. Verification morphological analysis is carried out by observing macroscopic and microscopic characteristics, as well as observing peloton. The results of the analysis showed that the characterization of Yogyakarta isolates had similarities with *Ceratobasidium*. These character equations include colony color, cell length, core number, and cell width. The ~~results~~ examination of ~~the induction of~~ orchid roots ~~mycorrhiza~~ also showed ~~a~~ the presence of pelotons ~~structure~~ in the cortical~~ex~~ cells. ~~of orchid root.~~ This study confirms that the fungal isolates of orchid~~s~~ mycorrhiza from Yogyakarta, Indonesia ~~are~~ were *Ceratobasidium* based on morphological and molecular analysis. This research is one of the efforts to preserve native orchids in Indonesia using myccorhiza fungi as a biocontrol agent. ~~a conservation and natural protection effort of native orchids in Indonesia using biocontrol agent.~~ This study is the first to report regarding *Ceratobasidium* isolated from native orchids in Indonesia based on morphological analysis.

Keywords: *Ceratobasidium*; mycorrhiza; morphological analysis; *Phalaenopsis*; Indonesia

Comment [R1]: What is the need for morphological characterization after a more relevant molecular characterization.

Comment [R2]: How was this determined?? The genus *Ceratobasidium* also contains plant pathogens.

1. Introduction

Orchid is a type of ornamental plant that has a high aesthetic value and is most in demand by the community (Mose et al., 2020). The moth orchid ~~{[~~*Phalaenopsis amabilis* (L.) Blume~~]}~~ is one of Indonesia's national flowers, namely the charm that was set through Presidential Indonesia Decree Number 4/1993. Disease~~s~~ ~~infections~~ are still the main obstacle in the cultivation and development of ~~the potential of~~ natural orchids (Mahfut et al., 2019; Mahfut, 2020; Mahfut et al., 2020[b]; Mahfut et al., 2021).

Endophytic mycorrhiza is a form of symbiosis between fungi and plant roots during a certain period of their life cycle, forming colonies in plant tissues without endangering their hosts. In general, ~~endophytic~~ mycorrhizal fungi help germination of orchid seeds in the presence of ethylene and various vitamins. Another role is to support efforts to provide nutrition for plant growth and development (Haro and Benito, 2019), and to assist in the formation of more buds and flowers. In plant resistance, mycorrhiza fungi serve as a biological agent of control in plant protection against pathogenic infections~~the role of endophytic mycorrhiza can be said to be a biological agent of control in plant protection against pathogenic infections~~ (Safarini et al., 2020; Song et al., 2020).

In ~~the~~ a previous study, Mahfut et al. (2020[a]) ~~was~~ reported one *Ceratobasidium* isolate from Yogyakarta, Indonesia ~~was reported to have been successfully isolated~~ based on rDNA-ITS molecular analysis. The results show ~~bands~~ sequences measuring 600-750 bp DNA products located on the ITS1-5.8S-ITS4 region. Reconstruction of phylogenetic trees resulted in Indonesian isolates having undergone speciation and separated from C*eratobasidium* isolates from other countries. This research is a follow-up study which aims to clarify the identification of these isolates as *Ceratobasidium* based on morphological analysis. Furthermore, this research can be used as a ~~database~~ benchmark in developing strategy for conserving ~~the~~ moth orchid ~~conservation strategy~~ through protection ~~of~~ against plant~~s against infectious~~ diseases.

2. Materials and Methods

2.1. Collection of Healthy Orchid Root Samples and Endophytic Mycorrhizal Isolation

Healthy root of *P. amabilis* ~~(L.) Blume~~ collected from Yogyakarta, Indonesia on coordinates 8º 30' - 7º 20' LS 109º 40' - 111º 0' BT. The sample was taken to the Biotechnology Laboratory of the Faculty of Biology UGM to isolate endophytic mycorrhiza following the Chand et al. (2020) method. Isolation of endophytic fungi was then carried out by cutting the roots and culturing on PDA medium. The ~~isolates~~ fungal colonies obtained from the isolation were then purified by sub-culturinge aseptically and incubating for ~~ed~~ 7 days at room temperature (20-25℃). It is possible that the isolates obtained are pure and single isolates that are not contaminated by other fungi.

Comment [R3]: It is not necessary to cite the authorities at every mention.

2.2. Macroscopic Characteristic Observation

To observe the macroscopic characteristics of ~~endophytic~~ the isolated fungi ~~isolated~~, small ~~fragments~~ portion of isolates (± 1 mm^2) were placed ~~± 1 mm^2 inoculated~~ in the middle of the PDA medium. Furthermore, the growth of the isolates were observed and measured ~~the growth of their colonies~~ every day for 7 days. Furthermore, observations of colony morphological characteristics such as colony color, colony base color, ~~and~~ the surface color of young colonies, the appearance of colonies, and the growth rate of the colony ~~colony characteristics~~ were carried out.

2.3. Making Slide Culture Preparations

Observation of microscopic characteristics was also carried out using the slide culture method ~~as delivered by~~ (Stoian et al. ~~(~~2019) with a slight modification. This method ~~is~~ was carried out to monitor microscopic characteristics such as the color of hyphae, bulk~~head~~ in hyphae, angle of branching of hyphae, and number of cell nuclei. ~~While for observing t~~The number of nuclei was observed after treating with ~~using~~ safranin and 3% KOH ~~3%~~.

2.4 Observation of ~~Endophytic Mycorrhizal Hyphae (~~Pelotons in roots~~)~~

Observation of peloton was carried out by making squash preparations on root samples which had been treated with previous endophytic fungal inoculations. Root samples are sliced and soaked in tryp~~h~~an blue dye for 15 minutes. Observations were carried out using a light microscope at 100 x magnification. ~~The peloton will appear in the cell portion of the root cortex.~~

3. Results

3.1. Collection of Healthy Orchid Root Samples and Endophytic Mycorrhizal Isolation

~~The results of isolation were obtained by 1 isolate of~~One endophytic fungus~~i~~, namely Yogyakarta was isolated from root samples of *P. amabilis* ~~(L.) Blume~~ in Yogyakarta, Indonesia in Figure 1.

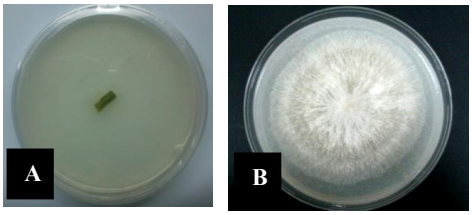


Figure 1. A) Root samples of MP1 ~~*P. amabilis* (L.)~~ cultured on PDA medium, B) Colonies of fungi that grow on 7 days old orchid roots

Identification results based on macroscopic, microscopic, and molecular~~–~~ characteristics showed that the isolate was *Ceratorhiza* which is one of the types of *Rhizoctonia* endophytic mycorrhiza. According to Chand et al. (2020), *Ceratorhiza* is *Rhizoctonia* grouped by cell hyphae and moniloid, and many are found to be associated with orchid roots.

3.2. Macroscopic Characteristics ~~Observation~~

~~Identification of Yogyakarta isolates was carried out by observing colony color, shape of branching hyphae, diameter of hyphae, number of cell nuclei, clamp connection, monoloid cell shape and size.~~

~~-~~Special characteristics of Yogyakarta isolate was identified as having branching types that formed elbows or 90º angles and hyphae cells with a cell nucleus 2 (binucleate). These two characteristics are special characteristics of endophytic mycorrhiza. Endophytic fungi have 1-3 nuclei (binucleate) nuclei, whereas pathogenic fungi have more than 3 nuclei (multinucleates). This is in accordance with the opinion of Pannecoucque & Hofie (2009) that in addition to binucleates, the characteristics of endophytic fungi are branching which forms a 90ºC or T-shaped branches.

Overall, the results of identification of Yogyakarta isolates showed similarities with the types of *Ceratorhiza* mycorrhiza. The following characteristics of Yogyakarta isolates compared to supporting references are presented in Tables 1 and ~~Table~~ 2.

Table 1. Comparison of the characteristics of Yogyakarta isolates and *Cerathoriza* isolates according to Shan et al. (2002)

Characteristics	Yogyakarta Isolate	*Cerathoriza* Isolate
The surface color of young colonies	White to yellow	Yellow to white
The appearance of colonies	Like cotton	Like cotton
Hyphae color	~~Hialin~~Hyaline	~~Hialin~~Hyaline
Hyphae diameter (µm)	6,.20-6,.72	3,.8-7,.5
Monilioid cell form	Elongated, barrel shaped	Ellipsoidal or~~atau~~ elongate barrel shape
Monilioid cell size (µm)	(12,.22-14,.56) × ~~x~~ (2,.39-2,.91)	(7,.5-15,.0) ~~x~~ × (10,.0-25,.0)

Comment [R4]: Specify the characters.

Formatted: English (U.S.)

Comment [R5]: This should be in the methods.

Formatted: Font: Not Italic

Formatted: Font: Not Italic

Formatted: Font: Not Italic

Comment [R6]: Use symbol.

The growth rate of the colony (mm / hr)	0.72	0,42-0,52
Number of cell nuclei	Binucleate	Binucleate

Table 2. Comparison of the characteristics of Yogyakarta isolates and *Cerathoriza* isolates according to Currah and Zelmer (1992)

Characteristics	**Yogyakarta Isolate**	***Cerathoriza* Isolate**
Colony color	White to yellow	Cream, yellow, or brown
The appearance of colonies	Like cotton	Like cotton
The form of branching hyphae	90 °	90°
Air hyphae diameter	<3 µm	<4 µm
Number of cell nuclei per cell	Binucleate	Binucleate
Clamp connection	-	-
Surface of the colony	Like cotton	*Flat and waxy*
Cell mMonilioid formcells	*Elongate barrel shaped*	-
Length of cell mMonilioid cell lengths	12,22-14,56	-
Cell mMonilioid cell width	2,39-2,91	-

Based on observations of hyphae and cells using safranin O-KOH dye **(**Figure 2) it was found that Yogyakarta isolates were endophytic fungi of *Ceratorhiza* with specific characteristics having branching types that form elbows or 90°o angles, the color of vegetative hyphae is hyaline, with diameters of 6.20-6.72 µm, fungal colonies form cell monilioids with elongate barrel shape and monilioid size (12,22-14,56) µm x (2.39-2.91) µm. The number of nuclei in binucleic cells, according to the characteristics reported by Shan et al. (2002) and Currah and Zelmer (1992).

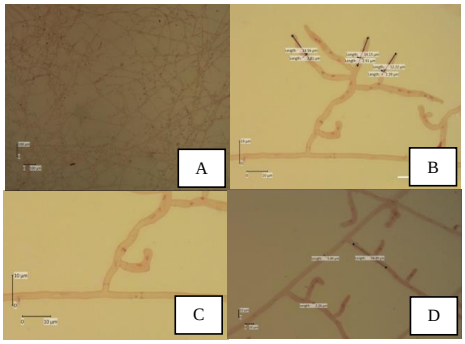


Figure 2. Microscopic observation of hyphae cells and moniliod cells *Ceratorhiza* from Yogyakarta isolates on the 7-day PDA medium using safranin O-KOH staining; (A) Colony, (B) Monilioid cell nucleus, (C) Binucleate, (D) Branching of hyphae 90º. Magnification (A) 10 x, (B, C, D) 40 x. Bar = 10 µm

3.4 Observation of Endophytic Mycorrhizal Hyphae (Peloton)

Observation of the cross section of the root sample found indicated the presence of a pelotons in the root cortex (Figure 3). Currah & Zelmer (1992) explain that the characteristic of endophytic fungal hyphae infecting colonizing plants will form a mass of dense hyphae in cortical cells called pelotons.

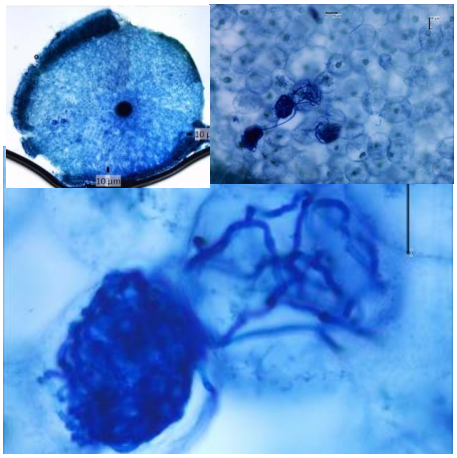


Figure 3. Peloton structure in the anatomy of the root cortex cells of MP1 ~~*P. amabilis* (L.) Blume~~ with Tryphan Blue staining. Magnification (A) 4 x, (B) 10 x, (C) 40 x. Bar = 10 µm

Formatted: Highlight

4. Discussion

Characteristics of endophytic mycorrhizal analysis were obtained and compared with the reference characteristics (Shan et al., 2002; Currah and Zelmer, 1992), it was found that Yogyakarta isolates were *Ceratorhiza* isolates. *Ceratorhiza* is an anamorphic phase of *Ceratobasidium*. According to Athipunyakom et al., (2004), the genus *Ceratorhiza* is a mycorrhizal isolate which is commonly found associated with orchids, where endophytic fungi of this type are *Rhizoctonia* which are grouped according to cell hyphae and moniloid. Growth of *Ceratorhiza* reached a diameter of 9 cm after 4 days ~~after~~ of incubation by forming a symmetrical zone. The ~~color of the~~ colony ~~will~~ turned yellowish after 7 days ~~with~~ and the colony surface ~~of the hyphae~~appeared as ~~clumping like~~ cotton~~y~~ clumps. The lumps ~~y hyphae~~ are aerial hyphae, this is known from the hyphae that grow on the surface of the agar media. These hyphae are fertile hyphae which play a role for reproduction. The length of moniloid cells is 12.22-14.56 µm, width 2.39-2.91µm and lancet-shaped. If the ratio of length and width is 3.5-4: 1 then it is called the lancet type. The number of nuclei in binucleic cells, according to the characteristics reported by Shan et al. (2002) and Currah and Zelmer (1992).

The peloton structure is evident in the roots of *P. amabilis* ~~(L.) Blume~~ which are associated with endophytic fungi (Figure 3). One of the parameters for the mechanism of resistance induction can be the formation of a peloton structure which shows that endophytic fungi penetrate the epidermal finger and into the cortex. In the cortex, the pulp will enter the cell space (intracellular) and form a peloton.

The presence of peloton proves that the endophytic fungi can penetrate into the root tissue of *P. amabilis* ~~(L.) Blume~~. According to Currah and Zelmer (1992), the stage of infection with endophytic fungi begins with the formation of appressorium which is an ~~thickening~~ inflated ~~of~~ hyphae. These hyphae originate from spores that germinate or externally hyphae at the root surface of infected host plants. Hyphae will then penetrate the root surface mechanically and enzymatically into the space between the root epidermal cells and into the cortex and beyond. Hyphae develop without destroying the cells of the root cortex. In cortex cells, hyphae form a peloton in the form of dense hyphae. In the platoon, the accumulation of organic materials includes protein, glycogen, fat, and nutrients produced by absorption from the soil.

5. Conclusion

The results showed that the characterization of endophytic mycorrhizae from the roots of *P. amabilis* ~~(L.) Blume~~ from Yogyakarta, Indonesia had similarities with the type of endophytic mycorrhizal *Ceratorhiza*. These character equations include colony surface color, colony appearance, hyphae color, hyphae diameter (µm), cell monilioid shape, monilioid cell size (µm), colony growth rate (mm / hour), core number, hyphae branching shape, and clamp connection. The results of mycorrhizal induction also produce a olpeloton structure in orchid root cortex cells. The peloton structure is evident in the roots of *P. amabilis* ~~(L.) Blume~~ which are associated with endophytic fungi. The presence of peloton proves that endophytic fungi can penetrate into the root tissue.

References

Chand K, Shah S, Sharma J, Paudel RM and Pant B. 2020. Isolation, characterization, and plant growth-promoting activities of endophytic fungi from a wild orchid *Vanda cristata*. *Plant Signaling & Behavior*, **15(5):** 1744294.

Currah RS and Zelmer C. 1992. A Key and Notes for the Genera of Fungi Mycorrhizal with Orchids and A New Species in the Genus Epulorhiza. *Rept. Tottori Mycology Institution*, **30:** 43-59.

Haro R and Benito B. 2019. The Role of Soil Fungi in K^+ Plant Nutrition. *International journal of molecular sciences*, **20(13):** 3169.

Mahfut. 2020. Identification and Detection Odontoglossum ringspot virus on Native Orchids Collection of Nurserys in Java, Indonesia. Proceeding of International Conference on Advanced Information Scientific Development (ICAISD). West Java, Indonesia. **1641:** 1-8.

Mahfut, Anggreiny A, Wahyuningsih S, Handayani TT and Sukimin. 2020[b]. Identification of Disease and Efforts to Protect Native Orchid Plants Against Bacteria Infection in Liwa Botanical Garden. Proceeding of International Conference on Advanced Information Scientific Development (ICAISD). West Java, Indonesia. **1641:** 1-7.

Mahfut, Daryono BS, Indrianto A and Somowiyarjo S. 2019. Effectiveness Test of Orchid Mycorrhizal Isolate (*Ceratorhiza* and *Trichoderma*) Indonesia and Its Role as a Biofertilizer. *Annual Research & Review in Biology*, **33(4):** 1-7.

Mahfut, Indrianto A, Somowiyarjo S and Daryono BS. 2020[a]. Molecular phylogeny of orchids mycorrhiza isolated from native tropical orchids in Indonesia. *Malaysian Journal of Microbiology*, **16(1):** 68-72.

Mahfut, Shafira H, Wahyuningsih S, Handayani TT and Sukimin. 2021. Identification of Virus Infection on Native Orchid in Liwa Botanical Garden. Proceeding of International Conference on Applied Science, Mathematics, and Informatics (ICASMI). Lampung, Indonesia. **1751:** 1-7.

Mose W, Daryono BS, Indrianto A, Purwantoro A and Semiarti E. 2020. Direct Somatic Embryogenesis and Regeneration of an Indonesian orchid Phalaenopsis amabilis (L.) Blume under a Variety of Plant Growth Regulators, Light Regime, and Organic Substances. *Jordan Journal of Biological Sciences.* **13(4):** 509-518.

Pannecoucque J and Hofie M. 2009. Interaction Between Cauliflower and *Rhizoctonia* Anastomosis Groups with Different Levels of Aggreeiveness. *BMC Plant Biology*, **9(95):** 1-12.

Safarini M, Rosyidah IH, Mertaniasih NM, Hamzah MNSB, Novianti KA, Primaharinastiti R, Rahmawaty D and Isnaeni I. 2020. In vitro Antibacterial Activity of Cell Free Fermentation Supernatant of *Passiflora edulis* forma flavicarpa Sims. Fruit Fermented by de Man, Rogosa and Sharp Media. *Jordan Journal of Biological Sciences.* **13(4):** 469-475.

Shan XC, Liew ECY, Weatherhead MA and Hodgkiss IJ. 2002. Characterization and Taxonomic Placement of *Rhizoctonia*-like Endophytes from Orchids Roots. *Mycologia*, **94(2):** 230-239.

Song Z, Bi Y, Zhang J, Gong Y and Yang H. 2020. Arbuscular mycorrhizal fungi promote the growth of plants in the mining associated clay. *Scientific Reports*, **10:** 2663.

Stoian V, Vidican R, Crisan I, Puia C, Sandor M, Stoian VA, Pacurar F and Vaida I. 2019. Sensitive approach and future perspectives in microscopic patterns of mycorrhizal roots. *Scientific Reports*, **9:** 10233.

Formatted: Font: Italic

Formatted: Font: Italic

Formatted: Font: Italic

6.

Review 2

Jordan Journal of Biological Sciences (JJBS)
ISSN 1995- 6673 (Print), 2307- 7166 (Online)
http://jjbs.hu.edu.jo

Scientific Research and Innovation Support Fund **Hashemite University**
Jordan Journal of Biological Sciences **Deanship of Scientific Research**

Manuscript Evaluation Report- Referee 2

Manuscript ID: JJBS 58/21/a8 **Due date: April 11, 2021**

MS Title: Morphological Identification of Orchid Mycorrhiza Fungi From Native Orchids in Indonesia

Type of Article: ☐ Review Article x **Research Paper** ☐ Case Report

PART A:

On a scale of 1 – 5 (1 being lowest and 5 being highest), rate the manuscript based on the following criteria;

NO.	Criteria	Score
1	**Is the topic of the manuscript within the scope of the journal?**	5
2	**Does the title clearly and sufficiently reflect its content?**	4
3	**Are the keywords and abstract sufficient and informative?**	4
4	**What is the scholarly quality of the manuscript?**	3
5	**Is this a new and/ or original contribution?**	3
6	**Is the research methodology utilized appropriate and properly administered?**	4
7	**Are the methods of data analysis acceptable?**	3
8	**Are the results and conclusions clear, adequately presented and organized in relation to rest of manuscript?**	3
9	**Are the illustrations and tables necessary and in acceptable format?**	4
10	**Are the interpretations/ conclusions sound and justified by the data?**	3
11	**Are the References in a proper format according to JJBS author Instruction?**	2
12	**Is the MS written in correct and satisfactory English?**	4

Please rate the priority for publication of this article (10 is the highest priority, 1 is the lowest priority)

7

PART B: Comments per Section of Manuscript:

Abstract	Title should be Morphological identification of orchid **mycorrhizal** fungi from native orchids in Indonesia.
Introduction	Good
Methodology	Good
Results	Results section should be improved. Fungal morphological characteristics were compared with only 2 references.
Discussion and Conclusion	Good
References	In some references cited journals were not abbreviated. The initials of the some authors names were not indicated.

PART C: Recommendation (Kindly Mark With An ✓)

Acceptable in its Present Form	
Acceptable with Minor Revision	X
Reconsidered after Major Revision	
Reject on Ground of (Please be Specific)	

PART D: Additional Comments:

Please add any other additional comments or specific suggestions on the enclosed comments sheet:

The results obtained from morphological studies should confirm from molecular (ITS-rDNA) analysis.

AUTHORS COMMENTS ON REVIEW RESULTS

Article title: **Morphological Identification of Orchid Mycorrhiza Fungi From Native Orchids in Indonesia**

Dear Reviewers,
Many thanks to make a valuable comment, improve and give us constructive inputs. I realized that this article (and experiment) have some weakness. However, the study on the isolation and characterization of mycorrhizal fungi associated with an epiphytic moth orchid *Phalaenopsis amabilis* is now a new emerging issue. Kindly find our comments for your review results. Waiting for your next responses, again we thank you.

	First reviewers **The corrections in text for 1st Reviewer are written in green**		
No	**Line No**	**Review results**	**Author comments**
1	2	Only one was examined in the present study	Its have been corrected
2	12	Not clear. Are you mentioning the association of this orchid with mycorrhizal fungi??	Yes. I have corrected it
3	14	What is the need for morphological characterization after a more relevant molecular characterization	Yes. . This study aims to identify these isolates based on morphological analysis.
4	16	Was there any fruiting bodies formed in culture?	The results of the analysis showed that the characterization of Yogyakarta isolates had similarities with *Ceratobasidium*
5	19	Not clear.	This research is one of the efforts to preserve native orchids in Indonesia using myccorhiza fungi as a biocontrol agent.
6	20	How was this determined?? The genus *Ceratobasidium* also contains plant pathogens.	With morphological characterization can determined it. Mycorrhizal Fungi have binuclei or multinuclei,
7	33	Rewrite for clarity.	In plant resistance, mycorrhiza fungi serve as a biological agent of control in plant protection against pathogenic infections (Safarini et al., 2020; Song et al., 2020).
8	45	It is not necessary to cite the authorities at every mention.	Healthy root of *P. amabilis* (L.) Blume collected from Yogyakarta, Indonesia on coordinates 8º 30' - 7º 20' LS 109º 40' - 111º 0' BT.
9	45	Provide the coordinates for the sampling site.	Healthy root of *P. amabilis* (L.) Blume collected from Yogyakarta, Indonesia on coordinates 8º 30' - 7º 20' LS 109º 40' - 111º 0' BT.
10	48	Specify the room temperature.	The fungal colonies obtained from the isolation were then purified by sub-culturing aseptically and incubating for 7 days at room temperature (20-25°C)
11	49	How was this determined?	It rewrite: It is possible that the isolates obtained are pure and single isolates that are not contaminated by other fungi.

12	55	Specify the characters.	It rewrite: ….the surface color of young colonies, the appearance of colonies, and the growth rate of the colony
13	60	What is this? Explain.	It rewrite: ….bulk in hyphae
14	83	Figures should be self-explanatory. Do not abbreviate the genus name	It rewrite: ….MP1
15	91	This should be in the methods.	I was delete this and move to the methods
16	101	Change as 6.20-6.72	It rewrite: ….6.20-6.72
17	101	??	It rewrite: ….or
18	101	Use symbol	It rewrite: ….×
19	110	Why do you include a discussion when there is a separate section for this?	I was delete this and move to the discuss

	Second Reviewer **The corrections for 2nd Reviewer are in blue**		
No	**Line no**	**Reviewe results**	**Author comments**
1	147	Formatted: Highlight	Corrected
2	200	Formatted: Font Italic	Corrected
3	201	Formatted: Font Italic	Corrected

7.

Review 3

Morphological Identification of Orchid Mycorrhizal Fungi From Native Orchid in Indonesia

Mahfut

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Lampung, Indonesia

Corresponding author e-mail: mahfut.mipa@fmipa.unila.ac.id

Abstract

Moth orchid [*Phalaenopsis amabilis* (L.) Blume] is a species native orchid from Indonesia. Thise orchid associates ~~tion of this orchid~~ with mycorrhizal fungi in nature which also act as biocontrol agent. In a previous study, one *Ceratobasidium* isolate was isolated from roots of *P. amabilis* growing in Yogyakarta, Indonesia and was successfully identified based on rDNA-ITS molecular analysis. This study aimed to identify the fungal~~se~~ isolates associated with *P. amabilis* based on morphological analysis to complement other identification data, namely anatomy and molecular. Verification of morphological analysis ~~is~~ was carried out by ~~observing macroscopic and~~ microscopic characterization~~stics~~, as well as observations of ~~ing~~ pelotons in roots. The results of the morphological analysis showed that the characterization of Yogyakarta isolates had similarities with *Ceratobasidium*. These characters ~~equations~~ observed included colony color, hyphal cell length, ~~core~~ nucleus number, and cell width. The examination of *P. amabilis*~~orchid~~ roots also showed the presence of pelotons in the cortical cells. This study confirms that the fungal isolates of orchid mycorrhiza in roots of *P. amabilis* from Yogyakarta, Indonesia were *Ceratobasidium* based on morphological and molecular analysis. This research is one of the efforts to preserve native orchids in Indonesia using myccorhizal fungi as a biocontrol agent. This study is the first to report regarding *Ceratobasidium* isolated from native orchids in Indonesia based on morphological analysis.

Keywords: *Ceratobasidium*; mycorrhiza; morphological analysis; *Phalaenopsis*; Indonesia

Comment [R1]: Avoid using words that are already in the title as key words.

1. Introduction

Orchids ~~are is a type of~~ ornamental plants that has a high aesthetic value and is in ~~most in~~ demand ~~by the community~~worldwide (Mose et al., 2020). The moth orchid [*Phalaenopsis amabilis* (L.) Blume] is one of Indonesia's national flowers, namely the charm that was set through Presidential Indonesia Decree Number 4/1993. Diseases are still the main obstacle in the cultivation and development of natural orchids in Indonesia (Mahfut et al., 2019; Mahfut, 2020; Mahfut et al., 2020[b]; Mahfut et al., 2021).

Endophytic mycorrhiza is a form of symbiosis between fungi and plant roots during a certain period of their life cycle, forming colonies in plant tissues without endangering their hosts. In general, mycorrhizal fungi help germination of orchid seeds in the presence of ethylene and various vitamins under in vitro conditions and essential for seedling development in nature. Another role is to ~~support efforts~~ to provide nutrition for plant growth and development (Haro and Benito, 2019), and to assist in the formation of more buds and flowers. In plant resistance, mycorrhiza fungi serve as a biological agent ~~of~~ in improving ~~control in~~ plant's resistance ~~protection~~ against pathogenic infections (Safarini et al., 2020; Song et al., 2020).

In a previous study, Mahfut et al. (2020) reported one *Ceratobasidium* isolate from Yogyakarta, Indonesia based on rDNA-ITS molecular analysis. The results of that study were based on ~~show~~ sequences measuring 600-750 bp DNA products located on the ITS1-5.8S-ITS4 region. Reconstruction of phylogenetic trees resulted in Indonesian isolates having undergone speciation and ~~separated~~ were distinct from C*eratobasidium* isolates from other countries. This research is a follow-up study which aims to clarify the identification of the~~se~~ isolates as *Ceratobasidium* associated with roots of *P. amabilis* based on morphological analysis. Furthermore, this research can be used as a benchmark in developing strategy for conserving moth orchid through protection against plant diseases.

2. Materials and Methods

2.1. ~~Collection of Healthy~~Sampling of Orchid Roots ~~Samples~~ and Endophytic Mycorrhizal Fungal Isolation

Healthy root of *P. amabilis* were collected from Yogyakarta, Indonesia ~~on coordinates~~(8º 30' - 7º 20' LS 109º 40' - 111º 0' BT). The sample was ~~taken~~ transfered to the Biotechnology Laboratory of the Faculty of Biology UGM to isolate endophytic mycorrhizal fungi following the protocol of Chand et al. (2020)~~ method~~. Isolation of endophytic fungi was then carried out by ~~cutting~~ sectioning the roots and culturing then on potato dextrose agar (PDA) medium at xxx°C for xxx days. The fungal colonies obtained ~~from the isolation~~ were then purified by sub-culturing aseptically and incubating for~~ 7~~ seven days at room temperature (20-25℃). It ~~is~~ was thus possible ~~that the isolates obtained are~~to obtain pure and single isolates that are not contaminated by other fungi.

Comment [R2]: Provide these details.

2.2. Macroscopic Characteristic Observation

To observe the macroscopic characteristics of the isolated fungi, small portion of ~~isolates~~ actively growing fungal colonies (± 1 mm^2) were placed in the middle of the PDA medium. Furthermore, the growth of the isolates were observed and measured every day for 7 days. Furthermore, observations of colony morphological characteristics such as colony color, colony base color, the surface color of young colonies, the appearance of colonies, and the growth rate of the colony were carried out.

2.3. Making Slide Culture Preparations

Observation of microscopic characteristics was also carried out using the slide culture method (Stoian et al. 2019) with a slight modification. This method was carried out to monitor microscopic characteristics such as the color of hyphae, bulk in hyphae, angle of branching of hyphae, and number of cell nuclei. The number of nuclei was observed after treating with safranin and 3% KOH.

2.4 Observation of Pelotons in roots

Observation of peloton was carried out by making squash preparations on root samples which had been treated with previous endophytic fungal inoculations. Root samples are sliced and soaked in trypan blue dye for 15 minutes. Observations were carried out using a light microscope at 100 x magnification.

Comment [R3]: What is this??

Comment [R4]: Safranin is not a nuclear stain.

Comment [R5]: Provide the method that was used to prepare root quashes.

Comment [R6]: Please be clear. Were the observations made on root squashes or sections??

3. Results

3.1. Collection of Healthy Orchid Root Samples and Endophytic Mycorrhizal Isolation

One endophytic fungus, namely Yogyakarta was isolated from root samples of *P. amabilis* in Yogyakarta, Indonesia ~~in~~ (Figure 1).

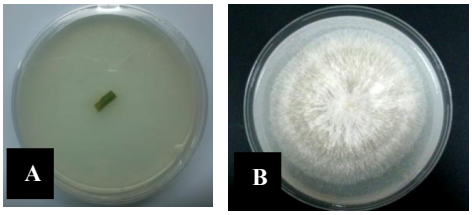


Figure 1. A) Root samples of MP1 cultured on PDA medium, B) Colonies of fungi that grow on 7 days old orchid roots

Comment [R7]: Insert scale bars in the images.

Identification results based on macroscopic, microscopic, and molecular characteristics showed that the isolate was *Ceratorhiza* which is one of the types of *Rhizoctonia* endophytic mycorrhiza. According to Chand et al. (2020), *Ceratorhiza* is *Rhizoctonia* grouped by cell hyphae and moniloid, and many are found to be associated with orchid roots.

3.2. Macroscopic Characteristics

Special characteristics of Yogyakarta isolate was identified as having branching types that formed elbows or 90º angles and hyphale cells ~~with a cell nucleus 2 (~~that were binucleate~~)~~. These two characteristics are special characteristics of endophytic mycorrhiza. Endophytic fungi have 1-3 nuclei ~~(binucleate)~~ nuclei, whereas pathogenic fungi have more than ~~3~~ three nuclei (multinucleate~~s~~). This is in accordance ~~with the opinion of~~ Pannecoucque & Hofie (2009) who suggested that in addition to binucleate conditions, the other characteristics ~~of~~ an endophytic fungi~~us~~ are the hyphal branching ~~which forms a~~ at 90ºC or the occurrence of T-shaped branches.

Overall, the results of identification of Yogyakarta orchid mycorrhizal fungal isolates showed similarities with the types of *Ceratorhiza* ~~mycorrhiza~~fungi. ~~The following characteristics of~~A comparison of Yogyakarta mycorrhizal fungal isolates compared to other isolated reported in literature ~~supporting references~~ are presented in Tables 1 and 2.

Table 1. Comparison of the characteristics of Yogyakarta *Cerathoriza* isolate ~~isolates~~ from roots of *Phalaenopsis amabilis* and *Cerathoriza* isolates according to Shan et al. (2002)

Characteristics	Yogyakarta Isolate	*Cerathoriza* Isolate
The surface color of young colonies	White to yellow	Yellow to white
The appearance of colonies	Like cotton	Like cotton
Hyphae color	Hyaline	Hyaline
Hyphae diameter (µm)	6.20-6.72	3.8-7.5
Monilioid cell form	Elongated, barrel shaped	Ellipsoidal or elongate barrel shaped
Monilioid cell size (µm)	(12.22-14.56) × (2.39-2.91)	(7.5-15.0) × (10.0-25.0)
The growth rate of the colony (mm / hr)	0.72	0.42-0.52
Number of cell nuclei	Binucleate	Binucleate

Comment [R8]: Combine Tables 1 and 2 into a single table.

Table 2. Comparison of the characteristics of Yogyakarta *Cerathoriza* isolate from roots of *Phalaenopsis amabilis* ~~isolate~~ and *Cerathoriza* isolates ~~according to~~reported by Currah and Zelmer (1992)

Characteristics	Yogyakarta Isolate	*Cerathoriza* Isolate
Colony color	White to yellow	Cream, yellow, or brown
~~The appearance of colonies~~Colony appearance	Like cotton	Like cotton
~~The form of branching hyphae~~Hyphal branching	90 °	90°
~~Air~~ Aerial hypha~~l~~e diameter	<3 µm	<4 µm
Number of nuclei per cell	Binucleate	Binucleate
Clamp connection	-	-
~~Surface of the colony~~Colony surface	~~Like cotton~~Cottony	Flat and waxy
Monilioid cells	Elongate and barrel shaped	-
Monilioid cell length	12.22-14.56	-
Monilioid cell width	2.39-2.91	-

Based on observations of the hyphae and cells using safranin O-KOH dye **(**Figure 2) it was found that Yogyakarta isolates were endophytic fungi of *Ceratorhiza* with specific characteristics having branching types that form elbows or 90° angles, the color of vegetative hyphae is hyaline, with diameters of 6.20-6.72 µm, fungal colonies form ~~cell~~ monilioids cell ~~with~~ that are elongate and barrel shape and ~~monilioid size~~measured ~~(~~12,22-14,56 ~~)~~ µm x ~~(~~2.39-2.91~~)~~ µm (length × width).

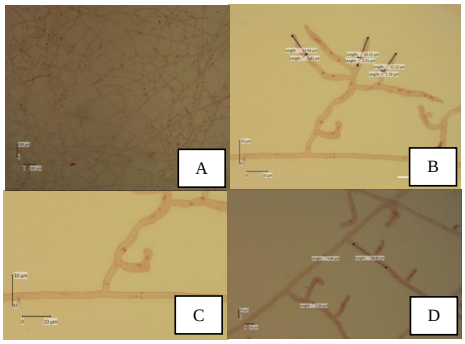
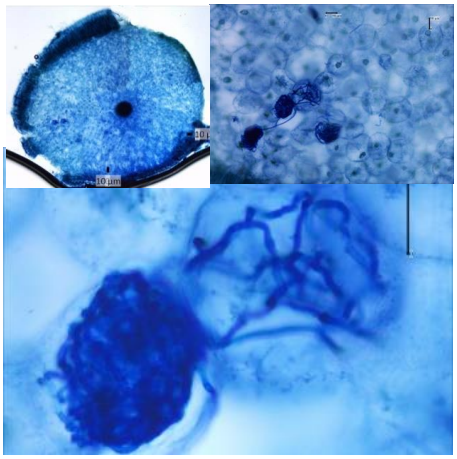


Figure 2. Microscopic observation of hypha~~l~~e ~~cells~~ and moniliod cells of *Ceratorhiza* isolated from roots of *Phalaenopsis amabilis* growing in Yogyakarta ~~isolates~~ on the 7th -day in potato dextrose agar ~~PDA~~ medium and stained with ~~using~~ safranin O-KOH staining; (A) Colony, (B) Monilioid cell nucleus, (C) Binucleate, (D) Branching of hyphae 90°. Magnification (A) 10 x, (B, C, D) 40 x. Bar = 10 µm

3.4 Observation of Endophytic Mycorrhizal ~~Hyphae~~ Structures in Roots ~~(Peloton)~~

Observation of the cross section of the root sample indicated the presence of pelotons in the root cortex (Figure 3). Currah & Zelmer (1992) explain that the characteristic of endophytic fungal hyphae colonizing plants will form a mass of dense hyphae in cortical cells called pelotons.



Formatted: Font: Not Italic

Formatted: Font: Not Italic

Formatted: Font: Not Italic

Formatted: Font: Not Italic

Comment [R9]: This is not a fungal stain. Fungi are generally stained with trypan blue, acid fuchsine, etc.

Comment [R10]: This is not a fungal stain.

Formatted: Superscript

Comment [R11]: This has been mentioned as squash in the methods.

Figure 3. Peloton structure in the anatomy of the root cortex cells of MP1 with Tryphan Blue staining. Magnification (A) 4 x, (B) 10 x, (C) 40 x. Bar = 10 µm

Comment [R12]: What is this?? Explain??

4. Discussion

Morphological Ccharacteristics of endophytic mycorrhizal fungi isolated from roots of *P. amabilis* analysis were obtained observed and compared with the characteristics of mycorrhizal fungi reported in the literature reference characteristics (Shan et al., 2002; Currah and Zelmer, 1992). This indicated, it was found that the Yogyakarta fungal isolates were obtained from the roots of *P. amabilis* belonged to *Ceratorhiza* isolates. *Ceratorhiza* is an anamorphic phase of *Ceratobasidium*. According to Athipunyakom et al., (2004), the genus *Ceratorhiza* is a mycorrhizal isolate which is commonly found associatesd with the roots of orchids. Previously these , where endophytic fungi of this type arewere termed *Rhizoctonia* which areand were grouped according to cell the hyphale and moniloid cell characteristics. Growth of *Ceratorhiza* reached a diameter of 9 cm after 4 four days of incubation by forming a symmetrical zone. The colony turned yellowish after 7 days and the colony surface appeared as cottony clumps. The clumps are of aerial hyphae , this is known from the hyphae that grow on the surface of the agar media. These hyphae are fertile hyphae which that play a role for in reproduction. The average length of moniloid cells is was 12.22-14.56 µm, and the width of the cells was 2.39-2.91µm and lancet-shaped. If the ratio of length and width is 3.5-4: 1 then it is called the lancet type. The cells were number of nuclei in binucleic cells, according to the characteristics similar to those reported by Shan et al. (2002) and Currah and Zelmer (1992).

Comment [R13]: Was conidia or other structures found in culture?

The presence of peloton structure is was evident in the roots of *P. amabilis* which arethat were associated with endophytic fungi (Figure 3). One of the parameters for the mechanism of resistance induction by the fungus in the orchid host can be the formation of a peloton structure which shows that the endophytic fungi penetrated the epidermal finger velamen and entered into the cortex. In the cortex, the pulp hyphae will transverse the intercellular spaces enters the cell space (intracellular) and forming a peloton.

The presence of peloton proves that the endophytic fungi can penetrate intocolonize the root tissue of *P. amabilis*. According to Currah and Zelmer (1992), the stage of infection colonization with endophytic fungi begins with the formation of appressorium which is an inflated hyphae on the root surface. The infecting hyphae could be the germinal se hyphae originatinge from the germinating spores that germinate or an externally hyphae at the root surface of originating from an infected host plants. Hyphae will then penetrate the root surface mechanically and enzymatically into the space between the root epidermal cells and enter into the cortex and beyond. The Hyphae development of the fungal structures happens without destroying the cells of the root cortex. In cortex cells, hyphae form a peloton in the form of dense hyphae. In tThe peloton latoon, the accumulateion of organic materials includes like proteins, glycogen, and fat, and synthesized from the nutrients produced by absorption from the soilthat is absorbed and translocated by the mycorrhizal fungi.

Comment [R14]: Stele is not colonizsed by mycorrhizal fungi.

5. Conclusion

The results showed that the characterization of endophytic mycorrhizae from the roots of *P. amabilis* from Yogyakarta, Indonesia had similarities with the type of endophytic mycorrhizal fungi *Ceratorhiza*. These character equations similarity include colony surface color, colony appearance, hyphae color, hyphae diameter (µm), cell monilioid shape, monilioid cell size (µm), colony growth rate (mm / hour), core number, hyphae branching shape, and clamp connection. The results of mycorrhizal induction also produce a peloton structure in orchidin the root corticalex cells. The peloton structure is evident in the roots of *P. amabilis* which are associated with endophytic fungi. The presence of peloton proves that endophytic fungi can penetrate into colonize the root tissue.

References

Chand K, Shah S, Sharma J, Paudel RM and Pant B. 2020. Isolation, characterization, and plant growth-promoting activities of endophytic fungi from a wild orchid *Vanda cristata. Plant Signaling & Behavior,* **15(5):** 1744294.

Currah RS and Zelmer C. 1992. A Key and Notes for the Genera of Fungi Mycorrhizal with Orchids and A New Species in the Genus Epulorhiza. *Rept. Tottori Mycology Institution,* **30:** 43-59.

Haro R and Benito B. 2019. The Role of Soil Fungi in K^+ Plant Nutrition. *International journal of molecular sciences*, **20(13):**

3169.

Mahfut. 2020. Identification and Detection Odontoglossum ringspot virus on Native Orchids Collection of Nurserys in Java, Indonesia. Proceeding of International Conference on Advanced Information Scientific Development (ICAISD). West Java, Indonesia. **1641:** 1-8.

Mahfut, Anggreiny A, Wahyuningsih S, Handayani TT and Sukimin. 2020[b]. Identification of Disease and Efforts to Protect Native Orchid Plants Against Bacteria Infection in Liwa Botanical Garden. Proceeding of International Conference on Advanced Information Scientific Development (ICAISD). West Java, Indonesia. **1641:** 1-7.

Mahfut, Daryono BS, Indrianto A and Somowiyarjo S. 2019. Effectiveness Test of Orchid Mycorrhizal Isolate (*Ceratorhiza* and *Trichoderma*) Indonesia and Its Role as a Biofertilizer. *Annual Research & Review in Biology*, **33(4):** 1-7.

Mahfut, Indrianto A, Somowiyarjo S and Daryono BS. 2020. Molecular phylogeny of orchids mycorrhiza isolated from native tropical orchids in Indonesia. *Malaysian Journal of Microbiology*, **16(1):** 68-72.

Mahfut, Shafira H, Wahyuningsih S, Handayani TT and Sukimin. 2021. Identification of Virus Infection on Native Orchid in Liwa Botanical Garden. Proceeding of International Conference on Applied Science, Mathematics, and Informatics (ICASMI). Lampung, Indonesia. **1751:** 1-7.

Mose W, Daryono BS, Indrianto A, Purwantoro A and Semiarti E. 2020. Direct Somatic Embryogenesis and Regeneration of an Indonesian orchid Phalaenopsis amabilis (L.) Blume under a Variety of Plant Growth Regulators, Light Regime, and Organic Substances. *Jordan Journal of Biological Sciences.* **13(4):** 509-518.

Pannecoucque J and Hofie M. 2009. Interaction Between Cauliflower and *Rhizoctonia* Anastomosis Groups with Different Levels of Aggreeiveness. *BMC Plant Biology*, **9(95):** 1-12.

Safarini M, Rosyidah IH, Mertaniasih NM, Hamzah MNSB, Novianti KA, Primaharinastiti R, Rahmawaty D and Isnaeni I. 2020. In vitro Antibacterial Activity of Cell Free Fermentation Supernatant of *Passiflora edulis* forma flavicarpa Sims. Fruit Fermented by de Man, Rogosa and Sharp Media. *Jordan Journal of Biological Sciences.* **13(4):** 469-475.

Shan XC, Liew ECY, Weatherhead MA and Hodgkiss IJ. 2002. Characterization and Taxonomic Placement of *Rhizoctonia*-like Endophytes from Orchids Roots. *Mycologia*, **94(2):** 230-239.

Song Z, Bi Y, Zhang J, Gong Y and Yang H. 2020. Arbuscular mycorrhizal fungi promote the growth of plants in the mining associated clay. *Scientific Reports*, **10:** 2663.

Stoian V, Vidican R, Crisan I, Puia C, Sandor M, Stoian VA, Pacurar F and Vaida I. 2019. Sensitive approach and future perspectives in microscopic patterns of mycorrhizal roots. *Scientific Reports*, **9:** 10233.

8.

Editing & Proofreading

Manuscript Title: **Morphological Identification of Orchid Mycorrhizal Fungi from Native Orchid in Indonesia**

Thank you for submitting your manuscript to Jordan Journal of Biological Sciences (JJBS). Please read the galley proof carefully and retain within 48 hr. **Our Language editor did not pass the parts that are marked in red. Please improve them**

If you have any corrections please mark in RED color. Please make sure that all References (the journal title should be abbreviated; for example we type Jordan J Biol Sci) are according to JJBS format, and they are in the text.

Please after correction use save icon only (**don't use save as icon it will change the document and we cannot get it back)**

Looking forward to your continuous cooperation with JJBS.

JJBS

Volume 14, Supplementary Issue,December 2021
ISSN 1995-6673
Pages xxx – xxx

Jordan Journal of Biological Sciences

Morphological Identification of Orchid Mycorrhizal Fungi from Native Orchid in Indonesia

Mahfut*

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Lampung, Indonesia

NODATE

Abstract

Moth orchid [*Phalaenopsis amabilis* (L.) Blume] is a species native orchid from Indonesia. The association of this orchid with mycorrhizal fungi in nature as biocontrol agent. In a previous study, one *Ceratobasidium* isolate from Yogyakarta, Indonesia was successfully identified based on rDNA-ITS molecular analysis. This study aimed to identify these isolates based on morphological analysis to complement other identification data, namely anatomy and molecular. Verification morphological analysis is carried out by observing macroscopic and microscopic characteristics, as well as observing peloton. The results of the analysis showed that the characterization of Yogyakarta isolates had similarities with *Ceratobasidium*. These character equations include colony color, cell length, core number, and cell width. The examination of orchid roots also showed the presence of pelotons in the cortical cells. This study confirms that the fungal isolates of orchid mycorrhiza from Yogyakarta, Indonesia were *Ceratobasidium* based on morphological and molecular analysis. This research is one of the efforts to preserve native orchids in Indonesia using myccorhiza fungi as a biocontrol agent. This study is the first to report regarding *Ceratobasidium* isolated from native orchids in Indonesia based on morphological analysis.

Keywords: *Ceratobasidium*; mycorrhiza; morphological analysis; *Phalaenopsis*; Indonesia

1. Introduction

Orchid is a type of ornamental plant that has a high aesthetic value and is most in demand by the community (Mose et al., 2020). The moth orchid [*Phalaenopsis amabilis* (L.) Blume] is one of Indonesia's national flowers, namely the charm that was set through Presidential Indonesia Decree Number 4/1993. Diseases are still the main obstacle in the cultivation and development of natural orchids (Mahfut et al., 2019; Mahfut, 2020; Mahfut et al., 2020[b]; Mahfut et al., 2021).

Endophytic mycorrhiza is a form of symbiosis between fungi and plant roots during a certain period of their life cycle, forming colonies in plant tissues without endangering their hosts. In general, mycorrhizal fungi help germination of orchid seeds in the presence of ethylene and various vitamins. Another role is to support efforts to provide nutrition for plant growth and development (Haro and Benito, 2019), and to assist in the formation of more buds and flowers. In plant resistance, mycorrhiza fungi serve as a biological agent of control in plant protection against pathogenic infections (Safarini et al., 2020; Song et al., 2020).

In a previous study, Mahfut et al. (2020) reported one *Ceratobasidium* isolate from Yogyakarta, Indonesia based on rDNA-ITS molecular analysis. The results show sequences measuring 600-750 bp DNA products located on the ITS1-5.8S-ITS4 region. Reconstruction of phylogenetic trees resulted in Indonesian isolates having undergone speciation and separated from C*eratobasidium* isolates from other countries. This research is a follow-up study which aims to clarify the identification of these isolates as *Ceratobasidium* based on morphological analysis. Furthermore, this research can be used as a benchmark in developing strategy for conserving moth orchid through protection against plant diseases.

2. Materials and Methods

2.1. Collection of Healthy Orchid Root Samples and Endophytic Mycorrhizal Isolation

Healthy root of *P. amabilis* collected from Yogyakarta, Indonesia on coordinates 8º 30' - 7º 20' LS 109º 40' - 111º 0' BT. The sample was taken to the Biotechnology Laboratory of the Faculty of Biology UGM to isolate endophytic mycorrhiza following the Chand et al. (2020) method. Isolation of endophytic fungi was then carried out by cutting the roots and culturing on PDA medium. The fungal colonies obtained from the isolation were then purified by sub-culturing aseptically and incubating for 7 days at room temperature (20-25℃). It is possible that the isolates obtained are pure and single isolates that are not contaminated by other fungi.

2.2. Macroscopic Characteristic Observation

To observe the macroscopic characteristics of the isolated fungi, small portion of isolates (± 1 mm^2) were placed in the middle of the PDA medium. Furthermore, the growth of the isolates was observed and measured every day for 7 days. Furthermore, observations of colony morphological characteristics such as colony color, colony

* Corresponding author e-mail: mahfut.mipa@fmipa.unila.ac.id.

base color, the surface color of young colonies, the appearance of colonies, and the growth rate of the colony were carried out.

2.3. Making Slide Culture Preparations

Observation of microscopic characteristics was also carried out using the slide culture method (Stoian et al. 2019) with a slight modification. This method was carried out to monitor microscopic characteristics such as the color of hyphae, bulk in hyphae, angle of branching of hyphae, and number of cell nuclei. The number of nuclei was observed after treating with safranin and 3% KOH.

2.4. Observation of Pelotons in roots

Observation of peloton was carried out by making squash preparations on root samples which had been treated with previous endophytic fungal inoculations. Root samples are sliced and soaked in trypan blue dye for 15 minutes. Observations were carried out using a light microscope at 100 x magnification.

3. Results

3.1. Collection of Healthy Orchid Root Samples and Endophytic Mycorrhizal Isolation

One endophytic fungus, namely Yogyakarta was isolated from root samples of *P. amabilis* in Yogyakarta, Indonesia in Figure 1.

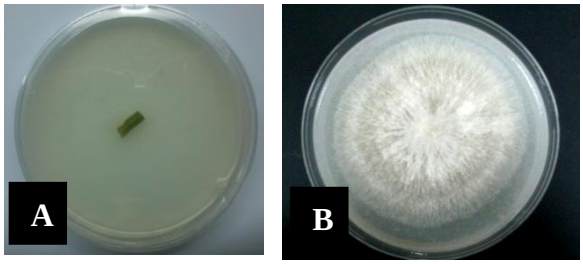


Figure 1. A) Root samples of MP1 cultured on PDA medium, B) Colonies of fungi that grow on 7 days old orchid roots

Identification results based on macroscopic, microscopic, and molecular characteristics showed that the isolate was *Ceratorhiza* which is one of the types of *Rhizoctonia* endophytic mycorrhiza. According to Chand et al. (2020), *Ceratorhiza* is *Rhizoctonia* grouped by cell hyphae and moniloid, and many are found to be associated with orchid roots.

3.2. Macroscopic Characteristics

Special characteristics of Yogyakarta isolate were identified as having branching types that formed elbows or 90º angles and hyphae cells with a cell nucleus 2 (binucleate). These two characteristics are special characteristics of endophytic mycorrhiza. Endophytic fungi have 1-3 nuclei (binucleate) nuclei, whereas pathogenic fungi have more than 3 nuclei (multinucleates). This is in accordance with the opinion of Pannecoucque & Hofie (2009) that in addition to binucleates, the characteristics of endophytic fungi are branching which forms a 90ºC or T-shaped branches.

Overall, the results of identification of Yogyakarta isolates showed similarities with the types of *Ceratorhiza* mycorrhiza. The following characteristics of Yogyakarta isolates compared to supporting references are presented in Tables 1 and 2.

Table 1. Comparison of the characteristics of Yogyakarta isolates and *Cerathoriza* isolates according to Shan et al. (2002)

Characteristics	Yogyakarta Isolate	*Cerathoriza* Isolate
The surface color of young colonies	White to yellow	Yellow to white
The appearance of colonies	Like cotton	Like cotton
Hyphae color	Hyaline	Hyaline
Hyphae diameter (µm)	6.20-6.72	3.8-7.5
Monilioid cell form	Elongated, barrel shaped	Ellipsoidal or elongate barrel shape
Monilioid cell size (µm)	(12.22-14.56) × (2.39-2.91)	(7.5-15.0) × (10.0-25.0)
The growth rate of the colony (mm / hr)	0.72	0.42-0.52
Number of cell nuclei	Binucleate	Binucleate

Table 2. Comparison of the characteristics of Yogyakarta isolate and *Cerathoriza* isolates according to Currah and Zelmer (1992)

Characteristics	Yogyakarta Isolate	*Cerathoriza* Isolate
Colony color	White to yellow	Cream, yellow, or brown
The appearance of colonies	Like cotton	Like cotton
The form of branching hyphae	90 º	90º
Air hyphae diameter	<3 µm	<4 µm
Number of nuclei per cell	Binucleate	Binucleate
Clamp connection	-	-
Surface of the colony	Like cotton	*Flat and waxy*
Monilioid cells	*Elongate barrel shaped*	-
Monilioid cell length	12.22-14.56	-
Monilioid cell width	2.39-2.91	-

Based on observations of hyphae and cells using safranin O-KOH dye (Figure 2), it was found that Yogyakarta isolates were endophytic fungi of *Ceratorhiza* with specific characteristics having branching types that form elbows or 90° angles, the color of vegetative hyphae is hyaline, with diameters of 6.20-6.72 µm, fungal colonies form cell monilioids with elongate barrel shape and monilioid size (12,22-14,56) µm x (2.39-2.91) µm.

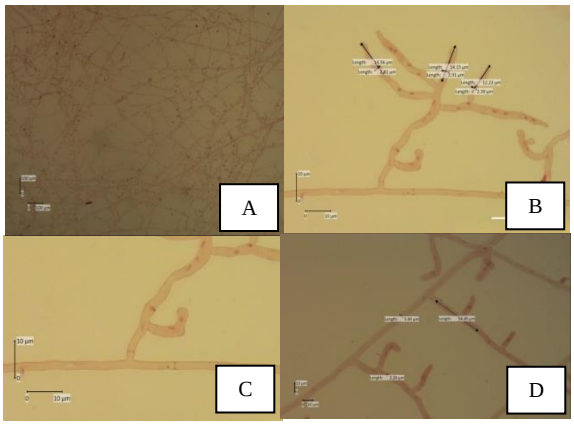


Figure 2. Microscopic observation of hyphae cells and moniliod cells *Ceratorhiza* from Yogyakarta isolates on the 7-day PDA medium using safranin O-KOH staining; (A) Colony, (B) Monilioid cell nucleus, (C) Binucleate, (D) Branching of hyphae 90º. Magnification (A) 10 x, (B, C, D) 40 x. Bar = 10 µm

3.3. *Observation of Endophytic Mycorrhizal Hyphae (Peloton)*

Observation of the cross section of the root sample indicated the presence of pelotons in the root cortex (Figure 3). Currah & Zelmer (1992) explain that the characteristic of endophytic fungal hyphae colonizing plants will form a mass of dense hyphae in cortical cells called pelotons.

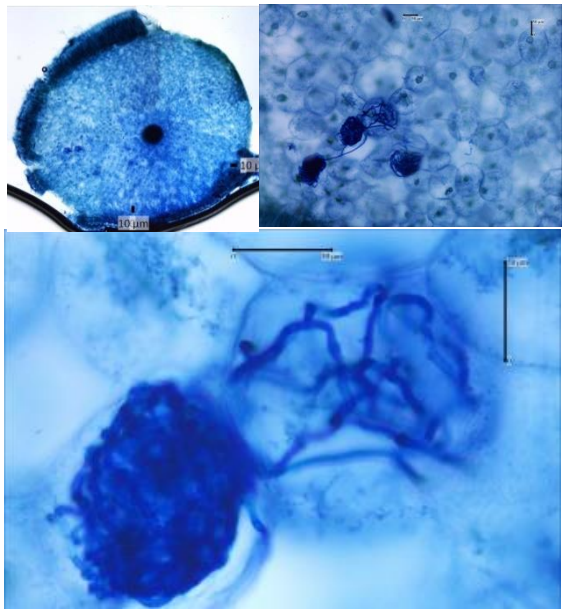


Figure 3. Peloton structure in the anatomy of the root cortex cells of MP1 with Tryphan Blue staining. Magnification (A) 4 x, (B) 10 x, (C) 40 x. Bar = 10 µm

4. Discussion

Characteristics of endophytic mycorrhizal analysis were obtained and compared with the reference characteristics (Shan et al., 2002; Currah and Zelmer, 1992); it was found that Yogyakarta isolates were *Ceratorhiza* isolates. *Ceratorhiza* is an anamorphic phase of *Ceratobasidium*. According to Athipunyakom et al. (2004), the genus *Ceratorhiza* is a mycorrhizal isolate which is commonly found associated with orchids, where endophytic fungi of this type are *Rhizoctonia* which are grouped according to cell hyphae and moniloid. Growth of *Ceratorhiza* reached a diameter of 9 cm after 4 days of incubation by forming a symmetrical zone. The colony turned yellowish after 7 days, and the colony surface appeared as cottony clumps. The lumps are aerial hyphae; this is known from the hyphae that grow on the surface of the agar media. These hyphae are fertile hyphae which play a role for reproduction. The length of moniloid cells is 12.22-14.56 µm, width 2.39-2.91µm and lancet-shaped. If the ratio of length and width is 3.5-4: 1 then it is called the lancet type. The number of nuclei in binucleic cells, according to the characteristics reported by Shan et al. (2002) and Currah and Zelmer (1992).

The peloton structure is evident in the roots of *P. amabilis* which are associated with endophytic fungi (Figure 3). One of the parameters for the mechanism of resistance induction can be the formation of a peloton structure which shows that endophytic fungi penetrate the epidermal finger and into the cortex. In the cortex, the pulp will enter the cell space (intracellular) and form a peloton.

The presence of peloton proves that the endophytic fungi can penetrate into the root tissue of *P. amabilis*. According to Currah and Zelmer (1992), the stage of infection with endophytic fungi begins with the formation of appressorium which is an inflated hyphae. These hyphae originate from spores that germinate or externally hyphae at the root surface of infected host plants. Hyphae will then penetrate the root surface mechanically and enzymatically into the space between the root epidermal cells and into the cortex and beyond. Hyphae develop without destroying the cells of the root cortex. In cortex cells, hyphae form a peloton in the form of dense hyphae. In the platoon, the accumulation of organic materials includes protein, glycogen, fat, and nutrients produced by absorption from the soil.

5. Conclusion

The results showed that the characterization of endophytic mycorrhizae from the roots of *P. amabilis* from Yogyakarta, Indonesia had similarities with the type of endophytic mycorrhizal *Ceratorhiza*. These character equations include colony surface color, colony appearance, hyphae color, hyphae diameter (µm), cell monilioid shape, monilioid cell size (µm), colony growth rate (mm / hour), core number, hyphae branching shape, and clamp connection. The results of mycorrhizal induction also produce a peloton structure in orchid root cortex cells. The peloton structure is evident in the roots of *P. amabilis* which are associated with endophytic fungi. The presence of peloton proves that endophytic fungi can penetrate into the root tissue.

References

Chand K, Shah S, Sharma J, Paudel RM and Pant B. 2020. Isolation, characterization, and plant growth-promoting activities of endophytic fungi from a wild orchid *Vanda cristata*. *Plant Signaling & Behavior*, **15(5):** 1744294.

Currah RS and Zelmer C. 1992. A Key and Notes for the Genera of Fungi Mycorrhizal with Orchids and A New Species in the Genus Epulorhiza. *Rept. Tottori Mycology Institution*, **30:** 43-59.

Haro R and Benito B. 2019. The Role of Soil Fungi in K^+ Plant Nutrition. *International journal of molecular sciences*, **20(13):** 3169.

Mahfut. 2020. Identification and Detection Odontoglossum ringspot virus on Native Orchids Collection of Nurserys in Java, Indonesia. Proceeding of International Conference on Advanced Information Scientific Development (ICAISD). West Java, Indonesia. **1641:** 1-8.

Mahfut, Anggreiny A, Wahyuningsih S, Handayani TT and Sukimin. 2020[b]. Identification of Disease and Efforts to Protect Native Orchid Plants Against Bacteria Infection in Liwa Botanical Garden. Proceedings of ICAISD Conference. University of Bina Sarana Informatika. West Java, Indonesia.

Mahfut, Daryono BS, Indrianto A and Somowiyarjo S. 2019. Effectiveness Test of Orchid Mycorrhizal Isolate (*Ceratorhiza* and *Trichoderma*) Indonesia and Its Role as a Biofertilizer. *Annual Research & Review in Biology*, **33(4):** 1-7.

Mahfut, Indrianto A, Somowiyarjo S and Daryono BS. 2020. Molecular phylogeny of orchids mycorrhiza isolated from native tropical orchids in Indonesia. *Malaysian Journal of Microbiology*, **16(1):** 68-72.

Mahfut, Shafira H, Wahyuningsih S, Handayani TT and Sukimin. 2021. Identification of Virus Infection on Native Orchid in Liwa Botanical Garden. Proceedings of ICASMI Conference. University of Lampung. Lampung, Indonesia.

Mose W, Daryono BS, Indrianto A, Purwantoro A and Semiarti E. 2020. Direct Somatic Embryogenesis and Regeneration of an Indonesian orchid Phalaenopsis amabilis (L.) Blume under a Variety of Plant Growth Regulators, Light Regime, and Organic Substances. *Jordan Journal of Biological Sciences.* **13(4):** 509-518.

Pannecoucque J and Hofie M. 2009. Interaction Between Cauliflower and *Rhizoctonia* Anastomosis Groups with Different Levels of Aggreeiveness. *BMC Plant Biology*, **9(95):** 1-12.

Safarini M, Rosyidah IH, Mertaniasih NM, Hamzah MNSB, Novianti KA, Primaharinastiti R, Rahmawaty D and Isnaeni I. 2020. In vitro Antibacterial Activity of Cell Free Fermentation Supernatant of *Passiflora edulis* forma flavicarpa Sims. Fruit Fermented by de Man, Rogosa and Sharp Media. *Jordan Journal of Biological Sciences.* **13(4):** 469-475.

Shan XC, Liew ECY, Weatherhead MA and Hodgkiss IJ. 2002. Characterization and Taxonomic Placement of *Rhizoctonia*-like Endophytes from Orchids Roots. *Mycologia*, **94(2):** 230-239.

Song Z, Bi Y, Zhang J, Gong Y and Yang H. 2020. Arbuscular mycorrhizal fungi promote the growth of plants in the mining associated clay. *Scientific Reports*, **10:** 2663.

Stoian V, Vidican R, Crisan I, Puia C, Sandor M, Stoian VA, Pacurar F and Vaida I. 2019. Sensitive approach and future perspectives in microscopic patterns of mycorrhizal roots. *Scientific Reports*, **9:** 10233.