Identification of a Potential IAA-Producing Fungus Isolated From *Alpinia* Sp. Rhizome in Hutan Sibayak, North Sumatera

Adrian Hartanto*, Anisa Lutfia, Erman Munir and Yurnaliza

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Indonesia

*erman@usu.ac.id

**Abstract.** Medicinal plant from members of Zingiberaceae have been used as traditional remedies by marginal communities. To date, information and potential of these endophytes, especially endophytic fungi is still limited to certain taxa and properties. The present study investigates one of plant growth promoting properties possessed by endophytic fungi, which is the ability to produce extracellular Indole-3-acetic acid (IAA). Rhizome of *Alpinia* sp. sampled from Hutan Sibayak, North Sumatera was used as source of isolation of endophytic fungi. The endophytic fungal isolates were grown in Czapek-dox broth supplemented with 0.1% (w/v) L-tryptophan for 7 days to induce secretion of extracellular IAA. IAA concentration was measured colorimetrically using Salkowsky’s solution by comparing absorbance (A530) of standard pure IAA with culture filtrates from each isolates. The study obtained 5 endophytic fungal isolates from *Alpinia* sp. rhizome differentiated morphologically. The highest IAA content in culture filtrate (42.91±1.41 μg.mL⁻¹) was produced by isolate Al04SU followed with Al02SU, Al05SU and Al01SU with IAA concentration of 26.25, 19.55 and 17.26 μg.mL⁻¹, respectively. Molecular identification of isolate Al04 based on ITS-DNA similarity showed that isolate was closely related to *Aspergillus flavus* and confirmed through phylogenetic tree construction. The presence of IAA-producing endophytic fungi from *Alpinia* indicates that medicinal plant may harbor bioprospective strains which could be used in crop improvement with further and deeper investigation.

1. Introduction

Stable relationship between microorganisms and plants have been documented in the form of endophytism with mutualistic behaviors [1]. Endophytic microorganisms may be explored from medicinal plants, refering to its ability in producing bioactive metabolites and novel compounds [2]. Endophytes originating from medicinal plants are also known to produce nitrogen-containing compound with prospect as antimicrobial, anticancer, antiviral and plant growth promotion [3]. Plant growth promotion is an example of beneficial impact exhibited by the endophytes to the growth and survival of plants [4]. Plant growth promoting activities by endophytic fungi are explored through synthesis of phytohormones, e.g. Auxin or Indole-3-acetic acid (IAA) which promote the early growth phase of roots in plant seedling [5].

*Alpinia* spp., as a member of medicinal herbs Zingiberaceae, have been studied extensively for its phytochemical constituents. Ethnomedicinal plant, *Alpinia officinarum* was used as herbal ingredient with biological properties as anti-inflammatory, antioxidant, antiviral, antimicrobial etc [6]. Furthermore, some *Alpinia* species have also been used in traditional remedies by native people in
Indonesia to treat general health disorders [7–10]. Recent investigation revealed that *Alpinia* may harbor diverse endophytic microorganisms with prospective biological properties [11–14].

Endophytic fungi were reported to inhabit the rhizome part of medicinal and wild Zingiberacean species in North Sumatra. In our preliminary studies, we successfully recovered some antagonistic fungal strains from *Amomum*, *Elettaria*, *Etlingera*, and *Globba* sampled from tropical forest in North Sumatra [15–18]. Here, we evaluate the fungal isolates of *Alpinia* in producing extracellular IAA as one of plant growth promoting activities possessed by endophytic fungi.

2. Materials and Methods

2.1. Fungal isolates

Endophytic fungi used in this study were . The endophytic fungi were isolated through surface-sterilized healthy rhizome part of *Elettaria* sampled from Hutan Sibayak, North Sumatera. Fungal isolates preserved in agar slants were subcultured into fresh Potato Dextrose Agar (PDA) prior experimentation.

2.2. Fermentation condition

All fungal isolates that have been subcultured on PDA were subjected to fermentation to produce exogenous IAA. Two agar plugs of fungal colonies were inoculated into 250-mL flask containing 100 mL Czapek-Dox broth supplemented with L-tryptophan (0.1% w/v). Medium were fermented for 8 d under ambient condition without agitation. After incubation, culture filtrates were filtered using Whatman filter paper No.1 to remove mycelial masses and centrifuged at 10,000 × g for 10 min [19].

2.3. IAA quantification

One mL of supernatants was reacted with 2–4 mL Salkowsky (150 mL H$_2$SO$_4$($q$), 250 mL distilled water, 7.5 mL 0.5 M FeCl$_3$.H$_2$O) and incubated in dark condition for 30 min. Presence of pinkish to reddish color in solution indicate positive result of IAA while orangish to yellowish color indicate low to negative result of IAA [20]. IAA was measured by comparing its absorbance (A$_{530}$) with standard IAA concentration (0–100 µg.mL$^{-1}$) plotted from linear regression between quantity of pure IAA (Sigma, USA) in µg.mL$^{-1}$ and its absorbances (Figure 1). All experiments were done in triplicate.

2.4. Data analysis

Data were presented in mean ± S.D from triplicate experiments. Mean results were analyzed using one-way ANOVA for statistical differences (P < 0.05). Graphical image was produced using GraphPad Prism 8.0.2. Qualitative result of experiment were documented in photograph.

2.5. Molecular identification of potential strain

Extraction fungal genome is based on Wizard® Genomic DNA Purification Kit Protocol. Isolate AI04SU mycelium were sampled and crushed using micropistilles in SDS Tris-HCl buffer pH 8.0 (600 µL) and Phenol:Chloroform (600 µL). Amplification of fungal genome within ITS-DNA region was using the universal primer for fungi identification, ITS-1F (5' - CTTGGTCATTTAGAGGAAGTAA - 3') and ITS-4R (5' - TCCTCCGCTTATTGATATGC - 3') [21]. PCR reaction using thermal cycler was specified as follows: Pre-denaturation (95°C, 3 min), Denaturation (95°C, 45 sec), Annealing (55°C , 45 sec), Elongation (72°C, 45 sec), and Final extension (72°C, 7 min) within 35 cycles. The ITS-DNA amplicons was visualized and assessed for its quality under UV illumination on agarose gel. High quality DNA extracts were sequenced using Macrogen services.

2.6. Bioinformatics study

The ITS-DNA sequence of isolate AI04SU was analyzed by comparing with other fungal ITS sequences retrieved from National Centre for Biotechnology Information (NCBI) databases (https://blast.ncbi.nlm.nih.gov/). The sequences were checked using Basic Local Alignment Search
Tool for nucleotide (BLASTn) Sequence pools were aligned using MUSCLE in software MEGA7.0 [22,23]. Phylogenetic tree is constructed based on neighbor-joining method with bootstrap replication 1000x [24,25].

3. Results and Discussion

The results obtained four endophytic fungal species namely which produced IAA in different concentrations namely Al01SU, Al02SU, Al04SU and Al05SU (Figure 2). The highest IAA concentration was produced by Al04SU with 42.91 µg.mL⁻¹, followed by isolates Al02SU, Al05SU and Al01SU with IAA concentration of 26.25, 19.55, and 17.26 µg.mL⁻¹ respectively. Only one isolate, Al03SU which did not produce any traceable amount of IAA based on colorimetry method. Isolate Al04SU was then subjected to species-level identification based on its ITS-DNA similarity with database. The isolate was identified as Aspergillus flavus with 90% bootstrap value (BV) which was statistically reliable based on Kimura-2 parameter method of phylogenetic construction [26] (Figure 2).

![Image](image_url)

**Figure 1.** Mean IAA concentration (µg.mL⁻¹) produced by each endophytic fungal species of Alpinia sp. Error bars indicate S.Ds. Bars sharing different letters are significantly different (Tukey test; *P*<0.05).

Mutualistic relationship between endophytic microorganisms and plant host may be elucidated by studying the biological properties of microbial associates. Beneficial impacts given by endophytic microbes in Zingiberaceae may be in the form of plant growth promotion as previously reported from Zingiberaceae members. Endophytic fungal species, Eupenicillium, Fusarium, Glomerella, Phomopsis, Phyllosticta, Pyricularia, and Talaromyces isolated from Amomum siamense were reported to increase soil phosphorus (P) availability to plant host [27]. Associated endophytic fungi from Alpinia malaccensis was reported to produce extracellular enzymes: Amylase, Cellulase, Pectinase and Asparaginase activity by fungal species of Aspergillus, Cladosporium, Colletotrichum, Diaporthe, Exophiala, Guignardia, Penicillium and Pyricularia [28].
Figure 2. Phylogenetic relationship of *Aspergillus flavus* strain A04SU isolated from *Alpinia* sp. rhizome based on the ITS-DNA region compared to databases retrieved from BLASTn. Databases are shown in the form of accession number followed by species names and strains. The neighbor-joining tree (NJ) was constructed using MEGA7.0. Bootstrap value (BV) through 1000 replications supports on the nodes represent ML ≥ 70%. The newly generated sequences in this study are presented by black-filled dots (•)
to gain better understanding of its role as endophytic fungus. Based on these evidences, our isolate may be explored further in future experimentation for its prospect as biofertilizers in promoting local plants from North Sumatra.

4. Conclusion
Four endophytic fungal strains isolated from *Alpinia* rhizome were able to produce extracellular IAA with a range of 17.26–42.91 µg·mL\(^{-1}\) based on colorimetry quantification. Isolate Al04SU produced the highest IAA concentration among isolates in which later identified as *Aspergillus flavus* based on ITS-DNA similarities.

Acknowledgement
This research is fully funded by Universitas Sumatera Utara through TALENTA funding scheme with contract number: 35/UN5.2.3.1/PPM/KP-TALENTA USU/2019.

References
[1] Strobel G 2018 The Emergence of Endophytic Microbes and Their Biological Promise *J. Fungi* 4 57
[2] Alvin A, Miller K I and Neilan B A 2014 Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds *Microbiol. Res.* 169 483–95
[3] Puri S K, Habbu P V, Kulkarni P V and Kulkarni V H 2018 Nitrogen Containing Secondary Metabolites from Endophytes of Medicinal Plants and their Biological/Pharmacological Activities- A Review *Syst. Rev. Pharm.* 9 22–30
[4] Ramawat K G 2017 *Endophytes: Crop Productivity and Protection* vol 16
[5] Mehmoood A, Hussain A, Irshad M, Hamayun M, Iqbal A and Khan N 2019 In vitro production of IAA by endophytic fungus *Aspergillus awamori* and its growth promoting activities in *Zea mays* *Symbiosis* 77 225–35
[6] Abubakar I B, Malami I, Yahaya Y and Sule S M 2018 A review on the ethnomedicinal uses, phytochemistry and pharmacology of *Alpinia officinarum* Hance *J. Ethnopharmacol.* 224 45–62
[7] Setyowati F M and Wardah 2007 Diversity of medicinal plant by Talang Mamak tribe in surrounding of Bukit Tiga Puluh *BIODIVERSITAS* 8 228–32
[8] Hartanto S F 2014 Studi Etnobotani Famili Zingiberaceae dalam Kehidupan Masyarakat Lokal di Kecamatan Pangean Kabupaten Kuantan Singingi, Riau *Biosaintifika* 6 98–108
[9] Wahidah B F and Husain F 2018 Etnobotani Tumbuhan Obat Yang Dimanfaatkan Oleh Masyarakat Desa Samata *Life Sci.* 7 56–65
[10] Auliani A, Fitmawati and Sofiyanti N 2014 Studi etnobotani famili zingiberaceae dalam kehidupan masyarakat lokal di kecamatan siak hulu kabupaten kampar *Jom Fmipa* 1 526–33
[11] Taechowisan T, Chanaphat S, Ruensamran W and Phutdhawong W S 2012 Antifungal activity of 3-methylcarbazoles from *Streptomyces* sp. LJK109; An endophyte in *Alpinia galanga* J. *Appl. Pharm. Sci.* 2 124–8
[12] Shubin L, Juan H, Renchoa Z, Shiru X, Yuanxiao J and Klopfenstein N B 2014 Fungal endophytes of *Alpinia officinarum* rhizomes: Insights on diversity and variation across growth years, growth sites, and the inner active chemical concentration *PLoS One* 9 1–21
[13] Taechowisan T, Wanbanjob A, Tuntiwachwuttikul P and Taylor W C 2006 Identification of *Streptomyces* sp. Te022, an endophyte in *Alpinia galanga*, and the isolation of actinomycin D *Ann. Microbiol.* 56 113–7
[14] Sunitha V H, Ramesha A, Savitha J and Srinivas C 2012 Amylase production by endophytic fungi *Cylindocephalum* sp. isolated from medicinal plant *Alpinia calcarata* (Haw.) Roscoe *Brazilian J. Microbiol.* 43 1213–21
[15] Lutfia A, Munir E and Yumaliza 2019 Antagonistic Endophytic Fungi of *Hedychium coronarium* Roxb. from Hutan Sibayak and Taman Hutan Raya, North Sumatra against
Staphylococcus aureus ATCC® 29213 TM IOP Conf. Ser. Earth Environ. Sci. 305 012002

[16] Luftia A, Munir E and Yurnaliza 2019 Antagonistic Endophytic Fungi of Globba pendula Roxb. from Taman Hutan Raya, North Sumatra against Staphylococcus aureus ATCC® 29213™ IOP Conf. Ser. Earth Environ. Sci. 305 012003

[17] Munir E, Luftia A and Yurnaliza 2019 Records of Culturable Endophytic Fungi Inhabiting Rhizome of Elettaria in Hutan Sibayak, North Sumatera IOP Conf. Ser. Earth Environ. Sci. 305 012004

[18] Luftia A, Munir E, Yurnaliza Y and Hartanto A 2019 Antagonistic Fungal Endophytes Colonizing Rhizome of Amomum centrocephalum A.D. Poulsen from North Sumatera, Indonesia Int. J. Adv. Sci. Eng. Inf. Technol. 9

[19] Mehmood A, Khan N, Irshad M, Hamayun M, Husna I, Javed A and Hussain A 2018 IAA Producing Endophytic Fungus Fusarium oxysporum wb Colonize Maize Roots and Promoted Maize Growth Under Hydroponic Condition Eur. J. Exp. Biol. 08 1–7

[20] Meadt W J and Gaines T P 1967 Studies on the oxidation of Indole-3-acetic acid by peroxidase enzymes. I. colorimetric determination of Indole-3-acetic acid oxidation products Plant Physiol. 42 1395–9

[21] Manter D K and Vivanco J M 2007 Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis J. Microbiol. Methods 71 7–14

[22] Edgar R C 2004 MUSCLE: Multiple sequence alignment with high accuracy and high throughput Nucleic Acids Res. 32 1792–7

[23] Kumar S, Stecher G and Tamura K 2016 MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets Mol. Biol. Evol. 33 1870–4

[24] Saitou N and Nei M 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4 406–25

[25] Felsenstein J 1985 Confidence Limits on Phylogenies: An Approach Using the Bootstrap Society 39 783–91

[26] Kimura M 1980 A Simple Method for Estimating Evolutionary Rates of Base Substitutions Through Comparative Studies of Nucleotide Sequences J. Mol. Evol. 16 111–20

[27] Bussaban B, Lumyong S, Lumyong P, McKenzie E H C and Hyde K D 2001 Endophytic fungi from Amomum siamense Can. J. Microbiol. 47 943–8

[28] Uzma F, Konappa N M and Chowdappa S 2016 Diversity and extracellular enzyme activities of fungal endophytes isolated from medicinal plants of Western Ghats, Karnataka Egypt. J. Basic Appl. Sci. 3 335–42

[29] Fu S F, Wei J Y, Chen H W, Liu Y Y, Lu H Y and Chou J Y 2015 Indole-3-acetic acid: A widespread physiological code in interactions of fungi with other organisms Plant Signal. Behav. 10

[30] Amaike S and Keller N P 2011 Aspergillus flavus Annual Review of Phytopathology 49 107–33

[31] Hedayati M T, Pasqualotto A C, Warn P A, Bowyer P and Denning D W 2007 Aspergillus flavus: Human pathogen, allergen and mycotoxin producer Microbiology 153 1677–92

[32] Ludovici G M, Cenciarelli O, Carestia M, Gabbarini V, Malizia A and Gaudio P 2017 Mycotoxins: A New Concern for Biosecurity? Biomed. Prev. 3 140–2

[33] Chowdhury F T, Sarker M, Islam M S, Nur H P, Islam M R and Khan H 2018 Investigation of Antimicrobial Activity and Identification of Bioactive Volatile Metabolites of Jute Endophytic Fungus Aspergillus flavus Bioresearch Commun. 4 476–82

[34] Patil M P, Patil R H and Maheshwari V L 2015 Biological Activities and Identification of Bioactive Metabolite from Endophytic Aspergillus flavus L7 Isolated from Aegle marmelos Curr. Microbiol. 71 39–48

[35] Ismail, Hamayun M, Hussain A, Afzal Khan S, Iqbal A and Lee I J 2019 Aspergillus flavus promoted the growth of soybean and sunflower seedlings at elevated temperature Biomed Res. Int. 2019 1–13