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Diversity of Endophytic Fungi from Red Ginger (*Zingiber officinale* Rosc.) Plant and Their Inhibitory Effect to *Fusarium oxysporum* Plant Pathogenic Fungi

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Indonesia has been known as a country with high medicinal plant diversity. One of the most common medicinal plant from Indonesia is red ginger (*Zingiber officinale* Rosc.). Nevertheless, limited studies of endophytic fungi associated with these medicinal plants are hitherto available. The objectives of this research were to study the diversity of endophytic fungi on red ginger and to analyze their potential as a source of antifungal agent. All parts of plant organs such as leaf, rhizome, root, and stem were subjected for isolation. Fungal identification was carried out by using a combination of morphological characteristic and molecular analysis of DNA sequence generated from ITS rDNA region. Thirty endophytic fungi were successfully isolated from leaf, rhizome, root, and stem of red ginger plant. Antagonistic activity was tested against *Fusarium oxysporum*, a pathogenic fungus on plants, using an antagonistic assay. Based on this approach, the fungi were assigned as *Acremonium macroclavatum, Beltraniella* sp., *Cochliobolus geniculatus* and its anamorphic stage *Curvularia affinis, Fusarium solani, Glomerella cingulata* and its anamorphic stage *Colletotrichum gloeosporoides, Lecanicillium kalimantanense, Myrothecium verrucaria, Neonectria punicea, Periconia macrospinosa, Rhizopycnis vagum,* and *Talaromyces assiutensis*. *R. vagum* was found specifically on root whereas *C. affinis, L. kalimantanense,* and *M. verrucaria* were found on stem of red ginger plant. *A. macroclavatum* was found specifically in red ginger plant's organ which located under the ground, whereas *C. affinis* was found from shoot or organ which located above the ground. The antagonistic activity of isolated endophytic fungi against *F. oxysporum* varied with the inhibition value range from 1.4 to 68.8%. *C. affinis* (JMbt7), *F. solani* (JMd14), and *G. cingulata* (JMr2) had significantly high antagonistic activity with the value above 65%; and *R. vagum* (JMa4) and *C. geniculatus* (JMbt9) had significantly low antagonistic activity with the range value 0-10%.

Keywords: medicinal plant, *Zingiber officinale*, endophytic fungi, biodiversity, antagonistic activity

INTRODUCTION

Almost all plants are mutually or neutrally associated with one or more endophytic fungi (Schulz & Boyle 2006). In the symbiosis, endophytic fungi produce bioactive compounds that can stimulate growth and enhance host plant resistance to abiotic and biotic stress factors such as drought, and pests and diseases, while plant as their host provides an ecological niche for growth and development of the endophytic fungi (Dai *et al*. 2008; Shipunov *et al*. 2008).

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In the symbiotic phase, the host plant serves as the environmental factor for the growth of the endophytic fungi. When the host plant suffer from parasite or pest attack, it makes environmental stress condition on the fungi grown inside the host plant and stimulate them to produce bioactive compounds. The source of fungal bioactive compound could be a result of fungal metabolism itself or the fungi gaining precursors from the host plant which were used for the production of the bioactive compound.

Endophytic fungi are also able to produce bioactive compound in the free-living system grown in synthetic medium. The product, however, could be same like

those in the symbiosis system and/or different compounds. Production of endophytic fungal bioactive compounds in free-living system using synthetic medium has several advantages compared to that of the symbiotic system. In the free-living system, it is easy to standardize either the growth medium or bioactive compound produced by the fungi, as well as it requires shorter time for incubation compare to the symbiotic system which depends upon host plant.

Medicinal plant is one of the potential sources of fungal endophytic diversity. It was expected that whole plant organs-inhabited by various endophytic fungi. It has been reported by Strobel and Daisy (2003) that endophytic fungi were potential source of producing novel secondary metabolites. It has been also stated that bioactive compound from endophytic fungi can be used in various field such agricultural industries (Schulz *et al*. 2002). In agriculture, endophytic fungi have been widely used as a biological control of pathogenic fungi in various cultivated plants (Campanile *et al*. 2007). For example endophytic fungi *Trichoderma*, *Pestalotiopsis*, *Curvularia*, *Tolypocladium*, and *Fusarium* from cacao were active against pathogenic fungi *Phytophtora palmivora*. Furthermore, *Colletotrichum trunctatum* isolated from *Jatropha curcas* was able to control growth of *Fusarium oxysporum* and *Scleorotinia sclerotiorum* plant pathogens (Hanada *et al*. 2010; Kumar & Kaushik 2013). Antifungal belong to the cytochalasin family, chaetoglobosin A and chaetoglobosin C produced by endophytic fungi *Chaetomium globosum*, could suppressed the growth of pathogenic fungi *Setosphaeria turcica* in maize (Zhang *et al*. 2013). Larkin *et al*. (1996) used endophytic fungi *F. oxysporum* nonpathogenic to control fusarium wilt of water melon.

Plant pathogenic fungi *F. oxysporum* causes fusarium wilt disease on a broad host range of agriculture plants at any age. A few of most susceptible agricultural plants were tomato, tobacco, legumes, cucurbits, sweet potatoes, and banana. Plant pathogenic fungi *F. oxysporum* generally produces symptoms such as wilting, chlorosis, necrosis, premature leaf drop, browning of the vascular system, stunting, and damping-off. Fusarium wilt is the most damaging and prevalent plant disease. It was expected that the endophytic fungi from red ginger (*Zingiber officinale* Rosc.) plant could have ability to control *F. oxysporum*. The mechanism of controling the patogenic fungi by endophytic fungi could be attributed to their production of antifungal compounds, aggresive growth habit, and high nutrient competition. For example, metabolite 13-oxo-9,11-octadecadienoic acid produced by endophytic fungi *Paraconiothyrium variabile* isolated from medicinal plant *Cephalotaxus harringtonia*, supressed plant pathogenic fungi *F. oxysporum* by controling production of most potent mycotoxin, beauvericin, by the pathogen (Combès *et al*. 2012). The bioactive compound 13-oxo-9,11-octadecadienoic acid reduced production of beauvericin which led to reduction of *F. oxysporum* pathogenic fungal growth and development.

There was no report on the endophytic fungi associated with red ginger plant so far, therefore, this research aimed to study the diversity of endophytic fungi on red ginger medicinal plant and to analyse their potential as a source of antifungal agent.

MATERIALS AND METHODS

Plant Materials. Endophytic fungi were isolated from various parts of red ginger plant organs, collection of Indonesian Medicinal and Aromatic Crops Research Institute, Bogor, Indonesia. Three stands of fresh and healthy plant were carefully harvested from the collection field by digging up the whole plant organs. The plants were washed carefully by tap water to remove adhering soil and followed up by rinsing them with sterile reverse osmosis water three times. Roots, stems, rhizomes, and leaves were cut separately, and each plant organ was pulled together to make a composite sample for each organ. Samples then put in clean plastic bags and stored at 10 ^{o}C before used.

Isolation of Endophytic Fungi. Each plant organ was cut into the size of 2 x 2 cm^2 for leaf, 1 cm for root and stem, and 2 x 2 x 2 cm^3 for rhizome. Fifty pieces of each plant organ were surface sterilized by immersing the sample in 70% ethanol for 1 min, rinsed three times with sterile water, then soaked again in hypochlorite solution (NaOCl) 0.5% for 5 min and rinsed in sterile water for six times. Samples were then dried on sterile filter paper for 12 hours. All preparations were carried out in biosafety cabinet. Isolation of endophytic fungi followed the method of Hallmann *et al*. (2007) using potato dextrose agar (PDA, difco) containing rose bengal fungistatic (30 mgl^{-1}) and chloramphenicol antibiotic (0.5 gl^{-1}) and incubated at 28 ^{o}C for 7 to 21 days. After the fungus was growing out from the plant organ, the hyphal tip arising from the fungal colony was cut and transferred on PDA without supplemented with either rose bengal or chloramphenicol. In the same organ, only colonies having different characteristics were selected.

Identification of Endophytic Fungi. Isolated endophytic fungi were identified by combining morphological characteristic and molecular analyses.

The morphological characteristics used in this study were colony and spore characteristics (Barnett & Hunter 1998).

Molecular identification of the isolated fungi were carried out by analyzing sequence of ITS rDNA using universal primer pair of ITS1 (forward) (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (reverse) (52 -TCCTCCGCTTATTGATATGC-32) including 5.8 region (White *et al*. 1990). When the DNA did not amplified successfully using ITS1 and ITS4 primers, then the pair of ITS5 (5' -GGAAGTAAAA GTCGTAACAAGG-3') and ITS2 (5' -GCTGCGTT CTTCATCGATGC-3'), or ITS3 (5' -GCATCGATG AAGAACGCAGC-3'), and NL4 (5'-GGTCCGTGT TTCAAGACGG-3') (White *et al*. 1990) were employed.

Fungal genomic DNA was isolated from mycelium grown in potato dextrose broth (PDB, difco) that incubated in a shaker at 120 rpm and 28 °C for 7 days. Mycelia were harvested by vacuum filtration through sterile filter paper, and immediately frozen in liquid nitrogen, ground in a sterile mortar. The genomic DNA extraction was done by CTAB-based extraction method. About 0.5 g biomass powder were transferred to 1.5 ml Eppendorf tube which contained warm extraction buffer (600 µl PVP and 1.2 µl CTAB). The tube was inverted and incubated at 65 °C for 30 min, and followed by incubation on ice for 5 min. About 600 µl chloroform:isoamyl alcohol (24:1) was added to the tube, and then inverted. After 10 min centrifugation at 10 °C, 25,000 × g, the aqueous phase was removed to a new tube, and was extracted with one volume of phenol:chloroform:isoamyl alcohol (25:24:1), followed by inverted the tube, centrifugation at 4 °C, 25,000 × g for 5 min. Supernatant was transferred to a new tube, and was added with an equal volume of 2M NaOAc pH 5.2 and 2x volume of cold EtOH and incubated for 30 min in 20 °C. DNA pellets were collected by centrifugation at 25,000 × g, 4 °C for 30 min. The DNA pellets were then washed with 500 µl 70% cold ethanol, centrifugation at 4 °C, 25,000 × g for 5 min. DNA pellets were dried brieûy in a vacuum, resuspended in 20 µl of sterile double-distilled water and 0.2x volume of RNAse, then incubated at 37 °C for 10 min. Inactivation of RNAse was done at 70 °C for 10 min. Fungal DNA was stored in a freezer until used.

DNA amplification was performed on a volume of 60 ml which consisted of 42.6 ml sterile ddH_2O, 6 µl buffer (10x), 1.2 µl 2 mM dNTP, 1.5 µl 10 pmol of each primer ITS1 (forward) and ITS4 (reverse), 1.2 ml 5 U *Taq* DNA polymerase, and 6 µl DNA template. Amplification reaction was performed as follows: pre-denaturation for 5 min at 95 °C 1x and 35 cycles of denaturation conditions for 30 seconds at 94 °C, annealing for 30 seconds at a temperature of 52 °C, extension for 1 min at 72 °C, and at the end of the cycle performed final re-extension for 5 min at 72 °C, the last stored for 10 min at 25 °C using a Gene Amp 9700 thermal cycler (Applied Biosystems, USA). An amount of 5 ml of PCR products were checked by electrophoresis using 1% agarose gel in buffer 1x TAE for 30 min (Sambrook & Russell 2000), stained with 0.5 mg/ml ethidium bromide and observed under UV light. When primers ITS1 and ITS4 did not anneal to the template we used primer ITS5 pairs with ITS2 or ITS3 with NL4 (White *et al*. 1990) with the same volume and reaction as above, except the annealing was carried out at a temperature of 58 °C.

PCR products were sent to FirstBase (Malaysia) for purification and sequencing using the same primer. The results of sequence were analyzed by using the BioEdit Ver.7.0.0 (Hall 1999) and aligned using ClustalW (Thompson *et al*. 1994). Sequence were determined by using available DNA fungal sequence at MycoBank search (http://www.mycobank.org) and BLAST search (http://www.blast.ncbi.nlm.nih.gov/blast).

Screening of Antagonistic Isolates. Antagonistic activity of endophytic fungi was tested against plant pathogenic fungi *F. oxysporum* (IPBCC.88.0.12 or CBS 254.52) using antagonists assay followed the method of Morton and Stroube (1955) by using dual culture technique on PDA medium. Briefly, the method was as follows: a 5 mm^2 diameter of endophytic fungal colony grown on PDA for 7 days was placed on one side of the Petri dish containing PDA, and was incubated at room temperature for 4 days. After 4 days incubation, each Petri dish was inoculated with a 5 mm^2 diam colony of *F. oxysporum* at an opposite distance of 5 cm from the endophytic fungus. In the control treatment, endophytic fungi were replaced by a piece of 5 mm^2 diam. PDA without fungal mycelia. Cultures were incubated at room temperature for 7 days. The radial growth of *F. oxysporum* was measured at day 4 and day 7 after inoculation. The magnitude of the inhibitory activity was calculated with the formula: PI = (100 × (R1 - R2) / R1), where PI is percentage inhibition of radial growth, R1 is radial growth of *F. oxysporum* in the control treatment, and R2 is radial growth of *F. oxysporum* toward endophytic fungi in dual culture with endophytic fungi. All of the endophytic fungi obtained in this study were tested and each assay was repeated for five times. Statistical analysis was done using MSTAT program (University of Wisconsin-Madison) and means value were analysis by DMRT ($P < 0.05$).

Table 1. The endophytic fungi associated with red ginger (*Zingiber officinale*) plant organs based on morphological characteristics

Root		Rhizome		Stem		Leaf	
Endophytic fungi	Spore characteristics	Endophytic fungi	Spore characteristics	Endophytic fungi	Spore characteristics	Endophytic fungi	Spore characteristics
Acremonium sp. 1 JMa6	Conidiophore and phialides slender, simple, conidia hyaline, 1 celled, collecting in a slime drop, size 3.1-5.5 x 1.2-2.5 µm	*Acremonium* sp. 3 JMr5	Conidiophore and phialides slender, simple, conidia hyaline, 1 celled, collecting in a slime drop, size 2.5-4.6 x 2.1-4.0 µm	*Colletotrichum* sp. 2 JMbt13	Conidiophores simple, elongate, conidia hyaline, 1 celled, ovoid or oblong, size 19.7-24.8 x 5.1-6.6 µm	*Colletotrichum* sp. 3 JMd1	Conidiophores simple, elongate, conidia hyaline, 1 celled, ovoid or oblong, size 5.6-13.1 x 2.4-4.0 µm
Acremonium sp. 2 JMa8	Conidiophore and phialides slender, simple, conidia hyaline, 1 celled, collecting in a slime drop, size 3.6-5.9 x 1.8-2.0 µm	*Acremonium* sp. 4 JMr7	Conidiophore and phialides slender, simple, conidia hyaline, 1 celled, collecting in a slime drop, size 3.0-6.2 x 1.6-2.4 µm	*Curvularia* sp. 1 JMbt4	Conidiophores brown, simple, bearing spores apically or on new sympodial growing points, conidia dark, end cells lighter, 3-5 celled, one of the central cells enlarged, size 14.1-19.4 x 6.9-9.7 µm	*Colletotrichum* sp. 4 JMd4	Conidiophores simple, elongate, conidia hyaline, 1 celled, ovoid or oblong, size 2.1-3.2 x 3.5-5.6 µm
Cylindrocarpon sp. 1 JMa1	Conidiophores hyaline, slender and terminating in slender phialides, conidia 2-4 celled, hyaline, cylindris, size 52.0-100 x 9.9-13.9 µm	*Beltraniella* sp. J Mr1	Separating cell, obovoid, smooth, hyaline, denticulate at each end, size 5.4-9.2 x 1.7-2.6 µm	*Curvularia* sp. 2 JMbt7	Conidiophores brown, simple, bearing spores apically or on new sympodial growing points, conidia dark, end cells lighter, 3-5 celled, typically bent, one of the central cells enlarged, size 16.5-20.3 x 7.8-10.7 µm	*Colletotrichum* sp. 5 JMd5	Conidiophores simple, elongate, conidia hyaline, 1 celled, ovoid or oblong, size 3.6-6.7 x 9.3-14.4 µm
Fusarium sp. 1 JMa3	Conidiophore hyaline, slender and simple, conidia hyaline, two kinds, macroconidia, several celled , slighty curved, 1-2 celled, microconidia, 1-2 celled, ovoid, oblong or slight curved, size macroconidia 20.5-35.1 x 4.2-5.6 ìm. microconidia 11.1-15.8 x 3.0-5.1 µm	*Colletotrichum* sp. 1 JMr2	Conidiophores simple, elongate, conidia hyaline, 1 celled, ovoid or oblong, size 10.4-15.2 x 2.5-4.1 µm	*Curvularia* sp. 3 JMbt8	Conidiophores brown, simple, bearing spores apically or on new sympodial growing points, conidia dark, end cells lighter, 3-5 celled, typically bent, one of the central cells enlarged, size 42.8-58.9 x 14.2-19.4 µm	*Colletotrichum* sp. 6 JMd12	Conidiophores simple, elongate, conidia hyaline 1 celled, ovoid or oblong, size 28.7-34.1 x 8.9-10.1 µm

Table 1. Continue

Root		Rhizome		Stem		Leaf	
Endophytic fungi	Spore characteristics	Endophytic fungi	Spore characteristics	Endophytic fungi	Spore characteristics	Endophytic fungi	Spore characteristics
Fusarium sp. 2 JMa5	Conidiophore hyaline, slender and simple, conidia hyaline, two kinds, macroconidia, several celled slighty curved, 1-2 celled, microconidia, 1-2 celled, ovoid, oblong or slight cutved, size macroconidia 37.3-74.4 x 3.2-9.5 ìm, microconidia 15.2-25.8 x 3.9-9.2 µm	*Cylindrocarpon* sp. 2 JMr3	Conidiophores erect, Conidiophores erect, slender, hyaline, terminating in slender phialides, conidia 2-4 celled, hyaline, cylindrical, size 25.2-49.5 x 5.3-7.4 µm	*Curvularia* sp. 4 JMbt9	Conidiophores brown, simple, bearing spores apically or on new sympodial growing points, conidia dark, end cells lighter, 3-5 celled, typically bent, one of the central cells enlarged, size 15.5-25.8 x 5.9-10.4 µm	*Curvularia* sp. 5 JMd13	Conidiophores brown, simple, bearing spores apically or on new sympodial growing points, conidia dark, end cells lighter, 3-5 celled, typically bent,one of the cells enlarged, size 21-28.5 x 8.8-10.7 µm
Mycelia sterilia 1 JMa4	No conidia observed	Mycelia sterilia 2 JMr4	No conidia observed	*Lecanicillium* sp. JMbt10	Conidiophores produced on erect or along prostrate aerial hypae, slender, tapering toward the apex, conidia slightly curved, size 4.8-10.7 x 2.1-3.1 µm	*Fusarium* sp. 3 JMd14	Conidiophores hyaline, slender and simple, conidia hyaline, two kinds, macroconidia, several celled slightly curved, microconidia, several celled slightly curved, 1-2 celled, microconidia 1-2 celled, ovoid, oblong or slight curved, size macroconidia 16.9-21.2 x 2.6-3.4 µm, microconidia 5.3-8.6 x 1.6-3.6 µm

Table 1. Continue

Root		Rhizome		Stem		Leaf	
Endophytic fungi	Spore characteristics	Endophytic fungi	Spore characteristics	Endophytic fungi	Spore characteristics	Endophytic fungi	Spore characteristics
Periconia sp. JMa2	Conidiophores dark, simple, enlarged at apex, conidia dark, 1 celled, globose, size 2.8-3.7 x 2.4-3.3 µm			Mycelia sterilia 3 JMbt2	No conidia observed	*Glomerella* sp. JMd3	Conidiophores simple, elongate, conidia hyaline, 1 celled, ovoid or oblong, size 3.7-5.0 x 1.7-2.8 µm, produce young ascocarp
Talaromyces sp. 1 JMa7	Conidiophores arising from the mycelium singly, conidia hyaline, 1 celled, mostly ellipsoidal, size 3.2-3.8 x 2.1-3.1 µm, produce young ascocarp 8 26.7			*Talaromyces* sp. 2 JMbt3	Conidiophores arising from the mycelium singly, conidia hyaline, 1 celled, mostly ellipsoidal, size 2.9-4.0 x 2.1-3.2 µm, produce young ascocarp	Mycelia sterilia 4 JMd9	No conidia observed
Number of isolates	8	6		8		8	
Percentage (%)	26.7	20.0		26.7		26.7	
Total isolates				30			

RESULTS

Diversity and Distribution of Endophytic Fungi on Red Ginger Plant. Thirty isolates of endophytic fungi having different colony characteristics were obtained from leaf, rhizome, root, and stem of red ginger plant. The number and their distribution in each plant organ studied are presented in Table 1 and 3. The root, stem, and leaf had the same number of endophytic isolate. Each of them was occupied by eight isolates. The rhizome, however, was inhabited by six isolates, which was the lowest number compare to the other plant organs studied. Based on morphological characteristics, 30 morphotypes of the endophytic fungi were determined from this study which belong to 4 mycelia sterilia and 9 genera with *Colletotrichum* were considered as anamorphic stage of *Glomerella* (Table 1).

Further identification to species level was conducted using sequence analysis of ITS1-5.8S - ITS2 rDNA. Based on BLAST search, similarity of the isolated fungi to the closest species available in MycoBank and GenBank varied from 76 to 100% (Table 2). The thirty isolates were identified into 11 species and 2 mycelia sterilia. The *Colletotrichum gloeosporoides* and *Glomerella cingulata*, *Curvularia affinis* and *Cochliobolus geniculatus* are anamorphic-teleomorphic stage, thus they were considered as two instead of 4 species. The list of species is presented in Table 2.

Molecular analysis indicated that isolates having different morphology were not always belong to different species (Table 1 & 2). For example, *Acremonium* sp. 1 (JMa6), *Acremonium* sp. 2 (JMa8), *Acremonium* sp. 3 (JMr5), and *Acremonium* sp. 4 (JMr7) differed in morphological characteristics,

Table 2. Endophytic fungi isolated from red ginger (*Zingiber officinale*) plant based on molecular identification

Fungal identification	Fungal code	No access GenBank references	Maximum score	% Similarity	Query coverage	E value
Acremonium macroclavatum	JMa6	HQ897806.1	710.1	95.0	98.3	0.0
Acremonium macroclavatum	JMa8	HQ897806.1	721.2	95.7	98.6	0.0
Acremonium macroclavatum	JMr5	HQ897806.1	651.4	95.6	82.6	0.0
Acremonium macroclavatum	JMr7	HQ897806.1	708.5	95.9	98.1	0.0
*Cochliobolus geniculatus***	JMbt4	JN943416.1	1027.0	99.0	98.0	0.0
Cochliobolus geniculatus	JMbt9	JN943416.1	825.8	99.6	98.0	0.0
Cochliobolus geniculatus	JMd13	JN943416.1	919.3	99.5	99.8	0.0
Colletotrichum gloeosporioides	JMd1	EU552111.1/AJ301908.1	871.7	98.6	99.7	0.0
Colletotrichum gloeosporoides	JMd4	EU552111.1/AJ301908.1	903.4	99.3	99.5	0.0
Colletotrichum gloeosporioides	JMbt13	EF423519.1	819.0	100.0	94.5	0.0
Curvularia affinis	JMbt7	GQ352486.1	914.5	99.7	98.5	0.0
Curvularia affinis	JMbt8	GQ352486.1	841.6	96.2	98.0	0.0
*Fusarium oxysporum**	JMr4	GQ365156.1	351.2	79.1	99.0	4e-93
Fusarium solani	JMa3	HQ608044.1	863.3	100.0	95.1	0.0
Fusarium solani	JMa5	FJ345352.1	1450.2	98.6	98.9	0.0
Fusarium solani	JMd14	FJ345352.1	884.4	97.9	99.3	0.0
*Glomerella cingulata****	JMd3	AB042317.1	860.6	97.2	99.5	0.0
Glomerella cingulata	JMd5	AB042317.1	857.5	98.1	99.5	0.0
Glomerella cingulata	JMd12	AB042315.1	908.2	99.8	99.3	0.0
Glomerella cingulata	JMr2	AB042317.1	958.0	98.0	78.3	0.0
Lecanicillium kalimantanense	JMbt10	DQ682584.1	659.3	89.5	99.7	0.0
*Leiosphaerella lycopodina**	JMr1	JF440975.1	614.0	86.0	99.0	3e-172
Myrothecium verrucaria	JMbt2	HQ607996.1	798.7	95.5	99.5	0.0
*Neonectria punicea*****	JMa1	HM534901.1	1443.0	90.0	99.0	0.0
Neonectria punicea	JMr3	HM534901.1	1472.0	89.0	99.0	0.0
Periconia macrospinosa	JMa2	AJ246159.1	895.5	98.8	89.6	0.0
*Periconia macrospinosa**	JMd9	AJ246159.1	340.0	76.0	91.0	8e-90
Rhizopycnis vagum	JMa4	AF022786.1	828.9	99.4	99.4	0.0
Talaromyces assiutensis	JMa7	JN899320.1	901.8	98.8	100.0	0.0
Talaromyces assiutensis	JMbt3	JN899320.1	901.8	98.5	99.3	0.0

*morphological identification data was used because their E value were very low, therefore, JMr4, JMd9, and JMr1 were mycelia sterilia 2, mycelia sterilia 4, and *Beltraniella* sp., respectively; **teleomorph stage of *Curvularia affinis;* ***teleomorph stage of *Colletorichum gloeosporioides;* ****teleomorph stage of *Cylindrocarpon* sp.

however, based on DNA sequence data, they were belong to the same species, namely *Acremonium macroclavatum*. Similarly with morphotype of *Colletotrichum*, *Fusarium*, *Curvularia,* and *Talaromyces* (Table 1 & 2). In addition, this data showed that molecular analysis is a powerful tool for the identification of unidentified fungi. Mycelia sterilia 1 JMa4 isolate derived from root was successfully identified as *Rhizopycnis vagum* with 99.4% of similarity to the sequence available in MycoBank. Mycelia sterilia 3 JMbt2 isolate derived from stem was successfully identified as *Myrothecium verrucaria* with 95.5% of similarity to the sequence available in MycoBank. However, the other two mycelia sterilia fungi, JMr4 isolate derived from rhizome and JMd9 isolate derived from leaf, could not be identified using the same approach. Hence, for the last three isolates, we used data of morphological identification instead of molecular identification (Table 2).

Based on data presented in Table 3, all plant organs of red ginger were inhabited by endophytic fungi. Some isolated endophytic fungi could only be found in one or at the most in three plant organs. For example, *A. macroclavatum* grow only in root and rhizome; *C. gloeosporiodes* in stem and leaf, and its teleomorphic stage *G. cingulata* in rhizome and leaf; *Fusarium solani* in root and leaf, *Neonectria punicea* in root and rhizome; *Talaromyces assiutensis* in root and stem; *C. affinis* in stem and its teleomorphic stage *C. geniculatus* in leaf and stem. The endophytic fungi that found in a specific plant organ were *R. vagum* which obtained from the root, whereas *Lecanicillium kalimantanense* and *M. verrucaria* were determined from stem, and *Beltraniella* sp. was found on rhizome of red ginger plant. When the host plant was divided into above ground (shoot) and under ground (root system), it showed interesting separation of the isolated fungi. Some fungi were found in shoot such as *C. affinis* and its teleomorphic stage *C. geniculatus*, while *A. macroclavatum* and *N. punicea* in root system (Table 3).

Table 3. Distribution of endophytic fungi on red ginger (*Zingiber officinale*) plant organs

Endophytic fungi	Isolate code	Total isolate number in a plant	Number of isolate in root	Number of isolate in rhizome	Number of isolate in stem	Number of isolate in leaf
Acremonium macroclavatum	JMa6, JMa8, JMr5, JMr7	4	2	2	-	-
Beltraniella sp.	JMr1	1	-	1	-	-
Cochliobolus geniculatus[a]	JMbt4, JMbt9, JMd13	3	-	-	2	1
Curvularia affinis[b]	JMbt7, JMbt8	2	-	-	2	-
Colletotrichum gloeosporiosides[a]	JMd1, JMd4, JMbt13	3	-	-	1	2
Glomerella cingulata[b]	JMd3, JMd5, JMd12, JMr2	4	-	1	-	3
Fusarium solani	JMd14, JMa3, JMa5	3	2	-	-	1
Lecanicillium kalimantanense	JMbt10	1	-	-	1	-
Myrothecium verrucaria	JMbt2	1	-	-	1	-
*Neonectria punicea**	JMa1, JMr3	2	1	1	-	-
Periconia macrospinosa	JMa2	1	1	-	-	-
Rhizopynis vagum	JMa4	1	1	-	-	-
Talaromyces assiutensis	JMa7, JMbt3	2	1	-	1	-
Mycelia sterilia 2	JMr4	1	-	1	-	-
Mycelia sterilia 4	JMd9	1	-	-	-	1
Total isolat number		30	8	6	8	8

[a] and [b] are anamorphic-teleomorphic relationship, and *teleomorphic stage of *Cylindrocarpon.*

Table 4. Inhibition activity of fungal endophyte derived from red ginger (*Zingiber officinale*) plant against *F. oxysporum*

Fungal identification	Fungal code	Organ	% inhibition
Acremonium macroclavatum	JMa6	Root	23.6 jkl
Acremonium macroclavatum	JMa8	Root	19.4 klm
Acremonium macroclavatum	JMr5	Rhizome	25.6 ij
Acremonium macroclavatum	JMr7	Rhizome	24.3 ijk
Beltraniella sp.	JMr1	Rhizome	54.1 bcd
Cochliobolus geniculatus	JMbt4	Stem	25.6 ij
Cochliobolus geniculatus	JMbt9	Stem	1.4 o
Cochliobolus geniculatus	JMd13	Leaf	50.9 cd
Colletotrichum gloeosporioides	JMbt13	Stem	58.1 b
Colletotrichum gloeosporoides	JMd1	Leaf	30.0 hi
Colletotrichum gloeosporoides	JMd4	Leaf	53.5 bcd
Curvularia affinis	JMbt7	Stem	68.8 a
Curvularia affinis	JMbt8	Stem	48.6 de
Fusarium solani	JMa3	Root	45.1 ef
Fusarium solani	JMa5	Root	55.6 bc
Fusarium solani	JMd14	Leaf	65.9 a
Glomerella cingulata	JMd3	Leaf	35.2 gh
Glomerella cingulata	JMd5	Leaf	50.9 cd
Glomerella cingulata	JMd12	Leaf	58.3 b
Glomerella cingulata	JMr2	Rhizome	65.1 a
Lecanicillium kalimantanense	JMbt10	Stem	24.8 ijk
Mycelia sterilia 2	JMr4	Rhizome	39.2 g
Mycelia sterilia 4	JMd9	Leaf	16.7 m
Myrothecium verrucaria	JMbt2	Stem	40.4 fg
Neonectria punicea	JMa1	Root	27.8 ij
Neonectria punicea	JMr3	Rhizome	24.1 ijk
Periconia macrospinosa	JMa2	Root	54.6 bcd
Rhizopycnis vagum	JMa4	Root	10.0 n
Talaromyces assiutensis	JMa7	Root	26.3 ij
Talaromyces assiutensis	JMbt3	Stem	18.1 lm
Coefficient variation (%)			8.6

Values of % inhibition are means from 5 replications. Means followed by the same letter are not significantly different in DMRT ($P < 0.05$).

Antagonistic Activity of Isolated Endophytic Fungi. All isolated endophytic fungi showed inhibition activity against *F. oxysporum*. The percentage of inhibition, however, varied ranging from of 1.4 to 68.8% and the differences were statistically significant. The ranges of inhibition value of endophytic fungi derived from root, rhizome, stem and leaf against *F. oxysporum* were 10.0 to 55.6%, 24.1 to 65.1%, 1.4 to 68.8%, 16.7 to 65.9%, respectively (Table 4).

The inhibition value of the endophytic fungi against *F. oxysporum* could be divided into low, medium and high categories based on statistical analysis. High category was for inhibition activity equal and more than 65%, moderate was for more than 10% to less than 65%, low was for equal and less than 10% (Table 4). The endophytic fungi with high significant inhibition include *C. affinis* (JMbt7), *F. solani* (JMd14), and *G. cingulata* (JMr2). Besides that, *G. cingulata* (JMd12) and *C. gloeosporioides* (JMbt13) significantly had no different inhibition activity with *F. solani* (JMa5), *Periconia macrospinosa* (JMa2), *G. cingulata* (JMd5), *Bertraniella* sp. (JMr1), and *C. gloeosporoides* (JMd4), but significantly had lower inhibition activity than *C. affinis* (JMbt7), *F. solani* (JMd14), and *G. cingulata* (JMr2), and higher inhibition activity than the other. The endophytic fungi with low inhibition include *R. vagum* (JMa4) and *C. geniculatus* (JMbt9). There was no indication that endophytic fungi showing high inhibition activity grown in a certain plant organ (Table 4). Endophytic fungi having high, medium and low inhibition activities against *F. oxysporum* were spread at all plant organs of red ginger. There was a tendency that antagonistic activity from the same species were different regardless the plant organs used for isolation, except for *A. macroclavatum* (Table 4). For example, two isolates of *C. affinis* obtained from stem, three isolates of *F. solani* isolated from root and leaf, and *T. assiutensis* derived from root and stem had had different value of inhibition percentage.

DISCUSSION

Diversity and Distribution of Endophytic Fungi on Red Ginger Plant Organs. It is showed that red ginger plant is inhabited by diverse endophytic fungi. The number of the endophytic fungi on red ginger obtained in this experiment, however, could still be underestimated since only culturable fungi were recovered by isolation method using PDA medium employed in this experiment. There were many endophytic fungi were obligate symbiont that could not be isolated and cultured using conventional isolation method. Almost all of the endophytic fungi isolated in this experiment were belong to mitosporic fungi and phylum Ascomycota. This finding is in agreement with Yuan *et al*. (2010) who worked on identity and diversity of endophytic fungi on rice root using isolation and environmental-PCR based method by direct DNA isolation from root. Low number of fungi belonging to phylum Basidiomycota were found as endophytes when isolation method using MEA medium was employed compare to that of environmental-PCR based method. Furthermore, diversity and total number of species obtained using the later method were significantly higher since it could determine both culturable and unculturable endophytic fungi living in the host plant.

Identification by ITS sequence analysis is generally used for identifying fungi to the species level, especially if the fungi do not sporulate (Chen *et al*. 2008). Most of the isolated endophytic fungi were successfully identified by combining morphological and molecular analysis, however, still there were three isolates unsuccessfully identified due to their very low E value from BLAST searched results or no available fungal sequence matched to these sequences. Two isolates were mycelia sterilia and one isolate morphologically identified to genus level, *Beltraniella*. This need further investigation by employing others primers or regions for DNA amplification to identify into species level.

Some of the fungi obtained from red ginger in this experiment such as *Acremonium, Curvularia, F. solani, G. cingulata,* and *Talaromyces* had been reported as endophytic on important agricultural plants such as *Zea mays, Theobroma cacao,* and *Theobroma grandiflorum* (Hadane *et al*. 2010; Banerjee 2011). There was no report on *Beltraniella* and *L. kalimantanense* obtained in this experiment however, as endophytic fungi particularly on Zingiberaceae (Bussaban *et al*. 2001). *C. gloeosporiodes* was the most frequent endophytes in the plants study. *C. gloeosporiodes* was found in stem and leaf, whereas *G. cingulata*, teleomorph of *C. gloeosporoides* was found in rhizome of red ginger plant. This results supported the data reported by Bussaban *et al*. (2001) which found that *C. gloeosporoides* and its teleomorphic stage *Glomerella* spp. and *Phomopsis* spp. were dominant endophytes on wild ginger *Amomum siamense*. Furthermore, Chen *et al*. (2011) reported that *Glomerella* and *Colletotrichum* were the most isolated strain in medicinal plant *Huperzia serrata* in China, whereas Khan *et al*. (2010) found that *Aspergillus alternata* was the most dominant endophyte in medicinal plant *Withania somnifera*.

In this experiment, *R. vagum* was isolated specifically on root, whereas *L. kalimantanense* and *M. verrucaria* were discovered specifically on stem. Beside that, *A. macroclavatum* determined specifically from under ground organs and *C. geniculatus* found from shoot or above ground organs of red ginger plant.

Antagonistic Activity of Isolated Endophytic Fungi. In this study, *C. gloeosporioides* and its teleomorph *G. cingulata* were dominant endophyte fungi and these fungi had high ability to control the growth of plants pathogenic fungi, *F. oxysporum*. Gong and Guo (2009) reported that *Fusarium* spp. was the most dominant genus in medicinal plants *Dracaena cambodiana* and *Aquilaria sinensis* in China and this fungi showed the most potent antimicrobial activity. In addition, Kishore *et al.* (2007) reported that crude extract of *G. cingulata* had potential activity to control the growth of *Rhizopus oryzae*, *Chrysoporium tropicum*, and *Beauveria bassiana* but no antifungal activity against *Alternaria tenuissima* and *Aspergillus niger*.

The inhibition ability of fungal isolates against *F. oxysporum* was not affected by the origin of plant organs as source of endophytic fungi isolation. All endophytic fungi were able to inhibit growth of pathogenic fungi *F. oxysporum* tested, and the inhibition activities were varied from low to high (Table 4). These results indicated that all red ginger plant organs harbor diverse endophytic fungi and their inhibition effects on growth of *F. oxysporum* were also varied. This finding showed that not only rhizome of red ginger inhabited by endophytic fungi with high antifungal activities, but also other organs. Therefore, this is important results for fungal endophytic study on medicinal plant for targeting novel bioactive compound. The isolation of targeted endophytic fungi are not necessarily carried out from plant organ used in herb which commonly called as functional organ of the host plant studied.

The functions of endophytic fungi grown in the symbiosis inside the host plant are protecting host plants from fungal pathogenic attack by direct and indirect mechanisms. The direct mechanism is though direct interaction between the endophyte with fungal pathogen and occupying ecological niche, while the indirect mechanism is by increasing plant resistance. In direct interaction mechanism, the endophyte produces antibiotics and lytic enzymes that will suppress growth or kill pathogens. Some endophytic fungi grown in synthetic medium produced secondary metabolites that were powerful in suppressing the growth of pathogenic fungi (Gunatilaka 2006). This paper shows that red ginger plant is occupied by diverse endophytic fungi in all part of the plant including functional organ that usually used in traditional medicine. Furthermore, the endophytic fungi are potential sources of antifungal compound, particularly for controlling plant pathogenic fungi, *F. oxysporum*.

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