



Growth Response of Four Leguminous Trees to Native Arbuscular Mycorrhizal Fungi from Tropical Forest in Indonesia

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AFM, MT, TS and KT designed and performed the experiments. Authors AFM, MT, TS, YH, WC and KT drafted the manuscript and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To isolate and identify arbuscular mycorrhizal fungi (AMF) from forest soils in Indonesia and to clarify their influence on the growth of four leguminous tree species.

Study Design: Isolation of AMF from five forest soils in Indonesia and inoculation of five AMF to four leguminous trees under pot culture condition.

Place and Duration of Study: Laboratory of Plant Nutrition and Soil Science, Faculty of Agriculture, Yamagata University, between 2015 to 2016.

Methodology: Soils were collected from five forests in Indonesia. *Paraserianthes falcataria*,

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Calliandra calothyrsus, and *Cassia siamea* were grown on each soil for 90 days. Sixty-one spore morphotypes were collected from the soil and propagated using the respective tree species. Propagated spores were identified and used in the inoculation of *P. falcataria*, *C. calothyrsus*, *C. siamea*, and *Sesbania grandiflora*. Shoot fresh weight (SFW), root fresh weight, shoot P concentration, and AM colonization rate were measured 60 days after the inoculation. Mycorrhizal dependency (MD) was calculated on the basis of SFW.

Results: Five isolates were identified: *Diversispora gibbosa* (M10-2), *Acaulospora* sp. (M11-1), Glomeraceae sp. (M44-3), *Ambispora appendicula* (M60-3), and *Glomus* sp. (S6-4). Mycorrhizal colonization ranged from 0% to 99%. SFW and shoot P content of the four leguminous trees inoculated with S6-4 were higher than those of trees inoculated with other AMF. The four leguminous trees inoculated with S6-4 showed higher MD than the trees inoculated with other AMF. *P. falcataria* and *C. calothyrsus* showed high MD upon inoculation with AMF.

Conclusion: Colonization by native AMF differed by leguminous tree and growth response differed depending on the AMF isolate.

Keywords: Arbuscular mycorrhizal fungi; leguminous tree; mycorrhizal dependency; tropical forest.

1. INTRODUCTION

Deforestation by land-use conversion into agricultural fields and plantations, open cast mining, and illegal logging is increasing in Indonesia [1]. Reforestation of tropical forests, such as those in Indonesia, requires human assistance to recover forest structure and species composition [2]. Rather than relying on natural forest recovery, human assistance would accelerate the speed of reforestation.

There are few methods for the remediation of degraded forests in Indonesia. The utilization of arbuscular mycorrhizal fungi (AMF), which can support plant growth through several mechanisms [3], is beneficial for plant survival in a degraded forest. Moreover, AMF domination in tropical forest suggests that AMF play an important role in tropical forest [4]. The utilization of AMF of some tree species to support reforestation efforts in Indonesia has been reported [5,6].

The effectiveness of AMF in promoting plant growth has been shown to vary [7]. AMF isolate and plant species are the main factors determining plant response to inoculation with AMF. Thus, the selection of the appropriate AMF isolate for a certain plant species is a strategy that would guarantee the success of reforestation efforts. Klironomos [8] found that native AMF isolates were more effective in promoting growth of local plants than foreign isolates. Therefore, the utilization of local AMF isolates for reforestation is important.

The selection of plant species is also a strategy for successful reforestation. After deforestation,

there are pioneer species that will grow naturally and cover deforested land. With human help, plant species composition could be adjusted to achieve faster reforestation [2]. In addition, problems related to environmental factors limiting plant growth, for example, low available P in soil, can be solved.

Leguminous trees are candidate plants for reforestation. *Paraserianthes falcataria* (L.) Nielsen, *Calliandra calothyrsus* Meisn., *Cassia siamea* (Lam.) Irwin et Barneby, and *Sesbania grandiflora* (L.) Poiret are common species in Indonesia. These are fast-growing species that serve many purposes. *P. falcataria* is profitable in a mixed plantation with crop species or a single-species plantation [9]. The wood of *P. falcataria* is a candidate for energy production [10]. *C. calothyrsus* is a source of high-protein forage and an intercropping plant with crop species [11]. *C. siamea* is a fallow tree and a good mulch for crop plant [12]. *S. grandiflora* is a candidate species for remediating Pb/Zn and Cu mine tailings [13]. The appropriate AMF isolates for these leguminous trees have not been isolated. The objectives of this study were to isolate AMF using these trees as trap plants and to clarify the effectiveness of the AMF isolates in promoting growth of these leguminous trees.

2. MATERIALS AND METHODS

2.1 Forest Soil Collection

Soils were collected from five forests in Indonesia with five replications in September 2012 (Table 1). Soils between 0–10 cm soil depth and within 0–5 cm distance from root of representative seedling or around the main stem

of representative tree species were collected after removing the organic layer. Two kilograms of soil was collected in one replication. The distance between replications in one forest was ≥ 20 m. The latitudinal coordinates of each forest were recorded.

2.2 Isolation of AMF from Forest Soil

Sand was acidified and sterilized by autoclaving at 80°C for 45 min. Forty (40) grams of sterilized sand was mixed with 40 g of forest soil and used as medium. Seeds of *C. calothyrsus*, *P. falcataria*, and *C. siamea* were sown on the sterilized sand and incubated in a growth chamber (Biotron LPH-350S, NK System, Japan) at 27°C with a 16-h photoperiod. One 2-leaf-stage seedling of each plant was transplanted onto the medium in a 50 mL syringe pot. Ten grams of sterilized sand was further added into the syringe pot to cover the seedling root system. All plants were grown in the growth chamber for 90 days at 27°C with a 16-h photoperiod. Five to ten milliliters of 1 mg L⁻¹ P nutrient solution (based on [14], hereinafter P1 solution) was applied once every 2 days. Twenty-five (25) pots were prepared for each plant species. Ninety (90) days after transplanting (DAT), application of P1 solution was stopped to induce sporulation. After another 30 days, plant roots along with the medium were collected. Spores were extracted

from the medium by wet sieving and decanting [15]. Spores were collected with forceps and grouped on the basis of spore diameter and color.

2.3 Spore Propagation and Observation of Morphological Characteristics

One to twenty (20) spores were used to inoculate respective plant species from which the spore was isolated. Plants were grown under the same conditions as those for the isolation experiment. Spores were extracted from the medium by wet sieving and decanting and mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG) and in a mixture of PVLG and Melzer's reagent. Spores were observed under a compound microscope (Eclipse 80i, Nikon, Japan).

2.4 DNA Extraction and Amplification

Spores of AMF were collected with forceps and put on the lid of a plastic tube containing 20 μ L of InstaGene™ Matrix (Bio-Rad, USA). The spores were crushed with the blunt end of a pipet tip, further mixed with 180 μ L of InstaGene™ Matrix, and vortexed. rDNA was extracted following the manufacturer's protocol for InstaGene™ Matrix. The extracted DNA was stored at -20°C until use.

Table 1. Forest sites where soils were collected

No	Forest	Location	Latitude	Remarks
1	Teak (<i>Tectona grandis</i> Linn. f.) monoculture forest	East Java, Java Island	07° 20.891' S, 111° 19.981' E	Planted in 1968 as a seed production area (SPA) of Perum Perhutani, a state-owned company
2	Mahogany (<i>Swietenia macrophylla</i> King) monoculture forest	Special Region of Yogyakarta, Java Island	07° 54.578' S, 110° 31.088' E	Planted in 1969 as a reforestation effort in Karst landscape
3	<i>Gmelina arborea</i> , <i>Artocarpus champeden</i> , and Dipterocarp mixed forest	East Kalimantan, Kalimantan Island	00° 59.050' S, 116° 55.228' E	Dipterocarp forest enriched in non-Dipterocarp species. Research forest of Wanariset Tropical Forest Research Station
4	Dipterocarp primary forest	East Kalimantan, Kalimantan Island	00° 59.363' S, 116° 56.792' E	Research forest of Wanariset Tropical Forest Research Station
5	<i>Macaranga</i> sp. secondary forest	East Kalimantan, Kalimantan Island	00° 59.519' S, 116° 57.241' E	<i>Macaranga</i> sp. grew naturally in Dipterocarp forest after forest fire. Research forest of Wanariset Tropical Forest Research Station

The sequence of the D1/D2 region was amplified using eukaryotic-specific primer LR1 (5'-GCATATCAATAAGCGGAGGA-3') [16] and fungal-specific primer FLR2 (5'-GTCGTTTAAAGCCATTACGTC-3') [17], with an Expand High Fidelity^{PLUS} PCR system (Roche, Germany) using the following composition: 4 µL of 5 x buffer with MgCl₂, 0.2 µL of DNA polymerase, 2 µL of 2.0 mM dNTP, 0.4 µL of ITS1F, 0.4 µL of ITS1R, 11 µL of MilliQ water, and 2 µL of DNA template. The reaction was performed in a Takara PCR Thermal Cycler Dice (Model TP600, Takara Bio, Japan) under the following conditions: initial denaturation at 94°C for 120 s; 30 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 60 s, and extension at 72°C for 80 s; and a final extension at 72°C for 600 s. The PCR products were separated on 1.0% agarose gel (D1 Agarose Low EEO, Conda, Spain) in 1x Tris-borate-EDTA buffer, stained with SYBR® Safe DNA Gel Stain (Invitrogen, USA), and viewed under blue light (470 nm, MBP-LED, Bio-Pyramid, USA). PCR-amplified fragments were purified using a MonoFas DNA Purification Kit (GL Science, Japan) following the manufacturer's protocol. Purified DNA was ligated into pT7Blue T-Vector (Novagen, USA) using a DNA Ligation Kit Ver 1 (Takara Bio, Japan). Twenty (20) microliters of IPTG (Takara Bio, Japan) and 35 µL of X-Gal (Takara Bio, Japan) were applied to Luria Bertani (LB) medium containing 100 mg L⁻¹ ampicillin. T-vector containing DNA was transformed into *Escherichia coli* JM109 (Takara Bio, Japan) by plating onto this LB medium. Plates with *E. coli* were incubated at 37°C for 16 hours.

Single colonies of *E. coli* were collected and DNA was amplified using primers T7 (5'-TAATACGACTCACTATAG-3') and U19 (5'-GTTTTCCAGTCACGACT-3'), with GoTaq® DNA Polymerase (Promega, USA) using the following composition: 2 µL of 5 x reaction buffer, 0.05 µL of DNA polymerase, 0.8 µL of 2.0 mM dNTP, 0.2 µL of T7, 0.2 µL of U19, 6.75 µL of MilliQ water, and a single colony of *E. coli*. The reaction was performed in a Takara PCR Thermal Cycler Dice (Model TP600, Takara Bio, Japan) under the following conditions: initial denaturation at 94°C for 120 s; 35 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 60 s, and extension at 72°C for 80 s; and a final extension at 72°C for 600 s. The PCR products were separated on 1.0% agarose gel in 1x Tris-borate-EDTA buffer, stained with SYBR® Safe DNA Gel Stain, and viewed under blue

light. The PCR products were used for sequencing.

Sequencing reactions were performed in a Bio-Rad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using an ABI BigDyeTM Terminator v3.1 Cycle Sequencing Kit with AmpliTaq DNA Polymerase (FS enzyme, Applied Biosystems, Japan) following the manufacturer's protocol. Single pass sequencing was performed on each DNA template using a T7 promoter. Fluorescent-labeled fragments were purified from the unincorporated terminators by adopting an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems, Japan).

2.5 Phylogenetic Analyses

Sequences of AMF were submitted for BLAST analysis [18]. The sequences and their corresponding BLAST top hits were aligned by MUSCLE [19]. The maximum likelihood method was performed by MEGA 7 (www.megasoftware.net) with 1000 replications of bootstrap analysis.

2.6 Screening for Effective AMF

Propagated spores were used in a screening experiment to clarify the effectiveness of AMF in promoting plant growth. Sand from spore propagation pot containing spore, hyphae, and colonized root was used as inoculum. Inoculum containing approximately 50 spores was mixed with 60 g of sterilized sand. One 2-leaf-stage seedling each of *C. calothyrsus*, *P. falcataria*, *C. siamea*, and *S. grandiflora* was transplanted onto inoculated or non-inoculated sand in a 50 mL syringe pot. Thirty (30) grams of sterilized sand was further added to cover the root system. Four replication pots each were prepared for inoculated and non-inoculated treatments. Plants were grown in the growth chamber at 27°C with a 16-hour photoperiod for 60 DAT. Ten milliliters of P1 solution was applied once every 2 days.

2.7 Plant Growth Parameters

Initial and final plant heights, number of leaves, shoot and root fresh weights, and shoot P concentration were measured after harvest. Fifty (50) to one hundred (100) milligrams of dried shoot samples was prepared for digestion. An acid mixture of HNO₃, HClO₄, and H₂SO₄ (5:2:1, v/v/v) was added to dried shoot in a digestion tube (Actac, Japan), and digestion was carried

out by heating at 200°C for 60 min. Shoot P concentration in the solution of digested shoot was determined colorimetrically with the vanadomolybdate-yellow assay [20] using a spectrophotometer at 410 nm absorbance (Hitachi, U-2900). Shoot P content was calculated by multiplying shoot P concentration by shoot dry weight. Mycorrhizal dependency (MD) of each plant species to AMF inoculation was calculated on the basis of shoot fresh weight (SFW) according to [21].

2.8 Assessment of Arbuscular Mycorrhizal Colonization

Roots were stained with aniline blue dye as described by [22]. First, the roots were cleaned by dipping into 10% (w/v) KOH solution and heated in a water bath at 80°C for 15 min or 5 min. Then, the roots were rinsed with tap water, acidified with 1% (w/v) HCl, and rinsed again with tap water. Finally, the roots were dipped into 0.05% aniline blue solution (Aniline blue, Wako, Japan) and heated again at 90°C for 5 min. After rinsing with tap water, the roots were transferred to a Petri dish and lactic acid-glycerol solution was added. Colonization was estimated by the gridline intersect method on 100 intersections [23].

2.9 Statistical Analysis

Statistical significance of AMF inoculation for plant growth was analyzed by one-way analysis of variance (ANOVA). Post hoc analysis was performed using the Tukey HSD test of Kaleida Graph 4.1 software (Synergy software 2012, USA). Three to four from initially four replication (See section 2.6) were survived until harvest and used for statistical analysis.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Propagation and identification of AMF isolates

Thirty-two (32), 14, and 15 spore morphotypes were collected from soils of *P. falcataria*, *C. calothyrsus* and *C. siamea*, respectively, and the spores were propagated using the respective tree species. Only five isolates produced new spores: isolates M10-2, M11-1, and S6-4 from *Gmelina arborea*, *Artocarpus champeden*, and Dipterocarp mixed forest with *P. falcataria* as trap plant; isolate M44-3 from *Macaranga* sp. secondary forest with *C. calothyrsus* as trap

plant; and isolate M60-3 from *Macaranga* sp. secondary forest with *C. siamea* as trap plant. Isolate S6-4 showed distinctive morphological characteristics by forming intraradical spores (Fig. 1).

Part of the LSU region of five AMF isolates were sequenced and aligned with reference sequences (Fig. 2). Based on the similarity of the 16 clones of each isolate with the reference sequences, the isolates were assigned to 4 families: Glomaceae (*Glomus* sp. isolate S6-4, Glomaceae sp. isolate M44-3), Acaulosporaceae (*Acaulospora* sp. isolate M11-1), Diversisporaceae (*Diversispora gibbosa* isolate M10-2) and Ambisporaceae (*Ambispora appendicula* isolate M60-3).

3.1.2 AMF colonization rate in root of leguminous tree species

Roots of *P. falcataria* were colonized by isolates M10-2 (66%), M11-1 (87%), and S6-4 (99%) (Table 2). Roots of *C. calothyrsus* were colonized by M10-2 (30%), M11-1 (53%), M60-3 (43%), and S6-4 (97%). Roots of *C. siamea* were colonized by M10-2 (8%), M11-1 (15%), M60-3 (28%), and S6-4 (91%). Roots of *S. grandiflora* were colonized by M11-1 (18%), M44-3 (3%), and S6-4 (84%). No colonization was detected in the roots of control treatment of all leguminous trees. No nodulation was observed in the roots of all leguminous trees after harvest.

3.1.3 Effect of AMF inoculation on plant growth

Plant height increment of *P. falcataria* and *C. siamea* inoculated with AMF was not significantly different from that of control plant (Table 2). Plant height increment of *C. calothyrsus* inoculated with M60-3 was 2.9-3.1 fold larger than that of *C. calothyrsus* inoculated with M10-2 or M44-3, but was not different with that of the other inoculated plants and the control plant. Plant height increment of *S. grandiflora* inoculated with S6-4 was 1.9 fold larger than that of control plant and 1.5 fold larger than that of *S. grandiflora* inoculated with M10-2 or M44-3, but was not different with that of other inoculated plants.

Number of leaves of *P. falcataria* inoculated with M11-1 was 1.5 fold larger than that of control plant, but was not different with that of other inoculated plants (Table 2). Number of leaves of *C. calothyrsus* inoculated with M11-1 or M60-3 was 1.8-2.1 fold larger than that of the other

inoculated plants and 6.0-6.3 larger than that of control plant. Number of leaves of *C. siamea* inoculated with S6-4 was not different with that of *C. siamea* inoculated with M10-2, but was 1.5-1.8 fold larger than that of the other inoculated

plants and the control plant. Number of leaves of *S. grandiflora* inoculated with S6-4 was 1.4-1.7 fold larger than that of control plant and *S. grandiflora* inoculated with M11-1, but was not different with that of other inoculated plants.

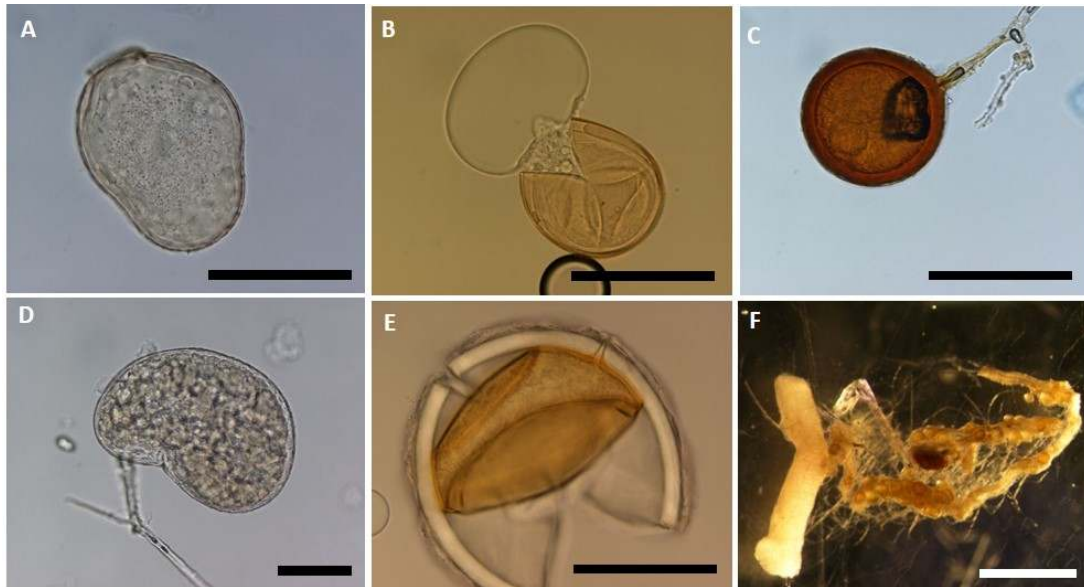


Fig. 1. Morphological characteristics of spores of M10-2 (A), M11-1 (B), M44-3 (C), M60-3 (D), and S6-4 (E), and intraradical spore of S6-4 (F). Black bar = 100 µm. White bar = 1000 µm

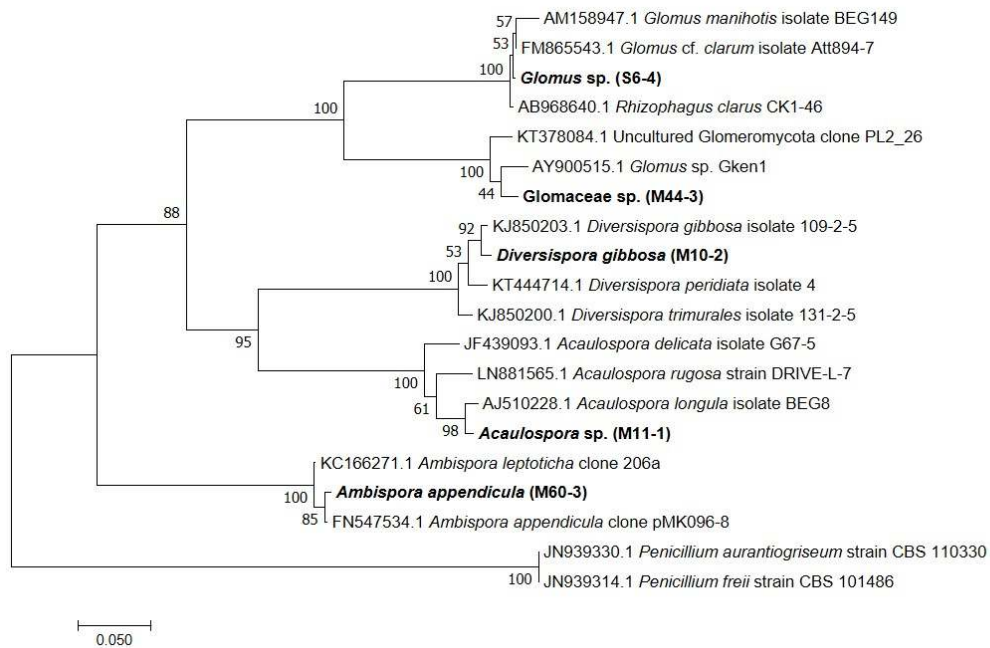


Fig. 2. Phylogenetic tree of five AMF isolates obtained by maximum likelihood analysis of LSU region of the ribosomal gene. The new sequences of the five AMF isolates are indicated in bold font. Bootstrap values are shown below the branch (1000 replications)

Table 2. AMF colonization in the root and growth response of four leguminous trees inoculated with or without AMF

Plant species	AMF isolate	Colonization (%)			Plant height increment (cm)			Leaf number (leaves/plant)			Shoot FW (mg/plant)			Root FW (mg/plant)			Shoot P concentration (mg/g)			Shoot P content (mg/plant)									
<i>Paraserianthes falcataria</i>	Control	0	±	0	c	1.2	±	0.2	a	6	±	0.6	b	399.4	±	60.5	b	812.5	±	98.9	a	0.89	±	0.08	b	0.11	±	0.02	c
	M10-2	66	±	4	b	1.4	±	0.1	a	8	±	0.3	ab	552.5	±	40.6	b	584.3	±	37.6	ab	1.39	±	0.14	a	0.21	±	0.01	b
	M11-1	87	±	2	a	1.7	±	0.1	a	9	±	0.9	a	973.6	±	64.2	a	789.0	±	17.3	a	1.31	±	0.04	a	0.36	±	0.02	a
	M44-3	0	±	0	c	1.3	±	0.2	a	7	±	0.3	ab	394.2	±	41.9	b	658.5	±	50.6	ab	0.79	±	0.04	b	0.10	±	0.01	c
	M60-3	0	±	0	c	1.2	±	0.1	a	7	±	0.3	ab	359.3	±	25.9	b	483.4	±	56.5	b	0.88	±	0.05	b	0.09	±	0.00	c
	S6-4	99	±	1	a	1.6	±	0.1	a	8	±	0.5	ab	1011.6	±	127.5	a	806.5	±	76.0	a	1.50	±	0.11	a	0.45	±	0.02	a
	Mean	42	±	1		1.4	±	0.1		7	±	0.5		615.1	±	60.1		689.0	±	56.2		1.13	±	0.08		0.22	±	0.01	
<i>Calliandra calothyrsus</i>	Control	0	±	0	c	1.5	±	0.5	ab	1	±	0.3	b	279.2	±	23.9	b	433.6	±	53.8	a	0.72	±	0.05	b	0.05	±	0.00	b
	M10-2	30	±	11	bc	1.1	±	0.2	b	5	±	0.6	b	438.6	±	32.1	ab	457.7	±	41.3	a	1.23	±	0.03	a	0.16	±	0.01	a
	M11-1	53	±	13	b	2.2	±	0.4	ab	9	±	1.2	a	653.9	±	92.2	ab	671.6	±	83.6	a	0.90	±	0.08	ab	0.14	±	0.03	ab
	M44-3	0	±	0	c	1.0	±	0.3	b	4	±	0.6	b	461.5	±	143.4	ab	497.1	±	129.9	a	1.10	±	0.12	ab	0.14	±	0.03	ab
	M60-3	43	±	6	b	3.2	±	0.3	a	8	±	0.8	a	714.6	±	100.8	a	750.2	±	88.6	a	0.94	±	0.09	ab	0.15	±	0.02	ab
	S6-4	97	±	1	a	2.6	±	0.4	ab	4	±	0.5	b	751.7	±	53.3	a	444.6	±	65.1	a	1.20	±	0.16	a	0.20	±	0.03	a
Mean	37	±	5		1.9	±	0.4		5	±	0.7		549.9	±	74.3		542.4	±	77.1		1.01	±	0.09		0.14	±	0.02		
<i>Cassia siamea</i>	Control	0	±	0	d	2.5	±	0.5	a	5	±	0.3	b	494.3	±	23.9	bc	568.5	±	53.8	a	0.43	±	0.05	c	0.07	±	0.00	b
	M10-2	8	±	11	cd	2.6	±	0.2	a	6	±	0.6	ab	710.1	±	32.1	b	536.2	±	41.3	ab	0.83	±	0.03	a	0.22	±	0.01	a
	M11-1	15	±	13	bc	2.9	±	0.4	a	5	±	1.2	b	441.0	±	92.2	bc	370.2	±	83.6	ab	0.66	±	0.08	ab	0.09	±	0.03	b
	M44-3	0	±	0	d	2.0	±	0.3	a	6	±	0.6	b	329.6	±	143.4	c	349.7	±	129.9	b	0.79	±	0.12	a	0.10	±	0.03	b
	M60-3	28	±	6	b	2.7	±	0.3	a	6	±	0.8	b	720.2	±	100.8	abc	502.7	±	88.6	ab	0.48	±	0.09	bc	0.12	±	0.02	b
	S6-4	91	±	1	a	2.7	±	0.4	a	8	±	0.5	a	1126.6	±	53.3	a	579.5	±	65.1	a	0.57	±	0.16	bc	0.24	±	0.03	a
Mean	24	±	5		2.6	±	0.4		6	±	0.7		636.9	±	74.3		484.5	±	77.1		0.63	±	0.09		0.14	±	0.02		
<i>Sesbania grandiflora</i>	Control	0	±	0	c	1.8	±	0.4	b	2	±	0.3	b	770.9	±	35.8	b	1247.9	±	92.4	a	0.59	±	0.06	c	0.12	±	0.02	c
	M10-2	0	±	0	c	2.4	±	0.1	b	3	±	0.0	ab	966.5	±	51.9	ab	1293.1	±	75.9	a	1.11	±	0.10	ab	0.24	±	0.02	ab
	M11-1	18	±	6	b	2.4	±	0.2	ab	3	±	0.3	b	808.7	±	86.4	ab	729.1	±	106.8	b	0.79	±	0.07	bc	0.18	±	0.02	bc
	M44-3	3	±	2	c	2.3	±	0.2	b	3	±	0.0	ab	947.1	±	93.0	ab	1365.6	±	43.0	a	1.27	±	0.12	ab	0.26	±	0.02	ab
	M60-3	0	±	0	c	2.5	±	0.1	ab	3	±	0.0	ab	965.1	±	103.2	ab	1327.6	±	89.3	a	1.32	±	0.14	a	0.28	±	0.01	ab
	S6-4	84	±	3	a	3.5	±	0.2	a	4	±	0.3	a	1137.2	±	42.6	a	1032.0	±	128.0	ab	0.97	±	0.14	abc	0.31	±	0.04	a
Mean	17	±	2		2.5	±	0.2		3	±	0.1		932.6	±	68.8		1165.9	±	89.2		1.01	±	0.11		0.23	±	0.02		

*Different letters indicate significant difference within the same plant species (Tukey HSD test, $P < .05$, $n = 3-4$)

SFW of *P. falcataria* inoculated with S6-4 or M11-1 was 1.8-2.8 fold higher than that of the other inoculated plants and the control plant (Table 2). SFW of *C. calothyrsus* inoculated with S6-4 or M60-3 was not different with that of the other inoculated plants, but was 2.6-2.7 fold higher than that of control plant. SFW of *C. siamea* inoculated with S6-4 was not different with that of *C. siamea* inoculated with M60-3, but was 1.6-3.4 fold higher than that of the other inoculated plants and the control plant. SFW of *S. grandiflora* inoculated with S6-4 was not different with that of other inoculated plants, but was 1.5 fold higher than that of control plant.

Root fresh weight (RFW) of control *P. falcataria* was not different with that of *P. falcataria* inoculated with M10-2, M11-1, M44-3 or S6-4, but was 1.7 fold higher than that of *P. falcataria* inoculated with M60-3 (Table 2). RFW of control *C. calothyrsus* was not different with the RFW of all inoculated plants. RFW of control *C. siamea* was not different with that of *C. siamea* inoculated with M10-2, M11-1, M60-3 or S6-4, but was 1.6 fold higher than that of *C. siamea* inoculated with M44-3. RFW of control *S. grandiflora* was not different with that of *S. grandiflora* inoculated with M10-2, M44-3, M60-3 or S6-4, but was 1.7 fold higher than that of *S. grandiflora* inoculated with M11-1.

Shoot P concentration of *P. falcataria* inoculated with M10-2, M11-1 or S6-4 was 1.5-1.9 fold higher than that of the other inoculated plants and the control plant (Table 2). Shoot P concentration of *C. calothyrsus* inoculated with M10-2 or S6-4 was not different with that of the other inoculated plants, but was 1.7 fold higher than that of control plant. Shoot P concentration of *C. siamea* inoculated with M10-2 or M44-3 was not different with that of *C. siamea* inoculated with M11-1, but was 1.4-2.0 fold higher than that of the other inoculated plants and the control plant. Shoot P concentration of *S. grandiflora* inoculated with M60-3 was the same as *S. grandiflora* inoculated with M10-2, M44-3 or S6-4 but 1.7-2.3 fold higher than other inoculated plant and the control plant.

Shoot P content of *P. falcataria* inoculated with S6-4 or M11-1 was 1.7-4.9 fold higher than that of the other inoculated plants and the control plant (Table 2). Shoot P content of *C. calothyrsus* inoculated with M10-2 or S6-4 was not different with that of the other inoculated plants, but was 3.6-4.4 fold higher than that of control plant. Shoot P content of *C. siamea* inoculated with

M10-2 or S6-4 was 1.8-3.2 fold higher than that of the other inoculated plants and the control plant. Shoot P content of *S. grandiflora* inoculated with S6-4 was not different with that of *S. grandiflora* inoculated with M10-2, M44-3 or M60-3, but was 1.7-2.6 fold higher than that of the other inoculated plants and the control plant.

3.2 Discussion

3.2.1 AMF isolation using leguminous trees from tropical forest in Indonesia

Five AMF morphotypes (Fig. 1) were successfully propagated from 61 spore morphotypes collected after trap culture. These five AMF were *D. gibbosa* (M10-2), *Acaulospora* sp. (M11-1), *Glomeromycota* sp. (M44-3), *A. appendicula* (M60-3), and *Glomus* sp. (S6-4) (Figs. 1 and 2). Isolate M10-2 closely matched *D. gibbosa* isolated by [24] from maritime sand dunes in Poland. Blaszkowski [24] described this species as *Glomus gibbosum*. Present study is the first to isolate and identify *D. gibbosa* from tropical forest soil. Isolate M60-3 closely matched *A. appendicula* (basonym= *Acaulospora appendicula*) isolated by Spain, Sieverd, and Schenk in 1984 [25]. *A. appendicula* was also isolated by [26] from the rhizosphere of a threatened native leguminous tree, *Pericopsis mooniana*, growing in a natural forest in Sulawesi Island, Indonesia. Isolates M44-3 and S6-4 closely matched *Glomus* species but were separated into different groups. Isolate S6-4 closely matched *Glomus* cf. *clarum* used in the experiment of [27]. Unfortunately, no clear information about the isolation of *Glomus* cf. *clarum* is available. Isolate S6-4 also closely matched *Glomus manihotis* found in the roots of sweet potato in China [28]. M11-1 closely matched *Acaulospora longula*. However, no details were provided regarding the isolation of *A. longula*.

3.2.2 Ability of AMF to promote leguminous tree growth

Two isolates, M11-1 and S6-4, colonized the four leguminous trees (Table 2). The other isolates colonized one to three leguminous trees. The results indicate that these AMF isolates have host preference. AMF host preference was addressed by [8], who inoculated ten plant species with 10 AMF isolates (two *Acaulospora*, two *Gigaspora*, four *Glomus*, and two *Scutellospora* species) and observed that the plants were either colonized or not colonized by AMF. As examples, *Glomus geosporum* and

Acaulospora morrowiae colonized eight and seven of the ten plant species, respectively. These results highlight the host preference of AMF.

S6-4 increased SFW and shoot P content of all leguminous trees and the shoot: root ratio of all leguminous trees except *C. calothyrsus*, compared to respective controls (Tables 2 and 3). S6-4 also promoted plant height increment of *S. grandiflora* and increased number of leaves of *C. siamea* and *S. grandiflora*. In contrast to trees inoculated with other AMF, trees inoculated with S6-4 showed similar or higher values of SFW, shoot P content, shoot: root ratio, and plant height. This may be due to the higher colonization rate of S6-4 (84–99%) than the other AMF. Based on the phylogenetic tree, S6-4 was considered as *Glomus* species. These results were in agreement with other studies that documented the ability of *Glomus* species to

promote leguminous tree growth. Kung'u et al [29] revealed that *Glomus etunicatum* and *Glomus macrocarpum* increased shoot dry weight, plant height, and number of leaves of *Senna spectabilis*. Guissou et al. [30] showed that *Glomus aggregatum* increased total dry weight and plant height of *Tamarindus indica*. Wulandari et al. [31] reported that *Glomus clarum* increased shoot dry weight, shoot P content, shoot height, and number of leaves of *Albizia saman*. However, isolate M44-3, which belongs to the same Glomaceae family as S6-4, showed the opposite result. M44-3 showed lower colonization rate (0–3%) than the other AMF isolates. All leguminous trees inoculated with M44-3 showed similar SFW, shoot: root ratio, plant height, and number of leaves to their respective controls. M44-3 increased only shoot P content of *S. grandiflora*. There were differences in the ability of Glomaceae species to promote leguminous tree growth.

Table 3. Shoot: root ratio and mycorrhizal dependency of four leguminous trees inoculated with or without AMF

Plant species	AMF isolate	Shoot: root ratio			Mycorrhizal dependency (%)		
<i>Paraserianthes falcataria</i>	Control	0.49	±	0.03	c	n.d.	
	M10-2	0.97	±	0.11	ab	28	± 11 ab
	M11-1	1.23	±	0.06	a	59	± 6 a
	M44-3	0.60	±	0.02	bc	-1	± 15 b
	M60-3	0.77	±	0.10	bc	-11	± 17 b
	S6-4	1.26	±	0.15	a	61	± 6 a
	Mean	0.89	±	0.08		27	± 11 xy
<i>Calliandra calothyrsus</i>	Control	0.67	±	0.12	b	n.d.	
	M10-2	0.96	±	0.04	b	36	± 5 c
	M11-1	0.97	±	0.06	b	57	± 4 ab
	M44-3	0.89	±	0.07	b	39	± 5 bc
	M60-3	0.95	±	0.07	b	61	± 3 a
	S6-4	1.76	±	0.16	a	63	± 3 a
	Mean	1.03	±	0.09		51	± 4 x
<i>Cassia siamea</i>	Control	0.89	±	0.12	b	n.d.	
	M10-2	1.31	±	0.10	ab	30	± 9 ab
	M11-1	1.16	±	0.37	b	-12	± 15 bc
	M44-3	0.98	±	0.10	b	-50	± 19 c
	M60-3	1.43	±	0.06	ab	31	± 9 ab
	S6-4	1.96	±	0.14	a	56	± 6 a
	Mean	1.29	±	0.15		11	± 12 y
<i>Sesbania grandiflora</i>	Control	0.63	±	0.07	a	n.d.	
	M10-2	0.76	±	0.08	a	20	± 4 ab
	M11-1	1.18	±	0.20	a	5	± 4 b
	M44-3	0.69	±	0.06	a	19	± 4 ab
	M60-3	0.74	±	0.09	a	20	± 4 ab
	S6-4	1.18	±	0.22	a	32	± 3 a
	Mean	0.86	±	0.12		19	± 4 y

*Different letters indicate significant difference within the same plant species (Tukey HSD test, $P < .05$, $n = 3-4$)

M11-1, which was considered an *Acaulospora* species, increased number of leaves of *P. falcataria* and *C. calothyrsus*, and SFW, shoot P content, and shoot: root ratio of *P. falcataria*. Kumar et al. [32] showed that *Acaulospora scrobiculata* increased plant height and shoot dry weight of a leguminous tree, *Leucaena leucocephala*. Other isolates also enhanced the growth of some leguminous trees. M10-2, which was considered as *D. gibbosa*, increased shoot P content of all leguminous trees. These results indicated that not only *Glomus* species but also other AMF isolates promoted leguminous tree growth depending on the leguminous tree species.

3.2.3 Response of leguminous trees to AMF inoculation

Positive correlations between colonization rate and shoot P content were observed for *P.*

falcataria ($R^2 = 0.87$, $P < .001$), *C. calothyrsus* ($R^2 = 0.41$, $P < .001$), *C. siamea* ($R^2 = 0.39$, $P = .0011$), and *S. grandiflora* ($R^2 = 0.13$, $P = .048$) (Fig. 3). *P. falcataria* showed higher correlation between colonization rate and shoot P content than the other leguminous trees. Smith and Smith [33] discussed in a review paper plant P uptake as a response to AMF colonization. This response was found to range from negative to positive depending on the plant species and the AMF isolate. We found that *P. falcataria* showed better response to AMF inoculation than the other leguminous trees, particularly in terms of shoot P uptake.

Despite the positive correlation between colonization rate and shoot P content for *P. falcataria*, *C. calothyrsus*, and *C. siamea*, the correlation varied among the different AMF. The colonization rates of the different AMF did not always correspond to shoot P content, such as

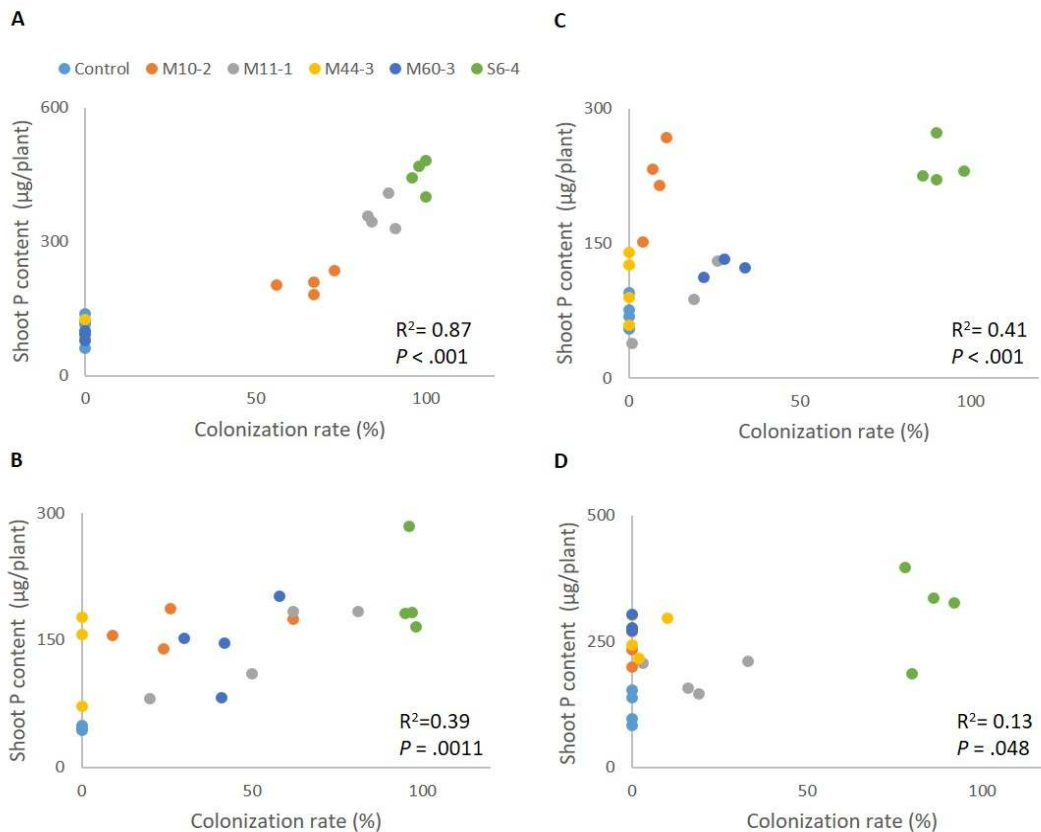


Fig. 3. Correlation between colonization rate and shoot P content of *P. falcataria* (A), *C. calothyrsus* (B), *C. siamea* (C), and *S. grandiflora* (D) inoculated with or without AMF isolates, at 60 days after transplanting

those of M10-2 and M11-1 in *C. siamea* roots. The colonization rates of the two AMF were the same, but shoot P content was higher in M10-2 inoculated plant than M11-1 inoculated plant. Smith et al. [34] inoculated two crop species, *Linum usitatissimum* and *Medicago truncatula*, with *Gigaspora rosea*, *Glomus caledonium* or *Glomus intraradices*. Colonization was observed in *M. truncatula* inoculated with *G. rosea* (84%), *G. caledonium* (77%) or *G. intraradices* (99%). However only *G. caledonium* and *G. intraradices* increased shoot P content. The colonization rate was not always followed by the change of shoot P content.

Positive correlations between shoot P content and SFW were also observed for *P. falcataria* ($R^2 = 0.88$, $P < .001$), *C. calothyrsus* ($R^2 = 0.47$, $P < .001$), *C. siamea* ($R^2 = 0.67$, $P < .001$), and *S. grandiflora* ($R^2 = 0.31$, $P = .003$). SFW of *P. falcataria* was more responsive to the change of shoot P content. In contrast, *S. grandiflora* was less responsive to the change of shoot P content. In addition, variations were noted in the correlation between the two parameters; for example, *P. falcataria* and *C. siamea* inoculated with M10-2 showed higher shoot P content than their respective control plants. However, the high shoot P content did not result in high SFW in those plants. In the study by Smith et al [34], shoot P content of *M. truncatula* inoculated with *G. rosea* was not different from that of control plant but shoot dry weight was decreased. The change in shoot P content was not always correlated with the change of shoot biomass although the correlation between those two parameters was generally significant.

Mycorrhizal dependency (MD) was calculated to understand the effect of AMF inoculation on SFW of the leguminous trees. Various ranges of MD for each leguminous tree were observed: *P. falcataria* (-1–61%), *C. calothyrsus* (36–63%), *C. siamea* (-12–56%), and *S. grandiflora* (5–32%) (Table 3). Mean MD of *C. calothyrsus* (51%) was not different from that of *P. falcataria* (27%) but was higher than that of *S. grandiflora* (19%) and *C. siamea* (11%), irrespective of AMF isolate. *C. calothyrsus* showed the same response as *P. falcataria* to AMF inoculation but a higher response than *S. grandiflora* and *C. siamea*, particularly in terms of SFW.

4. CONCLUSION

Five native AMF were isolated from different forest soils in Indonesia and propagated by native leguminous trees. These AMF differently

colonized the roots and increased P uptake and growth of four leguminous trees. Colonization by native AMF differed by leguminous tree and growth response differed depending on the AMF isolate. Inoculation of native AMF to native leguminous trees can be useful for reforestation in Indonesia.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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