Characterization of arbuscular mycorrhizal and endophytic fungi isolated from forest soils in Indonesia and its effect on plant growth

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CHAPTER 1

Introduction

1.1 Biodiversity of fungi in the tropics

Fungi are organisms which exists in most type of habitat, from forest which rich in resources to stressful environment (Colpaert and van Assche 1987, Li et al. 2012), terrestrial (Harley 1971) to aquatic habitat (Hyde et al. 1998, Shearer et al. 2007). Fungi not only can live on dead matter (Vossbrinck et al. 1979, Osono 2007) but also on or in living organism (Gianinazzi-Pearson and Gianinazzi 1983, Leger et al. 1986, Shah and Pell 2003).

Due to its wide distribution, some mycologists considered the estimation of its species abundance is important. From some estimation pronounced, 1.5 million of fungi by Hawksworth was widely accepted by many mycologist (Hawksworth 2001). By the development of molecular method, that estimation, considered very low and 5.1 million of fungi is possible number for fungal diversity (O'Brien et al. 2005, Blackwell 2011).

Described fungi on earth reaches 97330 species (Kirk et al. 2008), representing below 10% of 1.5 million accepted estimate. Considering the much higher estimate by the development of molecular method, the described fungi to date are even lower. This fact rises the question of where the 90% of undescribed fungi are.

Mueller et al. (2007) compiled data about global diversity of macrofungi and compare it with diversity of plants in the tropics and non-tropics. They proposed that the ratio of fungi to plant were 5:1 and 2:1 in the tropic and non-tropic, respectively. Then, they clarified the validity of this ratio using recorded number of plants and fungi, proving that

these ratio were acceptable. This study confirmed that the biodiversity of fungi in the tropics is likely to be higher than the non-tropics.

1.2 Exploration of fungi in the tropics

Tropical forest is a place where undescribed fungi could be found, another than unexplored habitats (Hawksworth and Rossman 1997), despite the well-known fact that tropics have the richest biodiversity (Gascon et al. 2004, Barthlott et al. 2005). In order to discover more fungal species the attention to collect new fungi should be sustained (Hawksworth, 2001, Hawksworth and Rossman 1997, Schmit and Mueller 2007). Moreover, ratio of fungi to plant in tropic and polar region could be higher than in temperate, thus more data from tropics are needed (Hawksworth 2001), since Hawksworth used ratio of fungi to plant in temperate region to make the 1.5 million estimate.

Exploration progress of fungi in the last 10–15 years has been some but less than would be ideal (Hawksworth 2004). One example of fungi collection from tropics is in Hong Kong, in which number of fungi has quadrupled in a decade with over 150 new species (Hyde 2001). Exploration from another part of tropical area, Guyana, was also successful to discover some new species of fungi (Henkel et al. 2002, Thacker et al. 2004, Henkel et al. 2011). Increasing studies in tropics is still needed not only to discover more fungi species but also to clarify the 1.5 million estimates whether applicable in the tropics (Aime and Brearley 2012).

Fungi associated with terrestrial plant were estimated to dominate the undescribed fungi (Schmit and Mueller 2007). Mycorrhizal fungi and endophytic fungi are among which needed to be studied more (Blackwell 2011). Higher diversity of vascular plant in

tropic (Barthlott et al. 2005) made this possibility to discover and collect new fungi species even higher.

1.3 Association between plant and fungi

Association between plant and soil-borne fungi began million years ago in plant terrestrialization (Strullu-Derrien et al. 2014). This is when aquatic plant move and habit the land. This early plant had no true roots and had problems in fulfilling the needs of water and nutrient. This is the start that drove the symbiosis with soil-borne fungi and form mutualistic symbiosis (Delaux et al. 2012). There are evidences about this association in well-preserved fossil of early plant (Strullu-Derrien et al. 2014).

1.4 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) is an obligate biotrophic fungi that form symbiosis with 80% plant species on earth (Smith and Read 2008). AMF species richness is the highest in tropical forest (Öpik et al. 2006). AMF is listed in phylum Glomeromycota and proved to be existed from million years ago based on fossil study (Bonfante and Genre 2008). AMF forms distinct feature when colonized the plant root: arbuscule and vesicle (Figure 1.1). Arbuscule is branching hyphae inside plant cortical cell and serves as exchange site between plant and fungi. Vesicle is bulbous end of hyphae and serves as storage for the fungi (Smith and Read 2008).

AMF are well-known in promoting plant growth. AMF helps plant in P uptake (Smith et al. 2011) by virtue of its small size hyphae to explore further and wider in soil. AMF can protect plant from endoparasitic nematode (Pinochet et al. 1996), increase plant tolerance to salt stress (Evelin et al. 2009), increase plant tolerance to heavy metal toxicity,

for example Zn, Pc and Cd (Göhre and Paszkowski 2006), and increase plant tolerance to drought (Khalvati et al. 2005).

1.5 **Endophytic fungi**

Endophytic fungi (EPF) are fungi that colonize plant tissue without causing any visible disease symptoms at any particular moment (Schulz and Boyle 2005). Practically, EPF colonize almost any plant tissue, including leaf, stem, and root (Rodriguez et al. 2009). Since their discovery, EPF have been studied in many types of plants, including non-vascular ones, such as mosses (e.g., Schulz et al. 1993) and algae (e.g., Zuccaro et al. 2008), and vascular ones, such as shrubs (e.g., Schulz et al. 1993) and trees (e.g., Arnold and Lutzoni 2007). EPF is a facultative biotrophic fungi. Most of the isolated EPF belong to Ascomycota and Basidiomycota (Schulz and Boyle 2005).

A conservative estimate of fungal diversity in the world of 1.5 million species has been accepted as the working hypothesis and the basis for the discovery of more fungal species (Hawksworth 2001). With only around 72,000 described species known so far, more than 1 million species are waiting to be found. EPF have been found in many plant species and considered an important component of fungal diversity (Rodriguez et al. 2009). Despite the increasing number of studies of EPF in many countries, studies of EPF in the tropics are still lacking.

Arnold et al. (2000) isolated 418 EPF morphospecies colonizing leaf of two understory tree species in a tropical forest in Panama, 59% of which were represented by single isolates. Cannon and Simmons (2002) isolated 64 EPF morphospecies colonizing leaf of 12 tree species in a tropical forest in Guyana, 29 of which were from single leaf samples. Taking into account the 5:1 ratio of fungi to plant in the tropics (Mueller et al.

2007), these two studies reflect the high diversity of EPF colonizing tree leaf in tropical forest.

The different environmental factors in forests are expected to influence fungal diversity (Saikkonen 2007). However, most studies of the diversity of EPF colonizing leaf in tropical forest are limited to one forest site (e.g., Arnold et al. 2000 and 2001, Cannon and Simmons 2002). Suryanarayanan et al. (2011) compared the EPF communities of 75 dicotyledonous trees belonging to 33 families from three tropical forest types in Southern India, namely, tropical dry thorn forest, dry deciduous forest, and montane evergreen forest. The type of forest appeared to have a larger effect on shaping the EPF community than the taxonomy of the host.

Studies of EPF in tropical forest are limited to EPF that colonize the above-ground part of plant, particularly leaf. EPF colonizing roots, further termed as root EPF, of tropical forest trees are rarely studied. Rodriguez et al. (2009) classified root EFP into two groups: class 2 EPF which colonize shoot, root and rhizome and class 4 EPF, also termed dark septate endophyte (DSE) which colonize root only. While class 1 and 3 are consisted of EPF that colonize above-ground part of plant. Apart from the colonization range in plant tissue, the difference between these two groups is also the morphology. The DSE is characterized by dark pigmented septate hyphae and hyaline hyphae. However, class 2 EPF was observed to only have the latter. In addition, the other distinctive feature of root EPF is microsclerotia (Figure 1.2). In a review by Jumpponen and Trappe (1998), they noted that DSE, colonized approximately 600 plant species representing 320 genera and 114 families, showing the abundance of DSE. However, DSE is not the only group of root EPF, indicating the possibility of an even higher abundance of root EPF in nature.

Root EPF are considered to play an important role in plant growth, similar to mycorrhizal fungi (Jumpponen and Trappe 1998). Some EPF were able to protect host against fungal pathogens (Narisawa et al. 2002, Maciá-Vicente et al. 2008) and increase plant nitrogen uptake (Usuki and Narisawa 2007). Meta-analysis of data from temperate and boreal areas showed that root EPF colonization had a negative, neutral or positive effect on plant growth (Mandyam et al. 2013, Mayerhofer et al. 2013, Newsham 2011). Evidence of the positive effect of root EPF along with the expectation of high EPF abundance in tropical forest has underscored the necessity to conduct more studies of root EPF in tropical forest. However, studies of root EPF in tropical area remain a rarity (Mandyam and Jumpponen 2005).

The difference in plant growth response to EPF colonization is governed by not only plant and EPF species but also environmental factors, particularly medium (Mayerhofer et al. 2013) Nutrient status may be an important factor as it also affects the relationship between plant and mycorrhizal fungi.

Murashige and Skoog (MS) medium is used to study the effect of EPF inoculation on plant growth (Hou and Guo 2009). Nutrient concentration variation in MS medium is expressed as the dilution strength of MS medium. Mandyam et al. (2013) used 1/10-strength MS medium and observed a positive response of plant growth to EPF inoculation. Lacercat-Didier et al. (2016) used full-strength MS medium and observed a positive response of plant growth to EPF inoculation.

1.6 Deforestation in Indonesia and methods for reforestation

Deforestation by land-use conversion into agricultural fields and plantations, open cast mining, and illegal logging is increasing in Indonesia (Abood et al. 2015).

Reforestation of tropical forests, such as those in Indonesia, requires human assistance to recover forest structure and species composition (Chazdon 2003). Rather than relying on natural forest recovery, human assistance would accelerate the speed of reforestation.

There are few methods to remediate degraded forest in Indonesia. Utilization of beneficial symbiotic fungi is an environmentally safe way to ensure the plant survival rate after transplantation to the field. Arbuscular mycorrhizal fungi and endophytic fungi are groups of root symbiotic fungi which reported to be able to promote plant growth.

The utilization of AMF, which can support plant growth through several mechanisms (Smith and Read 2008), 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 (Treseder and Cross 2006). The utilization of AMF of some tree species to support reforestation efforts in Indonesia has been reported (e.g., Graham et al. 2013, Wulandari et al. 2016).

The effectiveness of AMF in promoting plant growth has been shown to vary (Hoeksema et al. 2010). 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 (2003) 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.

There is no report yet about utilization of EPF for tree species in Indonesia. Some researcher in Indonesia has begun isolating this fungi from crop species to study about its diversity (Amin 2013a), degradative enzyme (Marlida et al. 2000) antagonism to pathogen (Suada et al. 2012) and anti-microbial substances (Kumala and Siswanto 2007,

Artanti et al. 2011) or from tree species to study about its effect on nematode (Amin 2013b). However, the utilization of EPF on promoting tree growth is very limited.

Increasing attention in studying tropical AMF and EPF should be sustained for the development of science. This would open greater opportunity in utilizing biodiversity as bioresources in a way that support sustainable environment. Efforts to get a good isolate able to promote certain plant species for reforestation purpose is one example.

1.7 Plant species for reforestation

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 (Chazdon 2003). 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 (Figure 1.3) 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 (Siregar et al. 2007). The wood of *P. falcataria* is a candidate for energy production (Amirta et al. 2016). *C. calothyrsus* is a source of high-protein forage and an intercropping plant with crop species (Kanmegne et al. 1999). *C. siamea* is a fallow tree and a good mulch for crop plant (Yobterik et al. 1994). *S. grandiflora* is a candidate species for remediating Pb/Zn and Cu mine tailings (Chan et al. 2003). The appropriate AMF isolates for these leguminous trees have not been isolated.

1.8 Objective

Study about AMF and EPF in the tropics is less than in temperate region despite higher biodiversity in the former. The importance is not limited to as an effort to support reforestation of Indonesian forest, but also for wider knowledge for example about role and mechanism of those fungal group in affecting plant growth. The objectives of this study were (1) to isolate AMF and EPF from forest in Indonesia, (2) to determine condition of screening of EPF, and (3) to screen effective isolates of AMF and EPF with tropical tree species.

CHAPTER 2

Characterization and screening of arbuscular mycorrhizal fungi isolated from forest soils in Indonesia

2.1 Introduction

Deforestation by land-use conversion into agricultural fields and plantations, open cast mining, and illegal logging is increasing in Indonesia (Abood et al. 2015). Reforestation of tropical forests, such as those in Indonesia, requires human assistance to recover forest structure and species composition (Chazdon 2003). Rather than relying on natural forest recovery, human assistance would accelerate the speed of reforestation.

There are few methods to remediate degraded forest in Indonesia. The utilization of arbuscular mycorrhizal fungi (AMF), which can support plant growth through several mechanisms (Smith and Read 2008), 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 (Treseder and Cross 2006). The utilization of AMF of some tree species to support reforestation efforts in Indonesia has been reported (e.g., Graham et al. 2013, Wulandari et al. 2016).

The effectiveness of AMF in promoting plant growth has been shown to vary (Hoeksema et al. 2010). 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 (2003) 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 (Chazdon 2003). 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 (Figure 1.3) 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 (Siregar et al. 2007). The wood of *P. falcataria* is a candidate for energy production (Amirta et al. 2016). *C. calothyrsus* is a source of high-protein forage and an intercropping plant with crop species (Kanmegne et al. 1999). *C. siamea* is a fallow tree and a good mulch for crop plant (Yobterik et al. 1994). *S. grandiflora* is a candidate species for remediating Pb/Zn and Cu mine tailings (Chan et al. 2003). The appropriate AMF isolates for these leguminous trees have not been isolated. The objectives of this chapter were (1) to isolate AMF using these trees as trap plants, and (2) to clarify the effectiveness of the AMF isolates in promoting growth of these leguminous trees.

2.2 Materials and methods

2.2.1 Spore propagation by soil culture

Five forest soils in Indonesia were used for isolation of AMF (Table 2.1, Figure 2.1, Maulana 2015). Five AMF were successfully isolated from forest soils in Indonesia: 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.

Sand was acidified and sterilized by autoclaving at 80 ºC for 45 min and used as growth medium. Eighty (80) grams of sterilized sand was added to 50 mL syringe pot. Approximately 20 to 50 spores were added to the sterilized sand and covered again by 5 g sterilized sand. Twenty (20) seeds of *Trifolium repens* (cv. California ladino, Snow brand seed, Japan) were sown and covered again by 5-mm-depth sterilized sand. Twenty milliliters of tap water were added to syringe pot. Plants were grown in the growth chamber (Biotron LPH-350S, NK System, Japan) at 27 ºC with a 16-hour photoperiod for 90 days after sowing (DAS). The plants were watered with 5–10 ml of P1 nutrient solution containing (1 mg L^{-1} P ($NaH_2PO_4 \cdot 2H_2O$), 40 mg L^{-1} NH_4NO_3-N (NH_4NO_3), 20 mg L^{-1} NO_3-N ($NaNO_3$), 60 mg L^{-1} K (K_2SO_4), 80 mg L^{-1} Ca ($CaCl_2 \cdot 2H_2O$), 40 mg L^{-1} Mg ($MgSO_4 \cdot 7H_2O$), 2 mg L^{-1} Fe ($FeSO_4 \cdot 7H_2O$), 1 mg-L^{-1} Mn ($MnSO_4 \cdot 5H_2O$), 0.01 mg L^{-1} Cu ($CuSO_4 \cdot 5H_2O$), 0.005 mg L^{-1} Mo ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$), 0.4 mg L^{-1} B (H_3BO_3), and 0.2 mg L^{-1} Zn ($ZnCl_2$) (based on Wagatsuma et al 1988), once every two days. Watering was stopped at 90 DAS and plants were let dry for another 30 days to induce AMF sporulation in the growth chamber and then harvested. Spores were extracted from 5 g of medium by wet sieving and decanting (Gerdemann and Nicolson, 1963), observed under dissecting microscope (SMZ800, Nikon, Japan) and number of new spore was

counted. These procedures were repeated 2 times and then spore density in the medium was calculated.

2.2.2 Spore propagation by root organ culture

2.2.2.1 Growth medium and hairy root for root organ culture

A Modified Strullu-Romand (MSR) medium (Cranenbrouck et al. 2005) was used in propagation by root organ culture. The composition of this medium in 1 L deionized water were as follows: 79 g $MgSO_4.7H_2O$; 7.6 g KNO_3; 6.5 g KCl; 0.41 g KH_2PO_4; 35.9 g $Ca(NO_3)_2.4H_2O$; 0.09 g calcium panthotenate; 0.0001 g biotin; 0.1 g nicotinic acid; 0.09 g pyridoxine; 0.1 g thiamine; 0.04 g cyanocobalamine; 0.16 g NaFeEDTA; 1.225 g $MnSO_4.4H_2O$; 0.14 g $ZnSO_4.7H_2O$; 0.925 g H_3BO_3; 1.1 g $CuSO_4.5H_2O$; 0.12 g $Na_2MoO_4.2H_2O$; 1.7 g $(NH_4)_6Mo_7O_{24}.4H_2O$. The pH was adjusted to 5.5. Five gram of Phytagel™ (Sigma, USA) was added before autoclaving at 121 °C for 15 min.

A hairy root of flax (*Linum usitatissimum*), transformed by mediation with *Agrobacterium rhizogenes*, purchased from Glomeromycota in vitro collection, Belgium (GINCO-BEL), was used as root organ in this experiment. Two to three-weeks-old hairy roots of flax was re-cultured by transferring an excised 1–2-cm-long root apex onto MSR medium in 85-mm Petri dish. The plates were sealed with Parafilm™ and incubated in inverted condition, in a dark room at 25 °C.

2.2.2.2 **Inoculation of AMF to root organ culture**

Spores were put into sterilized deionized water in sterilized tube. Spores were treated in an ultrasonic bath (Yamato 3510, Branson, USA), to remove the soil debris from surface of the spore. The water in the tube was removed and the same procedure was

repeated until three times. Spores were then sterilized by soaking them into 2% chloramine T with two drops of Tween 20 for 15 minutes (Declerck et al. 2005). The spores were rinsed with sterilized deionized water for three times and soaked into antibiotic for 10 minutes. The sterilized spores were transferred to MSR medium before inoculation.

Five surface-sterilized spores were transferred onto MSR medium in 85-mm Petri dish at 3-cm-distance from 2-weeks-old excised 1-cm-long root apex of flax (Figure 2.2). The Petri dish were sealed with Parafilm^{TM} and incubated in a dark room at 25 ^{o}C. Twenty (20) to forty-two (42) replications were made for each AMF isolates. The spores and root were observed under dissecting microscope every 15 to 30 days. Parameters in the observation were contamination, spore germination, presence of running hyphae, and formation of new spores. Observation were done with understanding in the order of structure presence which are spore germination, formation of running hyphae, and followed by formation of new spores (Karandashov et al. 2000). Further, new spores from this culture were used for single spore inoculation by transferring one spore to new medium with new root.

2.2.3 Observation of morphological characteristics

Spores were mounted on glass slide (76 mm x 52 mm) containing polyvinyl alcohol-lactic acid-glycerol (PVLG) or a mixture of PVLG and Melzer's reagent (Bills and Foster 2004). The spores were observed under a compound microscope (Eclipse 80i, Nikon, Japan). Another 100 spores were randomly chosen and used for measurement of spore size under dissecting microscope. Spore size was divided into classes (20 µm width, based

on International Culture Collection of (Vesicular) Arbuscular Mycorrhiza (INVAM), USA) to know the distribution of spore size.

2.2.4 Identification of AMF based on molecular method

2.2.4.1 DNA extraction and amplification

Spores were collected with forceps and put on the lid of a 200 µL plastic tube containing 20 µL of InstaGene^{TM} Matrix (Bio-Rad, USA) (Maki et al. 2008). The spores were crushed with the blunt end of a pipet tip, further mixed with 180 µL of InstaGene^{TM} Matrix, and vortexed. rDNA was extracted following the manufacturer's protocol for InstaGene^{TM} Matrix. The extracted DNA was stored at −20 ºC until use.

The sequence of the D1/D2 region was amplified using eukaryotic-specific primer LR1 (5'-GCATATCAATAAGCGGAGGA-3') (Van Tuinen et al. 1998) and fungal-specific primer FLR2 (5'-GTCGTTTAAAGCCATTACGTC-3') (Trouvelot et al. 1999), with an Expand High Fidelity^{PLUS} PCR system (Roche, Germany) using the following composition: 4 µL of 5 x buffer with $MgCl_2$, 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 ºC for 120 s; 30 cycles of denaturation at 94 ºC for 15 s, annealing at 50 ºC for 60 s, and extension at 72 ºC for 80 s; and a final extension at 72 ºC 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 ºC for 16 hours.

Single colonies of *E. coli* were collected and DNA was amplified using primers T7 (5'-TAATACGACTCACTATAG-3') and U19 (5'-GTTTTCCCAGTCACGACT-3') (Ikenaga et al. 2016), 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 ºC for 120 s; 35 cycles of denaturation at 94 ºC for 15 s, annealing at 50 ºC for 60 s, and extension at 72 ºC for 80 s; and a final extension at 72 ºC 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 BigDye™ 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).

2.2.4.2 Phylogenetic analyses

Sequences of AMF were submitted for BLAST analysis (Altschul et al. 1990). The sequences and their corresponding BLAST top hits were aligned by MUSCLE (Edgar 2004). The maximum likelihood method was performed by MEGA 7 (www.megasoftware.net) with 1000 replications of bootstrap analysis.

2.2.5 Screening of effective AMF

2.2.5.1 Inoculation of AMF to leguminous tree species

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. Seeds of *Caliandra calothyrsus*, *Paraserianthes falcataria*, *Cassia siamea*, and *Sesbania grandiflora* were sown on sterilized sand and incubated in a growth chamber at 27 ºC with a 16-hour photoperiod. 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 ºC with a 16-hour photoperiod for 60 days after transplanting (DAT). Ten milliliters of P1 solution was applied to sand once every 2 days.

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 HNO_3, $HClO_4$, and H_2SO_4 (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 ºC for 60 min with a DKL Heating Digester complemented with SMS Scrubber and Recirculating Water Aspirator (VELP Scientifica, Italy). Shoot P concentration in the solution of digested shoot was determined colorimetrically with the vanadomolybdate-yellow assay (Olsen and Sommers 1982), using a spectrophotometer at 410 nm absorbance (U-2900, Hitachi, Tokyo). 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 Plenchette et al. (1983).

2.2.5.2 Assessment of AMF colonization

Roots were stained with aniline blue dye as described by Tawaraya et al. (1998). First, the roots were cleared by dipping into 10% (w/v) KOH solution and heated in a water bath at 80 ºC 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 ºC 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 (Giovannetti and Mosse 1980).

2.2.5.3 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 replications were survived until harvest and used for statistical analysis.

2.3 Results

2.3.1 Propagation of AMF with soil culture and root organ culture

All five isolates were successfully propagated using *T. repens* (Table 2.2). Number of new spores per gram medium were ranged from 52–95 (M10-2), 32–77 (M11-1), 2–70 (M44-3), 15–30 (M60-3), and 19–63 (S6-4). Of the two (M11-1 and S6-4) of five isolates were successfully propagated by root organ culture (Table 2.3, Figure 2.3).

Successful rate of propagation by this method using 5 spores inoculum for M11-1 and S6-4 were 10% (2/20 plates) and 25% (5/20 plates), respectively (Tables 2.3 and 2.4). Number of new spores per plate were ranged from 75–145 (M11-1) and 15–551 (S6-4). Spores in some plates germinated. Running hyphae was formed and they ceased to develop further until sporulation. In contrast, none of the spores of the other three isolates (M10-2, M44-3, M60-3) were germinated.

Utilization of propagated spore by this method for further propagation using single spore inoculum increased the successful rate of propagation for M11-1 to 14% (6/42 plates) (Table 2.5). Even so, germinated spores and running hyphae were also observed but ceased to develop further until sporulation.

2.3.2 Morphological characteristics of spore of five isolated AMF

AMF isolates M10-2, M11-1, M60-3, and S6-4 exhibited clear to yellowish-white color when mounted in PVLG (Figure 2.4), while M44-3 exhibited different color, a reddish-brown. AMF isolates M10-2, M11-1, and M60-3 exhibited faint yellow color when mounted in the mixture of PVLG and Melzer`s reagent, while there is no color change for M44-3. In contrast, S6-4 exhibited color change to reddish-brown with clearly distinctive ornamentation of the spore. This isolate also showed distinctive morphological characteristics by forming intraradical spores.

Range of spore size of M10-2, M11-1, M44-3, M60-3, and S6-4 were 75–134 µm (mean: 103 µm), 47–92 µm (mean: 64 µm), 68–109 µm (mean: 90 µm), 96–217 µm (mean: 150 µm), and 78–220 µm (mean: 134 µm), respectively. Among the classes of spore size for each AMF, highest number of spores were grouped in class 100–120 µm (M10-2, Figure 2.5), 60–80 µm (M11-1, Figure 2.6), 80–100 µm (M44-3, Figure 2.7), 140–160 µm (M60-3, Figure 2.8) and 120–140 µm (S6-4, Figure 2.9).

2.3.3 AMF identity based on DNA

Part of the LSU region of five AMF isolates were sequenced and aligned with reference sequences (Table 2.6, Figure 2.10). Based on the similarity of the 10–12 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).

2.3.4 AMF colonization rate in root of four leguminous trees

Roots of *P. falcataria* were colonized by isolates M10-2 (66%), M11-1 (87%), and S6-4 (99%) (Table 2.7). 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.

2.3.5 AMF colonization, and growth of *Paraserianthes falcataria*, *Calliandra calothyrsus, Cassia siamea* and *Sesbania grandiflora*

Plant height increment of *P. falcataria* and *C. siamea* inoculated with AMF was not significantly different from that of control plant (Table 2.7). 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 and 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 2.7). 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.

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 2.7). 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 2.7). 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 2.7). 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 2.7). 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.

2.4 Discussion

2.4.1 *Trifolium repens* as a host plant for propagation of AMF by soil culture

T. repens was proven to be a good host plant in the present study for propagation of AMF. *T. repens* and another species in the genera of *Trifolium*, *T. pretense*, were recorded to be colonized by AMF in many studies and also used for trap culture host plant (Sanders 1992, Gamper et al. 2005, Velázquez and Cabello 2011). Not only *Trifolium* plants, utilization of other crop species, such as maize (*Zea mays*), bahiagrass (*Paspalum notatum*), sudangrass (*Sorghum bicolor* ssp. *drummondii*), millet (*Pennisetum*

americanum), and chickpea (*Cicer arietinum*), for trap culture are common and proven to be suitable to get diverse isolates (Struble and Skipper 1988, Simpson and Daft 1990).

2.4.2 Factors affecting spore germination and formation of new spores in root organ culture

The spores of three AMF isolates were not germinated in the propagation by root organ culture. Spore without germination on monoxenic culture was also observed in another study. Douds (1997) reported that spores of *Glomus mosseae* did not germinated at pH 5.5 on unbuffered M medium. It exhibited highest germinated on M medium buffered with MES [2-(N-morpholino) ethansulfonic acid] at pH above 6.7 or with Tris [tris (hydroxymethyl) aminomethane] at pH 7.3 and 7.6. pH of medium may be the factor that caused the no germination of the spores of these three isolates. Another study by Juge et al. (2002) highlighted cold-storage as a pre-treatment to break the dormancy of spores of *Glomus intraradices*. Their study showed that this pre-treatment can increase the germination of the spores. However, the spores in the control, without the pre-treatment, was already germinated but lower than the spores that received the pre-treatment. Thus, it is not likely the cause for no germination in our results that showed zero germination in all spores.

We recorded varied number of new spores among different plates although the initial number of the spores as inoculum was the same. The same result was also observed in the study of Karandashov et al. 2000. They inoculated single spore of *Glomus caledonium* to carrot roots and grown on M medium. They also reported that number of new spores were vary among plates after 5–7 weeks: 11–34 in first-generation culture, 2–43 spores in second-generation culture, and 14–91 in third-generation culture. Declerck et al. (2004)

observed that the sporulation of *Scutellospora reticulata* was parallel with extraradical fungal biomass. This could presumably be related to the resources uptake by extraradical mycelium for production of new spores. Unfortunately, present study did not measure the extraradical fungal biomass, thus its correlation with sporulation can`t be shown. However, extraradical fungal biomass may be the determinant for number of new spores produced in present study.

2.4.3 The difference in morphological characteristics of isolated AMF

Melzer`s reagent is a common chemical in mycology for spore staining. It is being used for observation of morphological characteristics of the spore. The reactions with this reagent were categorized based on color change: blue or black (amyloid), red-brown (pseudoamyloid or dextrinoid), and yellow or no color change (inamyloid) (Leonard 2006). However, the color change of the spore sometimes doesn`t fall for any of these categories. Thus, the color change should be recorded directly, for example, as blue, black, brown, red, or yellow (Castellano et al. 2004).

AMF isolates M10-2, M11-1, and M60-3 exhibited faint yellow color, while M44-3 exhibited no color change (Figure 2.4). It can be assumed that there is no reaction between the Melzer`s reagent with the spores of these four isolates. In contrast, S6-4 exhibited color change from yellowish-white to red-brown indicating a reaction with the reagent. Similar color change was observed in basidiomata after mounted in the reagent (Blackwell et al. 2001). It is suggested to be a reaction between the reagent and glycine betaine. The reason behind the color change in S6-4 spore might also be the same.

2.4.4 AMF isolation from tropical forest in Indonesia using leguminous trees

Five isolated AMF were identified on the basis of the LSU region of rDNA. 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) (Figure 2.10).

Isolate M10-2 closely matched *D. gibbosa* isolated by Blaszkowski 1997 from the rhizosphere of *Ammophila arenaria*, *Helichrysum arenarium*, *Hieracium umbelatum*, and *Petasites spurius*, in maritime sand dunes in Poland. Blaszkowski (1997) described this species as *Glomus gibbosum*. These plant species are grass plants that common in coastal area, very different with tree species in forest of the present study. In addition, to our knowledge, our study is the first to isolate and identify *D. gibbosa* from tropical forest soil.

Isolate M60-3 closely matched *A. appendicula* (basionym= *Acaulospora appendicula*) isolated by Spain, Sieverd, and Schenck in 1984 (Schenck et al. 1984, Walker 2008) from the the rhizosphere of native grasses and tropical kudzu, *Pueraria phaseoloides*, in Colombia. *A. appendicula* was also isolated from the rhizosphere of a threatened native leguminous tree, *Pericopsis mooniana*, growing in a natural forest in Sulawesi Island, Indonesia (Husna et al. 2014). This species was also isolated from a tropical coast in Brazil (Jobim and Goto 2016).

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 (Stockinger et al. 2009). 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 (Farmer et al. 2007).

M11-1 closely matched *Acaulospora longula*. However, no details were provided regarding the isolation of *A. longula*. This species was also isolated from the rhizosphere of a perennial forb, *Solidago missouriensis*, in tallgrass prairie in USA (Eom et al. 2000) and also from the rhizosphere of a tree species, *Diospyros blancoi*, in West Java of Indonesia (Ningsih et al. 2013).

2.4.5 Effect of AMF on promoting leguminous tree growth

Two isolates, M11-1 and S6-4, colonized the four leguminous trees (Table 2.7). 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 Klironomos (2003), 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 2.7 and 2.8). 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. (2008) revealed that *Glomus etunicatum* and *Glomus macrocarpum* increased shoot dry weight, plant height, and number of leaves of *Senna spectabilis*. Guissou et al. (2009) showed that *Glomus aggregatum* increased total dry weight and plant height of *Tamarindus indica*. Wulandari et al. (2014) 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.

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. (2017) 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.

2.4.6 Response of leguminous trees to AMF inoculation

Positive correlations between colonization rate and shoot P content were observed for *P. falcataria* ($R^2 = 0.87$, $P < 0.001$, Figure 2.11), *C. calothyrsus* ($R^2 = 0.41$, $P < 0.001$, Figure 2.12), *C. siamea* ($R^2 = 0.39$, $P = 0.0011$, Figure 2.13), and *S. grandiflora* ($R^2 =$

0.13, P = 0.048, Figure 2.14). *P. falcataria* showed higher correlation between colonization rate and shoot P content than the other leguminous trees. Smith and Smith (2012) 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 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. (2003) 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 < 0.001), *C. calothyrsus* (R^2 = 0.47, P < 0.001), *C. siamea* (R^2 = 0.67, P < 0.001), and *S. grandiflora* (R^2 = 0.31, P = 0.003). 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. (2003), 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 2.8). 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.

Mycorrhizal dependency (MD) of several leguminous trees in other studies, calculated based on the SDWs, were 81% for *S. spectabilis* (Kung`u et al. 2008), 34–65% for *A. saman* and 44–48% for *P. falcataria* (Wulandari et al. 2014). Mycorrhizal dependency (MD) of several non-leguminous trees in other studies, calculated based on the SDWs, were 39–62% for *Olea europaea*, an Oleaceae species (Porras-Soriano et al. 2009), 93% for *Cedrella fissilis*, a Meliaceae species and 95% for *Cecropia pachystachya*, a Cecropiaceae species (Siqueira and Saggin-Júnior 2001). MDs in present study were lower or the same as that of these studies.

CHAPTER 3

Effect of culture condition, nutrient solution, and agar medium on relationship between endophytic fungi and plant

3.1 Introduction

Endophytic fungi (EPF) are fungi that colonize plant tissue without causing any visible disease symptoms at any particular moment (Schulz and Boyle 2005). Practically, EPF colonize almost any plant tissue, including leaf, stem, and root (Rodriguez et al. 2009). Since their discovery, EPF have been studied in many types of plants, including non-vascular ones, such as mosses (e.g., Schulz et al. 1993) and algae (e.g., Zuccaro et al. 2008), and vascular ones, such as shrubs (e.g., Schulz et al. 1993) and trees (e.g., Arnold and Lutzoni 2007). Most of the isolated EPF belong to Ascomycota and Basidiomycota (Schulz and Boyle 2005).

EPF colonizing roots, further termed as root EPF, of tropical forest trees are rarely studied. Rodriguez et al. (2009) classified root EFP into two groups: class 2 EPF which colonize shoot, root and rhizome and class 4 EPF, also termed dark septate endophyte (DSE) which colonize root only. Apart from the colonization range in plant tissue, the difference between these two groups is also the morphology. The DSE is characterized by dark pigmented septate hyphae and hyaline hyphae. However, class 2 EPF was observed to only have the latter. In a review by Jumpponen and Trappe (1998), they noted that DSE, colonized approximately 600 plant species representing 320 genera and 114 families, showing the abundance of DSE. However, DSE is not the only group of root EPF, indicating the possibility of an even higher abundance of root EPF in nature.

Root EPF are considered to play an important role in plant growth, similar to mycorrhizal fungi (Jumpponen and Trappe 1998). Some EPF were able to protect host against fungal pathogens (Narisawa et al. 2002, Maciá-Vicente et al. 2008) and increase plant nitrogen uptake (Usuki and Narisawa 2007). Meta-analysis of data from temperate and boreal areas showed that root EPF colonization had a negative, neutral or positive effect on plant growth (Mandyam et al. 2013, Mayerhofer et al. 2013, Newsham 2011). Evidence of the positive effect of root EPF along with the expectation of high EPF abundance in tropical forest has underscored the necessity to conduct more studies of root EPF in tropical forest. However, studies of root EPF in tropical area remain a rarity (Mandyam and Jumpponen 2005).

The difference in plant growth response to EPF colonization is governed by not only plant or EPF species but also environmental factors, particularly experimental conditions Mayerhofer et al. 2013. Nutrient status may be an important factor as it also affects the relationship between plant and mycorrhizal fungi. The objective of this chapter was to determine screening condition of EPF.

3.2 Materials and methods

3.2.1 Effect of malt extract on EPF colonization rate and growth of *Brassica campestris*

3.2.1.1 Application of different concentration of malt extract for EPF culture

Bottom part of Plantbox (75 mm x 60 mm, BC-PB851-50, Biomedical science, Japan) containing 20 g of vermiculite was sterilized by autoclave at 121 ºC for 15 min. One 5-mm mycelial plug of 14-day-old EPF isolates 2614(4)PDA-1-2-1 and 2314(4)PDA cultured with ½-strength malt extract agar (MEA) medium were transferred to the surface

of vermiculite. Fifteen (15) milliliters of 1/10, ½ or full-strength liquid malt extract were applied to vermiculite. The Plantbox was covered with lid of Petri dish and sealed with Parafilm^{TM} and incubated in a dark room at 25 ºC. Diameter of colonies was measured, 4 and 6 days after inoculation (DAI). Isolate 2614(4)PDA-1-2-1 was chosen because in the previous study this isolate slightly increased the growth of *B. campestris* under application of low P (1 mg L^{-1}) or low N (4 mg L^{-1}) but normal concentration of other elements, with colonization rate less than 10%. Isolate 2314(4)PDA was chosen because in the previous study it slightly increased the growth of *B. campestris* under application of low P (1 mg L^{-1}, P1 solution) but normal concentration of other elements, with colonization rate less than 10%. These two isolates were isolated using potato dextrose agar (PDA) media.

3.2.1.2 Transplanting of *Brassica campestris*

The seeds of *Brassica campestris* (cv. Harusakari, Watanabe seed, Japan) were surface-sterilized by dipping into 5% NaClO for 3 mins. Then, the seeds were rinsed three times with sterilized deionized water. Seeds were sown on sterilized vermiculite in plastic box (18 cm x 11.5 cm x 11 cm), watered by sterilized deionized water and then wrapped by wrapping plastic. Sown seeds were incubated in growth chamber at 27 ºC with a 16-hours photoperiod.

Three 7-day-old seedlings of *B. campestris* were transplanted onto fungal colonies on vermiculite. Sixty (60) milliliters of ½-strength P8 nutrient solution containing 4 mg L^{-1} P ($NaH_2PO_4 \cdot 2H_2O$), 20 mg L^{-1} NH_4NO_3-N (NH_4NO_3), 10 mg L^{-1} NO_3-N ($NaNO_3$), 30 mg L^{-1} K (K_2SO_4), 40 mg L^{-1} Ca ($CaCl_2 \cdot 2H_2O$), 20 mg L^{-1} Mg ($MgSO_4 \cdot 7H_2O$), 1 mg L^{-1} Fe ($FeSO_4 \cdot 7H_2O$), 0.5 mg-L^{-1} Mn ($MnSO_4 \cdot 5H_2O$), 0.005 mg L^{-1} Cu ($CuSO_4 \cdot 5H_2O$), 0.0025

mg L^{-1} Mo ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$), 0.2 mg L^{-1} B (H_3BO_3), and 0.1 mg L^{-1} Zn ($ZnCl_2$) (based on Wagatsuma et al 1988) was applied to *B. campestris*. Root system of *B. campestris* was subsequently covered with 15 g of sterilized vermiculite. The Plantbox were closed with the upper part of Plantbox. The hole on top of Plantbox was covered by sterilized cotton. *B. campestris* was grown in growth chamber at 27 ºC with a 16-hours photoperiod, for 30 DAT.

3.2.1.3 Plant growth measurement

Plants were harvested at 30 DAT. Shoots and roots were separated and cleaned under running tap water and rinsed with deionized water. The shoots were weighed for shoot fresh weight (SFW) and then oven-dried at 70 ºC for 72 hours for shoot dry weight (SDW). The roots were weighed for root fresh weight (RFW) and then used for observation of EPF colonization rate. The significant difference among treatments were 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).

3.2.1.4 Assessment of EPF colonization

The roots were stained with aniline blue dye as described by Tawaraya et al. (1998). The roots of *B. campestris* were cleared by dipping into 10% (w/v) KOH solution and heated in a water bath at 80 ºC for 5 min. Then, the roots were rinsed with tap water, acidified with 1% (w/v) HCl, and rinsed again with tap water. The roots were dipped into 0.05% aniline blue solution (Aniline blue, Wako, Japan) and heated again at 90 ºC for 5 min. After rinsing with tap water, the roots were transferred to a Petri dish and lactic acid-glycerol solution was added. The roots were mounted on glass slides and covered with

cover glass. Colonization was observed under a microscope (Eclipse 80i, Nikon, Japan) at 200x magnification. The presence of fungal structures inside plant root indicated internal colonization. The presence of fungal structures on the surface of plant root indicated external colonization. Percentage colonization was estimated by the gridline intersect method on 100 intersections (Giovannetti and Mosse 1980).

3.2.2 Effect of different nutrient solution on EPF colonization rate and plant growth under Plantbox culture

The seeds of *B. campestris* were surface sterilized, sown on vermiculite and incubated in the growth chamber as described above. Plantbox (75 mm x 110 mm) containing 20 g of vermiculite were sterilized by autoclave at 121 ºC for 15 min. Three 7-day-old seedlings of *B. campestris* were transplanted onto the vermiculite. Two 5-mm mycelial plugs of 14-day-old 2614(4)PDA-1-2-1 cultured with ½-strength MEA medium were inoculated to each seedling at the same time as seedling transplanting by placing them adjacent (1–5 mm) to root system. Root system of *B. campestris* was subsequently covered with 15 g of sterilized vermiculite. Sixty (60) milliliters of P1 solution or ½-strength MS solution was applied to *B. campestris*. *B. campestris* was grown in growth chamber at 27 ºC with a 16-hours photoperiod, for 30 DAT.

Plants were harvested at 30 DAT. Shoots and roots were separated and cleaned under running tap water and rinsed with deionized water. Harvested plants were weighed for SFW, SDW and RFW. The roots were also used for observation of EPF colonization rate as described above. The significant difference among treatments were 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).

3.2.3 Effect of different concentration of agar medium under Petri dish culture or nutrient solution under Plantbox culture on growth of *Brassica campestris*

The seeds of *B. campestris* were surface sterilized as described above, sown on water agar (1%) and incubated in the growth chamber. Two 7-day-old seedlings of *B. campestris* were transplanted onto 20 ml water agar, 1/100-strength MS, 1/10-strength MS, 1/5-strength MS or full-strength MS medium, in 85-mm Petri dish. *B. campestris* were grown in growth chamber for 14 DAT. Harvested plants were weighed for SFW, SDW and RFW. Root length was also calculated based on Newman (1966).

Three 7-day-old seedlings of *B. campestris* were also transplanted to vermiculite in Plantbox with application of sterilized deionized water, 1/100-strength P8, 1/10-strength P8, 1/5-strength P8 or full-strength P8 nutrient solution. *B. campestris* were grown in growth chamber for 30 DAT. Harvested plants were weighed for SFW, SDW and RFW. The significant difference among treatments were 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).

3.2.4 Effect of different concentration of MS medium on EPF colonization rate and plant growth under Petri dish culture

The seeds of *B. campestris* were surface sterilized as described above, sown on water agar (1%) and incubated in the growth chamber. Two 7-day-old seedlings of *B. campestris* were transplanted onto 20 ml water agar, 1/100-strength MS, 1/10-strength MS, 1/5-strength MS or full-strength MS medium, in 85-mm Petri dish, and grown in growth chamber. Two 5-mm mycelial plugs of 14-day-old 2614(4)PDA-1-2-1 or 2531(3)WA-2-1 cultured with ½-strength MEA medium were inoculated to each seedling

7 DAT, by placing them adjacent (1–5 mm) to root system. *B. campestris* were grown again in growth chamber until 21 DAT. Five replications were made for each treatment. Isolate 2614(4)PDA-1-2-1 was chosen because in the previous study it slightly increased the growth of *B. campestris* under application of low P (1 mg L^{-1}) or low N (4 mg L^{-1}) but normal concentration of other elements. As comparison, isolate 2531(3)WA-2-1 was chosen because in the previous study it decreased the growth of *B. campestris*. Isolate 2614(4)PDA-1-2-1 and 2531(3)WA-2-1 were isolated using PDA and water agar (WA) media, respectively.

Plants were harvested at 21 DAT. Shoots and roots were separated and cleaned under running tap water and rinsed with deionized water. Harvested plants were weighed for SFW, SDW and RFW. The significant difference among treatments were 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).

3.3 Results

3.3.1 Growth of EPF isolates on vermiculite applied with different liquid medium

Colony diameter of both EPF were increased by the increase of concentration of liquid malt extract (Table 3.1, Figure 3.1). Colony diameter of both EPF on 6 DAI applied with ½-strength malt extract were almost 2-fold larger than that applied with 1/10-strength malt extract. Colony diameter of both EPF applied with full-strength malt extract were 1.1-1.4-fold larger than that applied with ½-strength malt extract. Growth rate of both EPF were not different.

3.3.2 Growth of *Brassica campestris* transplanted on pre-grown EPF colony

Survival rate of *B. campestris* inoculated with both EPF were decreased by the increase of concentration of liquid malt extract (Table 3.2, Figure 3.2). Shoot fresh and dry weight of *B. campestris* per plant were not different with control plant. Root fresh weight of *B. campestris* per plant were not different with control plant. There was no internal colonization observed in all treatments. External colonization of both EPF were increased by the increase of concentration of liquid malt extract.

3.3.3 Shoot growth of *Brassica campestris* applied with P1 agar and ½-strength MS agar medium

Shoot fresh and dry weight of control *B. campestris* grown on ½ MS agar were higher than that grown on P1 agar (Table 3.3). Shoot fresh and dry weight of control *B. campestris* grown on both medium were higher than that inoculated with 2614(4)PDA-1-2-1. Root fresh weight of control *B. campestris* grown on ½ MS agar were higher than that grown on P1 agar. Root fresh weight of control *B. campestris* grown on both medium were higher than that inoculated with 2614(4)PDA-1-2-1.

3.3.4 Growth of *Brassica campestris* on different medium under different culture condition

Shoot fresh and dry weight, root fresh weight of *B. campestris* were increased by the increase of concentration of MS agar medium under Petri dish culture (Table 3.4, Figure 3.3). Shoot fresh and dry weight of *B. campestris* grown on 1/100 MS were not different with that grown on water agar. Shoot fresh and dry weight of *B. campestris* grown on 1/5 MS were not different with that grown on 1/10 MS. Shoot fresh and dry weight of *B.*

campestris grown on MS were higher than that grown on other medium. Root fresh weight of *B. campestris* grown on MS medium were higher than that grown on water agar but not different with that grown on other MS medium. Root length of *B. campestris* were not different in all medium.

Shoot fresh weight of *B. campestris* were increased by the increase of concentration of P8 nutrient solution under Plantbox culture (Table 3.5, Figure 3.4). Shoot fresh weight of *B. campestris* applied with 1/100 P8 nutrient solution were not different with that applied with water. Shoot fresh weight of *B. campestris* applied with 1/5 P8 nutrient solution were not different with that applied with 1/10 P8 nutrient solution. Shoot fresh weight of *B. campestris* applied with P8 nutrient solution were higher than that applied with other nutrient solution. Root fresh weight of *B. campestris* applied with 1/10 P8, 1/5 P8 or P8 nutrient solution were higher than that applied with 1/100 P8 nutrient solution or water. Root dry weight of *B. campestris* applied with 1/10 P8 nutrient solution were higher than that applied with P8 nutrient solution but not different with that applied with other nutrient solution.

3.3.5 Growth of *Brassica campestris* on different concentration of MS medium after inoculation with EPF

B. campestris inoculated with 2531(3)WA-2-1 grown on water agar, 1/10 MS, 1/5 MS and full-strength MS medium did not survived until harvest (Table 3.6, Figure 3.5). *B. campestris* inoculated with 2614(4)PDA-1-2-1 grown on full-strength MS medium did not survived until harvest. Shoot fresh weight of *B. campestris* inoculated with 2614(4)PDA-1-2-1 grown on 1/10 MS and 1/5 MS were lower than that of control plant.

Shoot fresh and dry weight of *B. campestris* inoculated with both EPF grown on 1/100 MS were not different with that of control plant.

3.4 Discussion

3.4.1 Effect of malt extract on relationship between EPF and plant

Higher concentration of malt extract increased growth of both EPF. Malt extract is a common composition of medium for fungi (Mueller et al. 2004). As for study about EPF, 2% MEA was used in isolation of EPF because it was favorable for growth of diverse isolates (e.g. Fröhlich and Hyde 1999, Zhao et al. 2002, Urairuj et al. 2003, Arnold et al. 2003). The higher concentration of malt extract was more favorable for growth of both EPF. Further, external colonization on *B. campestris* of both EPF were higher in higher concentration of malt extract. To our knowledge, present study is the first to clarify the effect of different concentration of malt extract on relationship between EPF and host plant.

Survival rate and growth of *B. campestris* inoculated with both EPF were lower than that of control plant. In the previous study, growth of *B. campestris* inoculated with both fungi were no different with or slightly increased than control plant. The internal and external colonization rate of both EPF were less than 10%. EPF inoculation and *B. campestris* transplanting was done in the same time. Thus the growth of EPF was too less to colonize B. campestris and reach colonization rate higher than 10%. In this experiment, the EPF was grown first and then *B. campestris* seedlings were transplanted on the EPF colony. We expected to get higher EPF colonization rate with higher growth promotion in *B. campestris*. However, the results clearly showed that the higher colonization rate of both EPF decreased the growth of *B. campestris*. Hacquard et al. (2016) inoculated a

beneficial EPF, *Colletotrichum tofieldiae*, to *Arabidopsis thaliana* grown on ½-strength MS medium without sucrose containing high P (625 µM KH_2PO_4) or low Pi (50 µM KH_2PO_4). Growth promotion was only observed when grown on medium without P. However, their results confirmed that the growth promotion was obtained because *A. thaliana* allow the colonization to some extent that *A. thaliana* could get benefit from the EPF. In this symbiosis, the plant was the symbiont that actively responded to the change of the environment. Regarding our results, *B. campestris* was not able to limit the colonization of EPF and decreased its growth. Thus the higher colonization by EPF may not always result in higher growth promotion in plant.

3.4.2 Effect of different concentration of MS medium on relationship between EPF and plant

Growth of *B. campestris* inoculated with both EPF were not different with that of control plant when grown on 1/100-MS medium. The study of Hacquard et al. (2016) also highlighted the importance of environmental factors, especially medium, in modifying the response of plant colonized by EPF. Nutrient concentration in 1/100 MS medium may be in a condition that limit the growth of the EPF thus reduced the risk of pathogenicity to the plant. However further experiments are needed to explain how the plant growth was not affected even by a pathogenic EPF, 2531(3)WA-2-1, either because of the limited growth of EPF or the active response of plant in limiting the growth of EPF. Unfortunately, there were no studies about effect of EPF inoculation on plant growth that use the same concentration as or lower concentration of MS medium than 1/100-strength MS medium. Other studies used 1/10-strength or higher concentration of MS medium. Thus the results can`t be compared to the results of those studies.

CHAPTER 4

Isolation and screening of endophytic fungi isolated from five forests in Indonesia

4.1 Introduction

Endophytic fungi (EPF) are fungi that colonize plant tissue without causing any visible disease symptoms at any particular moment (Schulz and Boyle 2005). Practically, EPF colonize almost any plant tissue, including leaf, stem, and root (Rodriguez et al. 2009). Since their discovery, EPF have been studied in many types of plants, including non-vascular ones, such as mosses (e.g., Schulz et al. 1993) and algae (e.g., Zuccaro et al. 2008), and vascular ones, such as shrubs (e.g., Schulz et al. 1993) and trees (e.g., Arnold and Lutzoni 2007). Most of the isolated EPF belong to Ascomycota and Basidiomycota (Schulz and Boyle 2005).

A conservative estimate of fungal diversity in the world of 1.5 million species has been accepted as the working hypothesis and the basis for the discovery of more fungal species (Hawksworth 2001). With only around 72,000 described species known so far, more than 1 million species are waiting to be found. EPF have been found in many plant species and considered an important component of fungal diversity (Rodriguez et al. 2009). Despite the increasing number of studies of EPF in many countries, studies of EPF in the tropics are still lacking.

Arnold et al. (2000) isolated 418 EPF morphospecies colonizing leaf of two understory tree species in a tropical forest in Panama, 59% of which were represented by single isolates. Cannon and Simmons (2002) isolated 64 EPF morphospecies colonizing leaf of 12 tree species in a tropical forest in Guyana, 29 of which were from single leaf

samples. Taking into account the 5:1 ratio of fungi to plant in the tropics (Mueller et al. 2007), these two studies reflect the high diversity of EPF colonizing tree leaf in tropical forest.

The different environmental factors in forests are expected to influence fungal diversity (Saikkonen 2007). However, most studies of the diversity of EPF colonizing leaf in tropical forest are limited to one forest site (e.g., Arnold et al. 2000 and 2001, Cannon and Simmons 2002). Suryanarayanan et al. (2011) compared the EPF communities of 75 dicotyledonous trees belonging to 33 families from three tropical forest types in Southern India, namely, tropical dry thorn forest, dry deciduous forest, and montane evergreen forest. The type of forest appeared to have a larger effect on shaping the EPF community than the taxonomy of the host.

Studies of EPF in tropical forest are limited to EPF that colonize the above-ground part of plant, particularly leaf. EPF colonizing roots, further termed as root EPF, of tropical forest trees are rarely studied. Rodriguez et al. (2009) classified root EFP into two groups: class 2 EPF which colonize shoot, root and rhizome and class 4 EPF, also termed dark septate endophyte (DSE) which colonize root only. Apart from the colonization range in plant tissue, the difference between these two groups is also the morphology. The DSE is characterized by dark pigmented septate hyphae and hyaline hyphae. However, class 2 EPF was observed to only have the latter. In a review by Jumpponen and Trappe (1998), they noted that DSE, colonized approximately 600 plant species representing 320 genera and 114 families, showing the abundance of DSE. However, DSE is not the only group of root EPF, indicating the possibility of an even higher abundance of root EPF in nature.

Root EPF are considered to play an important role in plant growth, similar to mycorrhizal fungi (Jumpponen and Trappe 1998). Some EPF were able to protect host

against fungal pathogens (Narisawa et al. 2002, Maciá-Vicente et al. 2008) and increase plant nitrogen uptake (Usuki and Narisawa 2007). Meta-analysis of data from temperate and boreal areas showed that root EPF colonization had a negative, neutral or positive effect on plant growth (Mandyam et al. 2013, Mayerhofer et al. 2013, Newsham 2011). Evidence of the positive effect of root EPF along with the expectation of high EPF abundance in tropical forest has underscored the necessity to conduct more studies of root EPF in tropical forest. However, studies of root EPF in tropical area remain a rarity (Mandyam and Jumpponen 2005).

The difference in plant growth response to EPF colonization is governed by not only plant or EPF species but also environmental factors, particularly experimental conditions Mayerhofer et al. 2013. Nutrient status may be an important factor as it also affects the relationship between plant and mycorrhizal fungi.

Murashige and Skoog (MS) medium is used to study the effect of EPF inoculation on plant growth. Nutrient concentration variation in MS medium is expressed as the dilution strength of MS medium. Mandyam et al. (2013) used 1/10-strength MS medium and observed a positive response of plant growth to EPF inoculation. Lacercat-Didier et al. (2016) used full-strength MS medium and observed a positive response of plant growth to EPF inoculation. The objectives of this chapter were (1) to isolate root EPF from five forests soils in Indonesia and identify them on the basis of the rDNA ITS region, (2) to compare EPF community among different forests, and (3) to clarify the effect of nutrient concentrations in growth medium on the relationship between host plants and EPF.

4.2 Materials and Methods

4.2.1 Isolation of EPF from tree and crop species

Paraserianthes falcataria (L.) Nielsen and *Sorghum bicolor* (L.) Moench were used in this study. *P. falcataria* was used because this species would be used as the target species in this study and the EPF isolation rates for the four tree species were not markedly different in the previous study. This is a fast-growing tree and a candidate species for reforestation efforts (Otsamo 2002, Wulandari et al. 2016). This species is also economically important due to being profitable in a mixed plantation with crop species or as single-species plantation (Siregar et al. 2007, Krisnawati et al. 2011). Its wood is a candidate for energy production (Amirta et al. 2016). *S. bicolor* was chosen because it had the highest EPF isolation rate among all species (Maulana 2015).

Sand was acidified and sterilized by autoclaving at 80 ºC for 45 min. Forty (40) grams of sterilized sand was mixed with 40 g of forest soil and used as growth medium. Seeds of *P. falcataria* were sown on sterilized sand and incubated in a growth chamber (Biotron LPH-350S, NK System, Japan) at 27 ºC with a 16-hour photoperiod. One of two-leaf-stage seedling of *P. falcataria* was transplanted onto the medium in a 50-ml syringe pot. Three seeds of *S. bicolor* (cv. New Sorgo 2, Pasturage seed, Japan) were sown onto the same medium. Ten grams of sterilized sand was further added into the syringe pot to cover the root system of seedlings or seeds. All plants were grown in the growth chamber for 90 days at 27 ºC with a 16-hour photoperiod. Five to ten milliliters of tap water was applied once every two days, minimizing the nutrient input to mimic the original condition. Twenty-five (25) pots were prepared for each plant species. Number of seedling per pot, plant height, number of leaves, and symptoms of nutrient deficiency on leaf of *P. falcataria* and *S. bicolor* were recorded before harvest (Tables 4.1 and 4.2,

Figures 4.1 and 4.2). Plants showing good growth were harvested and EPF were isolated from the roots.

P. falcataria and *S. bicolor* were harvested 90 days after transplanting or sowing. Fresh roots were extracted from each soil and washed under running tap water. The roots were surface-sterilized following the method of Verma et al. (2012) by dipping in 90% EtOH (1 min), 5% NaClO (5 min), and 90% EtOH (10 s) and rinsing three times with sterilized deionized water. The roots were dried with sterilized Kimtowels^{TM} and left to air dry. The air-dried roots were cut into 5 mm. Five pieces were plated on ½-strength malt extract agar (MEA) containing 100 µg mL^{-1} Penicillin-Streptomycin (Lonza Biowhittaker, Penicillin-Streptomycin Mixture) (modified from Verma et al. 2012) and water agar. Five replication plates were made for each medium for roots from one pot for a total of 50 plates per pot. The plates were sealed with Parafilm^{TM} and incubated in a dark room at 25 ^{o}C. Emerging colony from root within 2-months-period of observation was cultured was cultured on new ½-strength MEA medium. Isolation rates of EPF were calculated by dividing the number of isolates by the number of initial plates.

4.2.2 Identification of EPF by molecular method

4.2.2.1 DNA extraction and amplification

EPF was subcultured on ½-strength MEA for 1–3 weeks depending on the growth rate of each isolate. Hyphae of each isolate were collected with forceps and placed on the lid of a plastic tube containing 20 µL of InstaGene^{TM} Matrix (Bio-Rad, USA) (Maki et al. 2008). Hyphae were crushed with a pipet tip having a blunt end, further mixed with 180 µL of InstaGene^{TM} Matrix, and vortexed. rDNA was extracted following the

manufacturer's protocol for InstaGene^{TM} Matrix. The extracted DNA was stored at −20 ^{o}C until use.

The internal transcribed spacer (ITS) region of the fungi was amplified by using universal primers, ITS1F (5'-GTAACAAGGTTTCCGT-3') and ITS1R (5'-CGTTCTTCATCGATG-3') (Fujita et al. 2010), with an Expand High Fidelity^{PLUS} PCR system (Roche, Germany) at the following composition: 4 µL of 5 x buffer with $MgCl_2$, 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 Milli-Q 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 ºC for 120 s; 30 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 60 s, and extension at 72 ºC for 60 s; and final extension at 72 ºC for 420 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 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 ºC for 16 hours.

Single colonies of *E. coli* were collected and DNA was amplified using primers T7 (5'-TAATACGACTCACTATAG-3') and U19 (5'-GTTTTCCCAGTCACGACT-3') (Ikenaga et al. 2016) with GoTaq® DNA Polymerase (Promega, USA) at 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 Milli-Q 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 ºC for 120 s; 35 cycles of denaturation at 94 ºC for 15 s, annealing at 50 ºC for 60 s, and extension at 72 ºC for 80 s; and final extension at 72 ºC 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 BigDye^{TM} 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 re-suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems, Japan).

4.2.2.2 Phylogenetic analysis

Sequences of EPF isolates were submitted for BLAST analysis (Altschul et al. 1990). The sequences and their corresponding BLAST top hits were aligned by MAFFT (Katoh et al. 2002) through http://guidance.tau.ac.il. Maximum parsimony method was performed by MEGA 7 (www.megasoftware.net) with 1000 replications of bootstrap analysis.

4.2.3 Inoculation of host plants with EPF under Petri dish culture

4.2.3.1 Inoculation of *Brassica campestris* and *Paraserianthes falcataria* with EPF

B. campestris and *P. falcataria* were used in this experiment. *B. campestris* was reported to be responsive to EPF colonization (Usuki and Narisawa 2007, Lee et al. 2011, Xie et al. 2016). *P. falcataria* is a target plant for reforestation in Indonesia (Otsamo et al. 1995, 1997). The seeds of *B. campestris* (cv. Harusakari, Watanabe seed, Japan) and *P. falcataria* were surface-sterilized by dipping into 5% NaClO for 3 or 10 min, respectively. Then, the seeds were rinsed three times with sterilized deionized water. The surface-sterilized seeds were sown on water agar (1% agar) and grown in a growth chamber (Biotron LPH-350S, NK system, Japan) at 27 ºC with a 16-hour photoperiod. Two 7-day-old seedlings of *B. campestris* and one 7-day-old seedling of *P. falcataria* was transplanted onto 1/100- and 1/10-strength MS medium in an 85-mm-diameter plastic Petri dish (Figures 4.3 and 4.4, modified from Mandyam et al. 2010) and grown in the growth chamber at 27 ºC with a 16-hour photoperiod. A piece of sterilized filter paper (± 1 cm x 2 cm) (No 1, Whatman, USA) was placed on top of *P. falcataria* seedling to fix the roots to the medium (Figure 4.4). One 5-mm-diameter mycelial plug of 14-day-old EPF isolate cultured with ½-strength MEA medium was inoculated at the distance of 5 mm from the most distant root of one seedling, 7 days after transplanting (DAT). The Petri dish was sealed with Parafilm™ and kept in the growth chamber until 28 or 37 DAT for *B. campestris* or *P. falcataria*, respectively. The use of 1/10-strength MS medium was based on Mandyam et al. (2010, 2013). However, in the present study, we included not only the basal salt but also sucrose in the medium composition. Thirty-three (33) EPF isolates were used in this experiment. Some EPF isolates showed dark mycelium (Figure 4.5). These EPF isolates were isolated from the roots of *P. falcataria* and *S. bicolor* that grow on forest soils in Indonesia. These forest soils were from three forests in Kalimantan

Island (*Gmelina arborea*, *Artocarpus champeden*, and Dipterocarp mixed forest, Dipterocarp primary forest, and *Macaranga* sp. secondary forest) and two forests in Java Island (*Tectona grandis* monoculture forest and *Swietenia macrophylla* monoculture forest).

4.2.3.2 Plant growth measurement

Plants were harvested at 28 DAT or 37 DAT for *B. campestris* or *P. falcataria*, respectively. Shoots and roots were separated and cleaned under running tap water and rinsed with deionized water. The shoots were weighed for shoot fresh weight (SFW) and then oven-dried at 70 ºC for 72 hours for shoot dry weight (SDW). Plant response (PR) to EPF inoculation onto 1/100- and 1/10-strength MS medium was calculated using the equation for mycorrhizal dependency formulated by Plenchette et al. (1983): PR = [SDW (inoculated) – SDW (control)] / SDW (inoculated).

4.2.3.3 Assessment of EPF colonization

The roots were stained with aniline blue dye as described by Tawaraya et al. (1998). The roots of *B. campestris* or *P. falcataria* were cleared by dipping into 10% (w/v) KOH solution and heated in a water bath at 80 ºC for 5 min or 15 min, respectively. Then, the roots were rinsed with tap water, acidified with 1% (w/v) HCl, and rinsed again with tap water. The roots were dipped into 0.05% aniline blue solution (Aniline blue, Wako, Japan) and heated again at 90 ºC for 5 min. After rinsing with tap water, the roots were transferred to a Petri dish and lactic acid-glycerol solution was added. The roots were mounted on glass slides and covered with cover glass. Colonization was observed under a microscope (Eclipse 80i, Nikon, Japan) at 200x magnification. The presence of fungal

structures inside plant root indicated internal colonization. The presence of fungal structures on the surface of plant root indicated external colonization. Percentage colonization was estimated by the gridline intersect method on 100 intersections (Giovannetti and Mosse 1980).

4.2.3.4 Statistical analysis

The significant difference in PR between 1/100- and 1/10-strength MS medium and the significant difference in SDW between inoculated plant and respective control were determined by the Student's t-test using Kaleida Graph 4.1 software (Synergy software 2012, USA). Two of the 33 isolates used to inoculate *B. campestris* were excluded from statistical analysis because less than three replication plants survived until harvest. Those isolates were 2613(5)-1 and 2655(2). Thus, only 31 isolates were included in the statistical analysis.

4.2.4 Inoculation of host plant with EPF under Plantbox culture

The seeds of *B. campestris* were surface sterilized as described above, sown on water agar (1%) and incubated in growth chamber at 27 ºC with a 16-hours photoperiod. Plantbox (75 mm x 110 mm, BC-PB851-50, Biomedical science, Japan) containing 40 g of vermiculite were sterilized by autoclave at 121 ºC for 15 mins. Three *B. campestris* seedlings were transplanted to the vermiculite. Two 5-mm mycelial plugs cultured with ½-strength MEA medium were inoculated for each seedling at the same time as seedling transplanting by placing them adjacent (1–5 mm) to root system. Root system of *B. campestris* was subsequently covered with 15 g sterilized vermiculite. Sixty (60) milliliters of 1/100 MS solution was applied to *B. campestris*. The hole on top of Plantbox

was covered by sterilized cotton. *B. campestris* was grown in growth chamber for 21 DAT. Four EPF isolates used in this experiment were 2312(3), 2331(2), 2332(5), and 2334(2). These isolates were chosen because *B. campestris* showed positive response to inoculation of these isolates.

Plants were harvested at 21 DAT. Shoots and roots were separated and cleaned under running tap water and rinsed with deionized water. Harvested plants were processed as described above. EPF colonization were determined as described above. The significant difference among treatments were 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).

4.2.5 Effect of concentration of C, N, and P in agar medium and EPF inoculation on plant growth under Petri dish culture

The seeds of *B. campestris* were surface sterilized as described above, sown on water agar (1%) and incubated in growth chamber at 27 ^{o}C with a 16-hours photoperiod. Two 7-day-old seedlings of *B. campestris* were transplanted onto 20 ml of agar medium in 85-mm Petri dish and grown in growth chamber. Two 5-mm mycelial plugs cultured with ½-strength MEA medium were inoculated to each seedling, 7 DAT, by placing them adjacent (1–5 mm) to root system. *B. campestris* were grown again in growth chamber until 28 DAT. Five replications were made for each treatment. The medium used in this experiment were 1/100 MS without sugar, 1/10 MS without sugar, 1/100 MS high sugar, 1/10 MS low sugar, 1/100 high N, and 1/100 high P. Concentration of high sugar, N and P were referred to concentration in 1/10 MS. NH_4NO_3 or KH_2PO_4 was added for 1/100 high N or 1/100 high P MS, respectively. Concentration of low sugar was referred to

concentration in 1/100 MS. Isolates 2312(3), 2331(2), 2332(5), or 2334(2) were used in these experiments. Isolates 2632(1), 2633(1) and 2354(1)-2 were also used only in 1/100 MS high sugar and 1/10 low sugar. Isolates 2632(1) and 2633(1) were chosen because *B. campestris* showed positive response to inoculation of these isolates. While 2354(1)-2 was chosen as a comparison because *B. campestris* showed negative response to inoculation of this isolate.

Plants were harvested at 21 DAT. Shoots and roots were separated and cleaned under running tap water and rinsed with deionized water. Harvested plants were weighed for SFW, SDW and RFW. The roots were used for observation of EPF colonization rate. The significant difference among treatments were 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).

4.3 Results

4.3.1 Isolation of EPF

Leaf necrosis were detected in some seedlings of *S. bicolor* (Figure 4.1). Seedlings with higher plant height and leaf number, and less leaf necrosis in 12 of totally 25 pots, were selected and used for isolation of EPF (Table 4.1). Twenty-one (21) EPF were isolated from the roots of *S. bicolor*. Among the 21 EPF, 5, 2, 7, and 7 EPF were isolated from soils of *T. grandis* monoculture, *S. macrophylla* monoculture, *G. arborea*, *A. champeden*, and Dipterocarp mixed, and *Macaranga* sp. secondary forest, respectively. EPF isolation rate for *S. bicolor* was 35%.

Leaf necrosis were not detected in all seedlings of *P. falcataria* (Figure 4.2). Seedlings with higher plant height and leaf number in 10 of totally 25 pots, were selected and used

for isolation of EPF (Table 4.2). Twelve (12) EPF were isolated from the roots of *P. falcataria*. Among the 12 EPF, 2, 7, and 3 EPF were isolated from soils of *T. grandis* monoculture, *G. arborea*, *A. champeden*, and Dipterocarp mixed, and *Macaranga* sp. secondary forest, respectively. EPF isolation rate for *P. falcataria* was 24%, which was higher than that of the first isolation of EPF.

4.3.2 EPF identity based on DNA

The sequences of the ITS regions of all the isolates were submitted to BLAST without limitation to uncultured or environmental samples. The similarity scores between isolated EPF and their closest relatives in GenBank were between 84–100% (Table 4.3).

Seven genotypes of EPF isolated from the roots of *P. falcataria* closely matched fungi identified to the species level (Table 4.3, Figure 4.6). One genotype closely matched fungus identified to the phylum level. The remaining four genotypes closely matched uncultured fungi. Based on National Center for Biotechnology Information/NCBI (https://www.ncbi.nlm.nih.gov) database, the identified fungi were classified in order Pleosporales, Hypocreales, Eurotiales, and Chaetothyriales.

Nine genotypes of EPF isolated from the roots of *S. bicolor* closely matched fungi identified to the species level (Table 4.3, Figure 4.6). One genotype closely matched fungus identified to the genus level (Fusarium). One genotype closely matched fungus identified to the family level (Clavicipitaceae). One genotype closely matched fungus identified to the order level (Sordariales). One genotype closely matched fungus identified to the class level (Dothideomycetes). There was one genotype that closely matched unidentified fungus (Fungal sp. voucher). The remaining seven genotypes

closely matched uncultured fungi. Based on NCBI database, the identified fungi were classified in order Pleosporales, Hypocreales, Eurotiales, and Magnaporthales.

4.3.3 Response of host plant to EPF inoculation under Petri dish culture

4.3.3.1 Response of *Brassica campestris* to EPF inoculation

Two isolates increased, eight isolates decreased, and 21 isolates did not affect SDW of *B. campestris* grown on 1/100-strength MS medium (Table 4.4). Two isolates increased, three isolates decreased, and 26 isolates did not affect SDW of *B. campestris* grown on 1/10-strength MS medium. The number of isolates that increased SDW of *B. campestris* grown on 1/100-strength MS medium was the same as that grown on 1/10-strength MS medium. The number of isolates that decreased SDW of *B. campestris* grown on 1/100-strength MS medium was larger than that grown on 1/10-strength MS medium.

SDW of *B. campestris* grown on 1/100-strength MS medium inoculated with 2312(3) or 2334(2) was 1.6- or 1.8-fold significantly higher than control, respectively. SDW of *B. campestris* grown on 1/10-strength MS medium inoculated with 2334(2) was 1.4-fold significantly higher than control. SDW of *B. campestris* grown on 1/10-strength MS medium inoculated with 2312(3) and that grown on 1/100- and 1/10-strength MS medium inoculated with 2331(2) or 2332(5) were not significantly different from control. Even so, SDW of *B. campestris* grown on 1/100-strength MS medium inoculated with 2331(2) or 2332(5) was 1.3- or 1.4-fold higher than control. SDW of *B. campestris* grown on 1/10 strength MS medium inoculated with 2312(3), 2331(2) or 2332(5) was 1.1-, 1.3- or 1.2-fold higher than control, respectively.

B. campestris inoculated with three isolates showed higher PR in the medium with low nutrient concentration than the one with high nutrient concentration (Figure 4.7). *B.*

campestris inoculated with 26 isolates showed the same PR in both nutrient concentrations. *B. campestris* inoculated with four isolates exhibited lower PR in the medium with low nutrient concentration than the one with high nutrient concentration.

4.3.3.2 EPF colonization of *Brassica campestris* root

Internal colonization was not always observed in the roots of inoculated *B. campestris* (Table 4.4). The number of intersections for colonization rate determination was between 11 and 100 depending on root availability. *B. campestris* inoculated with 2312(3) (Figure 4.3) or 2334(2) exhibited internal and external colonization, and both showed significantly higher SDW than control. Internal and external colonization was also observed in inoculated *B. campestris* that showed significantly lower SDW than control, for example, in *B. campestris* grown on 1/10-strength MS medium inoculated with 2655(2).

4.3.3.3 Response of *Paraserianthes falcataria* to EPF inoculation

One isolate increased, no isolate decreased, and 32 isolates did not affect SDW of *P. falcataria* grown on 1/100-strength MS medium (Table 4.5). No isolate increased, 11 isolates decreased, and 22 isolates did not affect SDW of *P. falcataria* grown on 1/10-strength MS medium. The number of isolates that decreased SDW of *P. falcataria* grown on 1/100-strength MS medium was smaller than that grown on 1/10-strength MS medium.

P. falcataria inoculated with eight isolates showed higher PR in the medium with low nutrient concentration than in that with high nutrient concentration (Figure 4.8). *P. falcataria* inoculated with 24 isolates exhibited the same PR in both nutrient

concentrations. *P. falcataria* inoculated with one isolate showed lower PR in the medium with low nutrient concentration than in that with high nutrient concentration.

4.3.3.4 EPF colonization of *Paraserianthes falcataria* root

Internal colonization was not always observed in the roots of inoculated *P. falcataria* (Table 4.5). The number of intersections for colonization rate determination was between 30 and 100 depending on root availability. *P. falcataria* inoculated with 2312(3) exhibited internal and external colonization (Figure 4.4) although there was no significant difference in SDW between the inoculated plant and control. Internal colonization was not observed in *P. falcataria* inoculated with 2651(4), which showed significantly higher SDW than control. Internal and external colonization was observed in inoculated *P. falcataria* that showed significantly lower SDW than control, for example, in *P. falcataria* grown on 1/10-strength MS medium inoculated with 2352(5).

4.3.4 Response of *Brassica campestris* to EPF inoculation under Plantbox culture

Shoot fresh weight of *B. campestris* inoculated with four EPF were not different compared with that of control plant (Table 4.6, Figure 4.9). However, shoot dry weight of *B. campestris* inoculated with 2312(3) were higher than that of control plant. Root fresh weight of *B. campestris* inoculated with 2331(2), 2332(5), and 2334(2) were higher than that of control plant but not different with that inoculated with 2312(3). External colonization of 2312(3) were higher than other EPF. Shoot: root ratio all inoculated *B. campestris* were lower than that of control plant.

4.3.5 Growth of *Brassica campestris* on MS medium with different concentration of C, N, and P after inoculation with EPF

Shoot dry weight of *B. campestris* inoculated with all EPF grown on all medium were not different with that of control, except *B. campestris* inoculated with 2331(2), 2354(1)2, 2632(1) and 2633(1) grown on 1/10 MS low sugar which was lower than control (Table 4.7). Internal and external colonization were generally higher in 1/100 MS high sugar than other medium. Plant response of *B. campestris* to EPF inoculation grown on 1/100 MS high N were the same as that grown on 1/100 MS high P but higher than that grown on other medium (Figure 4.10). Plant response of *B. campestris* to EPF inoculation grown on 1/10 MS low sugar was lower than that grown other medium.

4.4 Discussion

4.4.1 Preference of EPF for host plant and forest site

EPF in order Pleosporales, Hypocreales, Eurotiales, Magnaporthales, and Chaetothyriales were isolated in the present study (Table 4.3). EPF in order Pleosporales, Hypocreales, and Eurotiales were isolated from both host plants *P. falcataria* and *S. bicolor*, whereas EPF in order Magnaporthales and Chaetothyriales were specifically isolated from *P. falcataria* and *S. bicolor*, respectively. In the order level, most of the isolated EPF showed low host preference. EPF in these five orders were also isolated from the roots of mouse barley, *Hordeum murinum*, in Ireland (Murphy et al. 2015). EPF in two orders Pleosporales and Hypocreales were also isolated from the roots of plants growing in places with different environmental conditions, such as desert grass *Bouteloua gracilis* in New Mexico (Porras-Alfaro et al. (2008) and tropical shrub *Sophora*

tonkinensis in China (Yao et al.. 2017), showing the wide distribution of EPF in these two orders in nature.

EPF in genera *Acrocalymma*, *Fusarium*, *Tolypocladium*, *Penicillium*, *Talaromyces*, *Exophiala*, *Dictyosporium, Pseudochaetosphaeronema*, *Mariannaea*, *Trichoderma*, and *Mycoleptodiscus* were also reported in other studies. Jin et al. (2017) isolated *Acrocalymma vagum* from roots of tobacco, *Nicotiana tabacum*. Kwaśna et al. (2016) isolated *Talaromyces verruculosus* and *Trichoderma spirale* from roots of oak tree in a riparian forest, Poland. Lin et al. (2007) isolated *Dictyosporium* sp. from roots of a deciduous tree, *Camptotheca acuminata*, in a mountainous conservation area, China. Shubin et al. (2014) isolated *Mycoleptodiscus* sp. and *Penicillium* sp. from rhizome of *Alpinia officinarum*, China. Yao et al. (2017) isolated *Fusarium solani* from roots of a medicinal herb, *Sophora tonkinensis*, in a limestone mountainous areas, China. Zhang Q et al. (2017) isolated *Exophiala piscipila* from roots of Sorghum. Waipara et al. (1996) isolated *Mariannaea* sp. from roots of pasture plants in New Zealand. Zhang Y et al. (2017) isolated *Pseudochaetosphaeronema larense* from mangrove plant in China. Sánchez Márquez et al. (2010) isolated *Tolypocladium cylindrosporum* from leaves of a perennial grass, *Holcus lanatus*. However, to our knowledge, the present study is the first to isolate EPF in these genera from the roots of *P. falcataria* and *S. bicolor*. Amin (2013b) isolated EPF belonging a different genus, *Nigrospora* sp. (order Trichosphaeriales), from the roots of *P. falcataria*, and there is no study that isolated EPF from the roots of *S. bicolor*. Diene et al. (2010) and Zhang Q et al. (2017) inoculated DSE and confirmed EPF colonization in the roots of *S. bicolor*, indicating the possibility of isolating EPF from its roots.

Sixteen of the 33 isolates had the closest match to fungi identified to the species level (Table 4.3). Ninety-seven (97) percent similarity is widely used as the cut-off point

(O`Brien et al. 2005) to determine whether the isolates are identical to the species level or not. Thirteen of the 16 isolates were considered the same species with the closest match. The remaining three isolates, 2354(1)-2, 2624(5), and 2655(2), were close to *Exophiala calicioides* (84% similarity), *Pseudochaetosphaeronema martinelli* (95% similarity), and *Mycoleptodiscus terrestris* (94% similarity), respectively. These three isolates may be categorized in the same genera with the closest match.

Among the 16 isolates, 3 were specific to certain forest sites shared by *P. falcataria* and *S. bicolor*: *Fusarium solani* in *T. grandis* monoculture, *Talaromyces verruculosus* in *G. arborea*., *A. champeden* and Dipterocarp mixed, and *Talaromyces aculeatus* in *Macaranga* sp. secondary forest (Table 4.3). In addition, some of the isolates were specific to certain forest sites but were not shared by the two host plants, examples of which are *Dictyosporium heptasporum* in *T. grandis* monoculture, *Mariannaea camptospora* in *G. arborea*, *A. champeden*, and Dipterocarp mixed, and *Mycoleptodiscus* sp. in *Macaranga* sp. secondary forest. These results indicated that EPF had low or high preference for host plant as well as forest site. This phenomenon was also observed by Kernaghan and Patriquin (2011) in their study of Boreal area. Kernaghan and Patriquin (2011) isolated root EPF from *Betula papyrifera*, *Abies balsamea*, and *Picea glauca* and identified them by a molecular method. They revealed that some root EPF were found preferentially on a particular host whereas others were found specifically on a certain host. Jumpponen and Trappe (1998) and Mandyam and Jumpponen (2005) clarified that root EPF, specifically DSE, colonized 587 plants representing 320 genera and 114 families. Further inoculation experiments under natural and experimental conditions confirmed that DSE species had low host preference.

The numbers of isolates in *G. arborea*, *A. champeden*, and Dipterocarp mixed forest and *Macaranga* sp. secondary forest were higher than those in the other forest sites (Table

4.3). The dominant species in *G. arborea*, *A. champeden*, and Dipterocarp mixed forest were *G. arborea*, *A. champeden*, and Dipterocarp sp. The dominant species in *T. grandis* monoculture forest and *S. macrophylla* monoculture forest was only one species each, *T. grandis* and *S. macrophylla*, respectively. The number of plant species in each forest might be the reason why the number of isolates was higher in *G. arborea*, *A. champeden*, and Dipterocarp mixed forest than the other forest sites. The dominant species in *Macaranga* sp. secondary forest was also one species: *Macaranga* sp. The reason why the number of isolates was larger in *Macaranga* sp. secondary forest than *T. grandis* monoculture forest and *S. macrophylla* monoculture forest is not known.

Our results demonstrate that root EPF community differed among forest sites. Utilization of the trap culture method with the same host plant, *P. falcataria* or *S. bicolor*, still yielded different EPF among the five forests. Thus, differences in root EPF community were mainly due to differences in forest sites involving the plant community and environmental factors that resulted in the specific conditions in each forest.

4.4.2 EPF isolation by trap culture

The isolation of root colonizing microbes by trap culture is widely used in microbiological studies. However, the isolation from field-collected plant is a more common method in EPF studies, especially in studies that aim to isolate organic compounds for biotechnological applications (Strobel 2003). The trap culture method is common for studies that aim to clarify the role of EPF in protecting plants against soil pathogens and promoting plant growth (Amin 2013b; Narisawa et al. 2002, 2007) or to clarify the existence of certain EPF in the field (Ahlich et al. 1998).

Studies of root EPF by the isolation method might underestimate the number of species compared to studies that directly assess root samples by using the molecular method. However, the isolation method has one advantage, namely, the availability of culture for further studies. Brock et al. (2009) emphasized the importance of a herbarium or a fungal culture for the detailed assessment of unknown fungi.

P. falcataria and *S. bicolor* were not part of the plant community in the five forest sites. Nevertheless, EPF were still isolated from their roots. Moreover, different EPF were isolated from different forests, indicating that the trap culture method can be used to evaluate differences in EPF community among forests. Considering that both host plants were not part of the plant community in the five forest sites, the effect of original plant species on the identity of isolated EPF was minimal. Therefore, differences in EPF isolates among the forest sites reflect differences in EPF community among the forest sites.

4.4.3 Nutrient concentration in growth medium affects the relationship between host plant and EPF

Studies of EPF, particularly leaf EPF, in the tropics are increasing, as exemplified by studies conducted in Panama (Arnold et al. 2000, 2003) and India (Suryanarayanan et al. 2011), whereas studies of root EPF are scarce. Studies of root EPF in a temperate country by Mandyam et al. (2010, 2013) showed that EPF inoculation resulted in such PRs as parasitism and mutualism. In addition, Mandyam et al. (2013) and Lacercat-Didier et al. (2016) recorded positive PR upon EPF inoculation despite using MS medium with different nutrient concentrations. Based on those two studies, it seems that nutrient concentration in medium does not have any effect on the relationship between EPF and

plant. However, further work involving different nutrient concentrations under the same experimental conditions including plant species and EPF strain is needed to come to a definite conclusion. In the present study, our intent was to clarify the effect of inoculation of tropical EPF on plant growth, with nutrient concentration in medium as a possible factor determining PR to EPF inoculation. In addition, due to lack of studies of the effect of EPF on plant growth in the tropics, evidence obtained from studies in temperate countries was used to explain the results of the present study.

Two inoculated *B. campestris* and one inoculated *P. falcataria* exhibited increased SDW when grown on 1/100-strength MS medium but not on 1/10-strength MS medium. The number of inoculated *B. campestris* and *P. falcataria* with more positive PR was larger when grown on 1/100-strength MS medium than 1/10-strength MS medium. The difference between 1/100- and 1/10-strength MS medium was the concentrations of all the nutrients contained in the medium. Mutualism between *B. campestris* or *P. falcataria* and EPF was achieved when nutrient concentration was low. In the case of mycorrhizal association, particularly arbuscular mycorrhizal association, phosphorus (P) concentration in the medium is generally thought to be one of the important drivers of mutualism (Johnson et al. 1997). Some studies have documented the importance of P in the association between EPF and Brassicaceae species. Hiruma et al. (2016) inoculated *Arabidopsis thaliana* with *Colletotrichum tofieldiae* and grew it on ½-strength MS medium without sucrose and with two concentrations of P: 0.68 mg 100 g^{-1} (low P) and 8.51 mg 100 g^{-1} (high P). *C. tofieldiae* increased SFW of *A. thaliana* grown on MS medium with low P. Almario et al. (2017) inoculated *Arabis alpina* with Helotiales species and grew it on MS agar with two concentrations of P: 100 µM (low P) and 1000 µM (high P). The results corresponded to the present study and Hiruma et al.'s study (2016), namely, growth of *A. alpina* was promoted when conducted on medium with low

P. P concentration in the growth medium may have an effect on the relationship between EPF and host plant.

Nitrogen is also an important macronutrient that possibly exerts an effect on the relationship between EPF and plant. Usuki and Narisawa (2007) inoculated *B. campestris* with *Heteroconium chaetospira* and grew it on basal agar medium with different forms of nitrogen (NO_3, NH_4, glutamine, leucine, phenylalanine, and valine). *B. campestris* dry weight was increased by inoculating *H. chaetospira* when the medium contained organic nitrogen and not inorganic nitrogen. In the present study, we used only inorganic nitrogen in the medium and found that it may not affect the relationship between EPF and plant.

The increase of nitrogen and phosphorus in 1/100 MS medium, decreased the PRs of *B. campestris* inoculated with four EPF (Figure 4.10). These results clearly showed that nitrogen and phosphorus were the driver of the decrease of PRs in 1/10 MS medium compared to 1/100 MS. However, the fact that the PRs of *B. campestris* grown on either 1/100 high N or high P were lower than that grown on 1/10 MS, emphasize that there were other environmental factors affecting the PRs.

Some researchers proposed the mechanisms of plant growth promotion by root EPF. Hiruma et al. (2016) proposed that the root EPF, *C. tofieldiae* can take up P and transfer it to *A. thaliana*. They inoculated *A. thaliana* with *C. tofieldiae* and grown in 2-compartment system consisted of a root hyphal compartment (RHC) and a root-restricted, hyphae only compartment (HC) added with ^{33}P. They measured the ^{33}P concentration in the plant after harvest and then clarified that the inoculated *A. thaliana* had higher ^{33}P than control plant. While another study by Lee et al. (2011), inoculated *Piriformospora indica* to *B. campestris* and *A. thaliana*. They proposed that *P. indica* upregulated the genes related to auxin biosynthesis and signaling in roots of *B. campestris* and caused the growth promotion in *B. campestris*. However in the case of *A. thaliana*, those genes were

not upregulated although the growth were promoted. There may be other mechanisms in plant growth promotion by root EPF.

Most inoculated *B. campestris* and *P. falcataria* showed no difference in SDW even if those plants were grown on 1/100-strength MS medium. Diene et al. (2013) and Mahmoud and Narisawa (2013) inoculated EPF to *B. campestris* and also recorded the similar results. These findings indicate that EPF has functional diversity in promoting plant growth. These inoculated plants were colonized or not colonized by the EPF. The plants that were not colonized may not be affected by the EPF thus the SDWs were the same as that of control plants. The plants that were colonized were able to limit the adverse effect of EPF by activating the plant defense mechanism. Even if the fungi obtain carbon upon its colonization in plant roots, the effect on the plant growth might be minimum.

The number of inoculated *B. campestris* with decreased SDW when grown on 1/10-strength MS medium was larger than that grown on 1/100-strength MS medium. Nutrient concentration in the 1/10-strength MS medium was higher than that in the 1/100-strength MS medium. Besides the high P in the 1/10-strength MS medium, carbon from sucrose is a possible nutrient affecting the association between EPF and host plant. EPF can survive by being a biotroph or a saprotroph. In this regard, acquiring carbon from the growth medium without forming symbiosis with plant is possible for EPF. By acquiring carbon and other nutrients from the growth medium, EPF may indirectly limit nutrient availability for plant growth. This hypothesis may apply to EPF that are not parasitic. If the EPF are parasitic, when carbon and other nutrients are sufficient for the EPF, the EPF are likely to colonize and limit plant growth directly. However, further studies are needed to confirm this hypothesis.

Study by Hacquard et al. (2016) clarified the difference between closely related beneficial EPF, *C. tofieldiae*, and pathogenic EPF, *C. incanum*, when interacting with host plant. They found that *C. incanum* secreted higher candidate secreted effector proteins (CSEPs) and carbon active enzymes (CAZyme), especially related to cell wall polymer. Secretion of these effector and enzyme associated with plant cell death. These are the different characteristics that may possibly cause *C. incanum* to be a pathogenic EPF. In case of *C. tofieldiae*, secretion of these effector and enzymes were lower and plant responded in a way to limit the colonization of this EPF while still benefiting in P uptake.

4.4.4 Effect of EPF inoculation on growth of different plant species

SDWs of *B. campestris* and *P. falcataria* inoculated with same EPF isolates were increased, not affected, or decreased relative to control plant. EPF that increased SDW of *B. campestris* did not always increase SDW of *P. falcataria*. Different PRs to the inoculation of the same EPF were also observed by Mandyam et al. (2010). Mandyam et al. (2010) inoculated leek (*Allium porrum* L.) and C_4 grass (*Andropogon gerardii* Vitman) with *Microdochium* sp. and *Periconia macrospinosa*. Internal colonization of *A. porrum* root was observed but the total biomass was not affected by the EPF inoculation. Internal colonization of *A. gerardii* root was observed, but in contrast to *A. porrum*, the total biomass of *A. gerardii* was increased or not affected by the EPF inoculation. Mandyam et al. (2013) inoculated three genotypes of *Arabidopsis thaliana* (Col-0, Cvi-0, Kin-1) with four strains of *Microdochium* sp. and 34 strains of *Periconia* sp. Inoculation of the same EPF resulted in different PRs among the *A. thaliana* genotypes, underscoring the fact that PR to EPF inoculation differs with not only plant species but also plant genotype.

CHAPTER 5

Isolation and screening of endophytic fungi isolated from roots of *Santalum album* and *Swietenia macrophylla* in Indonesia

5.3 Introduction

Endophytic fungi (EPF) are fungi that colonize plant tissue without causing any visible disease symptoms at any particular moment (Schulz and Boyle 2005). Practically, EPF colonize almost any plant tissue, including leaf, stem, and root (Rodriguez et al. 2009). Since their discovery, EPF have been studied in many types of plants, including non-vascular ones, such as mosses (e.g., Schulz et al. 1993) and algae (e.g., Zuccaro et al. 2008), and vascular ones, such as shrubs (e.g., Schulz et al. 1993) and trees (e.g., Arnold and Lutzoni 2007). Most of the isolated EPF belong to Ascomycota and Basidiomycota (Schulz and Boyle 2005).

A conservative estimate of fungal diversity in the world of 1.5 million species has been accepted as the working hypothesis and the basis for the discovery of more fungal species (Hawksworth 2001). With only around 72,000 described species known so far, more than 1 million species are waiting to be found. EPF have been found in many plant species and considered an important component of fungal diversity (Rodriguez et al. 2009). Despite the increasing number of studies of EPF in many countries, studies of EPF in the tropics are still lacking.

Arnold et al. (2000) isolated 418 EPF morphospecies colonizing leaf of two understory tree species in a tropical forest in Panama, 59% of which were represented by single isolates. Cannon and Simmons (2002) isolated 64 EPF morphospecies colonizing

leaf of 12 tree species in a tropical forest in Guyana, 29 of which were from single leaf samples. Taking into account the 5:1 ratio of fungi to plant in the tropics (Mueller et al. 2007), these two studies reflect the high diversity of EPF colonizing tree leaf in tropical forest.

Studies of EPF in tropical forest are limited to EPF that colonize the above-ground part of plant, particularly leaf. EPF colonizing roots, further termed as root EPF, of tropical forest trees are rarely studied. Rodriguez et al. (2009) classified root EFP into two groups: class 2 EPF which colonize shoot, root and rhizome and class 4 EPF, also termed dark septate endophyte (DSE) which colonize root only. Apart from the colonization range in plant tissue, the difference between these two groups is also the morphology. The DSE is characterized by dark pigmented septate hyphae and hyaline hyphae. However, class 2 EPF was observed to only have the latter. In a review by Jumpponen and Trappe (1998), they noted that DSE, colonized approximately 600 plant species representing 320 genera and 114 families, showing the abundance of DSE. However, DSE is not the only group of root EPF, indicating the possibility of an even higher abundance of root EPF in nature.

Root EPF are considered to play an important role in plant growth, similar to mycorrhizal fungi (Jumpponen and Trappe 1998). Some EPF were able to protect host against fungal pathogens (Narisawa et al. 2002, Maciá-Vicente et al. 2008) and increase plant nitrogen uptake (Usuki and Narisawa 2007). Meta-analysis of data from temperate and boreal areas showed that root EPF colonization had a negative, neutral or positive effect on plant growth (Mandyam et al. 2013, Mayerhofer et al. 2013, Newsham 2011). Evidence of the positive effect of root EPF along with the expectation of high EPF abundance in tropical forest has underscored the necessity to conduct more studies of root EPF in tropical forest. However, studies of root EPF in tropical area remain a rarity

(Mandyam and Jumpponen 2005). The objectives of this chapter were (1) to isolate EPF from roots of *Santalum album* and *Swietenia macrophylla*, and (2) to screen the isolated EPF.

5.2 Materials and Methods

5.2.1 Forest site

Wanagama I forest was planted as a reforestation effort of bare land in a Karst landscape by Faculty of Forestry, Universitas Gadjah Mada. This forest is administratively located in Gunung Kidul Regency, DI Yogyakarta Province, Indonesia. *Santalum album* Linn. and *Swietenia macrophylla* King are two of several tree species planted in 1968/1969.

5.2.2 Collection of roots of *Santalum album* and *Swietenia macrophylla*

Five to eight-months-old, eight seedlings (15–25-cm-height) of *S. album* and seven seedlings (20–30-cm-height) of *S. macrophylla*, without any symptoms of nutrient deficiency on the leaf were uprooted carefully and immediately kept in an icebox with temperature < 10 ºC, on 18 January 2016. The roots of these seedlings were cut from the shoots and brought to Yamagata University, Japan.

5.2.3 Isolation of EPF from roots of *Santalum album* and *Swietenia macrophylla*

The roots were washed under running tap water and further surface-sterilized following the method of Verma et al. (2012) by dipping in 90% EtOH (1 min), 5% NaClO (5 min), and 90% EtOH (10 s) and rinsing three times with sterilized deionized water.

The roots were dried with sterilized Kimtowels™ and left to air dry. The air-dried roots were cut into 5 mm. Five pieces were plated on ½-strength malt extract agar (MEA) containing 100 µg mL^{-1} Penicillin-Streptomycin (Lonza Biowhittaker, Penicillin-Streptomycin Mixture) (modified from Verma et al. 2012). Ten replication plates were made for each seedlings. The plates were sealed with Parafilm™ and incubated in a dark room at 25 ºC. Emerging colony from root within 2-months-period of observation was cultured on new ½-strength MEA medium. Isolation rates of EPF were calculated by dividing the number of isolates by the number of initial plates.

5.2.4 Inoculation of host plants with EPF under Petri dish culture

5.2.4.1 Inoculation of *Brassica campestris* with EPF

The seeds of *B. campestris* (cv. Harusakari, Watanabe seed, Japan) were surface-sterilized by dipping into 5% NaClO for 3 min. Then, the seeds were rinsed three times with sterilized deionized water. The surface-sterilized seeds were sown on water agar (1% agar) and grown in a growth chamber (Biotron LPH-350S, NK system, Japan) at 27 ºC with a 16-hour photoperiod. Two 7-day-old seedlings of *B. campestris* and one 7-day-old seedling of *P. falcataria* were transplanted onto 1/100-strength MS medium in an 85-mm-diameter plastic Petri dish (Figure 5.1) and grown in the growth chamber at 27 ºC with a 16-hour photoperiod. One 5-mm-diameter mycelial plug of 14-day-old EPF isolate cultured with ½-strength MEA medium was inoculated at the distance of 5 mm from the most distant root of one seedling, 7 days after transplanting (DAT). The Petri dish was sealed with Parafilm™ and kept in the growth chamber until 28 DAT.

5.2.4.2 Plant growth measurement

Plants were harvested at 28 DAT. Shoots and roots were separated and cleaned under running tap water and rinsed with deionized water. The shoots were weighed for shoot fresh weight (SFW) and then oven-dried at 70 ºC for 72 hours for shoot dry weight (SDW). The roots were weighed for root fresh weight (RFW). Shoot: root ratio were calculated based on SFW and RFW. Plant response (PR) to EPF inoculation onto 1/100-strength MS medium was calculated using the equation for mycorrhizal dependency formulated by Plenchette et al. (1983): PR = [SDW (inoculated) – SDW (control)] / SDW (inoculated).

5.2.4.3 Statistical analysis

The significant difference in all parameters between inoculated plant and respective control were determined by the Student's t-test using Kaleida Graph 4.1 software (Synergy software 2012, USA).

5.3 Results

5.3.1 Isolation of EPF from *Santalum album* and *Swietenia macrophylla*

Totally 47 and 28 EPF were isolated from roots of *S. album* and *S. macrophylla*, respectively (Table 5.1). However only 36 and 24 EPF from roots of *S. album* and *S. macrophylla*, respectively, were successfully maintained without any contamination by fungi or bacteria. Mean number of EPF isolated from roots of *S. album* and *S. macrophylla* were 6 and 4 EPF, respectively. There was no significant difference in mean number of EPF isolated from both plants. Isolation rate of EPF from roots of *S. album* and *S. macrophylla* were 59% and 40%, respectively.

5.3.2 Growth response of *Brassica campestris* to EPF inoculation under Petri dish culture

All 36 and 24 EPF from roots of *S. album* and *S. macrophylla*, respectively, were used for inoculation experiments although showing the same morphological characteristics. Among 36 isolates from *S. album*, two isolates increase, 12 isolates decreased, and 22 isolates did not affect SDW of *B. campestris* grown on 1/100-strength MS medium (Table 5.2). SDW of *B. campestris* inoculated with SA 3-7 or SA 2-4 was 1.2- or 1.1-fold significantly higher than control, respectively.

Among isolates from *S. macrophylla*, four isolates increased, five isolates decreased, and 15 isolates did not affect SDW of *B. campestris* grown on 1/100-strength MS medium (Table 5.2). SDW of *B. campestris* inoculated with SM 5-2, SM 1-3, SM 6-3 or SM 6-6 was 1.5-, 1.1-, 1.2-, or 1.2-fold significantly higher than control, respectively. Among the isolates that increased SDW, only isolate SM 5-2 that also increased the SFW (Figure 5.1).

Plant response (PR) were not calculated for *B. campestris* inoculated with 17 EPF that did not survived until harvest (Figure 5.2). Response of *B. campestris* inoculated with 43 EPF were ranged from −0.37 to 0.32, with 22 EPF showed PR value higher than 0.00.

5.4 Discussion

5.4.1 EPF isolation from roots of *Santalum album* and *Swietenia macrophylla*

S. macrophylla is an exotic tree species in Indonesia with marketable wood for furniture or even building construction (Krisnawati et al. 2011). This species is also being studied for the antibacterial activity of its seed oil (Suliman et al. 2013), antimalarial

activity of its bark extract (Murningsih et al. 2005), and anticancer activity of its fruit extract (Goh and Kadir 2011).

Study about EPF from *S. macrophylla* is very few, focusing on isolation of bioactive compound from EPF colonizing aboveground parts (twigs, bark) of this species (e.g. Ramdanis et al. 2012, Dompeipen and Simanjuntak 2015). To the best of our knowledge, there is no study about root EPF from this species.

S. album is a native tree species in Indonesia and famous for the high economic value of the essential oil extracted from its wood. This species is listed in IUCN red list as vulnerable (www.iucnredlist.org) because of the decrease of population. This species is being studied from phytochemistry and pharmacological aspects such as antifungal, antibacterial, and antioxidant activity of its essential oil (Sindhu et al. 2010)

Study about EPF from *S. album* is also very few. Present study is not the first to isolate EPF from the root of *S. album*. Sun et al. (2014) isolated EPF from the roots of *S. album* in China.

5.4.2 Negative to positive response of *Brassica campestris* inoculated with EPF from roots of *Santalum album* and *Swietenia macrophylla*

Response of *B. campestris* to EPF inoculation was negative to positive. Other studies by Diene et al. (2013) and Mahmoud and Narisawa (2013) that inoculated root EPF to *B. campestris* also recorded similar response, although the inorganic medium used by those authors was different with that used in the present study. Value of positive response recorded in those studies were also lower than that in the present study. This is may be caused by the difference of medium used, highlighting the importance of medium in relationship between EPF and host plant. In addition, another study by Mandyam et al.

(2013) also recorded negative to positive response in *Arabidopsis thaliana*, a plant species from the same family as *B. campestris*.

CHAPTER 6

General discussion

6.1 Factors affecting propagation of AMF spore

In the present study *T. repens* was proven to be a good host plant in soil culture. Another *Trifolium* species, *T. pretense*, was recorded to be colonized by AMF in many studies and also used for trap culture host plant (Sanders 1992, Gamper et al. 2005, Velázquez and Cabello 2011). These results showed that *Trifolium* species are promising host plant for propagation of AMF spores.

The spores of three AMF isolates were not germinated in root organ culture. Spore without germination on monoxenic culture was also observed in another study. Douds 1997 reported that spores of *Glomus mosseae* did not germinated at pH 5.5 on unbuffered M medium but germinated on M medium buffered with MES at pH above 6.7 or with Tris at pH 7.3 and 7.6. pH of medium may be the factor that caused the no germination of the spores of these three isolates

6.2 AMF isolation from tropical forest in Indonesia using leguminous trees

Five isolated AMF were identified on the basis of the LSU region of rDNA. 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). Isolate M10-2 closely matched *D. gibbosa* isolated by (Blaszkowski 1997) from maritime sand dunes in Poland. To our knowledge, our study is the first to isolate and identify *D. gibbosa* from tropical forest soil. Isolate M60-3 closely matched *A. appendicula* (basionym= *Acaulospora appendicula*) isolated by Spain, Sieverd, and Schenk in 1984 (Walker 2008). *A.*

appendicula was also isolated from the rhizosphere of a threatened native leguminous tree, *Pericopsis mooniana*, growing in a natural forest in Sulawesi Island, Indonesia (Husna et al. 2014). 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 (Stockinger et al. 2009). Isolate S6-4 also closely matched *Glomus manihotis* found in the roots of sweet potato in China (Farmer et al. 2007). M11-1 closely matched *Acaulospora longula*. This species was also isolated from the rhizosphere of a perennial forb, *Solidago missouriensis*, in tallgrass prairie in USA (Eom et al. 2000) and also from the rhizosphere of a tree species, *Diospyros blancoi*, in West Java of Indonesia (Ningsih et al. 2013).

6.3 Effect of AMF on promoting leguminous tree growth

S6-4 increased growth of four leguminous trees. 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 in the study by Kung`u et al. (2008), Guissou et al. (2009), and Wulandari et al. (2014). 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. Other isolates also promoted growth of some tree species. M11-1 increased number of leaves of *P. falcataria* and *C. calothyrsus*, and SFW, shoot P content, and shoot: root ratio of *P. falcataria*. Other isolates also enhanced

the growth of some leguminous trees. M10-2 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.

Positive correlations between colonization rate and shoot P content were observed for *P. falcataria* ($R^2 = 0.87$, $P < 0.001$), *C. calothyrsus* ($R^2 = 0.41$, $P < 0.001$), *C. siamea* ($R^2 = 0.39$, $P = 0.0011$), and *S. grandiflora* ($R^2 = 0.13$, $P = 0.048$). *P. falcataria* showed higher correlation between colonization rate and shoot P content than the other leguminous trees. Smith and Smith (2012) 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.

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%). 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.

Mycorrhizal dependency (MD) of several leguminous trees in other studies, calculated based on the SDWs, were 81% for *S. spectabilis* (Kung`u et al. 2008), 34–65% for *A. saman* and 44–48% for *P. falcataria* (Wulandari et al. 2014). Mycorrhizal dependency (MD) of several non-leguminous trees in other studies, calculated based on the SDWs, were 39–62% for *Olea europaea*, an Oleaceae species (Porras-Soriano et al.

2009), 93% for *Cedrella fissilis*, a Meliaceae species and 95% for *Cecropia pachystachya*, a Cecropiaceae species (Siqueira and Saggin-Júnior 2001). MDs in present study were lower or the same as that of these studies.

6.4 Effect of malt extract on relationship between EPF and plant

The results clearly showed that the higher colonization rate of both EPF decreased the growth of *B. campestris*. Hacquard et al. (2016) clarified that the growth promotion was obtained because the host plant, *A. thaliana* allowed the colonization to some extent that *A. thaliana* could get benefit from the EPF. In this symbiosis, the plant was the symbiont that actively responded to the change of the environment. Regarding our results, *B. campestris* was not able to limit the colonization of EPF and the growth was decreased. Thus the higher colonization by EPF may not always result in higher growth promotion in plant.

6.5 Effect of different concentration of MS medium on relationship between EPF and plant

Growth of *B. campestris* inoculated with both EPF were not different with that of control plant when grown on 1/100 MS medium. The study of Hacquard et al. (2016) also highlighted the importance of environmental factors, especially medium, in modifying the response of plant colonized by EPF. Nutrient concentration in 1/100 MS medium may be in a condition that limit the growth of the EPF thus reduced the risk of pathogenicity to the plant. Unfortunately, there were no studies about effect of EPF inoculation on plant growth that use the same concentration as or lower concentration of MS medium than 1/100-strength MS medium.

6.6 Preference of EPF for host plant and forest site

P. falcataria and *S. bicolor* were not part of the plant community in the five forest sites but EPF were still isolated from their roots by trap culture. Moreover, different EPF were isolated from different forests, indicating that the trap culture method can be used to evaluate differences in EPF community among forests.

Sixteen of the 33 isolates had the closest match to fungi identified to the species level. Among the 16 isolates, 3 were specific to certain forest sites shared by *P. falcataria* and *S. bicolor*. In addition, some of the isolates were specific to certain forest sites but were not shared by the two host plants, examples of which are *Dictyosporium heptasporum* in *T. grandis* monoculture, *Mariannaea camptospora* in *Gmelina arborea*, *Artocarpus champeden*, and Dipterocarp mixed, and *Mycoleptodiscus* sp. in *Macaranga* sp. secondary forest. These results indicated that EPF had low or high preference for host plant as well as forest site. This phenomenon was also observed by Kernaghan and Patriquin (2011) in their study of Boreal area. Kernaghan and Patriquin (2011) isolated root EPF from *Betula papyrifera*, *Abies balsamea*, and *Picea glauca* and identified them by a molecular method. They revealed that some root EPF were found preferentially on a particular host whereas others were found specifically on a certain host.

6.7 Nutrient concentration in growth medium affects the relationship between host plant and EPF

Two inoculated *B. campestris* and one inoculated *P. falcataria* exhibited increased SDW when grown on 1/100-strength MS medium but not on 1/10-strength MS medium. The number of inoculated *B. campestris* and *P. falcataria* with more positive PR was larger when grown on 1/100-strength MS medium than 1/10-strength MS medium.

Mutualism between *B. campestris* or *P. falcataria* and EPF was achieved when nutrient concentration was low. Some studies have documented the importance of P in the association between EPF and Brassicaceae species. One of them is study of Hiruma et al. (2016) that inoculated *Arabidopsis thaliana* with *Colletotrichum tofieldiae* and grew it on ½-strength MS medium without sucrose and with two concentrations of P: 0.68 mg 100 g^{-1} (low P) and 8.51 mg 100 g^{-1} (high P). *C. tofieldiae* increased SFW of *A. thaliana* grown on MS medium with low P. Hiruma et al. (2016) proposed that the root EPF, *C. tofieldiae* can take up P and transfer it to *A. thaliana*. They inoculated *A. thaliana* with *C. tofieldiae* and grown in 2-compartment system consisted of a root hyphal compartment (RHC) and a root-restricted, hyphae only compartment (HC) added with ^{33}P. They measured the ^{33}P concentration in the plant after harvest and then clarified that the inoculated *A. thaliana* had higher ^{33}P than control plant. While another study by Lee et al. (2011), inoculated *Piriformospora indica* to *B. campestris* and *A. thaliana*. They proposed that *P. indica* upregulated the genes related to auxin biosynthesis and signaling in roots of *B. campestris* and caused the growth promotion in *B. campestris*. However in the case of *A. thaliana*, those genes were not upregulated although the growth were promoted. There may be other mechanisms in plant growth promotion by root EPF.

The increase of nitrogen and phosphorus in 1/100 MS medium, decreased the PRs of *B. campestris* inoculated with four EPF. These results clearly showed that nitrogen and phosphorus were the driver of the decrease of PRs in 1/10 MS medium compared to 1/100 MS. However, the fact that the PRs of *B. campestris* grown on either 1/100 high N or high P were lower than that grown on 1/10 MS, emphasize that there were other environmental factors affecting the PRs.

In another experiment, 50 EPF were isolated from *Santalum album* and *Swietenia macrophylla* from forest in Indonesia. *B. campestris* grown on 1/100 MS medium and

inoculated with these EPF exhibited negative to positive response. This experiment also clarified that the utilization of 1/100 MS medium for screening of EPF is reliable to determine the pathogenic and mutualistic EPF.

6.8 Effect of EPF inoculation on growth of different plant species

EPF that increased SDW of *B. campestris* did not always increase SDW of *P. falcataria*. Different PRs to the inoculation of the same EPF were also observed by Mandyam et al. (2010). Mandyam et al. (2010) inoculated leek (*Allium porrum* L.) and C_4 grass (*Andropogon gerardii* Vitman) with *Microdochium* sp. and *Periconia macrospinosa*. Internal colonization of *A. porrum* root was observed but the total biomass was not affected by the EPF inoculation. Internal colonization of *A. gerardii* root was observed, but in contrast to *A. porrum*, the total biomass of *A. gerardii* was increased or not affected by the EPF inoculation.

CHAPTER 7

Summary

Introduction: Deforestation by land-use conversion into agricultural fields and plantations, open cast mining, and illegal logging is increasing in Indonesia. Reforestation of tropical forests, such as those in Indonesia, requires human assistance to recover forest structure and species composition. There are few methods to remediate degraded forest in Indonesia. Utilization of beneficial symbiotic microorganisms is an environmentally safe way to ensure the plant survival rate after transplantation to the field. Arbuscular mycorrhizal fungi (AMF) and endophytic fungi (EPF) are groups of root symbiotic fungi which reported to be able to promote plant growth. AMF support plant growth through several mechanisms and is beneficial for plant survival in a degraded forest. However, the effectiveness of AMF in promoting plant growth has been shown to vary. AMF isolate and plant species are the main factors determining plant response to inoculation with AMF. Sselection of the appropriate AMF isolate for a certain plant species is a strategy that would guarantee the success of reforestation efforts. There is no report yet about utilization of EPF for tree species in Indonesia. Study about AMF and EPF in the tropics is less than in temperate region despite higher biodiversity in the tropics. The importance is not limited to as an effort to support reforestation of Indonesian forest, but also for wider knowledge for example about role and mechanism of those fungal group in affecting plant growth. The objectives of this study were (1) to isolate AMF and EPF from forest soil in Indonesia, (2) to determine condition of screening of EPF, and (3) to screen effective isolates of AMF and EPF with tropical tree species.

Materials and methods: Five AMF isolated from forest soils in Indonesia were propagated by inoculating to *Trifolium repens* (soil culture) and hairy root of *Linum usitatissimum* (root organ culture). Spores were mounted in PVLG and a mixture of PVLG and Melzer's reagent and their morphology was observed. The rDNA LSU region of the fungi was amplified from DNA of spores and identified. The spores were inoculated to four leguminous trees (*Calliandra calothyrsus*, *Paraserianthes falcataria*, *Cassia siamea*, *Sesbania grandiflora*) and grown in growth chamber with the application of low phosphorus (P) (1 mg L^{-1}) nutrient solution for screening of effective AMF isolates. EPF isolated from forest soils in Indonesia in the previous study was used to determine methods to clarify the factors affecting relationship between EPF and plant. *Brassica campestris* was used as the host plant. *B. campestris* was transplanted to pre-grown EPF colony supplied with different concentration of liquid malt extract. *B. campestris* was also inoculated with EPF and grown on different concentration of MS (Murashige and Skoog) medium. In other experiments, EPF were isolated from forest soils in Indonesia by trap culture using *P. falcataria* and *S. bicolor* as host plants. EPF were identified by extracting the rDNA ITS region. EPF were inoculated to *P. falcataria* and *B. campestris* grown on 1/100 MS and 1/10 MS medium to clarify the effect of nutrient concentration on relationship between EPF and host plant. Effective EPF were inoculated to *B. campestris* and grown on 1/100 MS and 1/10 MS medium with modified carbon, nitrogen (N) and P concentration. EPF were also isolated from roots of *Santalum album* and *Swietenia macrophylla* and screened using *B. campestris* on 1/100 MS medium.

Results: Five AMF isolates were propagated using *T. repens*. Two isolates were propagated using *L. usitatissimum*. Five AMF isolates were identified as *D. gibbosa* (M10-2), *Acaulospora* sp. (M11-1), Glomeromycota sp. (M44-3), *A. appendicula* (M60-3), and *Glomus* sp. (S6-4). In contrast to trees inoculated with other AMF, trees inoculated

with S6-4 showed similar or higher values of shoot fresh weight (SFW), shoot P content, shoot: root ratio, and plant height. Colonization rate of S6-4 was 84–99% and higher than that of another AMF. Isolate M44-3 belongs to the same Glomaceae family as S6-4 and showed the opposite result. M11-1, increased number of leaves of *P. falcataria* and *C. calothyrsus*, and shoot fresh weight SFW, shoot P content, and shoot: root ratio of *P. falcataria*. M10-2, increased shoot P content of all leguminous species. Mean mycorrhizal dependency (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. Positive correlations between colonization rate and shoot P content were observed for *P. falcataria* ($R^2 = 0.87$, $P < 0.001$), *C. calothyrsus* ($R^2 = 0.41$, $P < 0.001$), *C. siamea* ($R^2 = 0.39$, $P = 0.0011$), and *S. grandiflora* ($R^2 = 0.13$, $P = 0.048$). *P. falcataria* showed higher correlation between colonization rate and shoot P content than the other leguminous species. Higher concentration of malt extract increased growth of two EPF. External colonization on *B. campestris* of both EPF in higher concentration of malt extract were higher than that in control. Higher colonization decreased the survival rate and growth of *B. campestris* inoculated with EPF. Sixteen of the 33 EPF isolates had the closest match to fungi identified to the species level. Three isolates were specific to certain forest sites shared by *P. falcataria* and *S. bicolor*. Some isolates were specific to certain forest sites but were not shared by the two host plants; *Dictyosporium heptasporum* in *T. grandis* monoculture, *Mariannaea camptospora* in *Gmelina arborea*, *Artocarpus champeden*, and Dipterocarp mixed, and *Mycoleptodiscus* sp. in *Macaranga* sp. secondary forest. Two inoculated *B. campestris* and one inoculated *P. falcataria* exhibited increased shoot dry weight (SDW) when grown on 1/100 MS medium but not on 1/10 MS medium. The number of inoculated *B. campestris* and *P. falcataria* with higher positive PR was larger when grown on 1/100 MS medium than 1/10 MS medium.

The increase of N and P in 1/100 MS medium decreased the PRs of *B. campestris* inoculated with four EPF. These results showed that N and Ps were the driver of the decrease of PRs in 1/10 MS medium compared to 1/100 MS. EPF isolated from roots of *S. album* and *S. macrophylla* using *B. campestris* as host plant and 1/100 MS medium as medium yielded negative to positive plant response. These results suggest that (1) different leguminous species have different MD, (2) inoculation of AMF *Glomus* sp. (S6-4) promote growth of *P. falcataria* and *C. callothyrsus*, (3) 1/100 MS medium is a reliable medium for screening of EPF with *B. campestris* as host plant, and (4) concentration of N and P in medium affect the relationship between EPF and host plant.

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Table 2.1 Tree species, location, and latitude of five forests used for isolation of AMF

No	Forest	Location	Latitude	Remarks
1	Teak (*Tectona grandis* 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 (*Swietenia macrophylla* 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	*Gmelina arborea*, *Artocarpus champeden*, 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	*Macaranga* sp. secondary forest	East Kalimantan, Kalimantan Island	00° 59.519' S, 116° 57.241' E	*Macaranga* sp. grew naturally in Dipterocarp forest after forest fire. Research forest of Wanariset Tropical Forest Research Station

Table 2.2 Number of new spore of AMF isolates propagated using *Trifolium repens*, 90 days after transplanting

AMF isolates	Replication pots	Number of new spore per gram medium
M10-2	1	67
	2	48
	3	52
	4	56
	5	95
	Mean	64
M11-1	1	32
	2	72
	3	41
	4	45
	5	77
	Mean	53
M44-3	1	2
	2	3
	3	37
	4	70
	5	20
	Mean	26
M60-3	1	20
	2	30
	3	26
	4	15
	5	23
	Mean	23
S6-4	1	19
	2	63
	3	22
	4	38
	5	30
	Mean	34

Table 2.3 Spore germination, presence of running hyphae and formation of new spores in propagation of AMF isolate by root organ culture with five spores as inoculum

AMF isolate	Observation time (DAI)	Number of plate					
		Initial	Contaminated*	Not contaminated			
				No germination	Germinated spore	Running hyphae	Running hyphae and new spore
M10-2	82	20	-	20	-	-	-
	138	20	-	20	-	-	-
M11-1	82	20	10	3	2	3	2
	138	20	12	0	1	5	2
M44-3	82	20	-	20	-	-	-
	138	20	-	20	-	-	-
M60-3	82	20	-	20	-	-	-
	138	20	-	20	-	-	-
S6-4	82	20	7	0	4	3	6
	138	20	13	0	0	2	5

*Contamination by bacteria or other fungi.

Table 2.4. Number of new spores of AMF isolate propagated by root organ culture with five spores as inoculum

AMF isolate	Replication plates	Number of initial spore (/plate)	Number of new spores (spore/plate)
M11-1	1	5	145
	2	5	75
S6-4	1	5	15
	2	5	551
	3	5	287
	4	5	99
	5	5	296

Table 2.5 Spore germination, presence of running hyphae and formation of new spores in propagation of AMF isolate by root organ culture with one spore as inoculum

AMF isolate	Observation time (DAI)	Number of plate					
		Initial	Contaminated*	Not contaminated			
				No germination	Germinated spore	Running hyphae	Running hyphae and new spore
M11-1	15	42	0	29	13	0	0
	30	42	0	26	7	9	0
	60	42	2	26	6	6	2
	90	42	3	25	5	3	6
	120	42	6	11	13	6	6

*Contamination by bacteria or other fungi.

Table 2.6 List of AMF isolates with the closest relative based on sequence homology in National Center of Biotechnology Information (NCBI)

No	EPF isolate	Reps	Closest relative based on sequence homology	GenBank accession	Similarity
1	S6-4	1	Uncultured Glomeromycota clone RLC3 133	KM208325.1	99%
		2	Uncultured Glomeromycota clone RLC2 14	KM208384.1	100%
		3	Uncultured Glomeromycota clone RLC2 14	KM208384.1	99%
		4	Uncultured Glomeromycota clone RLC2 14	KM208384.1	99%
		5	Uncultured Glomeromycota clone 042	JN685253.1	99%
		6	Uncultured *Glomus* gene, clone N9-1-8-c	AB369757.1	99%
		7	Uncultured *Glomus* gene, clone N8-N9	AB369753.1	99%
		8	*Glomus* cf. *clarum* Att894-7	FM865539.1	99%
		9	Uncultured Glomeromycota clone RLC2 14	KM208384.1	99%
		10	*Glomus* cf. *clarum* Att894-7	FM865539.1	99%
		11	Uncultured Glomeromycota clone RLC3 133	KM208325.1	99%
		12	Uncultured Glomeromycota clone RLC2 14	KM208384.1	99%
2	M60-3	1	*Ambispora appendicula*	FN547527.1	99%
		2	*Ambispora appendicula*	FN547534.1	99%
		3	*Ambispora leptoticha* 206a	KC166271.1	99%
		4	*Ambispora appendicula*	FN547534.1	99%
		5	*Ambispora appendicula*	FN547531.1	98%
		6	*Ambispora appendicula*	FN547534.1	99%
		7	*Ambispora appendicula*	FN547534.1	99%
		8	*Ambispora appendicula*	FN547534.1	99%
		9	*Ambispora appendicula*	FN547534.1	99%
		10	*Ambispora appendicula*	FN547534.1	99%
		11	*Ambispora appendicula*	FN547534.1	99%
		12	*Ambispora appendicula*	FN547527.1	99%
3	M11-1	1	Uncultured *Acaulospora* clone AM159	KF849639.1	98%
		2	*Acaulospora longula* isolate BEG8	AJ510228.1	98%
		3	Uncultured *Acaulospora* clone AM159	KF849639.1	97%
		4	*Acaulospora longula* isolate BEG8	AJ510228.1	99%
		5	Uncultured *Acaulospora* clone AM159	KF849639.1	97%
		6	Uncultured *Acaulospora* clone AM159	KF849639.1	98%
		7	*Acaulospora longula* isolate BEG8	AJ510228.1	98%
		8	Uncultured *Acaulospora* clone AM159	KF849639.1	97%
		9	Uncultured *Acaulospora* clone AM159	KF849639.1	98%
		10	Uncultured *Acaulospora* clone AM159	KF849639.1	97%

Table 2.6 (Continued)

No	EPF isolate	Reps	Closest relative based on sequence homology	GenBank accession	Similarity
4	M10-2	1	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	95%
		2	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
		3	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
		4	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
		5	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
		6	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
		7	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
		8	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
		9	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
		10	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
		11	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
		12	*Diversispora gibbosa* isolate 109-2-5	KJ850203.1	99%
5	M44-3	1	Uncultured Glomeromycota clone PL2 26	KT378084.1	97%
		2	Uncultured Glomeromycota clone PL2 26	KT378084.1	97%
		3	Uncultured Glomeromycota clone PL2 26	KT378084.1	97%
		4	Uncultured Glomeromycota clone PL2 26	KT378084.1	96%
		5	Uncultured Glomeromycota clone PL2 26	KT378084.1	97%
		6	Uncultured Glomeromycota clone PL2 26	KT378084.1	97%
		7	Uncultured Glomeromycota clone PL2 26	KT378084.1	97%
		8	Uncultured Glomeromycota clone KP C078	JX276905.1	89%
		9	Uncultured Glomeromycota clone PL2 26	KT378084.1	97%
		10	Uncultured Glomeromycota clone PL2 26	KT378084.1	97%
		11	Uncultured Glomeromycota clone PL2 26	KT378084.1	97%
		12	Uncultured Glomeromycota clone PL2 26	KT378084.1	97%

Table 2.7 AMF colonization, shoot growth and shoot P concentration of four leguminous trees inoculated with or without AMF

Plant species	AMF isolate	Colonization (%)	Plant height increment (cm)	Number of leaves (leaves/plant)	Shoot fresh weight (mg/plant)	Root fresh weight (mg/plant)	Shoot P concentration (mg/g)	Shoot P content (mg/plant)
Paraserianthes falcataria	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
Calliandra calothrysus	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
Cassia siamea	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

Different letters indicate significant difference within the same plant species (Tukey HSD test, $P < 0.05$, n = 3-4)

Table 2.7 (Continued)

Plant species	AMF isolate	Colonization (%)	Plant height increment (cm)	Number of leaves (leaves/plant)	Shoot fresh weight (mg/plant)	Root fresh weight (mg/plant)	Shoot P concentration (mg/g)	Shoot P content (mg/plant)
Sesbania grandiflora	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, $P < 0.05$, n = 3-4)

Table 2.8 Shoot: root ratio and mycorrhizal dependency of four leguminous trees inoculated with or without AMF

Plant species	AMF isolate	Shoot: root ratio		Mycorrhizal dependency (%)	
Paraserianthes falcataria	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
Calliandra calothrysus	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
Cassia siamea	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
Sesbania grandiflora	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, $P < 0.05$, n = 3-4)

Table 3.1 Growth of EPF isolates 2614(4)PDA-1-2-1 and 2314(4)PDA on vermiculite applied with different liquid medium

EPF isolates	Liquid medium	Colony diameter (cm)	
		4 DAI	6 DAI
2614(4)PDA-1-2-1	1/10 strength malt extract	1.50	2.75
	1/2 strength malt extract	3.00	5.00
	full strength malt extract	3.75	5.65
2314(4)PDA	1/10 strength malt extract	1.00	2.25
	1/2 strength malt extract	3.00	4.35
	full strength malt extract	3.75	5.95

Table 3.2 Survival rate, shoot fresh and dry weight and root fresh weight of *Brassica campestris*, transplanted on pre-grown EPF colonies, 30 days after transplanting

EPF isolate	Liquid medium	Nutrient solution	Reps	Seedling number		Survival rate (%)	Shoot fresh weight		Root fresh weight		Shoot dry weight		Colonization rate (%)	
				at transplanting	at harvest		(mg/Plantbox)	(mg/plant)	(mg/Plantbox)	(mg/plant)	(mg/Plantbox)	(mg/plant)	Internal	External
Control	-	50% P8	1	3	3	100	282.1	94.0	8.9	3.0	23.1	7.7	0	0
			2	3	3	100	387.1	129.0	13.5	4.5	25.6	8.5	0	0
			3	3	3	100	533.9	178.0	34.3	11.4	28.4	9.5	0	0
			4	3	3	100	481.0	160.3	36.9	12.3	26.1	8.7	0	0
			Mean	3	3	100 a	421.0 a	140.3 a	23.4 a	7.8 a	25.8 a	8.6 a	0	0
2614(4)PDA-1-2-1	1/10 ME	50% P8	1	3	2	67	175.7	87.9	14.3	7.2	11.3	5.7	0	22
			2	3	1	33	179.4	179.4	11.7	11.7	9.9	9.9	0	18
			3	3	2	67	345.7	172.9	25.3	12.7	20.5	10.3	0	6
			4	3	3	100	295.6	98.5	25.6	8.5	21.8	7.3	0	8
			Mean	3	2	67 ab	249.1 ab	134.7 a	19.2 a	10.0 a	15.9 ab	8.3 a	0	14
	1/2 ME	50% P8	1	3	2	67	83.2	41.6	11.2	5.6	10.0	5.0	0	29
			2	3	1	33	119.4	119.4	6.2	6.2	10.4	10.4	0	27
			3	3	1	33	256.9	256.9	52.2	52.2	19.5	19.5	0	9
			4	3	1	33	216.2	216.2	27.0	27.0	15.8	15.8	0	9
			Mean	3	1	42 ab	168.9 b	158.5 a	24.2 a	22.8 a	13.9 ab	12.7 a	0	19
	Full ME	50% P8	1	3	0	0	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
			2	3	2	67	154.2	77.1	29.9	15.0	16.1	8.1	0	15
			3	3	1	33	14.3	14.3	1.6	1.6	2.2	2.2	0	90
			4	3	3	100	125.1	41.7	29.0	9.7	19.3	6.4	0	20
			Mean	3	2	50 ab	97.9 b	44.4 a	20.2 a	8.7 a	12.5 ab	5.6 a	0	42

Different letters indicate significant difference among treatments (Tukey HSD test, $P < 0.05$, n = 4); n.d = not determined.

Table 3.2 (Continued)

EPF isolate	Liquid medium	Nutrient solution	Reps	Seedling number		Survival rate (%)	Shoot fresh weight		Root fresh weight		Shoot dry weight		Colonization rate (%)	
				at transplanting	at harvest		(mg/Plantbox)	(mg/plant)	(mg/Plantbox)	(mg/plant)	(mg/Plantbox)	(mg/plant)	Internal	External
2314(4)PDA	1/10 ME	50% P8	1	3	3	100	272.1	90.7	18.1	6.0	20.5	6.8	0	7
			2	3	2	67	258.3	129.2	23.6	11.8	16.7	8.4	0	7
			3	3	1	33	84.6	84.6	1.8	1.8	6.8	6.8	0	20
			4	3	2	67	243.7	121.9	10.1	5.1	13.1	6.6	0	7
			Mean	3	2	67 ab	214.7 b	106.6 a	13.4 a	6.2 a	14.3 ab	7.1 a	0	10
	1/2 ME	50% P8	1	3	1	33	38.3	38.3	1.3	1.3	4.1	4.1	0	22
			2	3	2	67	94.0	47.0	4.3	2.2	12.8	6.4	0	22
			3	3	2	67	78.3	39.2	3.9	2.0	10.2	5.1	0	31
			4	3	0	0	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
			Mean	3	1	42 ab	70.2 b	41.5 a	3.2 a	1.8 a	9.0 b	5.2 a	0	25
	Full ME	50% P8	1	3	0	0	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
			2	3	0	0	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
			3	3	1	33	48.6	48.6	n.d	n.d	10.3	10.3	0	n.d
			4	3	0	0	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
			Mean	3	0	8 b	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

Different letters indicate significant difference among treatments (Tukey HSD test, $P < 0.05$, n = 4); n.d = not determined.

Table 3.3 Shoot fresh and dry weight and root fresh weight of *Brassica campestris* inoculated with or without EPF, applied with P1 nutrient solution and ½ strength MS solution, 30 days after transplanting

EPF isolate	Nutrient solution	Reps	Seedling number		Shoot fresh weight		Root fresh weight		Shoot dry weight		Colonization rate (%)	
			at transplanting	at harvest	(mg/Plantbox)	(mg/plant)	(mg/Plantbox)	(mg/plant)	(mg/Plantbox)	(mg/plant)	Internal	External
Control	P1	1	3	3	550.9	183.6	32.1	10.7	29.9	10.0	0	0
		2	3	3	510.3	170.1	34.9	11.6	28.6	9.5	0	0
		3	3	3	508.0	169.3	31.1	10.4	28.3	9.4	0	0
		4	3	3	480.9	160.3	27.1	9.0	29.1	9.7	0	0
		Mean	3	3	512.5 b	170.8 b	31.3 a	10.4 a	29.0 b	9.7 b	0	0
Control	1/2 MS	1	3	3	906.1	302.0	30.0	10.0	54.2	18.1	0	0
		2	3	3	723.9	241.3	29.4	9.8	46.8	15.6	0	0
		3	3	3	626.9	209.0	25.1	8.4	45.4	15.1	0	0
		4	3	0	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
		Mean	3	2	752.3 a	250.8 a	28.2 a	9.4 a	48.8 a	16.3 a	0	0
2614(4)PDA-1-2-1	P1	1	3	3	281.6	93.9	23.0	7.7	20.6	6.9	0	26
		2	3	3	339.1	113.0	16.9	5.6	20.2	6.7	0	10
		3	3	3	333.5	111.2	21.2	7.1	20.8	6.9	0	13
		4	3	3	257.3	85.8	16.5	5.5	17.6	5.9	0	18
		Mean	3	3	302.9 c	101.0 c	19.4 b	6.5 b	19.8 c	6.6 c	0	17
2614(4)PDA-1-2-1	1/2 MS	1	3	0	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
		2	3	0	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
		3	3	1	329.2	329.2	n.d	n.d	26.0	26.0	n.d	n.d
		4	3	0	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
		Mean	3	0.3	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

Different letters indicate significant difference among treatments (Tukey HSD test, $P < 0.05$, n = 4); n.d = not determined.

Table 3.4 Shoot fresh and dry weight, root fresh weight and length of *Brassica campestris* grown on different agar medium under Petri dish culture, 14 days after transplanting

Medium	Reps	Shoot fresh weight (mg/plant)		Root fresh weight (mg/plant)		Shoot dry weight (mg/plant)		Root length (cm/plant)	
Water agar	1	44.5		10.0		3.5		34.6	
	2	80.6		7.6		4.7		30.6	
	3	109.9		15.0		5.9		62.9	
	Mean	78.3	c	10.8	b	4.7	c	42.7	a
1/100 MS	1	60.6		11.2		4.2		37.7	
	2	126.4		16.4		7.2		77.4	
	3	96.5		13.2		5.5		69.5	
	Mean	94.5	c	13.6	ab	5.6	c	61.5	a
1/10 MS	1	189.8		12.0		10.8		54.6	
	2	290.0		37.5		15.5		124.5	
	3	199.8		27.4		11.5		76.2	
	Mean	226.5	b	25.6	ab	12.6	b	85.1	a
1/5 MS	1	258.8		21.6		15.0		59.7	
	2	293.2		34.9		17.1		80.1	
	3	*		*		*			
	Mean	276.0	b	28.3	ab	16.0	b	69.9	a
MS	1	452.8		29.1		32.6		34.2	
	2	530.3		35.0		40.0		40.9	
	3	437.4		31.3		38.6		46.4	
	Mean	473.5	a	31.8	a	37.0	a	40.5	a

Different letters indicate significant difference among treatments (Tukey HSD test, $P < 0.05$, n = 3); *contaminated by fungi.

Table 3.5 Shoot fresh and dry weight, root fresh weight of *Brassica campestris* grown on vermiculite in Plantbox, applied with different nutrient solution, 30 days after transplanting

Medium	Reps	Number of leaves		Shoot fresh weight (mg/plant)		Root fresh weight (mg/plant)		Shoot dry weight (mg/plant)		Root dry weight (mg/plant)	
		Green	Yellow, wilted								
Water	1	4	2	77.1		14.7		10.5		2.9	
	2	4	2	70.0		16.4		10.6		2.9	
	3	3	2	69.6		14.2		9.7		2.2	
	4	4	2	67.9		13.9		10.3		2.3	
	Mean	3.6	2.0	71.2	c	14.8	b	10.3	b	2.6	ab
1/100 P8	1	4	2	87.3		16.2		11.3		2.3	
	2	3	2	68.0		14.7		9.2		2.5	
	3	3	2	75.9		17.7		10.1		2.8	
	4	3	2	66.5		12.8		8.7		2.1	
	Mean	3.3	2.0	74.4	c	15.3	b	9.8	b	2.4	ab
1/10 P8	1	4	2	125.9		32.0		11.0		4.0	
	2	4	2	149.2		33.3		11.6		4.2	
	3	5	2	120.1		27.5		10.3		3.2	
	4	5	2	131.7		22.3		10.2		2.7	
	Mean	4.3	2.1	131.7	b	28.8	a	10.8	ab	3.5	a
1/5 P8	1	5	2	141.5		25.0		9.2		3.2	
	2	5	2	188.4		29.8		11.1		3.1	
	3	6	1	205.0		31.6		11.7		3.4	
	4	5	2	145.2		30.8		11.1		3.4	
	Mean	5.5	1.8	170.0	b	29.3	a	10.8	ab	3.3	ab
P8	1	8	0	251.4		21.1		12.5		1.8	
	2	8	0	243.9		22.0		11.9		2.2	
	3	9	0	226.3		20.4		12.2		1.9	
	4	8	1	278.6		33.6		15.0		3.4	
	Mean	7.7	1.2	250.1	a	24.3	a	12.9	a	2.3	b

Different letters indicate significant difference among treatments (Tukey HSD test, $P < 0.05$, n = 4)

Table 3.6 Shoot fresh and dry weight, root fresh and dry weight of *Brassica campestris* inoculated with or without EPF, grown on agar medium with different nutrient concentration, 21 days after transplanting

EPF isolate	Medium	Seedling number		Shoot fresh weight (mg/plant)		Root fresh weight (mg/plant)		Shoot dry weight (mg/plant)		Root dry weight (mg/plant)	
		at transplant	at harvest								
Control	Water agar	2.0	2.0	39.6 ± 3.0	d	22.1 ± 1.7	a	4.0 ± 0.2	c	1.6 ± 0.1	a
	1/100 MS	2.0	2.0	71.6 ± 2.5	cd	32.4 ± 5.5	a	7.3 ± 0.9	abc	2.1 ± 0.3	a
	1/10 MS	2.0	2.0	229.6 ± 7.6	a	49.2 ± 13.0	a	14.9 ± 2.9	a	3.4 ± 1.2	a
	1/5 MS	2.0	2.0	261.9 ± 27.2	a	46.4 ± 17.8	a	14.7 ± 3.5	ab	3.0 ± 1.5	a
	MS	2.0	2.0	153.3 ± 35.5	b	17.5 ± 1.1	a	8.1 ± 1.6	abc	0.8 ± 0.1	a
2531(3)WA-2-1	Water agar	2.0	0.0	n.d		n.d		n.d		n.d	
	1/100 MS	2.0	1.8	52.3 ± 4.4	d	n.d		7.0 ± 1.2	bc	n.d	
	1/10 MS	2.0	0.0	n.d		n.d		n.d		n.d	
	1/5 MS	2.0	0.0	n.d		n.d		n.d		n.d	
	MS	2.0	0.0	n.d		n.d		n.d		n.d	
2614(4)PDA-1-2-1	Water agar	2.0	2.0	46.1 ± 7.4	d	n.d		5.3 ± 1.0	c	n.d	
	1/100 MS	2.0	2.0	64.5 ± 6.7	cd	n.d		6.2 ± 0.6	c	n.d	
	1/10 MS	2.0	2.0	154.5 ± 10.7	b	n.d		7.4 ± 0.4	abc	n.d	
	1/5 MS	2.0	2.0	141.0 ± 9.1	bc	n.d		7.5 ± 0.6	abc	n.d	
	MS	2.0	0.0	n.d		n.d		n.d		n.d	

Different letters indicate significant difference among treatments (Tukey HSD test, $P < 0.05$, n = 4); n.d= not determined.

Table 4.1. Growth parameters of *Sorghum bicolor*, number of EPF isolates, and code of EPF isolated from *Sorghum bicolor*

No	Forest	Seedling number per pot	Plant height (cm/plant)	Number of leaves (leaves/pot)	Number of plates		Number of EPF isolate	Code of EPF isolate
					Initial	Colonized		
1	*Tectona grandis* monoculture	3 ± 0.2	29.2 ± 3.0	5 ± 0.6	10	9	5	2612(2), 2612(3)-1, 2612(4), 2613(5)-1, 2613(5)-2
2	*Swietenia macrophylla* monoculture	3 ± 0.3	24.3 ± 2.3	4 ± 0.7	5	3	2	2624(1), 2624(5)
3	*Gmelina arborea*, *Artocarpus champeden* and Dipterocarp mixed	2 ± 0.4	26.5 ± 3.4	4 ± 0.8	15	9	7	2632(1), 2632(5), 2633(1), 2633(2), 2633(5)-1, 2633(5)-2, 2635(4)
4	Dipterocarp primary	2 ± 0.4	17.3 ± 3.1	4 ± 0.9	5	0	0	
5	*Macaranga* sp. secondary	3 ± 0.2	30.7 ± 2.0	6 ± 0.5	25	8	7	2651(3), 2651(4), 2652(1), 2652(3), 2652(4), 2653(3)-1, 2655(2)
Total					60	29	21	
Isolation rate (%)							35	

Table 4.2. Growth parameters of *Paraserianthes falcataria*, number of EPF isolates, and code of EPF isolated from *Paraserianthes falcataria*

No	Forest	Seedling number per pot	Plant height (cm/plant)	Number of leaves (leaves/pot)	Number of plate		Number of EPF isolate	Code of EPF isolate
					Initial	Colonized		
1	*Tectona grandis* monoculture	1	3.1 ± 0.1	4 ± 1.0	10	3	2	2312(3), 2313(1)
2	*Swietenia macrophylla* monoculture	1	2.8 ± 0.1	5 ± 0.9	0	0	0	
3	*Gmelina arborea*, *Artocarpus champeden* and Dipterocarp mixed	1	3.2 ± 0.1	6 ± 1.0	20	8	7	2331(1)-1, 2331(1)-2, 2331(2), 2331(5), 2332(2), 2332(5), 2334(2)
4	Dipterocarp primary	1	2.7 ± 0.2	3 ± 0.4	0	0	0	
5	Macaranga sp. secondary	1	3.1 ± 0.2	6 ± 1.4	20	3	3	2352(5), 2354(1)-1, 2354(1)-2
Total					50	14	12	
Isolation rate (%)							24	

Table 4.3 EPF isolated from roots of *Paraserianthes falcataria* and *Sorghum bicolor* grown in forest soils of Indonesia

No	Host plant	Forest	EPF isolate	Closest relative based on sequence homology	Order	Family	GenBank accession	Similarity
1	*Paraserianthes falcataria*	*Tectona grandis* monoculture	2312(3)	*Acrocalymma vagum* strain CPC 24227	Pleosporales	Morosphaeriaceae	KP170637.1	100%
2			2313(1)	*Fusarium solani* isolate FJAT-30854	Hypocreales	Nectriaceae	KX229750.1	99%
3		*Gmelina arborea*, *Artocarpus champeden*, and Dipterocarp mixed	2331(1)-1	Uncultured fungus clone T2949	-	-	KF742561.1	84%
4			2331(1)-2	*Tolypocladium album* isolate 360Jb14	Hypocreales	Ophiocordycipitaceae	KU516596.1	99%
5			2331(2)	Ascomycota sp. Glum291	-	-	KM678363.1	98%
6			2331(5)	*Penicillium citrinum* strain P1.21	Eurotiales	Trichocomaceae	EU833213.1	100%
7			2332(2)	*Talaromyces verruculosus* isolate ATT281	Eurotiales	Trichocomaceae	HQ607919.1	100%
8			2332(5)	Uncultured fungus clone T2949	-	-	KF742561.1	85%
9			2334(2)	Uncultured Sordariomycetes clone OTU68	-	-	KU144662.1	99%
10		*Macaranga* sp. secondary	2352(5)	*Talaromyces aculeatus* C36-375	Eurotiales	Trichocomaceae	KJ439089.1	99%
11			2354(1)-1	Uncultured Ascomycota clone 736	-	-	HM162169.1	99%
12			2354(1)-2	*Exophiala calicioides* strain JCM9764	Chaetothyriales	Herpotrichiellaceae	AB007685.1	84%
13	*Sorghum bicolor*	*Tectona grandis* monoculture	2612(2)	Clavicipitaceae sp. MEXU 26354	Hypocreales	Clavicipitaceae	JQ811555.1	99%
14			2612(3)-1	Uncultured fungus clone 109A74984	-	-	JX386729.1	99%
15			2612(4)-4	*Dictyosporium heptasporum* CBS 396.59	Pleosporales	Dictyosporiaceae	DQ018090.1	98%
16			2613(5)-1	*Fusarium solani* isolate FJAT-30854	Hypocreales	Nectriaceae	KX229750.1	99%
17			2613(5)-2	Uncultured organism clone ciidir1004 C11	-	-	JN660770.1	92%

Table 4.3 (Continued)

No	Host plant	Forest	EPF isolate	Closest relative based on sequence homology	Order	Family	GenBank accession	Similarity
18	*Sorghum*	*Swietenia*	2624(1)-2	Sordariales sp. REF169	Sordariales	-	JN859389.1	95%
19	*bicolor*	*macrophylla* monoculture	2624(5)	*Pseudochaetosphaeronema martinelli* CBS 135986	Pleosporales	Pleosporales incertae sedis	NR_132930.1	95%
20		*Gmelina arborea*,	2632(1)	Uncultured Ascomycota clone 736	-	-	HM162169.1	99%
21		*Artocarpus*	2632(5)	*Talaromyces verruculosus* isolate P87-271	Eurotiales	Trichocomaceae	KJ439175.1	100%
22		*champeden*, and Dipterocarp mixed	2633(1)	Uncultured Ascomycota clone 736	-	-	HM162169.1	98%
23			2633(2)	*Mariannaea camptospora*	Hypocreales	Nectriaceae	AB112029.1	99%
24			2633(5)-1	Uncultured Ascomycota clone OMG C15	-	-	GU174012.1	97%
25			2633(5)-2	Fungal sp. voucher ARIZ:PS0824	-	-	KU977925.1	96%
26			2635(4)	*Trichoderma spirale* A725	Hypocreales	Hypocreaceae	KU529839.1	99%
27		*Macaranga* sp.	2653(3)-1	*Fusarium* sp. isolate E-179.5	Hypocreales	Nectriaceae	KU059845.1	99%
28		secondary	2655(2)	*Mycoleptodiscus terrestris* CBS 231.53	Magnaporthales	Magnaporthaceae	NR_145373.1	94%
29			2652(1)	Uncultured fungus clone RFLP14	-	-	GU187835.1	96%
30			2652(3)	Dothideomycetes sp. KO-group G 2014	-	-	AB986433.1	99%
31			2652(4)	*Trichoderma spirale* isolate A725	Hypocreales	Hypocreaceae	KU529839.1	99%
32			2651(3)	*Talaromyces aculeatus* C36-375	Eurotiales	Trichocomaceae	KJ439089.1	99%
33			2651(4)	Uncultured fungus clone 034A4289	-	-	JX321968.1	87%

Table 4.4 Shoot fresh and dry weight and internal/external colonization of *Brassica campestris* inoculated with or without endophytic fungi, 28 days after transplanting

EPF isolate	Shoot fresh weight (mg/plant)				Shoot dry weight (mg/plant)				Internal colonization (%)		External colonization (%)	
	1/100 MS		1/10 MS		1/100 MS		1/10 MS		1/100 MS	1/10 MS	1/100 MS	1/10 MS
Control	52.9		321.6		5.4		19.0		0.0	0.0	0.0	0.0
2312 (3)	85.6	**	359.7	ns	8.7	**	20.6	ns	14.1	23.9	27.9	44.3
2331 (2)	76.6	*	430.5	ns	7.2	ns	24.1	ns	90.8	78.6	0.0	5.2
2332 (5)	86.2	**	406.6	ns	7.5	ns	21.8	ns	0.0	1.7	25.9	43.9
2334 (2)	76.8	*	441.8	ns	9.9	**	28.2	*	57.0	57.8	15.4	17.6
Control	52.7		354.1		10.3		28.2		0.0	0.0	0.0	0.0
2313 (1)	52.9	ns	0.0		4.7	*	0.0		n.d.	n.d.	n.d.	n.d.
2331 (1) - 1	71.1	ns	291.5	**	6.8	ns	18.1	ns	0.0	0.0	74.0	90.0
2331 (1) - 2	67.0	ns	274.2	**	8.7	ns	18.7	ns	23.3	52.9	60.6	46.4
2332 (2)	63.0	ns	123.0	***	4.5	*	14.9	ns	n.d.	n.d.	n.d.	n.d.
2352 (5)	58.6	ns	168.3	***	4.6	*	17.6	ns	n.d.	n.d.	n.d.	n.d.
2354 (1) - 1	65.1	ns	197.4	***	10.0	ns	18.7	ns	2.4	10.7	20.4	40.6
2624 (5)	75.7	ns	317.8	ns	5.3	*	16.1	ns	n.d.	n.d.	n.d.	n.d.
2633 (5) - 1	60.4	ns	280.8	*	4.5	*	17.2	ns	n.d.	n.d.	n.d.	n.d.
2633(5) - 2	72.6	ns	301.8	**	9.0	ns	29.4	ns	0.0	31.9	11.3	19.7
Control	62.5		421.8		8.0		22.3		0.0	0.0	0.0	0.0
2632(1)	53.6	ns	260.4	*	7.7	ns	27.6	ns	0.5	2.0	39.0	6.6
2633 (1)	56.4	ns	294.1	ns	9.1	ns	29.2	*	3.3	10.5	47.6	34.3
2651 (3)	32.9	**	181.6	**	5.6	*	19.6	ns	n.d.	n.d.	n.d.	n.d.
2653 (3) - 1	55.2	ns	258.2	*	6.8	ns	24.8	ns	n.d.	n.d.	n.d.	n.d.

Table 4.4 (Continued)

EPF isolate	Shoot fresh weight (mg/plant)				Shoot dry weight (mg/plant)				Internal colonization (%)		External colonization (%)	
	1/100 MS		1/10 MS		1/100 MS		1/10 MS		1/100 MS	1/10 MS	1/100 MS	1/10 MS
Control	43.0		224.4		6.1		15.0		0.0	0.0	0.0	0.0
2612(4)	40.5	ns	204.5	ns	6.5	ns	15.9	ns	0.0	0.0	0.0	0.0
Control	34.0		244.4		4.5		16.4		0.0	0.0	0.0	0.0
2613(5)-1	13.14†		12.82†		2.53†		1.59†		n.d.	n.d.	n.d.	n.d.
2613(5)-2	35.5	ns	189.4	**	4.4	ns	15.2	ns	0.0	0.0	1.6	1.0
2624(1)	44.0	ns	222.7	ns	4.3	ns	15.6	ns	0.4	0.0	1.0	0.0
2632(5)	38.6	ns	113.9	**	4.6	ns	14.5	ns	0.0	0.0	4.9	0.0
2633(2)	39.4	ns	213.7	ns	5.7	ns	17.3	ns	0.0	0.0	31.4	22.4
2635(4)	26.7	ns	94.9	***	4.5	ns	13.1	ns	0.0	0.0	27.5	7.5
2655(2)	0†		22.76†		0†		2†		-	-	-	9.1
Control	67.2		331.9		12.1		31.0		0.0	0.0	0.0	0.0
2331(5)	76.5	ns	259.3	ns	10.5	n	27.3	ns	0.0	4.0	0.8	13.4
2354(1)-2	70.3	ns	343.6	ns	10.4	ns	21.4	*	0.0	0.0	10.6	4.6
2612(2)	71.5	ns	206.9	*	9.9	**	21.2	*	0.0	0.0	0.0	3.8
2612(3)-1	63.3	ns	233.2	*	10.6	n	23.6	ns	0.0	0.0	0.2	7.6
2652(4)	58.5	ns	230.1	*	8.2	*	21.6	*	0.0	0.0	65.6	32.6
Control	62.5		328.3		4.7		17.1		0.0	0.0	0.0	0.0
2651(4)	68.5	ns	323.4	ns	5.8	ns	19.2	ns	0.2	0.0	1.2	1.6
2652(1)	67.4	ns	346.1	ns	5.0	ns	19.4	ns	0.0	0.0	0.0	0.0
2652(3)	69.3	ns	334.9	ns	5.5	ns	18.2	ns	0.0	0.0	0.0	0.0

Significant difference between inoculated plant and respective control was determined by the Student's t-test (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not significant; n= 5). †The number of replication plants that survived until time of harvest was <3 and thus excluded from statistical analysis. Colonization rate could not be determined in some plants due to insufficient number of roots for observation, and is indicated by n.d. (not determined).

Table 4.5 Shoot fresh and dry weight and internal/external colonization of *Paraserianthes falcataria* inoculated with or without endophytic fungi, 37 days after transplanting

EPF isolate	Shoot fresh weight (mg/plant)		Shoot dry weight (mg/plant)		Internal colonization (%)		External colonization (%)	
	1/100 MS	1/10 MS	1/100 MS	1/10 MS	1/100 MS	1/10 MS	1/100 MS	1/10 MS
Control	74.1	119.8	23.0	51.0	0.0	0.0	0.0	0.0
2313(1)	65.9 ns	157.4 ns	21.7 ns	53.1 ns	0.0	0.0	0.3	14.3
2612(2)	82.8 ns	147.6 ns	25.5 ns	54.8 ns	0.0	0.0	0.0	2.8
2612(3)-1	68.0 ns	167.8 ns	22.1 ns	56.4 ns	0.0	0.0	0.0	0.5
2613(5)-1	69.7 ns	126.8 ns	22.7 ns	40.1 *	0.0	0.0	0.0	8.5
2633(2)	84.7 ns	148 ns	26.6 ns	46.7 ns	0.0	0.0	0.0	5.5
2633(5)-1	71.3 ns	157.9 ns	22.3 ns	50.7 ns	0.0	0.0	0.5	1.5
2652(1)	70.3 ns	105.5 ns	23.2 ns	37.7 *	0.0	0.0	0.0	2.3
Control	72.5	173.9	23.7	54.3	0.0	0.0	0.0	0.0
2331(1)-1	62.2 ns	165.7 ns	19.8 ns	50.6 ns	0.0	0.0	0.3	7.5
2331(1)-2	65.8 ns	148.7 ns	21.5 ns	48.9 ns	0.0	0.0	0.3	10.5
2354(1)-2	71.7 ns	181.7 ns	23.6 ns	58.6 ns	0.0	0.0	0.0	1.0
2613(5)-2	59.0 ns	176.2 ns	20.6 ns	58.1 ns	0.0	0.0	0.0	0.5
2624(5)	72.6 ns	156.3 ns	23.0 ns	52.3 ns	0.0	0.0	1.3	5.8
2632(1)	66.4 ns	142.7 ns	22.5 ns	51.2 ns	0.0	0.0	1.3	3.3
2633(5)-2	56.2 ns	177.6 ns	18.4 ns	55.7 ns	0.0	0.0	0.3	1.8

Significant difference between inoculated and respective control was determined by the Student's t-test (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not significant; n= 5).

Table 4.5 (Continued)

EPF isolate	Shoot fresh weight (mg/plant)				Shoot dry weight (mg/plant)				Internal colonization (%)		External colonization (%)	
	1/100 MS		1/10 MS		1/100 MS		1/10 MS		1/100 MS	1/10 MS	1/100 MS	1/10 MS
Control	76.9		181.7		21.9		58.8		0.0	0.0	0.0	0.0
2354(1)-1	83.1	ns	118.1	*	25.7	ns	51.8	ns	0.0	0.0	1.8	0.5
2612(4)	78.1	ns	56.2	***	24.0	ns	42.8	ns	0.0	0.0	0.0	0.3
2624(1)	63.6	ns	58.1	***	18.4	ns	49.8	*	0.0	0.0	0.0	0.0
2633(1)	71.6	ns	126.5	ns	21.4	ns	59.0	ns	3.5	0.5	4.0	1.0
2651(4)	95.4	ns	72.2	**	26.9	*	53.1	ns	0.0	0.0	0.3	0.3
2652(3)	96.2	ns	143.1	ns	27.7	ns	54.6	ns	0.0	0.0	0.0	0.0
2653(3)-1	72.5	ns	129.0	ns	22.1	ns	52.4	ns	5.5	7.3	10.8	5.5
2655(2)	69.4	ns	48.3	***	23.2	ns	44.5	*	23.5	0.5	0.0	1.0
Control	59.2		169.1		20.5		52.3		0.0	0.0	0.0	0.0
2331(5)	58.9	ns	149.9	*	18.3	ns	50.3	ns	5.5	41.3	1.5	18.8
2332(2)	64.4	ns	96.0	***	21.6	ns	28.0	***	0.0	0.0	0.0	0.0
2352(5)	47.0	ns	31.3	***	16.5	ns	15.4	**	3.3	6.3	5.6	25.0
2632(5)	55.8	ns	111.3	*	17.7	ns	39.1	*	4.3	3.8	3.0	5.3
2635(4)	64.4	ns	119.0	*	21.2	ns	38.1	*	0.0	0.5	4.5	16.8
2651(3)	43.5	ns	59.7	**	17.7	ns	23.5	**	6.6	0.9	10.8	13.4
2652(4)	61.1	ns	121.6	**	20.3	ns	37.3	*	0.0	0.0	11.0	24.3
Control	69.9		171.5		23.1		56.0		0.0	0.0	0.0	0.0
2312(3)	73.7	ns	180.9	ns	24.2	ns	58.8	ns	5.8	21.5	6.8	14.3
2332(5)	68.6	ns	191.0	ns	23.2	ns	63.3	ns	3.3	7.5	5.3	14.3
2334(2)	68.4	ns	164.5	ns	23.3	ns	52.1	ns	2.5	0.5	5.8	1.0
Control	77.3		196.1		25.3		62.3		0.0	0.0	0.0	0.0
2331(2)	64.3	*	179.5	*	21.1	ns	57.1	*	0.3	0.5	0.0	0.5

Significant difference between inoculated and respective control was determined by the Student's t-test (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not significant; n= 5).

Table 4.6 Growth and internal/external colonization of *Brassica campestris* inoculated with or without EPF under Plantbox culture, 28 days after transplanting

EPF isolate	Reps	Number of leaves (leaves/plant)	Shoot fresh weight (mg/plant)	Root fresh weight (mg/plant)	Shoot dry weight (mg/plant)	Internal colonization (%)	External colonization (%)	Shoot:root ratio
Control	1	3	40.7	13.8	8.0	0	0	2.9
	2	3	66.2	27.0	6.4	0	0	2.5
	3	3	65.6	22.7	7.2	0	0	2.9
	4	3	71.9	26.0	7.7	0	0	2.8
	Mean	3.0	61.1 a	22.4 b	7.3 b	0	0	2.8 a
2312 (3)	1	3	61.1	26.3	8.6	0	6	2.3
	2	3	62.4	27.1	8.4	1	4	2.3
	3	3	57.4	25.1	8.3	0	4	2.3
	4	3	63.5	31.3	9.3	1	9	2.0
	Mean	3.0	61.1 a	27.4 ab	8.7 a	1	6	2.2 b
2331 (2)	1	3	62.7	24.8	6.8	0	0	2.5
	2	3.3	69.0	32.7	6.8	0	0	2.1
	3	3	66.8	31.5	6.9	0	0	2.1
	4	3	75.5	35.7	7.4	3	0	2.1
	Mean	3.1	68.5 a	31.2 a	7.0 b	1	0	2.2 b
2332 (5)	1	3	68.8	29.4	7.2	0	2	2.3
	2	3.3	73.3	33.3	8.4	0	2	2.2
	3	3	67.3	31.9	7.4	0	1	2.1
	4	3	74.1	31.6	8.7	0	0	2.3
	Mean	3.1	70.9 a	31.5 a	7.9 ab	0	1	2.3 b
2334 (2)	1	3	67.7	31.9	7.2	1	1	2.1
	2	3	70.9	32.3	6.8	0	2	2.2
	3	3	78.3	35.7	8.4	0	1	2.2
	4	3	76.2	36.9	7.6	0	0	2.1
	Mean	3.0	73.3 a	34.2 a	7.5 ab	0	1	2.1 b

Different letters indicate significant difference among treatments (Tukey HSD test, $P < 0.05$, n = 4)

Table 4.7 Growth and internal/external colonization of *Brassica campestris* inoculated with or without EPF, grown on different MS medium, 28 days after transplanting

Medium	EPF isolate	Shoot fresh weight (mg/plant)	Root fresh weight (mg/plant)	Shoot dry weight (mg/plant)	Internal colonization (%)	External colonization (%)
1/100 MS without sugar	Control	56.8 a	20.9 a	4.6 a	0.0	0.0
	2312(3)	60.5 a	21.8 a	4.8 a	0.8	11.0
	2331(2)	51.3 a	17.6 a	4.0 a	0.0	0.0
	2332(5)	57.3 a	16.9 a	4.5 a	0.0	30.2
	2334(2)	56.5 a	21.3 a	3.9 a	0.6	3.8
1/10 MS without sugar	Control	117.0 a	13.0 a	6.4 a	0.0	0.0
	2312(3)	98.7 ab	10.6 ab	6.0 a	0.0	6.5
	2331(2)	72.4 b	7.7 b	4.9 a	1.3	1.5
	2332(5)	81.7 b	10.9 ab	5.0 a	0.0	31.9
	2334(2)	88.8 ab	10.4 ab	5.4 a	0.0	0.6
1/100 MS high sugar	Control	48.5 ab	54.3 a	6.7 a	0.0	0.0
	2312 (3)	46.0 b	50.5 a	6.1 a	51.6	57.0
	2332 (5)	54.1 a	63.0 a	6.1 a	10.0	27.2
	2334 (2)	49.6 ab	60.9 a	5.4 a	52.6	54.2
	Control	54.0 ab	65.5 a	6.9 a	0.0	0.0
	2331(2)	61.1 a	62.2 ab	7.2 a	24.8	7.2
	2354(1)-2	57.6 ab	65.7 a	6.3 a	0.0	7.0
	2632(1)	53.9 ab	55.6 ab	6.8 a	55.4	58.0
	2633(1)	50.0 b	49.3 b	6.0 a	64.6	65.6

Different letters indicate significant difference among treatments within the same medium (Tukey HSD test, $P < 0.05$, n = 5)

Table 4.7 (Continued)

Medium	EPF isolate	Shoot fresh weight (mg/plant)	Root fresh weight (mg/plant)	Shoot dry weight (mg/plant)	Internal colonization (%)	External colonization (%)
1/10 MS low sugar	Control	277.6 a	29.1 a	12.3 a	0.0	0.0
	2312 (3)	241.3 a	11.7 a	9.9 a	1.7	5.2
	2332 (5)	225.9 a	16.4 a	8.5 a	0.0	10.9
	2334 (2)	232.9 a	16.6 a	10.0 a	3.5	7.5
	Control	379.4 a	59.4 a	18.6 a	0.0	0.0
	2331(2)	336.0 a	27.4 b	11.7 b	13.8	2.6
	2354(1)-2	300.0 a	22.6 b	10.1 b	0.0	10.0
	2632(1)	281.9 a	28.1 b	12.3 b	0.0	1.3
	2633(1)	285.1 a	17.4 b	10.4 b	0.2	0.8
1/100 MS high N	Control	68.8 a	7.4 a	4.0 a	0.0	0.0
	2312 (3)	77.3 a	10.5 a	4.5 a	2.0	10.9
	2331 (2)	71.7 a	9.7 a	4.5 a	1.8	1.1
	2332 (5)	86.6 a	9.2 a	5.0 a	0.9	20.5
	2334 (2)	77.6 a	7.5 a	4.4 a	1.2	1.1
1/100 MS high P	Control	54.7 a	20.0 a	4.1 a	0.0	0.0
	2312 (3)	52.4 a	22.0 a	4.3 a	4.4	9.0
	2331 (2)	54.7 a	20.3 a	4.4 a	0.2	0.0
	2332 (5)	53.6 a	21.3 a	4.4 a	4.2	25.6
	2334 (2)	53.9 a	19.2 a	4.1 a	0.6	0.2

Different letters indicate significant difference among treatments within the same medium (Tukey HSD test, $P < 0.05$, n = 5)

Table 5.1 Number of EPF isolated from roots of *Santalum album* and *Swietenia macrophylla*

Plant species	Replication	Number of initial plates	Number of EPF isolate		
			Initial	Succesfully maintained	Not succesfully maintained*
Santalum album	1	10	1	1	0
	2	10	5	3	2
	3	10	8	8	0
	4	10	5	5	0
	5	10	8	4	4
	6	10	8	7	1
	7	10	6	5	1
	8	10	6	3	3
	Total	80	47	36	11
	Mean		6	5	1
	Isolation rate (%)	59			
Swietenia macrophylla	1	10	6	6	0
	2	10	5	3	2
	3	10	0	0	0
	4	10	4	4	0
	5	10	5	5	0
	6	10	7	5	2
	7	10	1	1	0
	Total	70	28	24	4
	Mean		4	3	1
	Isolation rate (%)	40			

*Cultures were contaminated by other fungi or bacteria

Table 5.2 Growth of *Brassica campestris* inoculated with or without EPF isolated from roots of *Santalum album* and *Swietenia macrophylla*, 28 days after transplanting

EPF isolate	Shoot fresh weight (mg/plant)		Root fresh weight (mg/plant)		Shoot dry weight (mg/plant)		Shoot: root ratio	
Control	54.0		33.7		4.6		1.6	
SA 2-5	0.0		0.0		0.0		n.d	
SA 3-7	42.0	*	31.3	ns	5.6	*	1.4	*
SA 4-1	24.2	**	13.7	***	5.8	ns	1.8	ns
SA 4-3	50.8	ns	17.4	***	6.0	ns	3.0	ns
SA 5-2	44.9	ns	23.2	ns	5.8	ns	2.2	ns
SA 6-3	25.9	***	21.5	*	4.9	ns	1.2	**
SM 1-5	0.0		0.0		0.0		n.d	
SM 4-2	0.0		0.0		0.0		n.d	
SM 5-2	67.7	**	33.9	ns	7.1	*	2.0	*
SM 5-3	0.0		0.0		0.0		n.d	
SM 5-4	59.3	ns	26.1	*	5.3	ns	2.3	*
SM 5-5	37.6	*	25.8	*	5.3	ns	1.5	ns
Control	57.9		28.7		4.6		2.1	
SA 2-3	33.0	**	23.1	ns	4.5	ns	1.4	ns
SA 3-1	0.0		0.0		0.0		n.d	
SA 3-3	36.9	**	20.6	ns	4.9	ns	1.8	ns
SA 3-6	35.9	**	22.0	ns	5.0	ns	1.6	ns
SA 3-8	0.0		0.0		0.0		n.d	
SA 6-2	55.0	ns	25.7	ns	4.6	ns	2.1	ns
SA 6-4	52.6	ns	22.6	ns	5.1	ns	2.3	ns
SA 7-1	26.5	***	20.7	ns	4.2	ns	1.4	ns
SA 7-5	35.3	*	16.8	*	4.7	ns	2.0	ns
SA 7-6	47.5	ns	25.4	*	4.3	ns	1.9	ns
SA 8-2	19.7	***	12.4	ns	4.1	ns	1.7	ns
SM 4-3	23.1	**	12.3	ns	4.0	ns	1.8	ns
SM 5-1	53.8	ns	25.6	*	4.9	ns	2.2	ns
Control	31.9		20.3		4.3		1.6	
SA 2-4	22.0	*	5.4	***	4.8	*	4.5	*
SA 3-2	0.0		0.0		0.0		n.d	
SA 4-5	22.9	ns	15.6	*	4.1	ns	1.4	ns
SA 5-4	0.0		0.0		0.0		n.d	
SA 6-5	0.0		0.0		0.0		n.d	

Significant difference between inoculated and respective control was determined by the Student's t-test (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not significant; n= 5); n.d = not determined.

Table 5.2 (Continued)

EPF isolate	Shoot fresh weight (mg/plant)		Root fresh weight (mg/plant)		Shoot dry weight (mg/plant)		Shoot: root ratio	
SA 6-7	48.2	*	31.5	**	4.9	ns	1.6	ns
SA 7-2	0.0		0.0		0.0		n.d	
SA 7-3	0.0		0.0		0.0		n.d	
SM 1-3	21.8	*	16.4	***	4.7	*	1.3	ns
SM 2-1	0.0		0.0		0.0		n.d	
SM 2-3	0.0		0.0		0.0		n.d	
SM 6-3	26.0	ns	25.3	ns	5.2	*	1.1	ns
SM 6-6	25.9	ns	22.8	ns	4.9	**	1.1	*
Control	49.4		29.7		5.2		1.7	
SA 1-4	0.0		0.0		0.0		n.d	
SA 3-4	55.6	ns	21.2	*	5.0	ns	2.8	ns
SA 4-2	63.8	*	30.3	ns	5.2	ns	2.1	ns
SA 5-7	51.5	ns	29.6	ns	4.7	ns	1.8	ns
SA 6-1	56.1	ns	29.2	ns	5.0	ns	1.9	ns
SA 6-6	0.0		0.0		0.0		n.d	
SA 8-3	0.0		0.0		0.0		n.d	
SM 1-2	48.6	ns	21.3	*	4.8	ns	2.4	ns
SM 1-6	33.6	ns	11.4	**	5.2	ns	3.3	ns
SM 2-2	66.8	*	31.2	ns	6.2	ns	2.2	ns
SM 4-1	57.4	ns	26.7	ns	4.9	ns	2.2	ns
SM 6-1	62.9	*	26.3	ns	5.3	ns	2.4	*
SM 6-5	31.7	*	20.5	***	4.4	ns	1.6	ns
Control	57.6		23.8		5.8		2.5	
SA 3-5	0.0		0.0		0.0		n.d	
SA 4-4	42.6	ns	15.2	*	6.8	ns	2.8	ns
SA 5-1	65.2	ns	18.7	ns	6.5	ns	3.5	ns
SA 8-4	67.6	ns	27.0	ns	6.7	ns	2.5	ns
SM 1-1	64.1	ns	32.5	*	6.2	ns	2.0	ns
SM 1-4	37.7	*	13.4	**	5.1	ns	2.9	ns
SM 4-4	41.6	ns	18.4	ns	4.3	ns	2.3	ns
SM 6-7	57.8	ns	18.6	ns	5.5	ns	3.2	ns
SM 7-1	58.0	ns	27.7	ns	5.4	ns	2.1	ns

Significant difference between inoculated and respective control was determined by the Student's t-test (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not significant; n= 5); n.d = not determined.

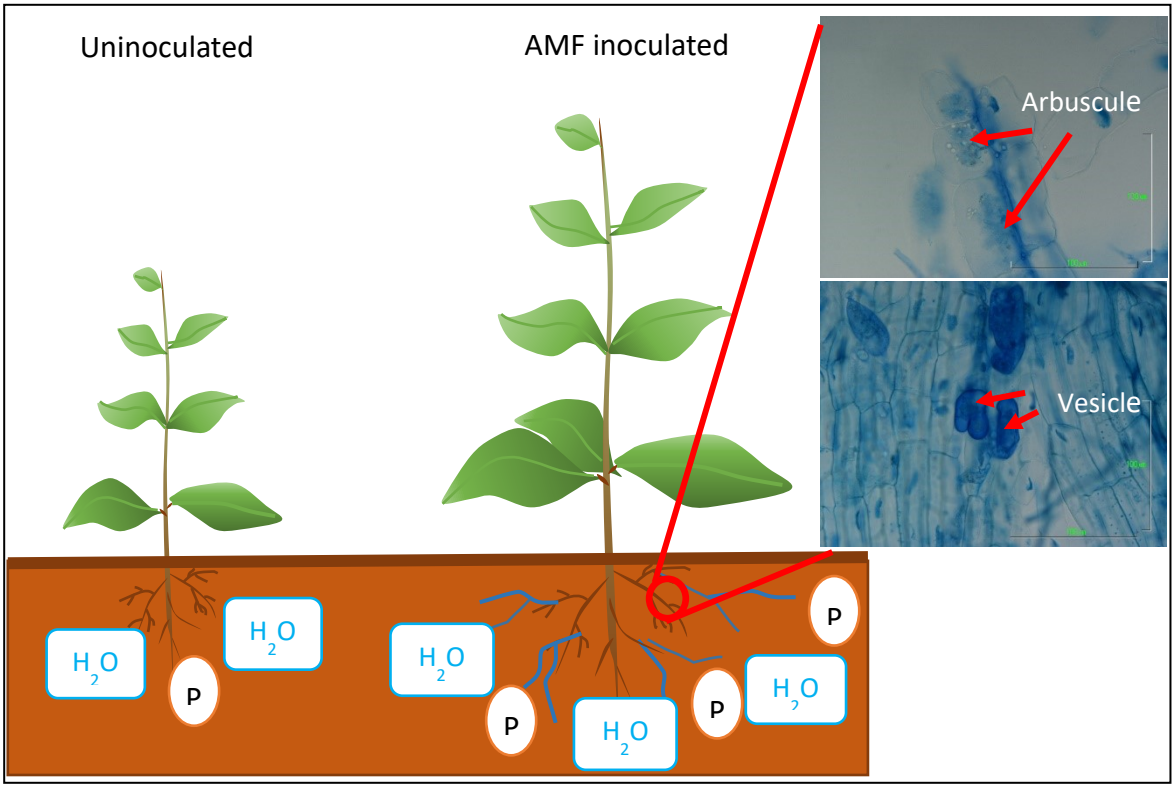

Figure 1.1 Growth, nutrient and water uptake and mycorrhizal structure of uninoculated and AMF-inoculated seedling

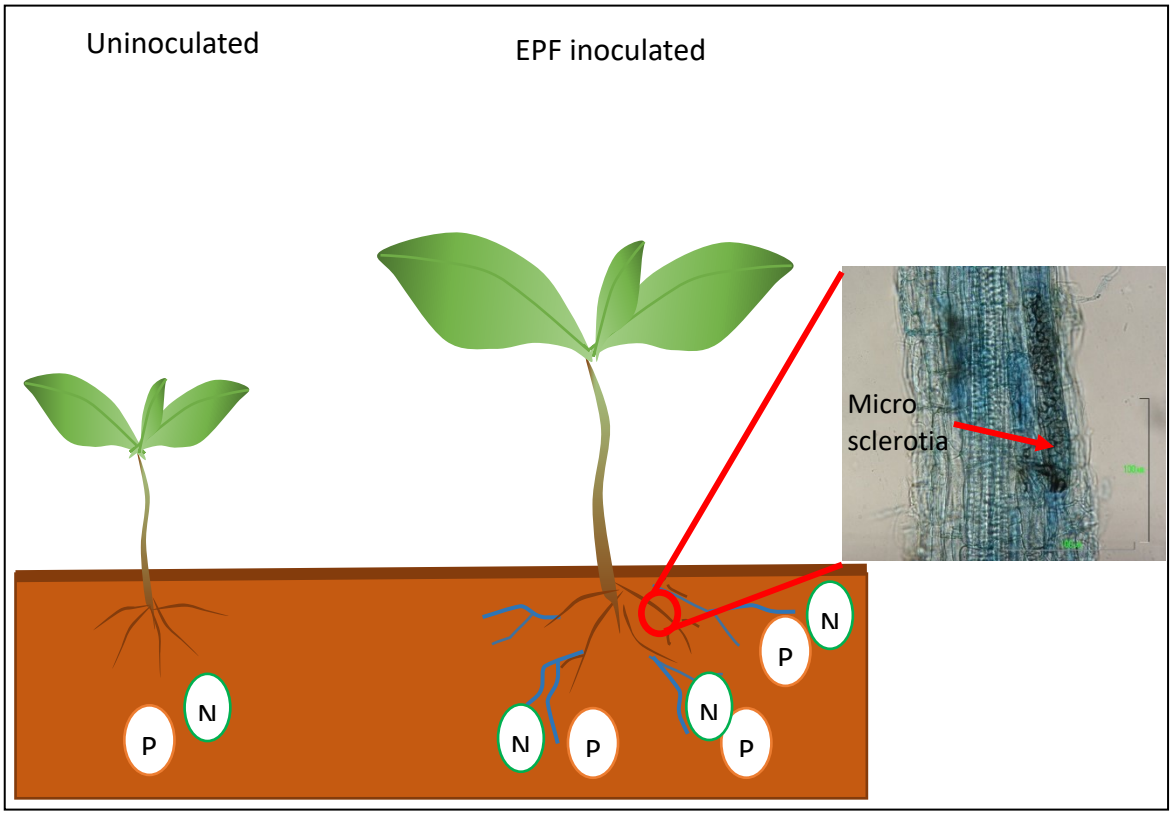

Figure 1.2 Growth, nutrient uptake and endophytic structure of uninoculated and EPF-inoculated seedling

Figure 1.3 Leguminous tree species from Indonesia: *Paraserianthes falcataria*, 67 days after sowing (A); *Calliandra calothyrsus*, 74 days after sowing (B); *Cassia siamea*, 74 days after sowing (C); and *Sesbania grandiflora*, 74 days after sowing (D)

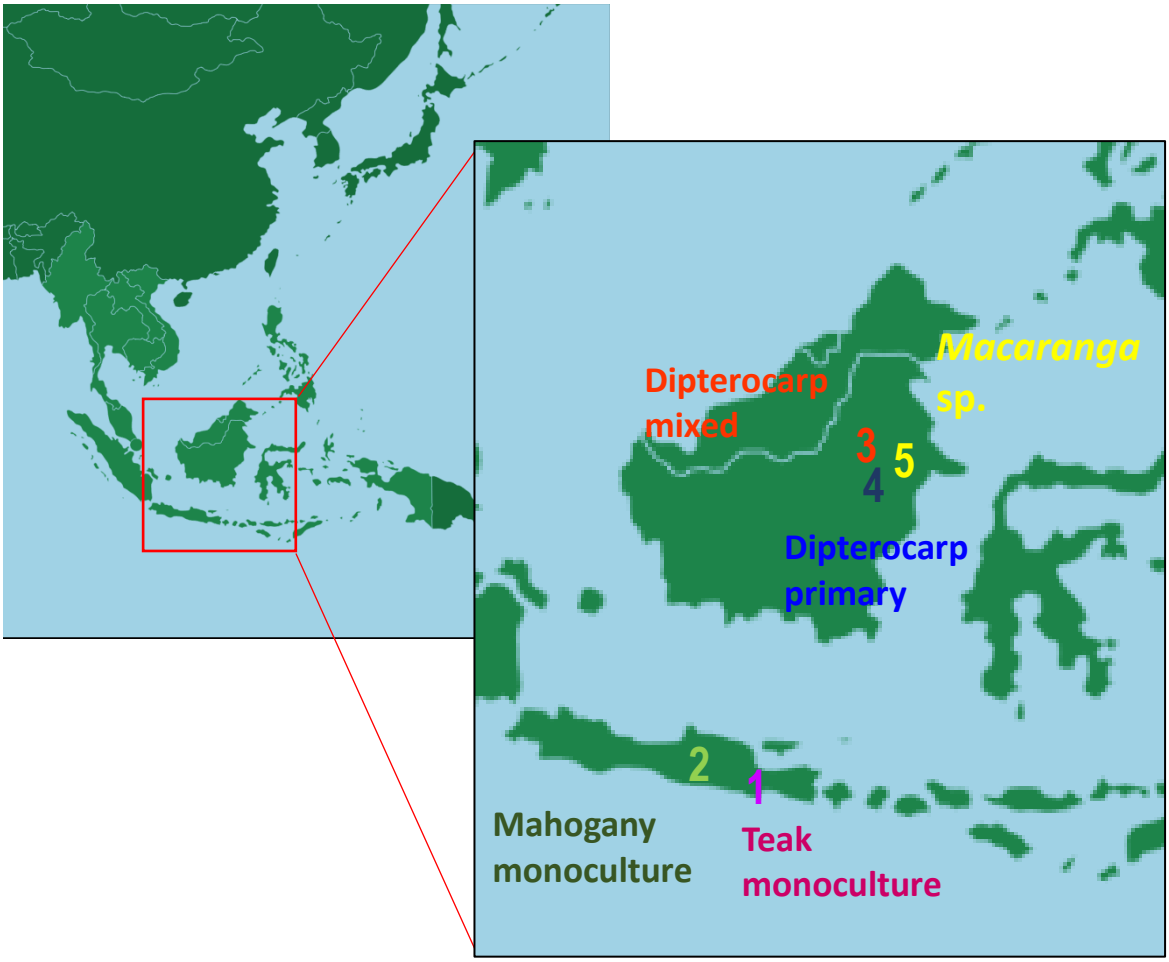

Figure 2.1 Location of forest sites in Indonesia

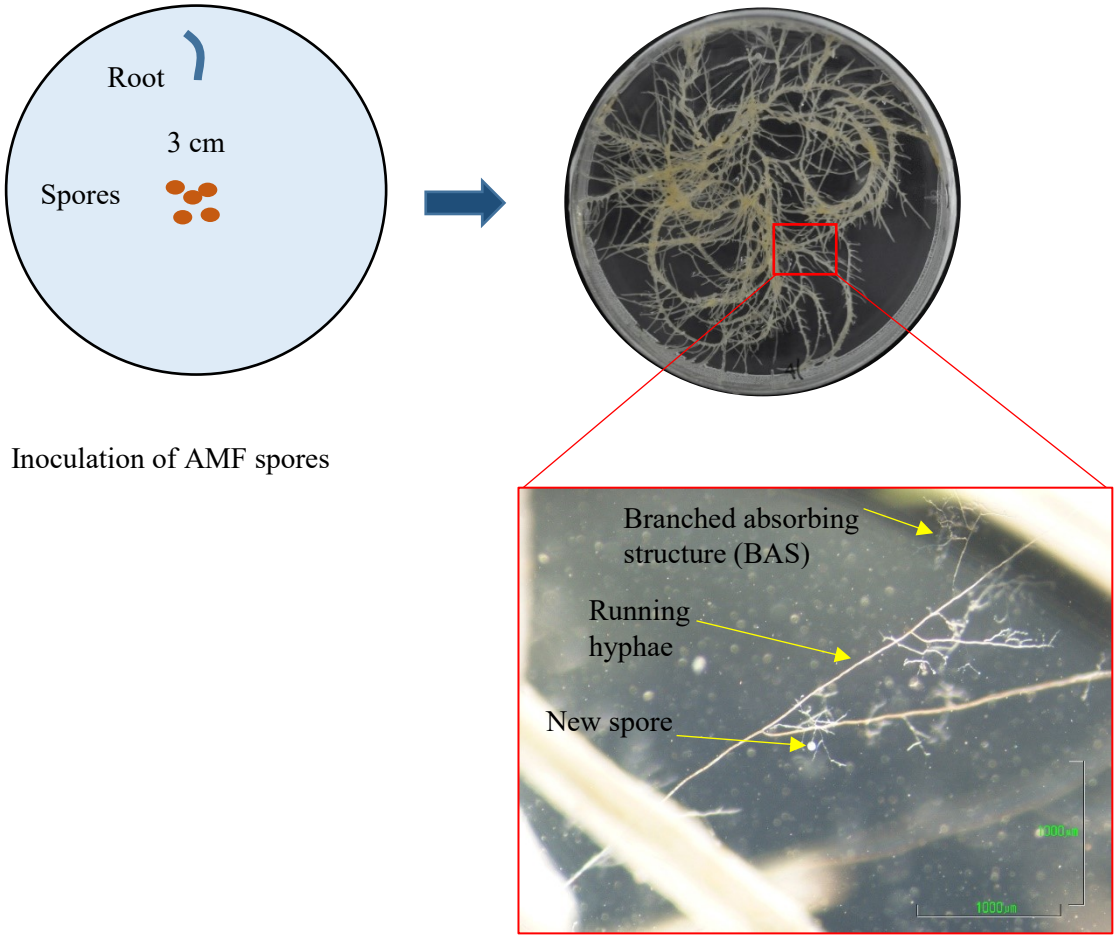

Figure 2.2 Schematic diagram of propagation of AMF isolates by root organ culture. Five AMF spores were placed on the center of Petri dish containing MSR medium, at 3-cm-distance from one-cm-long hairy root of *Linum usitatissimum*. Root growth and formation of running hyphae, branched absorbing structure and new spores after several weeks of incubation.

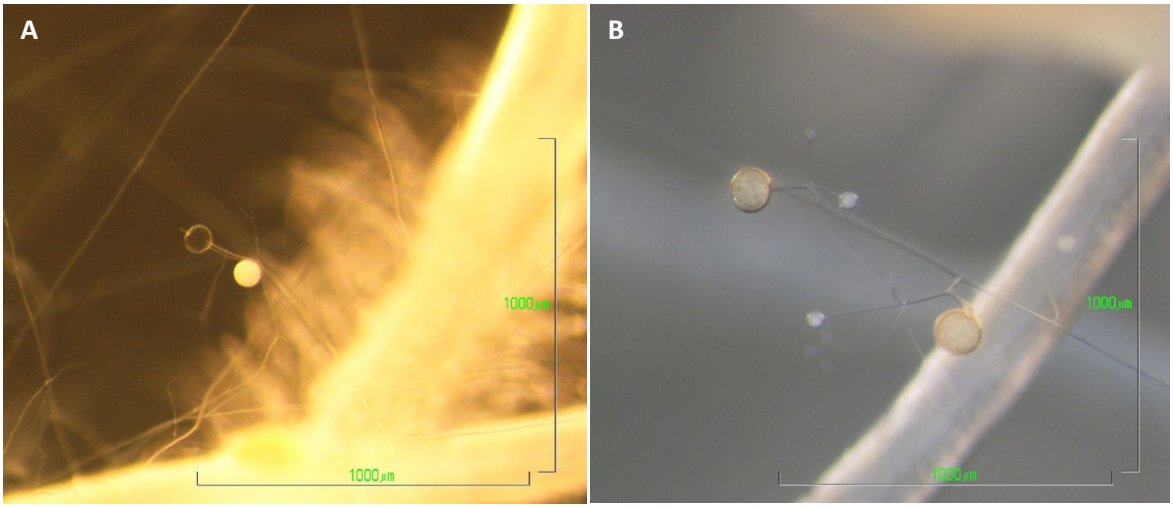

Figure 2.3 New spores of AMF isolate M11-1 (A) and S6-4 (B) propagated by root organ culture using hairy root of *Linum usitatissimum*, 82 days after inoculation

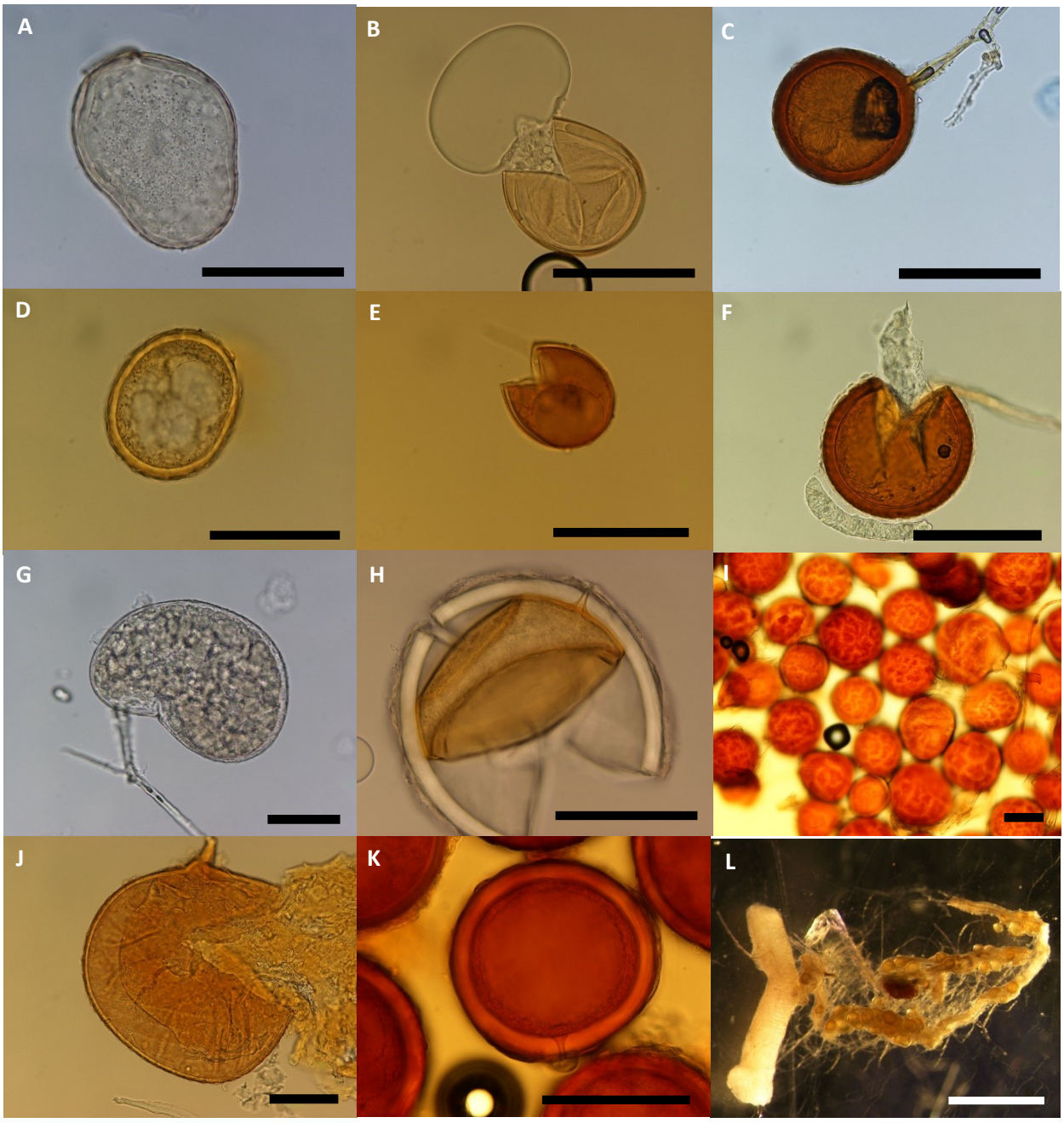

Figure 2.4 Morphological characteristics of spores of M10-2 in PVLG (A) and PVLG + Melzer (D), M11-1 in PVLG (B) and PVLG + Melzer (E), M44-3 in PVLG (C) and PVLG + Melzer (F), M60-3 in PVLG (G) and PVLG + Melzer (J), and S6-4 in PVLG (H) and PVLG + Melzer (K, I). Intraradical spore of S6-4 (L). Black bar = 100 µm. White bar = 1000 µm.

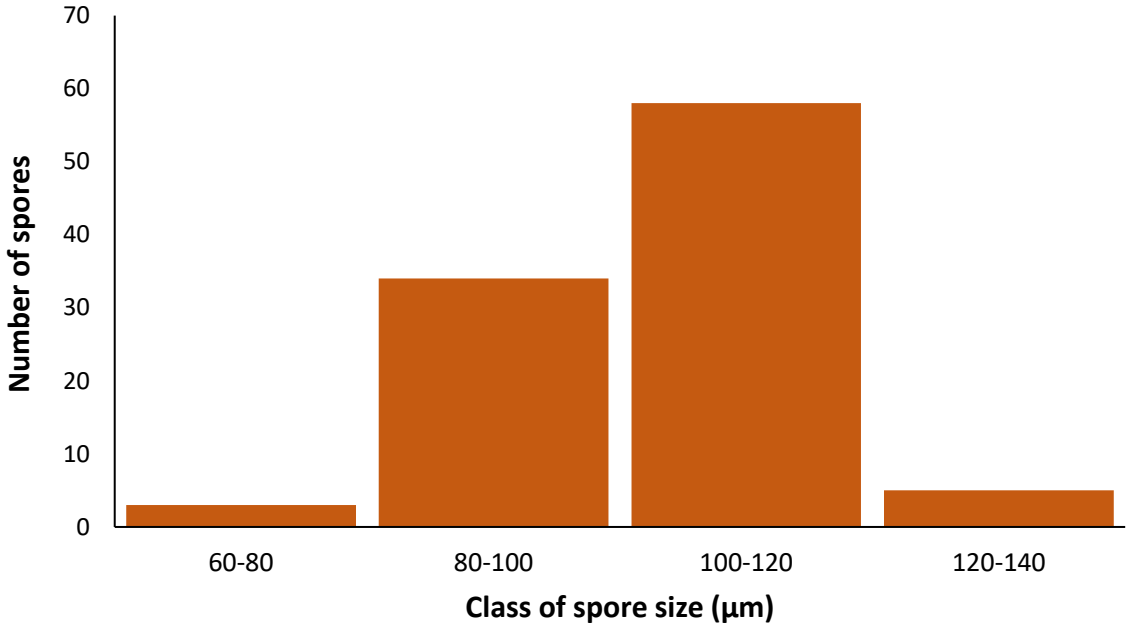

Figure 2.5 Class of spore size of AMF isolate M10-2 (n= 100)

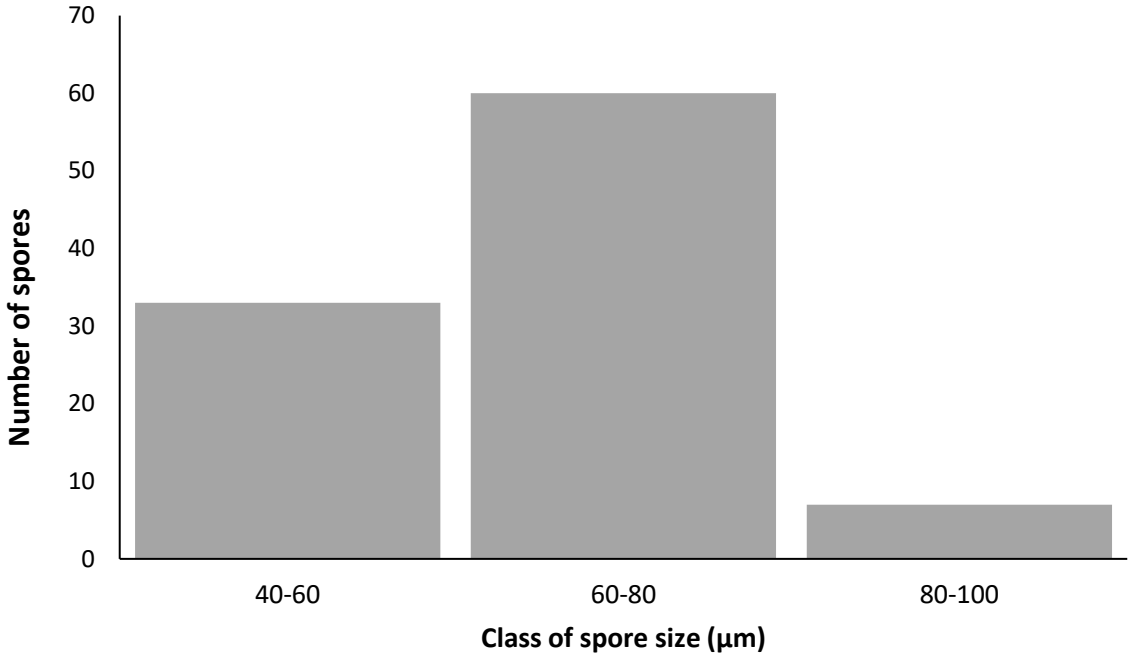

Figure 2.6 Class of spore size of AMF isolate M11-1 (n= 100)

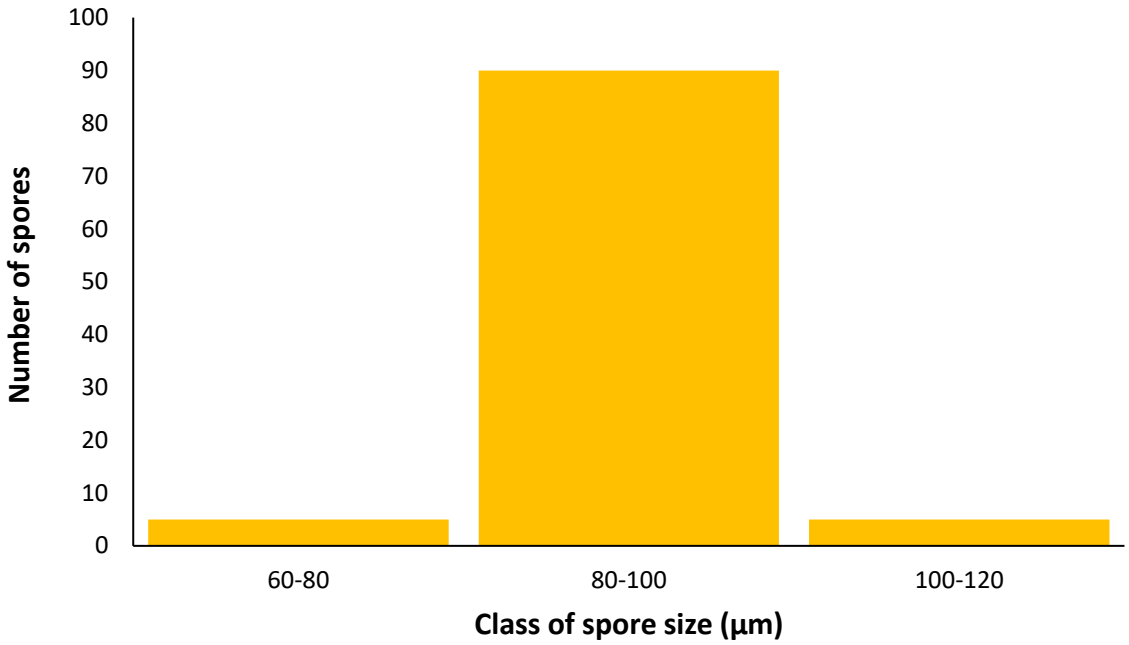

Figure 2.7 Class of spore size of AMF isolate M44-3 (n= 100)

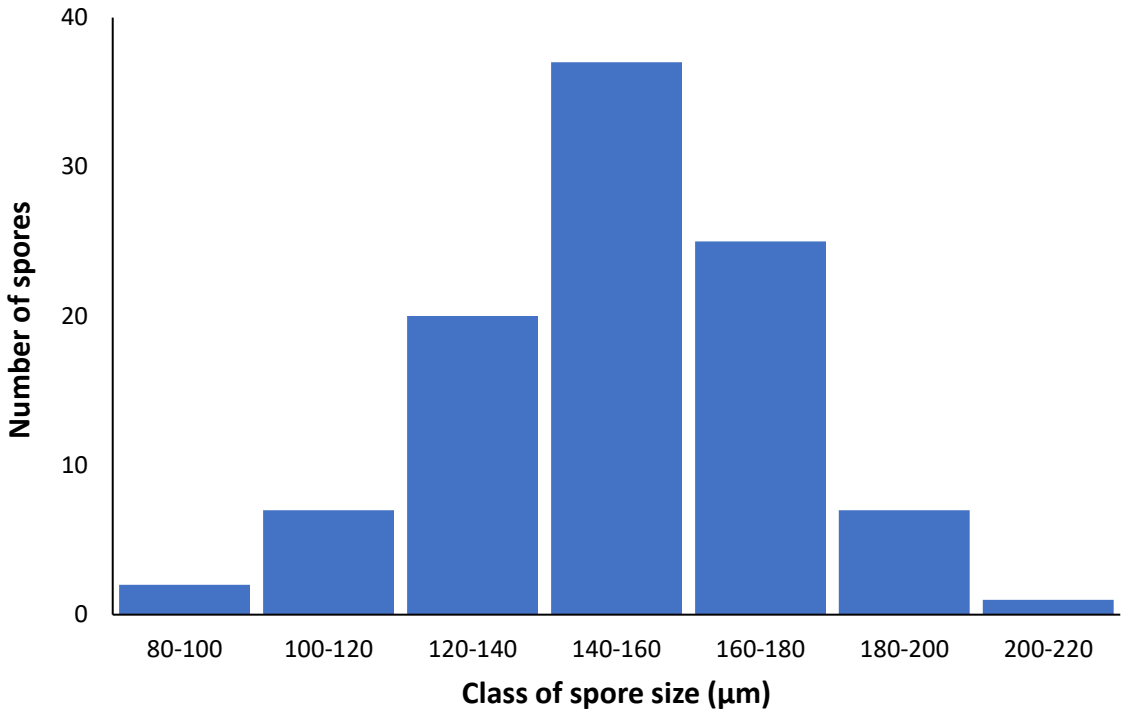

Figure 2.8 Class of spore size of AMF isolate M60-3 (n= 99)

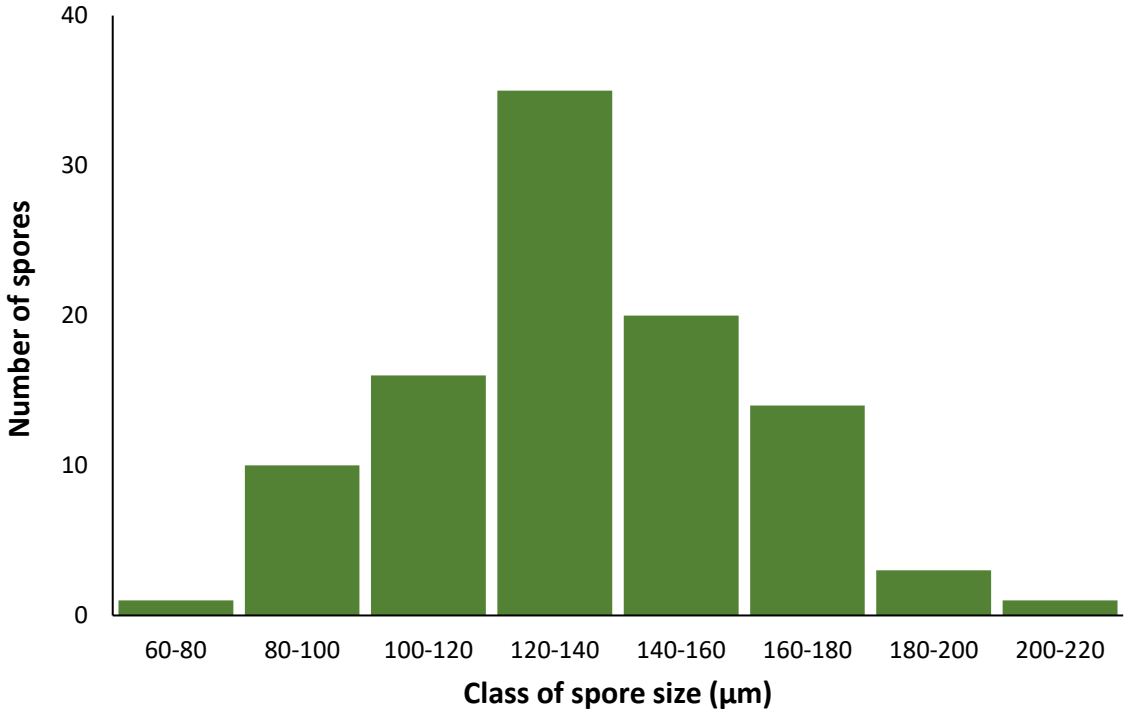

Figure 2.9 Class of spore size of AMF isolate S6-4 (n= 100)

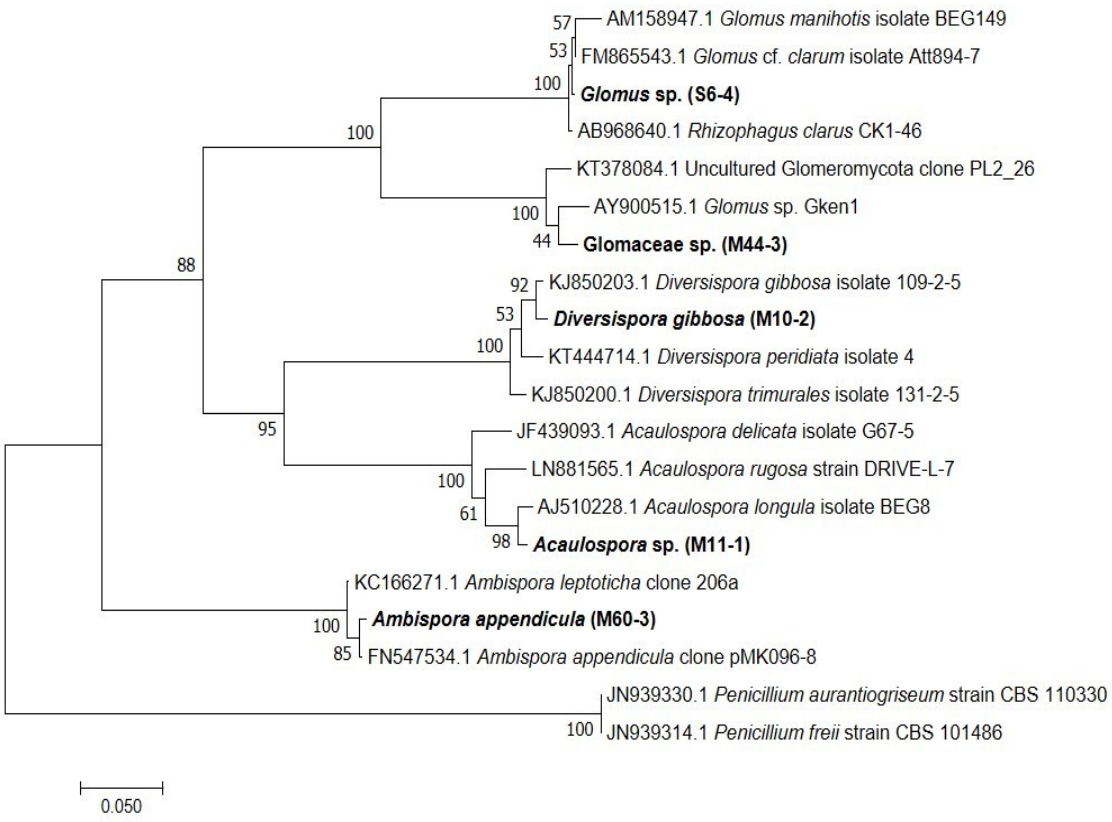

Figure 2.10 Phylogenetic tree of five AMF isolates obtained by maximum likelihood analysis of LSU region of the ribosomal gene. The new sequences of the five AMF isolates are indicated in bold font. Bootstrap values are shown below the branch (1000 replications).

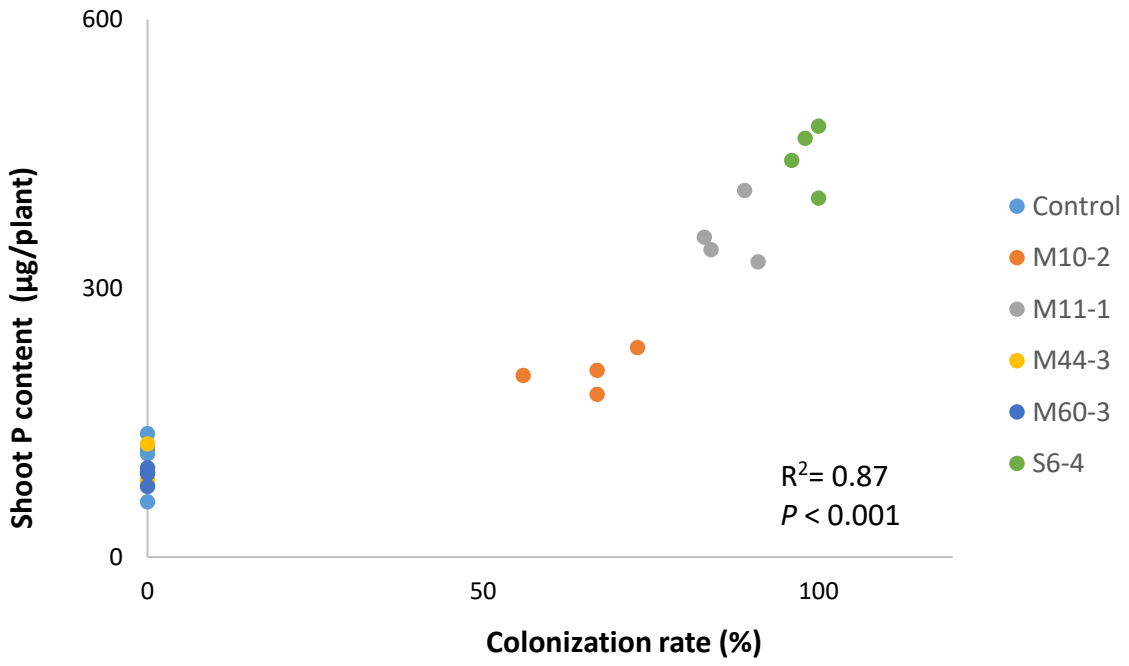

Figure 2.11 Correlation between colonization rate and shoot P content of *Paraserianthes falcataria* inoculated with or without AMF isolates, 60 days after transplanting

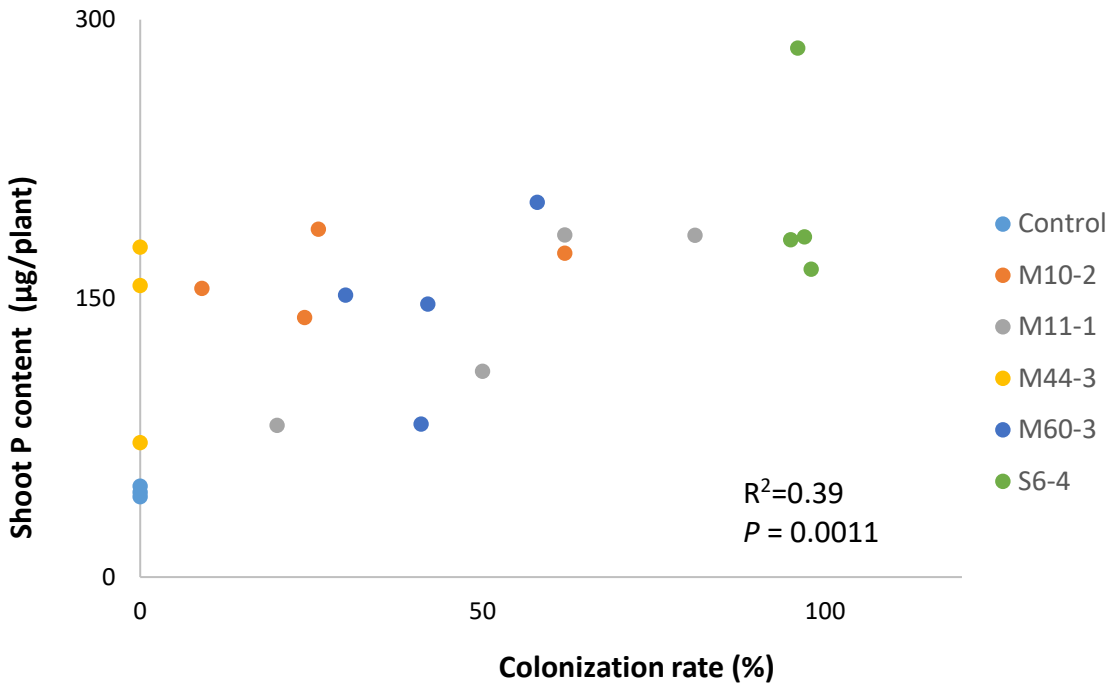

Figure 2.12 Correlation between colonization rate and shoot P content of *Calliandra calothyrsus* inoculated with or without AMF isolates, 60 days after transplanting

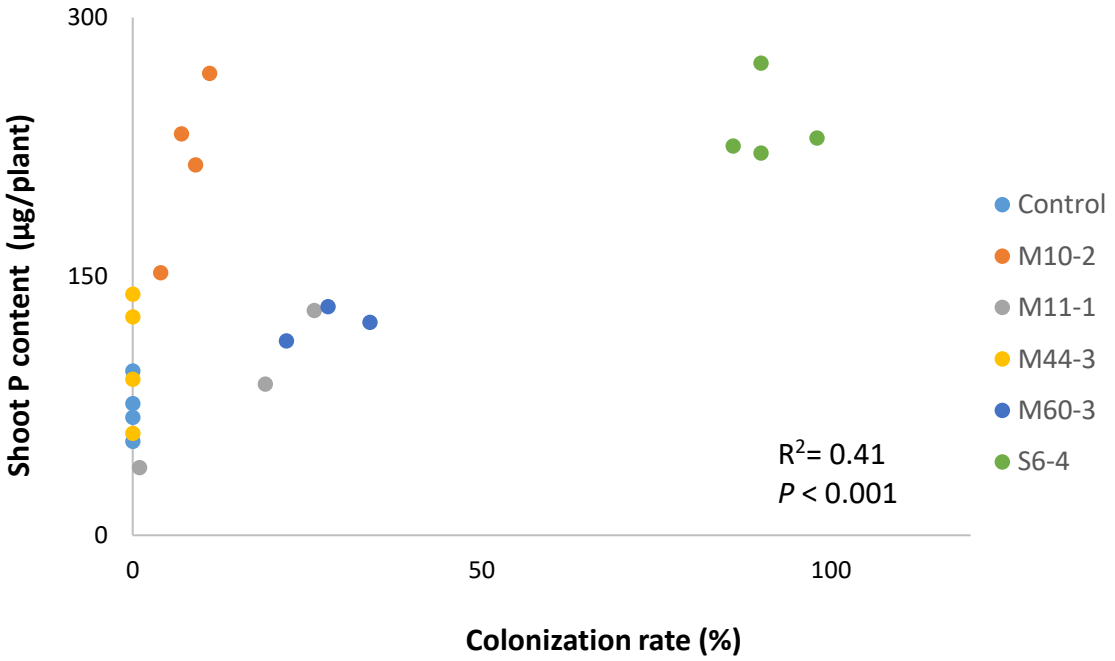

Figure 2.13 Correlation between colonization rate and shoot P content of *Cassia siamea* inoculated with or without AMF isolates, 60 days after transplanting

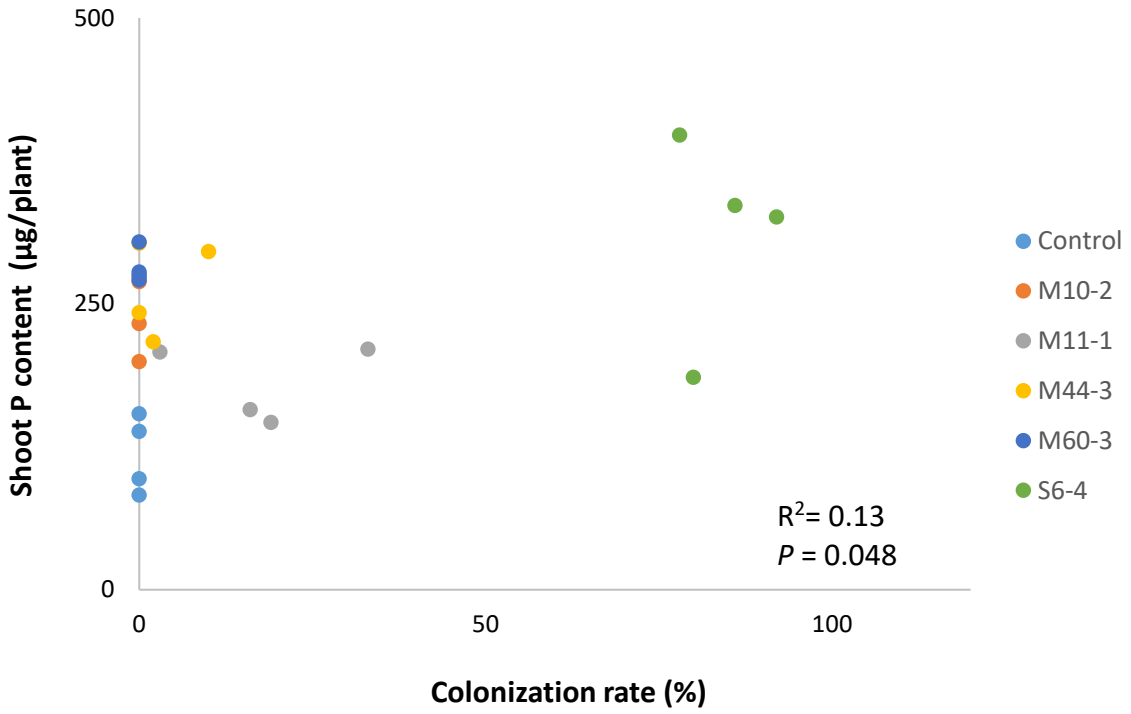

Figure 2.14 Correlation between colonization rate and shoot P content of *Sesbania grandiflora* inoculated with or without AMF isolates, 60 days after transplanting

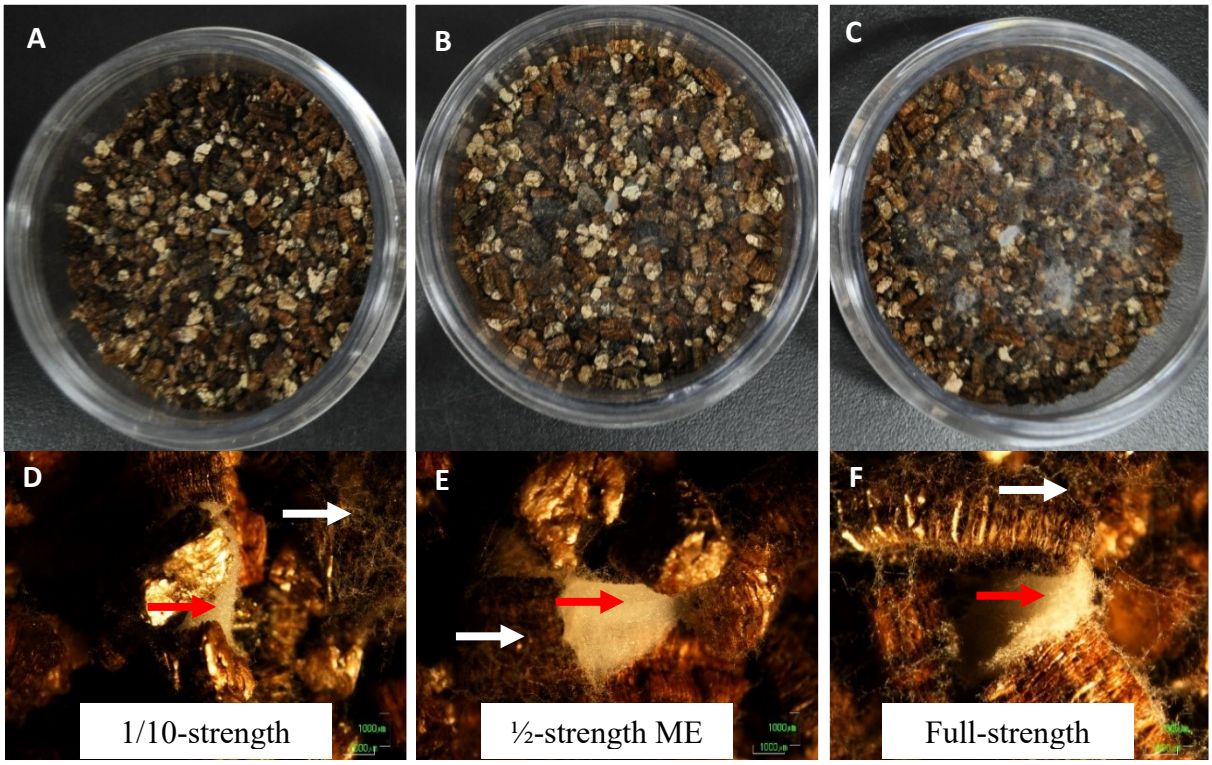

Figure 3.1 Growth of EPF isolate 2614(4)PDA-1-2-1 on vermiculite with application of 1/10-strength (A, D), ½-strength (B, E), and full-strength (C, F) liquid malt extract (ME). Mycelial plugs (red arrow) and new mycelia (white arrow) of 2614(4)PDA-1-2-1 on vermiculite with application of 1/10-strength ME (D), ½-strength ME (E), and full-strength ME (F), observed at 62x magnification.

Figure 3.2 *Brassica campestris* transplanted on pre-grown EPF colony of 2614(4)PDA-1-2-1 with application of 1/10-strength, ½-strength, and full-strength liquid malt extract (left to right), 30 days after transplanting

Figure 3.3 *Brassica campestris* grown on water agar and different concentration of Murashige and Skoog (MS) medium, 14 days after transplanting. Left to right: water agar, 1/100-strength MS, 1/10-strength MS, 1/5-strength MS, and full-strength MS.

Figure 3.4 *Brassica campestris* grown on vermiculite with application of sterilized deionized water and different concentration of P8 nutrient solution, 30 days after transplanting. Left to right: deionized water, 1/100-strength P8, 1/10-strength P8, 1/5-strength P8, and full-strength P8 nutrient solution.

Figure 3.5 *Brassica campestris* inoculated with 2531(3)WA-2-1 (A) and 2614(4)PDA-1-2-1 (B) grown on water agar, 1/100-strength MS, 1/10-strength MS, 1/5-strength MS, and full-strength MS medium (left to right), 21 days after transplanting

Figure 4.1 *Sorghum bicolor* grown in forest soils for trap culture of EPF, 90 days after sowing. *S. bicolor* with lower growth (left) and higher growth (right) in the selection of plants for EPF isolation. Only the plants with higher growth were harvested for EPF isolation.

Figure 4.2 *Paraserianthes falcataria* grown in forest soils for trap culture of EPF, 90 days after transplanting. *P. falcataria* with lower growth (left) and higher growth (right) in the selection of plants for EPF isolation. Only the plants with higher growth were harvested for EPF isolation.

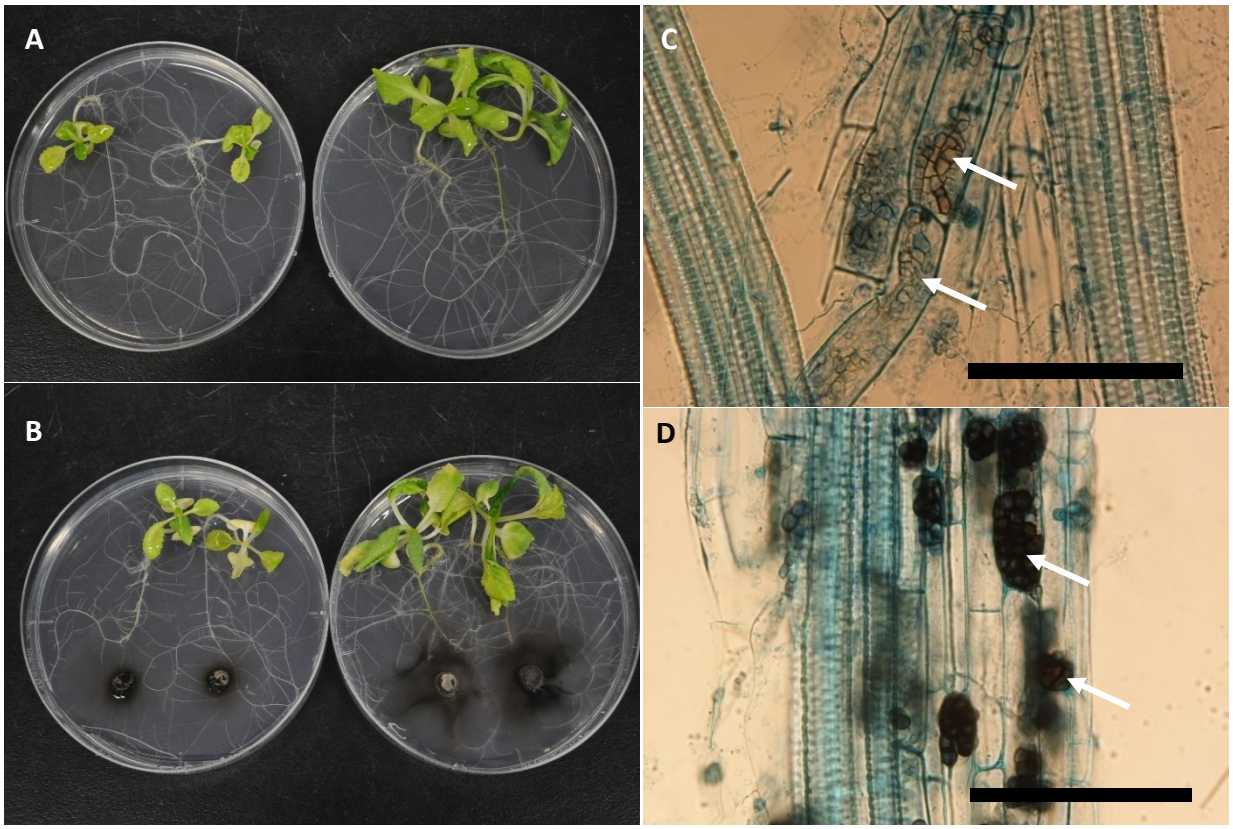

Figure 4.3 *Brassica campestris* inoculated without (A) or with isolate 2312(3) (B) and grown on 1/100 (left) and 1/10 (right) MS medium, 28 days after transplanting. Internal colonization (C, arrow) and external colonization (D, arrow) of *B. campestris* roots by isolate 2312(3). Black bar = 100 µm.

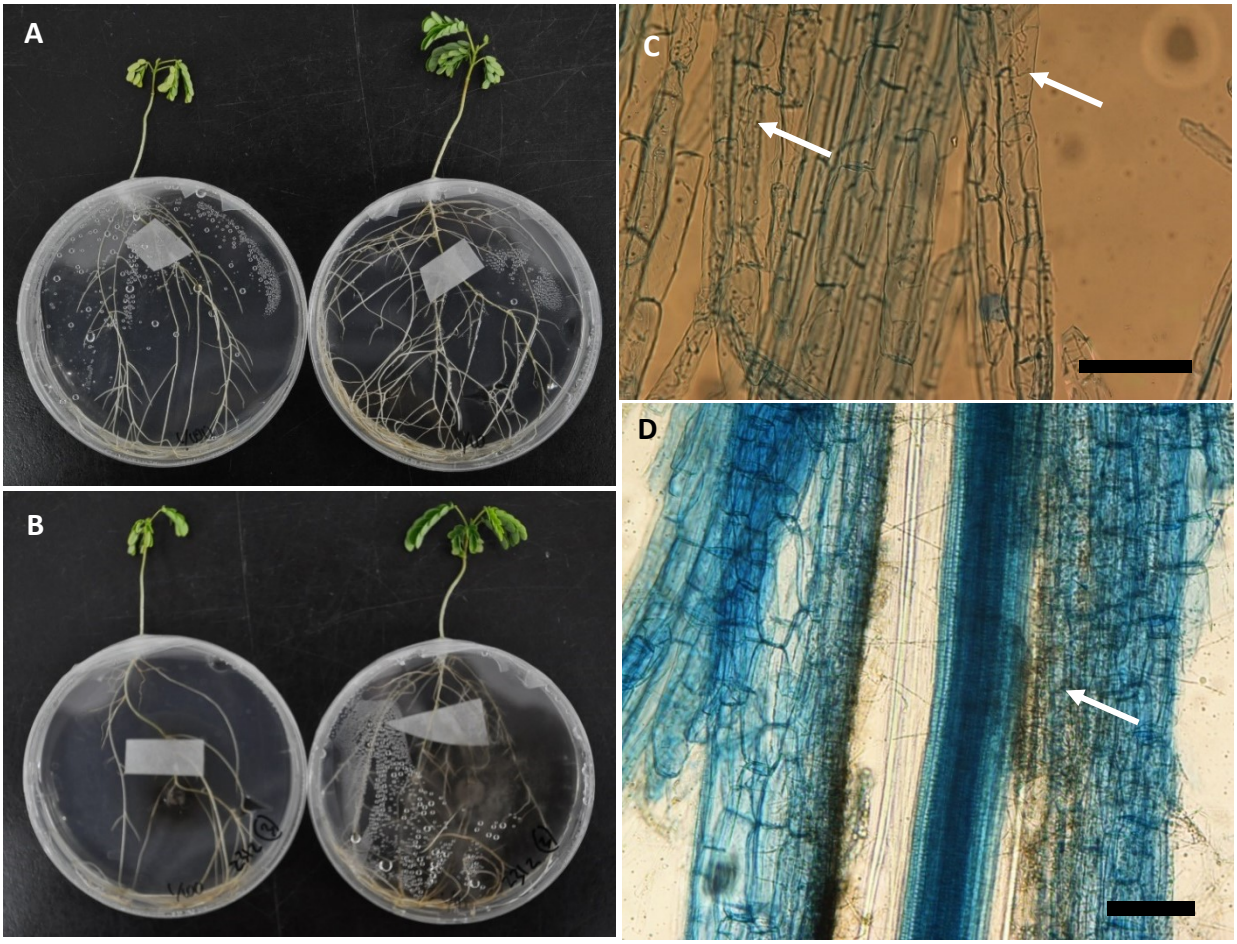

Figure 4.4 *Paraserianthes falcataria* inoculated without (A) or with isolate 2312(3) (B) and grown on 1/100 (left) and 1/10 (right) MS medium, 37 days after transplanting. Internal colonization (C, arrow) and external colonization (D, arrow) of *P. falcataria* roots by isolate 2312(3). Black bar = 100 µm.

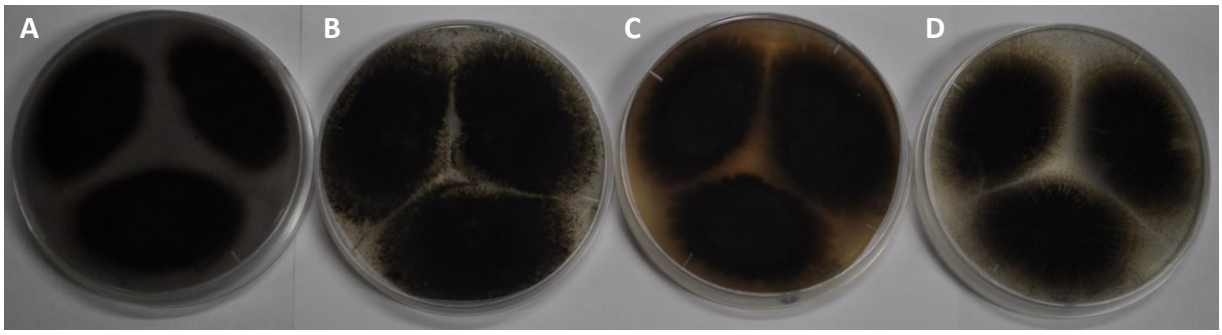

Figure 4.5 Fourteen-days-old colonies of EPF isolates 2312(3) (A), 2331(2) (B), 2332(5) (C), and 2334(2) (D) showing dark color mycelium.

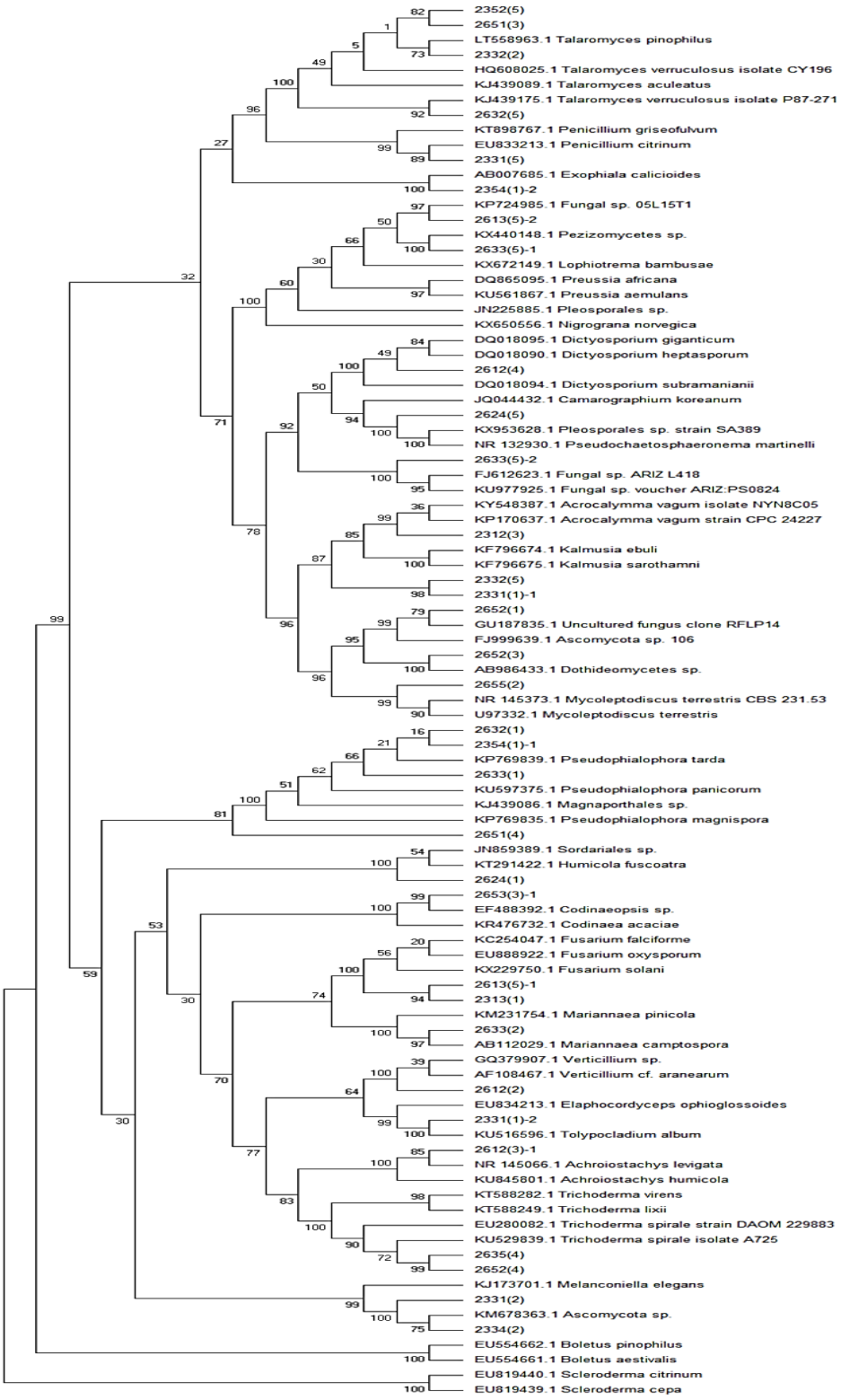

Figure 4.6 Maximum parsimony analysis of ITS1 region sequences of EPF. Values from bootstrap analysis (1000 replications) are shown.

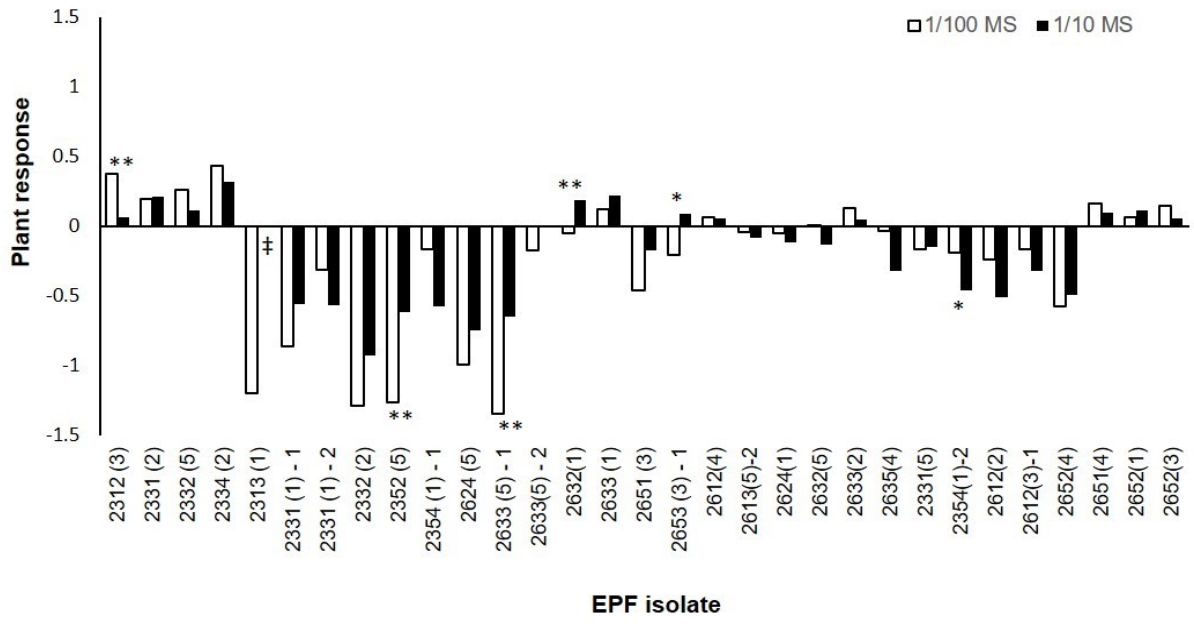

Figure 4.7 Response of *Brassica campestris* to inoculation with 31 EPF isolates and growth on 1/100-strength MS medium (open bar) or 1/10-strength MS medium (closed bar). Values higher (lower) than 0 on the x-axis indicate positive (negative) response. Significant difference in PR between plant grown on 1/100-strength MS medium and that grown on 1/10-strength MS medium was determined by the Student's t-test (** $P < 0.01$, * $P < 0.05$; n= 5). ‡ The denominator was zero.

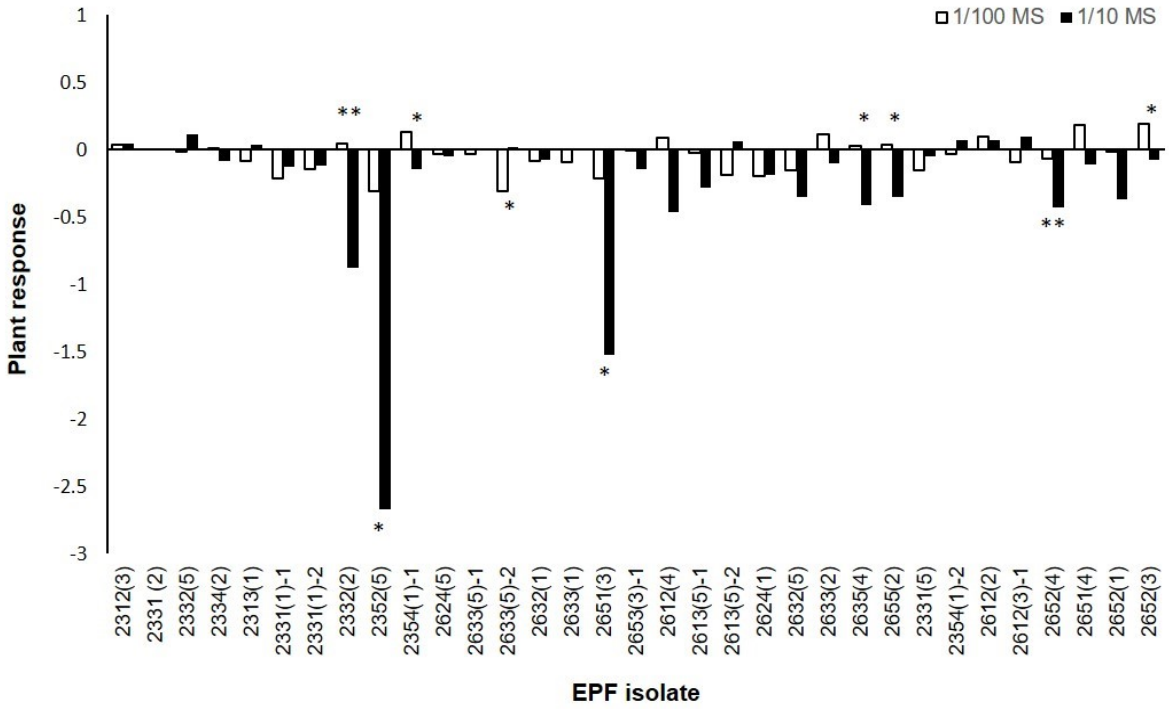

Figure 4.8 Response of *Paraserianthes falcataria* to inoculation with 33 EPF isolates and growth on 1/100-strength MS medium (open bar) or 1/10-strength MS medium (closed bar). Values higher (lower) than 0 on the x-axis indicate positive (negative) response. Significant difference in PR between plant grown on 1/100-strength MS medium and that grown on 1/10-strength MS medium was determined by the Student's t-test (** $P < 0.01$, * $P < 0.05$; n= 5).

Figure 4.9 *Brassica campestris* inoculated (left to right) without or with EPF isolate 2312(3), 2331(2), 2332(5) and 2334(2) with application of liquid 1/100-strength MS, 21 days after transplanting

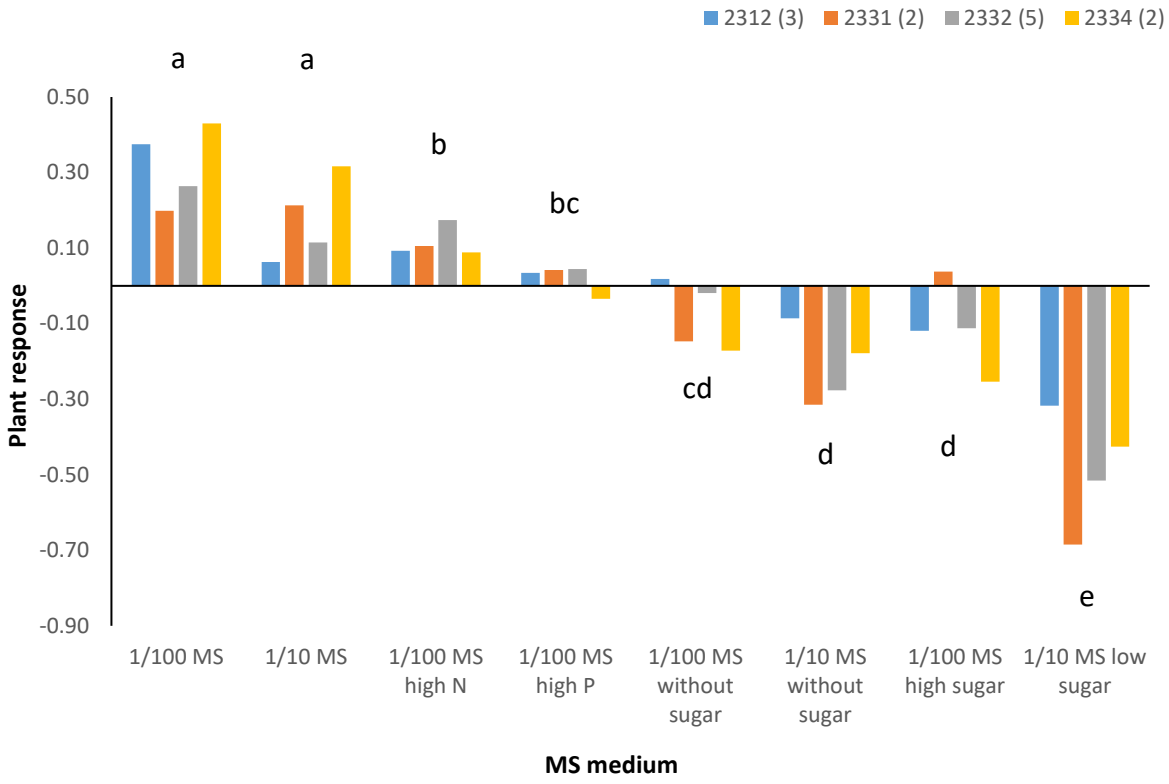

Figure 4.10 Response of *Brassica campestris* to inoculation with 4 EPF isolates on 1/100-strength MS medium. Values higher (lower) than 0 on the x-axis indicate positive (negative) response. Significant difference in PR between plant grown on different MS medium was determined Tukey HSD test ($P < 0.05$; n= 5).

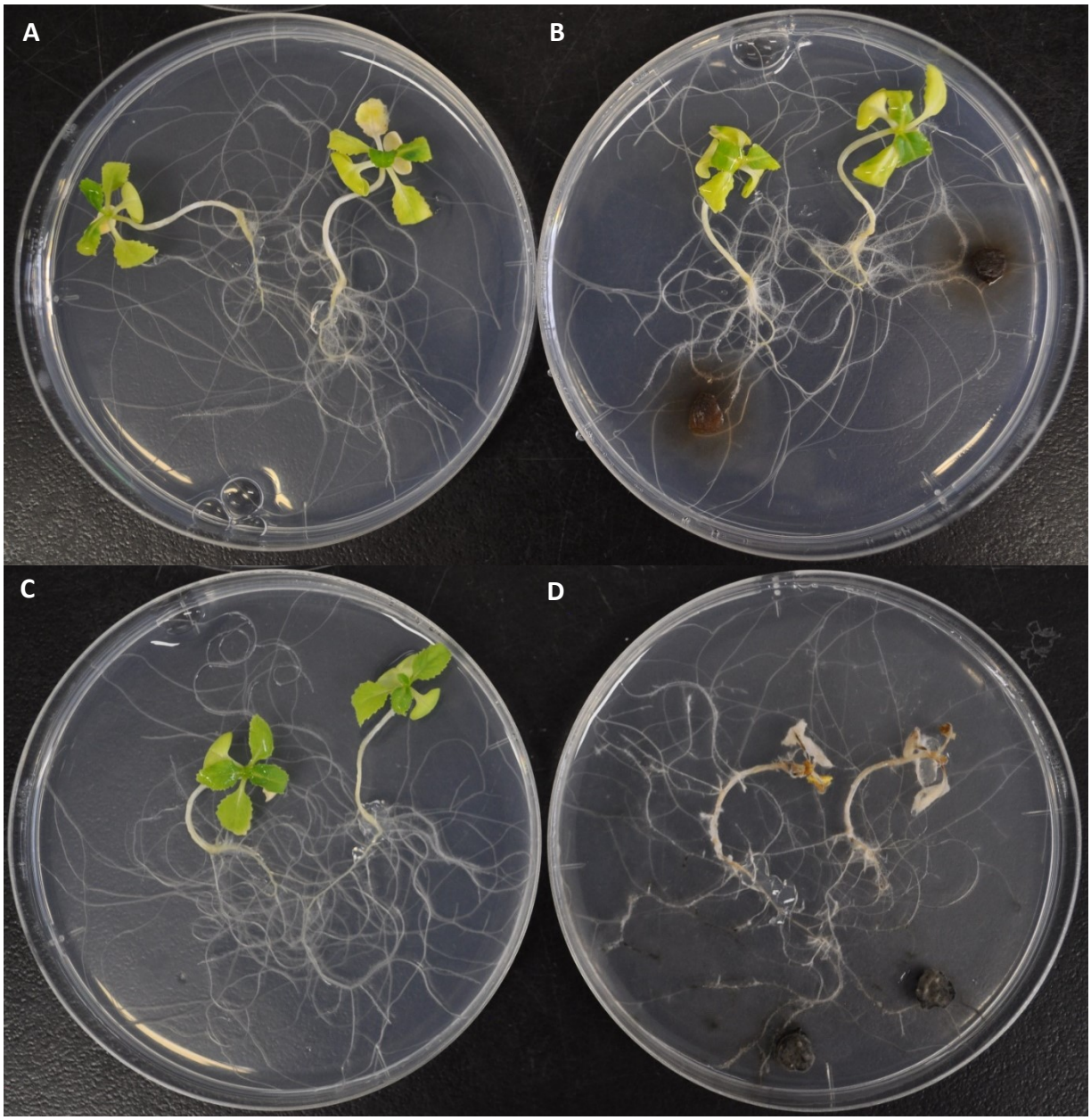

Figure 5.1 *Brassica campestris* inoculated without (A, C) or with isolate SM 5-2 (B) and isolate SM 2-3 (D), grown on 1/100-strength MS medium, 28 days after transplanting

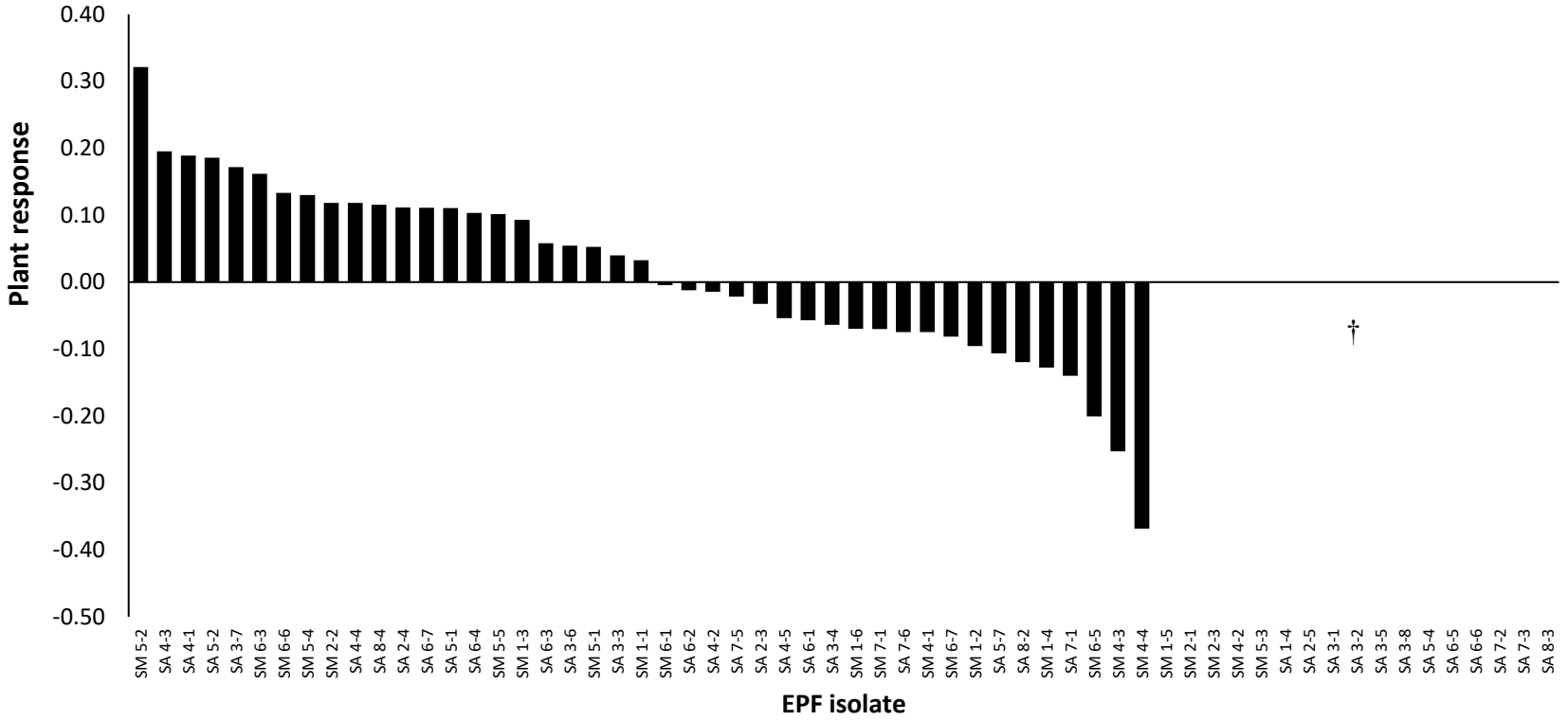

Figure 5.2 Growth response of *Brassica campestris* to inoculation with 50 EPF isolated from roots of *Santalum album* and *Swietenia macrophylla*, grown on 1/100 MS medium. Values higher (lower) than 0 on the x-axis indicate positive (negative) response. † Inoculated plants were not survived.