ARBUSCULAR MYCORRHIZAL FUNGI INCREASED PLANT GROWTH AND NUTRIENT CONCENTRATIONS OF MILKWOOD TROPICAL TREE SPECIES *Alstonia scholaris* UNDER GREENHOUSE CONDITIONS

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ABSTRACT

The objective of this study was to determine the effect of five arbuscular mycorrhizal (AM) fungi on the early growth of *Alstonia scholaris* (milkwood) seedlings. The seedlings were inoculated with *Glomus clarum* Nicholson & Schenk, *Gigaspora decipiens* Hall & Abbott, *Glomus* sp. ACA Tulasne & Tulasne, *Entrophospora* sp. Ames & Scheneider, and *Glomus* sp. ZEA Tulasne & Tulasne, and uninoculated (control) under greenhouse conditions. Percentage of AM colonization, plant growth, survival rate, mycorrhizal dependency (MD), shoot nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg) concentrations were measured after 150 days. Survival rates were higher in the AM-colonized seedlings at 150 days after transplantation than those in the control seedlings. Mycorrhizal Dependency (MD) values were 80, 78, 79, 78 and 78% in *A. scholaris* inoculated with *G. clarum, G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp., and *Glomus* sp. ZEA, respectively. Shoot N, P, K, Ca and Mg content of the seedlings were increased by AM fungi as much as 82-86, 81-86, 81-86, 88-91 and 85-90%, respectively. The percentage of AM colonization of *A. scholaris* ranged from 64 to 91 %. Colonization by five AM fungi increased plant height, diameter, total fresh weight, total dry weight and total length root. *Glomus clarum* was more effective in improving nutrient content and plant growth of *A. scholaris* than *G. decipiens, Entrophospora* sp., *Glomus sp.* ZEA and *Glomus* sp. ACA. Total root length of *A. scholaris* ranged from 1,180 to 1,310 cm. The results suggest that AM fungi can accelerate the establishment of the seedling stocks of *A. scholaris*. This finding would contribute to the effort of establishing *A. scholaris* plantation.

Keywords: *Glomus* sp., *Gigaspora decipiens, Entrophospora* sp., native mycorrhizal fungi, growth promotion, seedlings, greenhouse.

I. INTRODUCTION

The Apocynaceae family is important as they provide valuable timber (Turner, 2001). It consists of 164 genera, and some of these genera, i.e. *Tabernaemontana*, *Secamone*, *Ochrosia*, *Dyera*, and *Alstonia* produce timber and nontimber forest products (NTFPs). The genera of *Alstonia* consists of 40 species, with *A. angustiloba*, *A. angustifolia*, *A. macrophylla*, *A. pneumatophora* and *A. scholaris* producing milkwood (Soerianegara and Lemmens, 1994). *A. scholaris* is a medium-sized to large tree, which can grow up to 35 m in height. Species of *A. scholaris* is common in both primary and secondary lowland evergreen to deciduous rain forest

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of South Asia, Southeast Asia, Southern China and Northern Australia. Natural regeneration of *A. scholaris* occurs preferentially in open areas at forest edges and in secondary forest, and is considered to be a light-demanding species. Milkwood is suitable for boxes, pencils, crates, coffins, matches, drawing boards, and furniture components. Moreover, valuable latex is also harvested from the stem of *A. scholaris*. The latex can be used for cleaning wounds, traditional medicine and a good-quality chewing gum. In Southeast Asia *A. scholaris* is a popular medicinal plant. Its root bark is known for its antimalarial properties (Macabeo *et al*., 2005) and anticancer (Jagetia and Baliga, 2004). However, natural regeneration of *A. scholaris* is scarce, and seedlings found scattered or in groups, making it difficult to produce milkwood.

Deforestation rates on the Indonesian islands of Sumatra and Kalimantan are categorized the highest in the world (Linkie *et al*., 2004). It is necessary to accelerate reforestation and afforestation in degraded tropical forests and to enhance the commercial value of timber, pulp and NTFPs. *A. scholaris* is among species that, for its value, should be used in both rehabilitating logged over forest and development of plantation forests. The fast production of high quality seedling stocks in nurseries is valuable for replenishing degraded tropical forests. Furthermore, a lot of soils of tropical forests are infertile and the majority of Indonesian soils are ultisols, which are acid soils. Acid soils are severe environments for plants; high concentrations of Al, Mn and H, and low availability of N, P, K, Ca, and Mg will decrease the chance for many tree species to establish and survive (Postma *et al*., 2007).

Some studies on the effects of arbuscular mycorrhizal (AM) colonization on plant growth of some tropical trees have been well documented. Arbuscular mycorrhizal fungi increased plant growth of tropical fruit tree species of *Parkia biglobosa*, *Tamarindus indica*, and *Zizyphus mauritiana* at after inoculation (Guisso *et al*., 1998), *Tectona grandis* (Rajan *et al*., 2000), *Azadirachta indica* (Muthukumar *et al*., 2001) , and *Araucaria angustifolia* (Zandavalli *et al*., 2004). However, little is known about AM inoculation on growth of Apocynaceae species in tropical forests. Turjaman *et al.* (2006) reported that AM fungi increased growth of *Dyera polyphylla* seedlings, Guadarrama *et al.* (2004) reported that AM fungi increased growth of *Stemmadenia donnell-smithii,* Weber *et al.* (1995) reported that AM fungi increased growth of *Adenium obesum*, *Pachypodium lamerei* and *Plumeria obtuse.* Nevertheless, to the best of our knowledge, there are no reports on the effect of the growth of *Alstonia* tree species following AM fungal inoculation. The objective of this study was to determine whether five AM fungi, *Glomus clarum* Nicholson & Schenk, *Gigaspora decipiens* Hall & Abbott, *Glomus* sp. ACA Tulasne & Tulasne*, Entrophospora* sp. Ames & Scheneider, and *Glomus* sp. ZEA Tulasne & Tulasne increase early growth of *A. scholaris* under greenhouse conditions. Isolates of the five AM fungi are native to the peat swamp forests of Central Kalimantan.

II. MATERIALS AND METHODS

A. Seed preparation

Seeds of *A. scholaris* were collected from the arboretum of the Forest and Nature Conservation Research and Development Center (FNCRDC), Bogor, West Java. The seeds were soaked in water for two hours and then surface-sterilized by shaking in 5% NaClO solution for 5 min. They were then thoroughly rinsed twice in sterile distilled water. The seeds

were sown in a plastic flat containing autoclave-sterilized zeolite and grown under a 55% shading intensity net to control solar radiation. The seeds were allowed to germinate for 21 days after sowing.

B. Soil medium preparation

Soil used in the experiment was an ultisol collected from the Haurbentes Experimental Forest, Jasinga, West Java (6° 32'-33' S, 108° 26' E) and stored in a greenhouse. It was passed through a five mm sieve and then mixed with river sand (3:1, v/v) to improve drainage. The pH (H_2O) of the soil mixture was 4.8, available P (Bray-1) was 0.17 mg kg^{-1}, and total N (Kjeldahl) was 1.7 mg kg^{-1}. The soil mixture was sterilized at 121°C for 30 minutes. Polyethylene pots (15 x 10 cm^2) were filled with 500 g of sterilized soil mixture.

C. Arbuscular mycorrhizal fungal inoculum preparation

Five AM fungi *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp. and *Glomus* sp. ZEA were isolated from peat soil of Kalampangan, Palangkaraya, Central Kalimantan by trap culture. The pot cultures began as single spore cultures. They were propagated in pot cultures of *Pueraria javanica*. Plastic pots were filled with 175 g of sterilized zeolite and five g of AM fungal inoculum in the planting hole. Plastic pots were hung in iron racks (1.5x1x1.5m^3) under greenhouse floor and they were made a distance 15 cm between pot cultures to avoid contamination. The AM fungi in culture pots were checked as determined by morphological features every two weeks (for three months). A microbial filtrate was not applied to the controls to account for differences from other fungi or bacteria. A preliminary experiment showed that AM fungal inoculum was pure culture and effective without a microbial filtrate application. Two 6-day-old *P. javanica* seedlings were transplanted into the pots and grown under natural light greenhouse conditions with no temperature and humidity control. After 90 days, spores, external hyphae, and colonized roots of *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp. and *Glomus* sp. ZEA were observed in the zeolite.

D. Plants inoculation

One 21-day-old *A. scholaris* seedling was transplanted into the pots containing sterilized soil mixture medium. The AM inoculation was achieved by placing five gram of inoculum of each species 1-3 cm below seedlings. Control seedlings were not mock-inoculated because a preliminary experiment showed that the sterilized inoculum did not affect growth of the seedlings. Seedlings were watered daily with tap water to field capacity. No fertilizer was applied during the course of the experiment. Weeds and pests were removed manually. The seedlings were grown for five months in a greenhouse at the Forest and Nature Conservation Research and Development Center, Bogor, West Java (6° 36' S, 106° 45' E). Temperature varied from 26 to 35°C, relative humidity was 80-90% and the photoperiod was about 12 h.

E. Experimental design

The experiment consisted of six treatments of *A. scholaris* seedlings (a) control (no inoculation), (b) inoculation with *G. clarum*, (c) inoculation with *G. decipiens*, (c) inoculation with *G. decipiens*; (d) inoculation with *Glomus* sp. ACA; (e) inoculation with *Entrophospora* sp.;

(f) inoculation with *Glomus* sp. ZEA. There were ten replications per treatment. Shoot height and stem diameter at one cm from the soil surface were measured five months after transplantation. After harvest, shoots and roots were separated. They were oven-dried at 70° C for 72 h before weighing. Ground shoots were digested with H_2SO_4 and H_2O_2 solution (3:1, v/v). N and P concentration in the digested solution were determined by the semi-micro Kjeldahl method and vanadomolybdate-yellow assay (Olsen and Sommers, 1982), respectively. K, Ca and Mg were determined by means of an atomic absorption spectrophotometer (Heffernan, 1985). An additional 30 seedlings each of *A. scholaris* uninoculated or inoculated with *G. clarum* or *G. decipiens* or *Glomus* sp. ACA or *Entrophospora* sp. or *Glomus* sp. ZEA were grown under the same conditions as those of the seedlings in the above experiment. Numbers of viable seedlings were counted 5 months after transplanting. Survival rate was calculated as follows:

Survival rate (%) = (number of viable seedlings/number of initial seedlings) x 100

Roots of *A. scholaris* were washed gently over a two mm sieve under running tap water to separate them from soil particles. The roots were cleared in 100 g l^{-1} KOH for 1 h, acidified with diluted HCl and stained with 500 mg l^{-1} trypan blue in lactoglycerol (Brundrett *et al*., 1996). Roots were destained with 50% glycerol and 30 1-cm segments were viewed under a compound microscope at x200 magnification. Percentage of AM colonization was estimated using the gridline intersect method (Giovannetti and Mosse, 1980). Mycorrhizal dependency (MD) was calculated according to Plenchette *et al.* (1983): MD (%) = (dry weight of mycorrhizal plant-dry weight of non mycorrhizal plant) /dry weight of mycorrhizal plant x 100. Root length were measured by gridline millimeter block.

Data were statistically analyzed using analysis of variance with the statistical software StatView 5.0 (Abacus Concepts). Comparison of means was done using the least significant difference (LSD) method at the 5% probability level where the F-value was significant.

III. RESULTS AND DISCUSSION

The roots of *A. scholaris* were colonized by *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp. and *Glomus* sp. ZEA 150 months after transplantation under greenhouse conditions (Table 1.). There was no difference in percentage of colonization among *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp. and *Glomus* sp. ZEA. The control seedlings of *A. scholaris* were colonized by indigenous AM fungi. AM colonization by *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp. and *Glomus* sp. ZEA increased plant height, stem diameter, total fresh and dry weight of *A. scholaris* 5 months after transplantation (Table 1.) and ready for planting in the field.

Table 1. Arbuscular mycorrhizal (AM) colonization, shoot and root growth of *A. scholaris* inoculated with or without AM fungi

Treatment	Plant growth				AM Colonization (%)*
	Height (cm)*	Stem diameter (mm)*	Total fresh weight (g/plant)*	Total dry weight (g/plant)*	
No inoculation	16.1a	3.9a	5.3a	1.1a	5a
G. clarum	33.1b	6.5b	20.6b	5.6b	64b
G. decipiens	34.1b	6.9b	18.7b	5.1b	79b
Glomus sp. ACA	32.8b	6.8b	19.7b	5.2b	74b
Entrophospora sp.	36.1b	6.6b	19.2b	4.9b	79b
Glomus sp. ZEA	32.5b	6.5b	20.3b	5.0b	91b

*Values with the same letter are not significantly different (P<0.05)

This study clearly demonstrates that inoculation of five AM fungi increased the early growth and nutrient content of *A. scholaris* five months under greenhouse conditions. This species is an important tree species in tropical forest of Asia because it produces milkwood. Within the same family of Aponynaceae, the growth of AM seedlings of *A. scholaris* is better than AM seedlings of *Dyera polyphylla* (Turjaman *et al.*, 2006). The AM seedlings of *A. scholaris* can be planted in the field after five months in greenhouse or nursery. Shoot height, stem diameter, and total dry weight were also increased by inoculation of AM fungi. These parameters can determine the value of *A. scholaris* as the milkwood. The improvement of early growth of these species would increase production of the milkwood. Moreover, inoculation of AM fungi would be useful for production of *A. scholaris* because this species is often scarce and found scattered or in groups in natural tropical forests.

Nutrient concentrations were higher in shoots of *A. scholaris* inoculated with *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp., and *Glomus* sp. ZEA than those of control seedlings (Table 2.). The inoculation of *A. scholaris* by five AM fungi also increased their shoot N, P, K, Ca and Mg content. Arbuscular mycorrhizal colonization by five AM fungi increased shoot N, P, K, Ca and Mg content of *A. scholaris*. There was no difference in shoot N, P, K, Ca and Mg content of *A. scholaris* among *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp., and *Glomus* sp. ZEA. Shoot nutrient concentrations and content of *A. scholaris* were higher in the seedlings inoculated with *G. clarum* than *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp., and *Glomus* sp. ZEA treatments. Shoot nutrient concentrations of *A. scholaris* were higher in the AM seedlings than in the control seedlings, indicating that in the absence of AM associations, *A. scholaris* was not capable to absorb enough N, P, K, Ca and Mg from the soil and keep adequate levels in their tissues. Arbuscular mycorhizal seedling inoculated by *G. clarum* increased their shoot N, P, K, Ca and Mg concentrations by 27, 33, 30, 55, and 47%, respectively. Similar results, was reported by Zandavalli *et al.* (2004) that *Araucaria angustifolia*

inoculated by *G. clarum* increased their shoot N, P, K concentrations, except for shoot Ca and Mg concentrations of inoculated plants were not different with control seedlings. Soil bases such as Mg and Ca have a role in root colonization and sporulation of AM fungi (Jarstfer *et al.*, 1998). Moreover , high Mg/low Ca shoot concentrations induced premature root senescence, which may have disturbed the AM association process, indicating the importance of Ca for the maintenance of a functioning AM symbiosis. In addition, Cuenca and Azcón (1994) reported that AM fungi *G. etunicatum* was not only increasing the N, P, Ca, Mg and Zn uptake of tropical tree *Erythrina poeppigiana* in the presence of NO^-_3 fertilizer, but also P and Mg in the presence of NH^+_4 applications. The AM colonization by *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp. and *Glomus* sp. ZEA increased also the nutrient content. Shoot height, stem diameter, fresh and dry weight were also increased by inoculating those five AM fungi. Increases of these parameters may lead to increase benefit from the milkwood and medicinal plant of *A. scholaris*. Furthermore, AM fungal inoculation induces healthy and vigorous growth of seedlings, which would be helpful to reforestation and afforestation activities. AM colonization also increased nutrient content of N, P, K, Ca, and Mg of *A. scholaris*. Shoot N, P, K, Ca, and Mg content of the AM inoculated seedlings were increased by 82-86, 81-86, 81-86, 88-91, and 85-90%, respectively. Fertilizers are generally of benefit to the tree, not the site, and measurable permanent site improvement is only likely if the amount of nutrient applied is large in relation to the soil fertility (Miller, 1981). Fertilization has been generally applied to *A. scholaris* in nursery on the commercial scale. Fertilization had the most dramatic effect and caused a decrease of AM colonization and thereafter reduces growth improvement by AM fungi (Titus and Lepš, 2000). Effective AM fungal inoculum could be produced on the commercial scale and at low cost production, therefore application of AM fungi can minimize fertilization without diminish AM growth enhancement of *A. scholaris*.

Table 2. Shoot nutrient concentration and content of *A. scholaris* inoculated with or without AM fungi

Treatment	Shoot Nutrient Concentration					Shoot Nutrient Content				
	N (mg/g)	P (mg/g)	K (mg/g)	Ca (mg/g)	Mg (mg/g)	N (mg/plant)	P (mg/plant)	K (mg/plant)	Ca (mg/plant)	Mg (mg/plant)
Control	6.7a	1.4a	5.5a	2.9a	1.7a	4.5a	1.0a	3.7a	1.9a	1.1a
G. clarum	9.2c	2.1c	7.9c	6.4c	3.2c	32.1b	7.4b	27.6b	22.3b	11.1b
G. decipiens	8.8c	1.9b	7.7c	6.0c	2.9c	27.4b	6.1b	23.9b	18.8b	9.1b
Glomus sp. ACA	7.8b	1.8b	6.6b	5.7c	2.3b	25.0b	5.7b	21.3b	18.4b	7.4b
Entrophospora sp.	7.9b	1.7b	6.3b	5.0b	2.3b	24.8b	5.3b	19.6b	15.5b	7.3b
Glomus sp. ZEA	8.6c	1.8b	7.0b	6.0c	2.9c	26.3b	5.4b	21.2b	18.5b	8.8b

*Values with the same letter are not significantly different ($P<0.05$)

Entrophospora sp. and *Glomus* sp. ZEA increased the survival rates of *A. scholaris* 5 months after transplantation under greenhouse conditions. The survival rates of *A. scholaris* inoculated with *G. clarum* (93%), *G. decipiens* (100%), *Glomus* sp. ACA (100%), *Entrophospora* sp.(100%) and *Glomus* sp. ZEA(100%) were higher than control seedlings (80%). The *A. scholaris* seedlings inoculated with *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp. and *Glomus* sp. ZEA were not different in survival rates. The survival rate of *A. scholaris* seedlings is an evaluation decisive factor of success in reforestation and afforestation programs. These results also have a number implication for forest plantation management of *A. scholaris*. This species can be planted as monoculture or agroforestry systems because *A. scholaris* is a fast growing species and almost similar growth performance with plantation forest industry species like *Acacia*, *Eucalyptus* and *Gmelina*. In South Sumatra (Indonesia), a forest plantation company has a sharing with local farmers to establish forest plantation industry for supplying raw materials of pencil. They provide *A. scholaris* to farmers who are planting and maintaining the seedlings in their forest gardens. The forest plantation company or farmers may get yield and increase production of *A. scholaris* inoculated by AM fungi.

Arbuscular mycorhizal colonization by five AM fungi increased total root length, total first roots, total second roots and total third roots of *A. scholaris* (Table 3.). There was no difference in total root length, total first roots, total second roots and total third roots of *A. scholaris* among *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp. and *Glomus* sp. ZEA. There was no difference in total main root of *A. scholaris* between five AM fungi and control seedlings. The AM increased total root length of *A. scholaris*. Even though colonization by AM fungi characteristically results in more numerous lateral roots in the hosts, these are much shorter than in non-colonized root systems (Berta *et al.*, 1993). The AM seedlings can increase the length and fineness of their roots or the length and density of their root hairs in the response to P deficiency (Trolove *et al.*, 2003). AM seedlings are able to obtain more nutrients from nutrient-deficient soils than are control seedlings because hyphae exploit a greater volume of soil than roots alone (Entry *et al.*, 2002). AM enhances plant acquisition of nutrients by increasing the absorptive surface area of the uptake system. P uptake is most likely the best described process in the AM associations, with the fungi supplying host plants with P in exchange for carbon (Smith and Read, 1997). In a greenhouse study, Inoculation of AM fungi increased soil compaction reduced root length, AM formation, root dry weight, P content and shoot growth on *Cajanus cajan* L. (Yano et al., 1998), *Trifolium subterraneum* L. (Nadian *et al.*, 1997), *Trifolium pratense* L. (Li *et al.*, 1997). The proportion of root length colonized by the fungus increases with decreasing nutrient availability (Graham *et al.*, 1997).

Table 3. Total length roots of *A. scholaris* inoculated with or without AM fungi

Treatment	Root length (cm)				Total root length (cm)
	main root	1st root	2nd root	3rd root	
Control	15.4a	88.6 a	79 a	25.1 a	208.1 a
G. clarum	16.9a	410.8 b	524 b	230.1 b	1181.8 b
G. decipiens	17.5a	440.6 b	646.8 b	172.2 b	1277.1 b
Glomus sp. ACA	18.6a	384.6 b	588.5 b	249.5 b	1241.2 b
Entrophospora sp.	16.6a	363.6 b	528.8 b	240.7 b	1149.7 b
Glomus sp. ZEA	15.1a	378.7 b	664.3 b	251.6 b	1309.7 b

*Values with the same letter are not significantly different ($P<0.05$).

For a given fungus, Mycorrhizal Dependency (MD) values were 80, 78, 79, 78 and 78% in *A. scholaris* inoculated with *G. clarum*, *G. decipiens*, *Glomus* sp. ACA, *Entrophospora* sp. and *Glomus* sp. ZEA, respectively. The MD in acid soil was higher (>75%) in *A. scholaris* inoculated with AM fungi. *A. scholaris* was very highly dependent according to the MD categories defined by Habte and Manajunath (1991). *A. scholaris* responded to the AM colonization more largely. The MD proposed that AM inoculation would be valuable in production of vigorous seedlings in the nursery which might establish in the field conditions and might be more resistant to drought stress, nutrient deficiency and pathogenic infection (Wilson *et al*., 1991; Ghosh and Verma, 2006). Moreover , Cáceres and Cuenca (2006) reported that MD has other functions in two tropical species from Venezuela *Clusia minor* and *Clusia multiflora* in two soils with different pH. Both tree species were found to be highly dependent on AM fungi for their growth in acidic soil, and there was an inverse relationship between dependency of the species and the soil phosphorus content.

IV. CONCLUSIONS

This study is the first report of which we are aware concerning the inoculation of AM fungi on the growth of milkwood tropical tree species *A. scholaris*. Colonization by five AM fungi increased shoot nutrient content, plant growth, and survival rates of *A. scholaris* seedlings 5 months after transplantation under greenhouse conditions. *Glomus clarum* was more effective in improving nutrient content and plant growth of *A. scholaris* than *G. decipiens, Entrophospora* sp.*, Glomus sp.* ZEA, and *Glomus* sp. ACA. Field trials with AM fungal inoculation are required to monitor the growth and survival rates of *A. scholaris*. Inoculation techniques may be adopted by a large scale nursery jointly with reforestation and afforestation programs, thereby aiding in the increase of the milkwood production of such tropical tree species as *A. scholaris.* These results suggested that AM fungi increased the establishment of the planting stocks of *A. scholaris*, thereby sustaining the production of milkwood *A. scholaris.*

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