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Improvement of early growth of two tropical peat-swamp forest tree species *Ploiarium alternifolium* and *Calophyllum hosei* by two arbuscular mycorrhizal fungi under greenhouse conditions

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Abstract Tropical peat-swamp forests are one of the largest near-surface reserves of terrestrial carbon. However, many peat-swamp forest tree species have resulted in the reduction due to over-exploitation, forest fires and conversion into agricultural land in Indonesia. The objective of this study was to determine the effects of two arbuscular mycorrhizal (AM) fungi, *Glomus clarum* and *G. aggregatum*, on the early growth of two slow-growing peat-swamp forest tree species, *Ploiarium alternifolium* and *Calophyllum hosei*, under greenhouse conditions. Cuttings of *P. alternifolium* and *C. hosei* were uninoculated or inoculated with *G. clarum* and *G. aggregatum* and grown under greenhouse conditions for 6 months. Percentage AM colonization, plant growth, phosphorus (P) concentration and survival rate were measured. The AM colonization of *P. alternifolium* and *C. hosei* ranged from 27 to 32% and 18 to 19%, respectively. Colonization by *G. clarum* and *G. aggregatum* increased shoot height, stem diameter, leaf number, and shoot and root dry weights. Cutting shoot P content were increased by AM fungal colonization. The survival rates of inoculated plants were higher (100%) than those of control plants (67%). The results suggest that inoculation with AM fungi improves early growth of *P. alternifolium* and *C. hosei* in a tropical peat-swamp forest and can therefore contribute to rehabilitation of peat-swamps.

Keywords *Glomus* · Inoculation · Mycorrhiza · Phosphorus · Survival rate

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Introduction

Tropical peat-swamp forests in Southeast Asia have the large area of peat and support luxuriant growth of peat-swamp tree species overlying peat deposits up to 20 m thick (Page et al. 2002). In Indonesia, tropical peat-swamp forests are located at low altitudes in the coastal and sub-coastal lowlands of Irian Jaya (4.6 Mha), Sumatra (8.3 Mha) and Kalimantan (6.8 Mha) (Page et al. 1999). In Kalimantan, trees of the family Dipterocarpaceae dominate in terms of abundance, density and biomass (Appanah and Turnbull 1998). Slik et al. (2003) found that the 10 most abundant tree families after Dipterocarpaceae were Euphorbiaceae, Myrtaceae, Sapotaceae, Lauraceae, Myristicaceae, Burseraceae, Anacardiaceae, Ebenaceae, Annonaceae and Guttiferae. *Ploiarium* and *Calophyllum* are important genera of the family Guttiferae (Kobuski 1950; Soerianegara and Lemmens 1994). Timbers from *Calophyllum* are used in the construction of boats and furniture and as plywood, and those from *Ploiarium* are used to build houses and as firewood and charcoal by local people (Sosef et al. 1998). Several species of *Ploiarium* and *Calophyllum* are used in medicine because of their antimicrobial activity and of the presence of other biologically active compounds such as coumarins, xanthones, and terpenoids. Recently, *Calophyllum* species have received considerable attention from a pharmacological viewpoint because they contain compounds that strongly inhibit HIV (human immunodeficiency virus) reverse transcriptase (Reyes-Chilpa et al. 1997).

Tropical peat-swamp forests have diminished due to forest fires, over-exploitation and conversion into highways, industrial and agricultural lands, and plantation estates (Phillips 1998). Today, degraded peat-swamp forests require extensive rehabilitation and an annual supply of high-quality seedling stocks for reforestation. The major obstacle in the rehabilitation of peat-swamp forests is slow growth and high mortality of seedlings in the nursery. *Ploiarium* and *Calophyllum* are slow-growing tree species and appear to have irregular flowering and fruiting habits (Soerianegara and Lemmens 1994; Sosef et al. 1998). *Ploiarium* seeds germinate insufficiently when sown immediately after collection. Scarification may be required to germinate well. *Calophyllum* seeds have low germinative ability and are often lost in floods during the rainy season, further limiting reproduction (Nair and Seeni 2003).

Numerous studies of tropical rain forest mycorrhizas have indicated the dominance of arbuscular mycorrhiza (AM) (Janos 1980; Béreau and Garbaye 1994). The highest percentage AM colonization was reported in a tropical Mexican wet forest (Guadarrama and Álvarez-Sánchez 1999). AM colonization was approximately 40% in tree species in tropical heath forests and mixed Dipterocarpaceae forest in Brunei (Moyersoen et al. 2001). AM colonization was observed 17 of 22 wild seedlings of peat-swamp tree species in Central Kalimantan, Indonesia (Tawaraya et al. 2003). Dipterocarpaceae is one of most important families in peat swamp forests. It is well known that ectomycorrhiza is formed in the roots of Dipterocarpaceae (Lee and Lim 1989; Turner et al. 1993; Yazid et al. 1994). Little is known about the effects of AM colonization on the growth of tropical peat-swamp tree species. The objective of this study was to determine the effects of two AM fungi, *Glomus aggregatum* and *G. clarum*, on the early growth of two slow-growing peat-swamp forest tree species, *Ploiarium alternifolium* (Vahl) Melchior (synonym = *Hypericum alternifolium* Vahl) (Kobuski 1950) and *Calophyllum hosei* Ridley, under greenhouse conditions. The isolate of *G. aggregatum* originated in Japan and the isolate of *G. clarum* is native to the peat-swamp forest of Central Kalimantan, Indonesia. These fungi were chosen because *G. aggregatum* had been shown to be a highly effective symbiont with *Acacia crassicarpa* and *Eucalyptus* spp. (Turjaman et al. 2000), and *Khaya ivorensis* (Santoso et al. 2003) in previous study.

Materials and methods

Cutting propagation method

Cutting materials of *P. alternifolium* and *C. hosei* were collected from wildlings at Nyaru Menteng arboretum near Palangka Raya in Central Kalimantan, Indonesia (2°43′ S; 111°38′ E). Since the flowering and fruiting characteristics of those two tree species in peat-swamp forests are not well understood, it was difficult to obtain the seeds. The wildlings were 1–2 years old and their heights were approximately 0.5–1.5 m. The wildlings with orthotropic position were cut to a length of approximately 10 cm with two leaves. The leaf area was reduced to approximately half its original size. All cutting materials from Central Kalimantan were placed in an icebox and brought to greenhouse of Forest and Nature Conservation Research and Development Centre (FNCRDC) in Bogor, West Java (6°36′ S, 106°45′ E). Roots of the two seedling species were stimulated with the ROOTON (Ishihara Sangyo Co., Japan). Rooton contains 0.1% naphthyl acetic acid in powder type. Cut ends were dipped into the rooton. Cutting medium consisting of crushed coconut fiber mixed with rice husks (2:1, v/v) was sterilized in an autoclave at 121°C for 50 min. Subsequently, one cutting was planted in one hole of a 45-hole tray (52 × 29 × 5.6 cm). The volume of one hole was 140 ml. The trays were placed inside a propagator. The propagator is transparent plastic box (66 × 37 × 33 cm) to keep a constant temperature and relative humidity in the trays. Each species were prepared 45 cuttings. A fog evaporative cooling system was installed inside the greenhouse to lower temperature inside the propagator (Sakai et al. 2002).

Arbuscular mycorrhizal fungi

Spores of *Glomus clarum* Nicholson & Schenk were isolated from peat soil of Kalampangan, Palangka Raya, Central Kalimantan (2°13′ S, 113°56′ E) by trap culture (Brundrett et al. 1996). The pot culture was established as mixed propagule cultures. The mixed propagule consists of spores, external hyphae and roots colonized by *G. clarum*. *Glomus clarum* was propagated in pot cultures of *Pueraria javanica*. Plastic pots were filled with 175 g of sterilized zeolite and 5 g of AM fungal inoculum was placed in the planting hole. 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. Plastic pots were place 15 cm apart in a greenhouse to avoid contamination. A preliminary experiment showed that AM fungal inoculum was effective to give high AM fungal colonization in the roots of *P. javanica* without application of microbial filtrate. After 90 days, spores, external hyphae and roots colonized by *G. clarum* were observed in the zeolite. Spore numbers in the zeolite were 713 spores 10 g^{-1}. AM fungal inoculum of *G. aggregatum* Schenk & Smith was obtained from Research and Development Division, Osaka Gas Ltd., Japan.

Soil preparation and seedling inoculation

Ultisol was collected from Haurbentes Experimental Forest, Jasinga,West Java (6°32′–33′ S, 108°26′ E) and stored in a greenhouse. The soil was passed through a 5 mm sieve and mixed with river sand (3:1, v/v) to improve drainage. The pH (H_2O) of the mixed soil was 4.8 and available P (Bray-1) was 0.17 mg kg^{-1}. The mixed soil was sterilized in an autoclave at 121°C for 30 min.

Polyethylene pots (15 × 10 cm) were filled with 500 g of the sterilized soil mixture. AM fungal inoculation was accomplished by placing 5 g of inoculum of *G. clarum* or *G. aggregatum* 1–3 cm below the cuttings. One 90-day-old *P. alternifolium* or *C. hosei* cutting was transplanted into each pot. Control cuttings were not inoculated. The cuttings were watered daily with tap water to field capacity. All pots in each treatment were placed 15 cm apart on benches in a greenhouse to avoid contamination. Weeds and pests were removed manually. The cuttings were grown for 180 days in a greenhouse at the FNCRDC, Bogor,West Java. Daily temperatures varied between 26 and 35°C, relative humidity was 80–90% and photoperiod was approximately 12 h.

Growth measurement

The experiment consisted of three treatments of *P. alternifolium* and *C. hosei* cuttings: (1) control (no inoculation); (2) inoculation with *G. aggregatum*; and (3) inoculation with *G. clarum*. There were 15 replications per treatment. Shoot height and stem diameter at 1 cm from the soil surface were measured 180 days after transplantation. After 180 days, shoots and roots were separated and oven-dried at 70°C for 72 h before weighing. Shoots were ground and digested with H_2SO_4 and H_2O_2 solution (3:1, v/v). P concentration in the digested solution was determined by the vanadomolybdate yellow assay (Olsen and Sommers 1982). Shoot P content was calculated by multiplying the shoot P concentrations and shoot dry weights.

An additional 15 cuttings each of *P. alternifolium* and *C. hosei* uninoculated or inoculated with *G. aggregatum* or *G. clarum* were grown under the same conditions as those of the cuttings in the above experiment. Numbers of viable cutting were counted 180 days after transplantation. Survival rate was calculated as follows: Survival rate (%) = number of viable cuttings/number of initial cuttings × 100.

Arbuscular mycorrhizal colonization

Roots of *P. alternifolium* and *C. hosei* were washed over a 2 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 were viewed under a compound microscope at 200× magnification. Percentage AM colonization was examined 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] × 100.

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 method at 5% probability level where the F-value was significant. Data of AM colonization were transformed by arcsine (square root) before statistical analysis.

Results

Arbuscular mycorrhizal colonization

Roots of *P. alternifolium* and *C. hosei* were colonized by *G. aggregatum* or *G. clarum*, 6 months after transplantation under greenhouse conditions (Table 1). Percentage AM

Table 1 Arbuscular mycorrhizal (AM) fungal colonization, shoot and root dry weights, P content of *P. alternifolium* and *C. hosei* un inoculated or inoculated with AM fungi *G. aggregatum* and *G. clarum*, under greenhouse conditions

Tree species	Treatment	AM fungal colonization (%)	Shoot dry weight (g/plant)	Root dry weight (g/plant)	P content (mg P/plant)
P. alternifolium	Control	3 a*	1.34 ± 0.31 a	0.30 ± 0.03 a	2.17 ± 0.56 a
	G. aggregatum	32 b	2.76 ± 0.09 b	0.91 ± 0.10 b	5.89 ± 0.90 b
	G. clarum	27 b	3.05 ± 0.14 b	0.81 ± 0.12 b	5.74 ± 0.22 b
C. hosei	Control	3 a	0.21 ± 0.04 a	0.07 ± 0.008 a	0.36 ± 0.08 a
	G. aggregatum	18 b	0.53 ± 0.11 b	0.20 ± 0.05 b	0.50 ± 0.14 ab
	G. clarum	19 b	0.49 ± 0.13 b	0.14 ± 0.02 b	0.60 ± 0.16 b

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

colonization of *P. alternifolium* and *C. hosei* by *G. aggregatum* was not significantly different from that by *G. clarum*. Control cuttings of *P. alternifolium* and *C. hosei* were colonized by indigenous AM fungi as evidence by wind and/or insects carried the AM fungi inoculum.

Shoot phosphorus content

P content was higher in shoot of *P. alternifolium* inoculated with *G. aggregatum* and *G. clarum* than in control cuttings (Table 1). There was no significant difference in shoot P content between *P. alternifolium* inoculated with the two AM fungi. Inoculation of *G. clarum* increased shoot P content of *C. hosei*. However, there was no significant difference in shoot P content between *G. aggregatum* and control.

Plant growth

AM colonization increased shoot height, stem diameter and leaf number of *P. alternifolium* 6 months after transplantation (Fig. 1). The inoculation of *G. aggregatum* and *G. clarum* increased shoot dry weight and root dry weight (Table 1). There was no significant difference in stem diameter, leaf number, and shoot and root dry weights between *P. alternifolium* inoculated with the two fungi.

AM colonization by *G. aggregatum* increased shoot height and stem diameter but not leaf number of *C. hosei* (Fig. 2). By contrast, *G. clarum* colonization increased shoot height, stem diameter and leaf number of *C. hosei*. Inoculation of the two AM fungi increased shoot dry weight and root dry weight of *C. hosei* compared with control (Table 1). However, there was no significant difference in shoot dry weight and root dry weight between *C. hosei* inoculated with the two fungi.

Mycorrhizal dependency

For a given fungus, MD values varied depending on the host species. MD values obtained in this study with both cutting species exceeded 50%. The MD of *P. alternifolium* and *C. hosei* ranged from 55–58% and 57–63%, respectively.

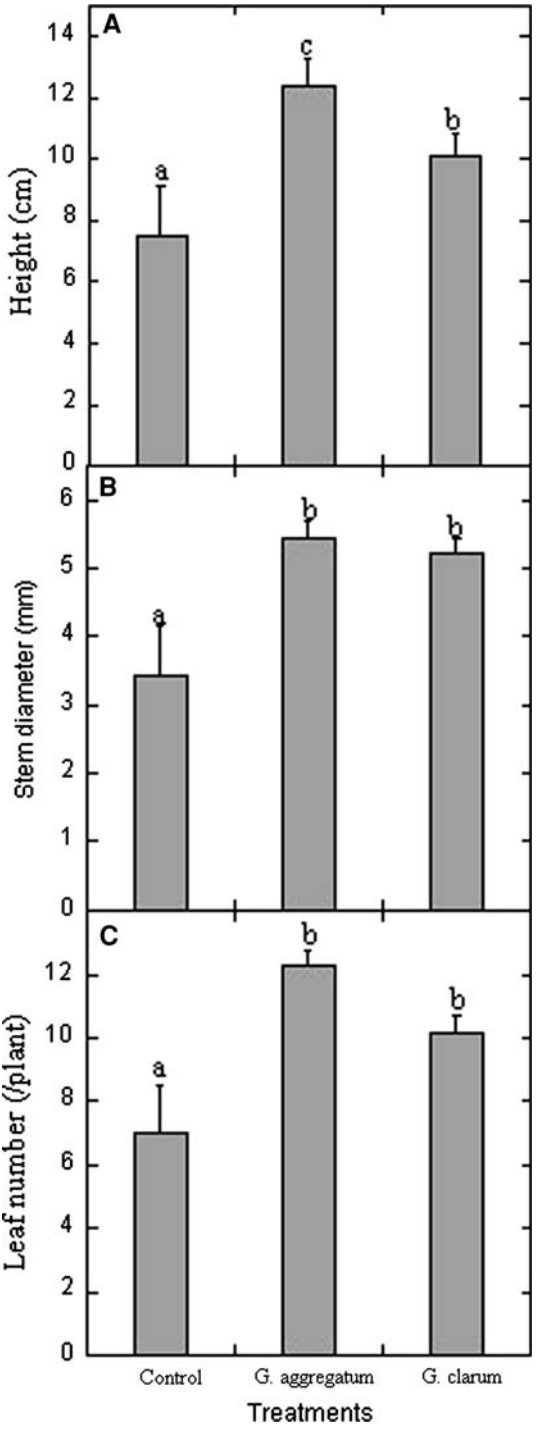

Fig. 1 Shoot height (**a**), stem diameter (**b**) and leaf number (**c**) of *P. alternifolium* uninoculated or inoculated with AM fungi, *G. aggregatum* and *G. clarum*, under greenhouse conditions

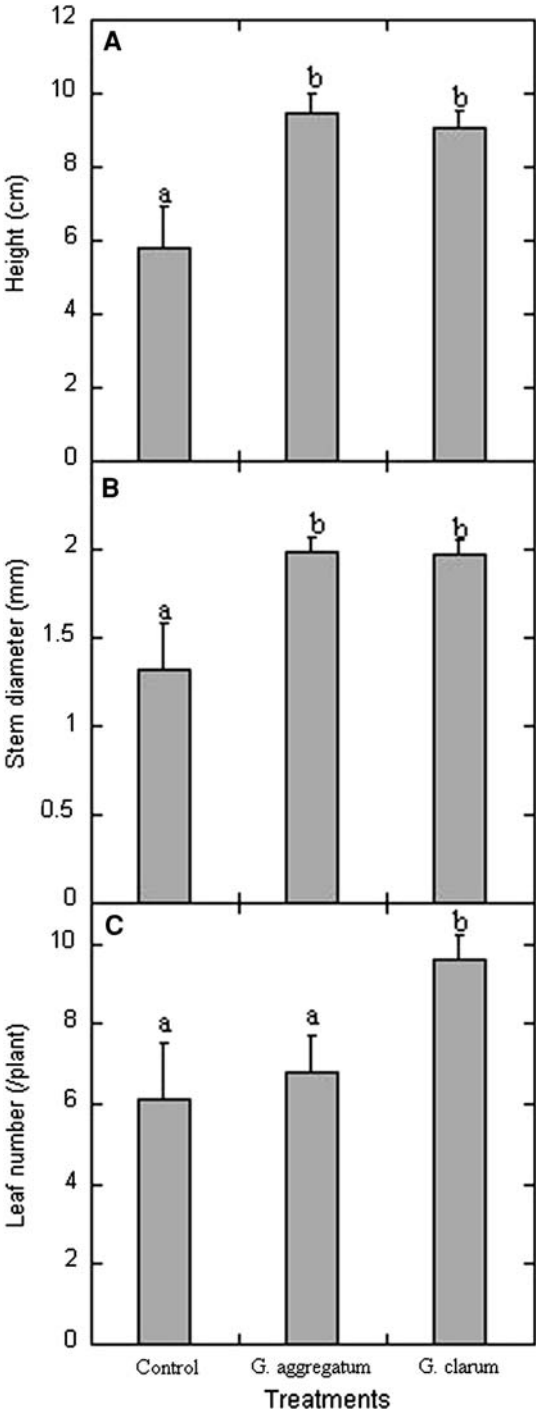

Fig. 2 Shoot height (**a**), stem diameter (**b**) and leaf number (**c**) of *C. hosei* uninoculated or inoculated with AM fungi, *G. aggregatum* and *G. clarum*, under greenhouse conditions

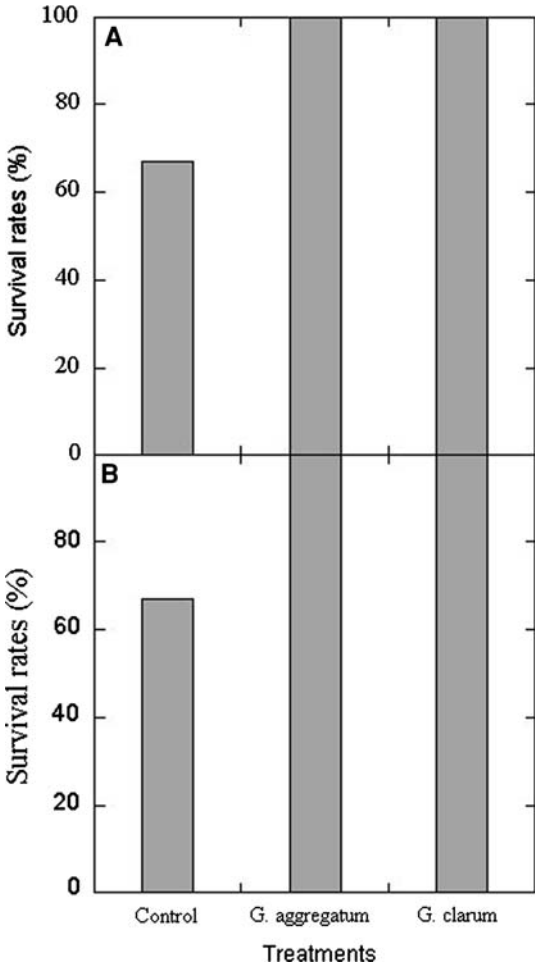

Fig. 3 Survival rate of *P. alternifolium* (**a**) and *C. hosei* (**b**) uninoculated or inoculated with AM fungi, *G. aggregatum* and *G. clarum*, under greenhouse conditions

Survival rate

The survival rates of *P. alternifolium* cuttings inoculated with *G. aggregatum* and *G. clarum* were higher than that of control cuttings 6 months after transplantation under greenhouse conditions (Fig. 3). The survival rates of *C. hosei* inoculated with the two fungi were also higher than that of control cuttings. No difference in survival rate was observed between cuttings inoculated with *G. aggregatum* and *G. clarum* for both host species.

Discussion

In this study, we showed the importance of AM fungi for early growth and shoot P nutrition of tropical peat-swamp forest tree species. We demonstrated for the first time the positive effects of two AM fungi, *G. aggregatum* and *G. clarum*, on *P. alternifolium* and *C. hosei*, and confirmed the previously reported AM colonization of a native tree genus of *Calophyllum* in a lowland tropical rain forest of Singapore (Burslem et al. 1995), and two

native tree species of *Calophyllum* in a tropical peat-swamp forest in Central Kalimantan, Indonesia (Tawaraya et al. 2003). To the best of our knowledge, this is the first demonstration of AM colonization in *P. alternifolium* and *C. hosei*.

The extent of AM colonization in the roots of *P. alternifolium* and *C. hosei* was comparable to that reported previously. Tawaraya et al. (2003) reported that natural AM colonization of *C. sclerophyllum* and *C. soulattri* growing in peat-swamp forest was between 18 and 60%. Natural AM colonization was observed in control *P. alternifolium* cuttings, but the extent was lower (3%) than that observed in cuttings colonized by *G. aggregatum* and *G. clarum*. Béreau et al. (2000) observed that AM colonization of *Dicorynia guianensis* control seedlings was very low (<10%). It is very likely that AM colonization of control seedlings originated from the soil underneath the pots since the pots were placed directly above the ceramic surface without any border. The direct contact between the bottom of pots and the greenhouse benches may be a reason for the natural AM fungal colonization. We confirmed that the AM fungi that colonized the control cuttings were not *G. aggregatum* or *G. clarum* by morphological features.

AM colonization by *G. aggregatum* and *G. clarum* increased the P contents of *P. alternifolium* and *C. hosei* by as much as 164–171% and 39–67%, respectively. There are some nutrient constrains in degraded peat-swamp forest and P is most limiting factor for early growth of both tree species. *P. alternifolium* and *C. hosei* are important trees in the tropical peat-swamp forests of Southeast Asia because they have medicinal properties and are used as timber. Shoot height, stem diameter and leaf number were also increased by inoculating with those two AM fungi. Increases of these parameters may lead to increased yield from the medicinal plant and timber of both species. Furthermore, AM fungal inoculation induces vigorous growth of seedlings, which would be advantageous to reforestation activities. This experiment was carried out in the greenhouse conditions, where there was no competition with other fungi. The field experiment is underway in degraded peat-swamp forest.

Studies on these tree species and other tropical tree species have indicated that AM fungal inoculation reduces the amount of chemical fertilizers required for seedling production (Habte et al. 1993; Siqueira et al. 1998; Muthukumar and Udaiyan 2006). The production of seedlings of fast growing species such as *Acacia*, *Eucalyptus* and *Gmelina* in commercial nursery scales requires application of large amounts of chemical fertilizers, which entails high cost. Without fertilizer application, intensively managed tree plantations generally have low harvest. Moreover, fertilization cost is highest for degraded forest lands (Mackensen and Föster 2000). However, heavy fertilization often reduces the formation of some AM (Johnson 1993; Titus and Leps 2000). If the production cost using AM inoculum is lower than that using fertilizers, AM fungal inoculation can replace or reduce the amount of chemical fertilizers used in nurseries and in the field, without loss of efficiency of AM fungal colonization.

AM colonization in cuttings of *P. alternifolium* and *C. hosei* species differed between both AM fungi. *P. alternifolium* showed better response to AM colonization than *C. hosei* because percentage AM colonization and root dry weight of *P. alternifolium* were higher than those of *C. hosei*. For example, *G. aggregatum* colonized *P. alternifolium* quite well, but colonized *C. hosei* only poorly. Nevertheless, both AM fungi stimulated the root system of both cuttings, suggesting that there is no clear relationship between the degree to which a cutting is colonized by AM fungi and the potential for the cutting to benefit from this. MD (Plenchette et al. 1983) was 55 and 58% in *P. alternifolium* inoculated with *G. aggregatum* and *G. clarum*, and 63 and 57% in *C. hosei*, respectively. *Ploiarium alternifolium* and *C. hosei* were considered to be highly dependent according to the MD

categories defined by Habte and Manajunath (1991). These values for AM dependency in both cutting species are also similar when compared with *Sesbania padulosa*, *Acacia radiana*, and *Rhynchosia minima* inoculated by *G. aggregatum* (Duponnois et al. 2001).

The survival rate of seedling stocks in the field is vital to reforestation. The survival rates of both AM-inoculated cuttings were 100% after 6 months (Fig. 3), and were higher than the survival rates of two tropical tree species from Panama inoculated with AM fungi, *Ochroma pyramidale* (97%) and *Luehea seemannii* (52%) (Kiers et al. 2000). Generally, 120% seedling stock is necessary for reforestation; however, our study shows that much more seedlings are needed when the seedlings are not inoculated with AM fungi, because field conditions are much more extreme than nursery conditions. The use of AM-inoculated seedlings might solve this problem: it would reduce significantly the cost of seedlings required for reforesting vast areas such as peat-swamp forests. However, it was difficult to get both species seedlings from the field to produce seedling stocks for planting activity. Among methods of vegetative propagation of grafting, air layering, tissue culture and cutting propagation, the latter is the most commonly used technique (Weinland 1998). Development of hedge orchard is a strategy to sustain the resource of cuttings and this technique has been used also to produce cuttings some species of dipterocarps. The hedge orchard can serve as a continuous supply for large-scale nursery seedling stocks.

We succeeded in making rootings of two peat-swamp tree cuttings, *P. alternifolium* and *C. hosei*, with the propagation cutting system, utilizing the greenhouse-use fogging system developed by Sakai et al. (2002). This technique has been used to produce seedlings of *Shorea leprosula*, *S. selanica* and *S. platyclados* (Dipterocarpaceae). To accelerate reforestation programs, Nair and Seeni (2003) proposed that *Calophyllum* seedlings be multiplied by tissue culture technology, but the *Calophyllum* roots grew slowly.

In conclusion, the inoculation of *G. aggregatum* and *G. clarum* improved plant growth and increased shoot P contents and survival rates of *P. alternifolium* and *C. hosei* cuttings 6 months after transplantation under greenhouse conditions. There was no significant difference in plant growth between the cuttings inoculated with the two AM fungi. Although *G. aggregatum* is an exotic species, it performed well in both tree species. When native species are unavailable, the use of exotic AM fungi should be considered provided that the inoculated cuttings show improved growth. *Glomus clarum* is beneficial if those two tree species are selected for reforestation activity. Further studies of field conditions are required to confirm this preliminary finding. It may also be possible to increase the reliability of inoculation by using mixed inoculum. Alexander and Lee (2005) suggested that it is best to use mixed AM fungal species inoculum rather than single AM fungal species as different species may have different functional roles. Taken together, our results suggest that the integration of the propagation cutting system with AM fungal inoculation technology can accelerate the establishment of cutting stocks on a large scale in nurseries, to rehabilitate tropical peat-swamp forests.

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