INCREASE IN EARLY GROWTH AND NUTRIENT UPTAKE OF *SHOREA SEMINIS* SEEDLINGS INOCULATED WITH TWO ECTOMYCORRHIZAL FUNGI

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TURJAMAN, M., TAMAI, Y., SEGAH, H., LIMIN, S. H., OSAKI, M. & TAWARAYA, K. 2006. Increase in early growth and nutrient uptake of *Shorea seminis* seedlings inoculated with two ectomycorrhizal fungi. Seedlings of *Shorea seminis* were inoculated with spores or mycelium of two ectomycorrhizal (ECM) fungi, *Pisolithus arhizus* and *Scleroderma columnare*, and were grown in pots containing sterilized peat soil for seven months. The percentage of ECM colonization was 35–37% in roots inoculated with mycelium and 61–65% in roots inoculated with spores. ECM colonization increased shoot height and biomass of *S. seminis*. It also increased shoot nitrogen (N) and phosphorus (P) uptakes in *S. seminis*. A positive relationship was found between N or P uptake and shoot biomass of *S. seminis* inoculated with both ECM fungi. This has implications for the successful regeneration of dipterocarps seedlings and rehabilitation in degraded tropical rain forests.

Keywords: Dipterocarpaceae, ectomycorrhiza, spore, mycelium, nutrient uptake, reforestation, tropical rain forest

TURJAMAN, M., TAMAI, Y., SEGAH, H., LIMIN, S. H., OSAKI, M. & TAWARAYA, K. 2006. Peningkatan pertumbuhan dan pengambilan nutrien bagi anak benih *Shorea seminis* yang diinokulasi dengan dua kulat ektomikoriza. Anak biji benih *Shorea seminis* diinokulasi dengan spora atau miselium dua kulat ektomikoriza (ECM) iaitu *Pisolithus arhizus* dan *Scleroderma columnare*. Anak benih itu kemudiannya dibiar tumbuh dalam pasu yang mengandungi tanah gambut yang terbasmi kuman selama tujuh bulan. Peratusan kolonisasi ECM ialah 35% hingga 37% bagi akar yang diinokulasi dengan miselium sementara bagi akar yang diinokulasi dengan spora, peratusannya ialah 61% hingga 65%. Kolonisasi ECM meningkatkan ketinggian pucuk dan biojisim *S. seminis*. Kolonisasi ECM juga meningkatkan pengambilan nitrogen (N) serta fosforus (P) pucuk *S. seminis*. Pengambilan N atau P serta biojisim *S. seminis* yang diinokulasi dengan kedua-dua ECM menunjukkan hubungan positif. Ini memberi implikasi kepada kejayaan pertumbuhan semula anak benih dipterokarpa dan pemulihan hutan hujan tropika terdegradasi.

INTRODUCTION

Presently dipterocarps predominate the international tropical timber market and, therefore, play a significant role in the economy of many South-East Asian countries (Appanah & Turnbull 1998). The dipterocarps also comprise important timbers for domestic needs in seasonal evergreen forests of Asia. In the intervening time, many scientists from all over the world focus on dipterocarp forests to offer models for sustainable forest management, ecology, and silviculture to ensure a continual supply of industrial wood in the future.

As with many *Shorea* (Dipterocarpaceae) species, *S. seminis* is in danger of extinction because of uncontrolled deforestation. *Shorea seminis* is abundant throughout Kalimantan and attains a height of 60 m and diameter of 120 cm (Soerianegara & Lemmens 1994). This species occurs in areas lower than 1000 m altitude, with a mean annual rainfall of more than 1600 mm and a dry season of below six months. *Shorea seminis* is economically important because it produces edible nuts and the prized light red meranti (lightweight hardwood). *Shorea seminis*

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has not yet been planted on a large scale. It is still propagated by seed. Its numbers in the wild have been decreasing and an effective method for its regeneration has yet to be developed. Since large tracts of dipterocarp forests have become overlogged and degraded, interest in planting dipterocarps either in plantations or by under planting in poor forests has gained momentum.

In the last 15 years, the rate of reforestation increased in all countries in South-East Asia. Simultaneously, the rate of forest destruction, forest exploitation, clear-cut forest areas, amended adverse sites, old agricultural lands, mineral mining, forest fire, conversion of natural forests into plantations, resettlement areas, and other uses are also increasing. A major problem faced in reforestation of disturbed tropical rain forest is having an established supply of planting stocks in nursery. This is especially so since the majority of *Shorea* species including *S. seminis* neither flower nor fruit regularly (Krishnapillay & Tompsett 1998). In aseasonal zones flowering and fruiting of this species occur at intervals of two to five years and their accurate prediction is impossible. Growth of *S. seminis* seedlings is habitually slow and in the nursery this species requires pots of large size for the first seven months before transplanting to the field.

The positive effects of ectomycorrhizal (ECM) fungi on plant nutrition have usually been recognized to the quantitative result of the extraradical mycelium on uptake of dissolved nutrients from the soil solution (Smith & Read 1997). ECM fungi may have great potential for the regeneration of tropical forests (Lee 1990, Smits 1994). ECM could play an important role in successful establishment of seedlings by increasing nutrient and water uptakes of plants and increasing their resistance to environmental stress (Lee 1990). Although many studies have documented the effects of ECM colonization on plant growth and nutrient uptake, especially nitrogen (N) and phosphorus (P), little of this information concerns tropical tree species (Harley & Smith 1983). Also it must be underlined that little information has been conducted on the very important aspect of mineral nutrient requirements of dipterocarps. It has been reported that foliar P concentration of *S. curtisii* and *S. leprosula* seedlings growing in a logged over forest site with low level of available P was significantly correlated with the extent of ECM colonization (Lee & Lim 1989). The level of ECM colonization of *S. macroptera* seedlings showed a positive correlation with growth and survival of unfertilized seedlings (Turner *et al.* 1993). Positive growth responses to ECM colonization were also reported for *Hopea helferi* and *H. odorata* (Lee & Alexander 1994). However, potted *Dipterocarpus kunstleri* seedlings did not show any positive growth response to nutrient additions although additional P increased the concentrations of K and Ca in the leaves (Burslem *et al.* 1995). In addition, litter supplement enhanced the growth of *Parashorea tomentella, H. nervosa* and *Dryobalanops lanceolata* and the ECM association of dipterocarps assisted right of entry to organic nutrient source (Brearley *et al.* 2003).

Pisolithus and *Scleroderma* species have been used to enhance the early growth of a number of tree species, both in the greenhouse and in the field (Chambers & Cairney 1999, Jeffries 1999). These species have the advantage over many other ECM fungi as their spores can be collected from mature fruiting bodies. Preparation of ECM inoculum from spores is comparatively easy with material collected from the field. The use of mycelium or spores as inoculum is a viable option for low technology nurseries currently producing seedlings for outplanting in developing countries. However, there are no records of these species forming ECM with *S. seminis*.

Vegetative mycelium ECM inoculum entrapped in alginate beads have been used for some temperate tree species (Nezzar-Hocine *et al.* 1998, Frey-Klett *et al.* 1999, Boukcim *et al.* 2002). In the Philippines, tablets made from a mixture of basidiospores of *Scleroderma cepa* and *P. tinctorius* have confirmed to be effective on several eucalypt species (de la Cruz 1990), but little work has been undertaken on the inoculation of dipterocarps with fungal spores and vegetative mycelium. The aim of this study was to (1) investigate the effects of ECM inoculation on nutrient uptake and plant growth of *S. seminis*, and (2) compare two types of inoculum, i.e. spore and mycelium.

MATERIALS AND METHODS

Seed and plant substrates

Seeds of *S. seminis* were obtained from Carita Beach Experimental Forest Site, West Java. Peat

soil used for the pot experiment was collected from a peat swamp forest in Kalampangan, Palangka Raya, Central Kalimantan. Peat soil was sterilized in a drum by heating over a wood fire for 1 hour. A preliminary experiment showed that this sterilization procedure got rid of most of the ECM and pathogenic fungi. Seeds of *S. seminis* were soaked for 2 hours and gently washed with running water. These seeds were sown in polyethylene pots (size 15 × 10 cm) containing 500 g sterilized peat soils. Pots containing seeds were transferred to a nursery at the University of Palangka Raya, Central Kalimantan (2° 13' S, 113° 56' E). One seedling was grown per pot under a 75% shading intensity net to control solar radiation.

Ectomycorrhizal inoculum

Fruit bodies of *P. arhizus* and *S. columnare* were obtained from basidiomes in the field under mature trees of *Pinus merkusii* in a mountain forest in Majenang, Central Java and *S. leprosula* at the Haurbentes Experimental Forest Site in Jasinga, West Java. *Pisolithus arhizus* was identified previously by S. Hadi and E. Santoso (pers. comm.) in 1995. Morphology of the fruit body and basidiomes of this species was different from *P. abditus* (Kanchanaprayudh *et al.* 2003) and *P. aurantioscabrosus* (Watling *et al.* 1995). *Scleroderma columnare* was identified by M. R. Rifai (pers. comm.) in 1988 and confirmed a revised key to the genus *Scleroderma* (Sims *et al.* 1995). Mature and dry basidiomes were selected. The basidiomes were crushed manually in plastic bags to ensure minimal loss of spores and cross-contamination between fungi. They were then pelletized in a tabletting machine (de la Cruz *et al.* 1990). The tabletting machine was used to standardize the size and weight of tablets; each tablet weighed 0.4 g.

The mycelium of ECM inoculum was grown in Pachlewski liquid medium (Pachlewski & Pachlewska 1974) for 1 month at 100 rpm in a dark room at room temperature (28–30 °C). The mycelia were harvested and rinsed with sterile distilled water and then homogenized. Following this, 5 g mycelia were mixed with 15 g coconut fiber in 2% sodium alginate. Then the inoculum paste was transferred to 5% calcium chloride solution by passing through a 4-mm mesh sieve to obtain a granular calcium alginate-entrapped inoculum (Mauperin *et al.* 1987). The granules were soaked in calcium chloride solution for 5 min and rinsed subsequently with sterile distilled water. The weight of each alginate bead was about 0.1 g. The beads were stored at room temperature.

Ectomycorrhizal inoculation

Inoculation of seedlings was carried out 10 days after germination. One tablet or two alginate beads of ECM fungal inoculum was applied to each seedling, 1 cm below soil surface. In addition, 0.5 g slow release fertilizer (14% N, 13% P_2O_5 and 13% K_2O) was added to each pot at the time of the ECM inoculation. The seedlings were irrigated with tap water everyday and weeds were removed. The following treatments were used: (1) control, (2) *P. arhizus* spores, (3) *S. columnare* spores, (4) *P. arhizus* mycelium and (5) *S. columnare* mycelium.

Plant harvest and ectomycorrhizal colonization

Shoots and roots were harvested seven months after being inoculated with ECM fungi. After measuring the fresh biomass of shoots, its dry biomass was determined after drying at 70 °C for 72 hours. Ground shoots were digested in H_2SO_4 and H_2O_2 solution (3:1, v/v). The N and P contents in the digested solution were determined by semi-micro Kjeldahl and vanado molybdate yellow methods (Olsen & Sommers 1982) respectively.

To calculate the percentage of ECM colonization, roots were cleaned using running water to separate them from the soil and then the root systems were spread on trays. The total number of root tips and the number of ECM short roots were counted under a dissecting microscope. Verification of ECM colonization was obtained by examining the cross section of root tips (cut manually) under a compound microscope for the presence of mantle and Hartig net (Brundrett *et al.* 1996). The data were subjected to analysis of variance and means were compared by the least significant difference method ($p = 0.05$) using the statistical software StatView 5.0 (Abacus Concepts).

RESULTS

Ectomycorrhizal colonization

All ectomycorrhizas found on roots of *S. seminis* were of the inoculant type, and there was no cross-contamination between treatments. At the end of the seven months in the nursery, *P. arhizus* and *S. columnare* also formed ECM in *S. seminis* seedlings (Table 1). ECM colonization of seedlings inoculated with spores was higher than those inoculated with mycelium. ECM colonization of *S. seminis* inoculated with mycelium was not significantly different from that of uninoculated seedlings, but a tendency to increase with ECM colonization was observed. Control seedlings were also colonized by indigenous ECM fungi.

Plant growth

Ectomycorrhizal colonization of *S. seminis* using *P. arhizus* and *S. columnare* increased plant growth of *S. seminis*. Both ECM increased the fresh biomass, dry biomass and height of shoot of *S. seminis* (Table 1). There was no significant difference in dry shoot biomass between *S. columnare* spores and control treatments. Furthermore, there was no significant difference in plant growth between fungi treatments.

Shoot nutrient uptake

Ectomycorrhizal colonization of *S. seminis* using mycelia from *P. arhizus* and *S. columnare* increased shoot N uptake of *S. seminis* (Table 2). ECM colonization with *P. arhizus* mycelium increased shoot P uptake of *S. seminis*. There was no significant difference in shoot N and P uptakes of *S. seminis* between spore inoculated and uninoculated treatments.

A strong positive relationship was found between N uptake and shoot dry biomass of *S. seminis* ($r = 0.84$, $p < 0.001$). Interaction between P uptake and shoot dry biomass of *S. seminis* was also significantly affected by ECM ($r = 0.59$, $p < 0.05$).

DISCUSSION

Pisolithus arhizus and *S. columnare* formed ECM colonization in *S. seminis* seedlings at the end of the seven-month study period in the nursery. ECM colonization with *P. arhizus* and *S. columnare* increased plant growth and nutrient uptake of

Table 1 Ectomycorrhizal colonization and shoot biomass and height of *Shorea seminis* seedlings inoculated with the ectomycorrhizal fungi, *Pisolithus arhizus* and *Scleroderma columnare*

Treatment	ECM colonization (%)	Shoot biomass		Height of shoot (cm)
		Fresh biomass (g/plant)	Dry biomass (g/plant)	
Control	19 a	6.1 a	2.2 a	21.8 a
P. arhizus spores	61 b	15.7 b	5.5 b	30.28 b
S. columnare spores	65 b	13.0 b	4.6 ab	26.28 ab
P. arhizus mycelium	35 ab	18.9 b	7.0 b	26.36 b
S. columnare mycelium	37 ab	16.0 b	6.1 b	26.60 b

Values are means of three seedlings per treatment.
Values with the same letter are not significantly different ($p < 0.05$).

Table 2 N and P uptake of *Shorea seminis* seedlings inoculated with the ectomycorrhizal fungi, *Pisolithus arhizus* and *Scleroderma columnare*

Treatment	Shoot nutrient uptake	
	N (mg/plant)	P (mg/plant)
Control	140.18 ± 73.94 a	22.41 ± 9.73 a
P. arhizus spores	224.78 ± 17.33 a	31.83 ± 1.41 a
S. columnare spores	200.10 ± 38.95 a	22.40 ± 4.54 a
P. arhizus mycelium	353.17 ± 28.66 b	68.74 ± 2.78 b
S. columnare mycelium	384.89 ± 40.22 b	36.30 ± 5.70 a

Values are means of three seedlings per treatment (± SE).
Values with the same letter are not significantly different ($p < 0.05$).

S. seminis. To the best of our knowledge, this is the first observation of ECM synthesis between *S. seminis* and *P. arhizus* or *S. columnare*.

The percentage of ECM colonization on *S. seminis* exceeded 35%. *Scleroderma columnare* spore had higher ECM colonization than *P. arhizus* spore or *P. arhizus* mycelium. Early colonization of dipterocarp seedlings was highly dependent on contact with living ECM roots of adult trees (Alexander *et al.* 1992). This suggests that controlled inoculation of dipterocarp seedlings in the nursery with selected efficient ECM fungal strains should be introduced in forest regeneration programmes. Growth measured in the present experiments with the exotic *P. arhizus* showed that a fungal strain isolated from a non-dipterocarp host outside the natural distribution of Dipterocarpaceae could form a completely functional ECM with *S. seminis*. These results suggest that in terms of specificity, *P. arhizus* and *S. columnare* have a broad range of ECM to host tree species (Chambers & Cairney 1999, Jeffries 1999).

Scleroderma columnare had higher N uptake than *P. arhizus* but *P. arhizus* had higher P uptake. Moreover, a strong positive correlation between N uptake or P uptake and shoot dry weight of *S. seminis* was significantly affected by ECM. Some mycorrhizal associations, notably the ericoid and ECM, definitely improve plant N uptake by accessing organic N which is inaccessible to the roots alone (Read & Perez-Moreno 2003). ECM colonization in *H. odorata* increased shoot P uptake and increased shoot and total dry weights to the same or greater extent than those of uncolonized plants growing on P-amended soil (Lee & Alexander 1994). In another report, the growth of *H. odorata* and *H. helferi* was stimulated by inoculation with a strain of *P. tinctorius* in pure culture and P uptake in the seedlings was also improved by ECM colonization (Yazid *et al.* 1994).

ECM colonization was also observed in control treatments of *S. seminis* seedlings in the nursery (19%). Therefore, it is possible that indigenous ECM fungi persisted in the nursery and slowly reached the seedling roots and formed ECM. However, the significant difference of all parameters ($p < 0.05$) between controls and inoculated plants was due to faster colonization by inoculated fungi so that either ECM fungi could prevent invasion by indigenous ECM fungi. The fungi on the control seedlings developed pink ectomycorrhiza roots and may be of the genus *Laccaria*. However, the indigenous ECM could not compete with *P. arhizus* and *S. columnare*.

There was a higher overall success rate for ECM inoculation with spore tablets (61–65%) than for mycelial alginate beads (35–37%). These results are comparable with the 49 and 35% values that were observed in spore suspensions and mycelial slurries used by Brundrett *et al.* (2005) to inoculate *Eucalyptus* seedlings. This was probably the result of slow establishment of *P. arhizus* or *S. columnare* from the time required for hyphae to spread from the inoculation point. However, the risk of introducing pathogens, or other uncontrolled microorganisms, was eliminated with mycelial (pure culture) inoculation. Liquid industrial fermentation process has been developed for entrapping the mycelium produced in a fermenter with ground peat in alginate beads (Kuek *et al.* 1992); this method is very suitable for use in commercial nurseries. Alginate beads inoculum can be utilized in large-scale nursery production of *Shorea* seedlings for the rehabilitation of degraded lands. The production of alginate beads can be carried out year-round.

However, a tablet of spores can still be used as an alternative to that of mycelium entrapped in alginate beads. Seedlings can be conveniently inoculated by simply adding one tablet per pot. It has been reported that spore tablets of *Pisolithus* and *Scleroderma* increased early growth of *Eucalyptus* seedlings (de la Cruz *et al.* 1990). However, another study has shown that *Pisolithus* spore tablet inoculum was considerably less effective for ECM root development of seedlings of four-needled pine species compared with vegetative inoculum (Hua *et al.* 1991). Spores can also be used to coat seeds by mixing them with a carrier such as clay (Marx *et al.* 1984) and this protocol is suitable for large-seed tree species such as pines. However, the problem in using spores is the inherent genetic diversity of the inoculum. Basidiomes of *P. arhizus* or *S. columnare* collected from different locations undoubtedly have different genetic traits (Tonkin *et al.* 1989, Burgess *et al.* 1994).

CONCLUSIONS

In our experiments colonization of roots of *S. seminis* by *P. arhizus* or *S. columnare* increased N and P uptakes and early growth of seedlings after seven months under nursery condition. A

positive relationship was found between N or P uptake and shoot dry biomass of *S. seminis* inoculated with both ECM fungi. Spore tablet inoculum was easy to apply and inexpensive relative to other tree seedling production costs and more effective than mycelial alginate beads. Both types of ECM inocula can replace soil inoculum on a large scale. With the present interest in establishing regeneration or plantation of *S. seminis* including other member dipterocarps, mycorrhization is a key technology to enhance or encourage rapid growth of seedlings on a large scale in commercial nurseries and to enable the seedlings to survive successfully in the field. Furthermore, it is important to consider that in Kalimantan alone *Shorea* comprises over 60 species with a broad range of growth rate and ecology, including preference for mineral soil, heath forest and peat swamp forest. It would be imprudent to make simplifications about the role of ECM in *Shorea* based on a few experiments on a single species. The results of this study showed that inoculation of ECM fungi can increase early growth of *S. seminis* grown in tropical rain forest and that this technique will accelerate and assure the achievement of reforestation programmes.

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