EFFECTS OF ECTOMYCORRHIZAL FUNGUS *ASTRAEUS ODORATUS* ON *DIPTEROCARPUS ALATUS* SEEDLINGS

T Kaewgrajang[1], U Sangwanit[2], K Iwase[3], M Kodama[4] & M Yamato[4, *]

[1]*The United Graduate School of Agricultural Science, Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan*
[2]*Department of Forest Biology, Faculty of Forestry, Kasetsart University, 50 Ngam Wong Wan Road, Ladyaow, Chatuchak, Bangkok 10900, Thailand*
[3]*Department of Natural and Environmental Science, Teikyo University of Science, Yatsusawa 2525, Uenohara 409-0193, Japan*
[4]*Fungus/Mushroom Resources and Research Center, Faculty of Agriculture, Tottori University, 4-101-Koyama-Minami, Tottori 680-8553, Japan*

Received March 2012

KAEWGRAJANG T, SANGWANIT U, IWASE K, KODAMA M & YAMATO M. 2013. Effects of ectomycorrhizal fungus *Astraeus odoratus* on *Dipterocarpus alatus* seedlings. *Astraeus odoratus* is an edible ectomycorrhizal (ECM) fungus found in natural dipterocarp forests. The efficacy of two different inoculum types of this fungus, namely, spore suspension and cultured mycelium in inducing ECM formation on pot-cultured seedlings of *Dipterocarpus alatus* was studied. Both types of inocula increased ECM formation. A positive correlation was found between the ECM rate and seedling growth. Both inoculation methods could be used to produce ECM seedlings of *D. alatus*.

Keywords: Spore suspension, cultured mycelium, inoculation, seedling growth, ectomycorrhizal root

KAEWGRAJANG T, SANGWANIT U, IWASE K, KODAMA M & YAMATO M. 2013. Kesan kulat ektomikoriza *Astraeus odoratus* terhadap anak benih *Dipterocarpus alatus*. *Astraeus odoratus* merupakan kulat ektomikoriza (ECM) yang boleh dimakan dan dijumpai di hutan dipterokarpa asli. Dua jenis inokulum daripada kulat ini iaitu ampaian spora dan miselium yang dikultur dikaji keberkesanannya mengaruh pembentukan ECM dalam anak benih *Dipterocarpus alatus* yang ditanam di dalam pasu. Kedua-dua jenis inokulum menggalakkan pembentukan ECM. Korelasi positif dicerap antara kadar pembentukan ECM dengan pertumbuhan anak benih. Kedua-dua kaedah penginokulatan boleh digunakan untuk menghasilkan anak benih *D. alatus* yang mengandungi ECM.

INTRODUCTION

Dipterocarpaceae is a family of trees that dominate canopies of rainforests in South-East Asia. In Thailand, 65 species from 8 genera are found in this family (Phengklai & Niyomdham 1999). *Dipterocarpus alatus* is recognised as the most economically important species producing not only good quality wood but also resin and oil (Boontawee 2001). Plantations of *D. alatus* have been established in Thailand with a total area of 2080 ha (Boontawee 1999, 2001). However, the establishment of dipterocarp plantations is difficult compared with other fast-growing tree species such as *Acacia* and *Eucalyptus*.

All dipterocarps form ectomycorrhizae on roots and many ectomycorrhizal (ECM) fungi have been found in dipterocarp forests (Brundrett et al. 1996). Fruiting bodies of ECM fungi have been found in Amanitales, Boletales, Cantharellales and Russulales in dipterocarp forests, which have common tree families including Euphobiaceae, Annonaceae, Dipterocarpaceae, Leguminasae and Burseraceae (Lee et al. 2002). A total at 33 ECM fungal species in 8 families (Thelephoraceae, Russulaceae, Amanitaceae, Cortinariaceae, Sclerodermataceae, Agaricaceae, Pisolitaceae and Boletaceae) were reported from ECM roots of 15 dipterocarp species (*Anisoptera costata*, *A. curtisii*, *Cotylelobium lanceolatum*, *D. alatus*, *D. baudii*, *D. kerrii*, *D. tuberculatus*, *D. turbinatus*, *Hopea ferrea*, *H. odorata*, *Parashorea stellata*, *Shorea farinose*, *S. guiso*, *S. obtusa*, *S. roxburghii*) in Thailand,

**m_yamato@muses.tottori-u.ac.jp*

in which *Amanita virosa*, *Tomentella* sp. and *Pisolithus* sp. were associated with *D. alatus* (Yuwa-Amornpitak et al. 2006). ECM formation induces enhancement of host–plant growth through increase of nutrient uptake (Smith & Read 2008). For dipterocarps, positive correlations were found between ECM formation and seedling growth (Lee & Lim 1989, Sangwanit & Sangthian 1991). In several dipterocarp species, ECM fungal inoculation was found to improve phosphorus uptake and seedling growth (Brearley 2006, Lee et al. 2008, Turjaman et al. 2011). Thus, ECM fungi can be beneficial in dipterocarp plantations.

Astraeus is a common ECM fungal genus found in various forests in the world. It associates with a wide range of host plants such as *Pinus*, *Eucalyptus*, *Pseudotsuga*, *Alnus* and *Castanea* (De Roman et al. 2005, Fangfuk et al. 2010). In Thailand, three species of *Astraeus,* namely, *A. odoratus*, *A. asiaticus* and *A. hygrometricus* are found in lowland dipterocarp forests (Phosri et al. 2004, Petcharat 2005, Phosri et al. 2007). The former two species were described from that country. In Thailand, *Astraeus* species are popular and profitable edible ECM mushrooms (Sanmee et al. 2003). ECM formation on roots of *D. alatus* by *A. hygrometricus* was studied using *in vitro* culture technique (Sangwanit & Sangthian 1993). Since it is relatively easy to grow *Astraeus* fungus on culture media, the fungus can be used as a practical inoculum for large-scale inoculation. We examined the effects of two different ECM fungal inoculum types of *A. odoratus*—spore suspension and cultured mycelium—on ECM formation in *D. alatus* seedlings in order to investigate the practical application of this fungus. To date, there have been no studies to compare the inocula for ECM formation and growth of dipterocarp seedlings.

MATERIALS AND METHODS

Seedling preparation

Seeds of *D. alatus* were collected from a natural tree stand (14° 29' N, 101° 56' E) in Nakhon Ratchasrima province, north-eastern Thailand. After removing the fruit wings, seeds were soaked in water overnight and then incubated in a sealed plastic bag at room temperature for 7–10 days. The germinating seed with 3–4 cm root length was transplanted into a polyethylene bag (10 cm × 15 cm), which was filled with 500 mL of fumigated sandy loam soil (1.8 g kg^{-1} of N, 2.0 g kg^{-1} of P, 47 mg kg^{-1} of K, 263 mg kg^{-1} of Ca, 73 mg kg^{-1} of Mg, pH 4.67). The soils were fumigated using methyl bromide 50 g m^{-3} for 48 hours and then left in the nursery for 14 days to release the excessive gas. Seedlings were irrigated daily with industrially bottled drinking water. After 1 month, uniform healthy and non-mycorrhizal seedlings were selected for fungal inoculation.

Fungal inoculation

Both spore suspension and mycelial culture of *A. odoratus* were prepared as inocula. The spore suspension was prepared by homogenising 51 g of air-dried fruiting bodies in 510 mL of distilled water and a few drops of liquid detergent using a blender. The spore density was 6.7×10^4 spores mL^{-1}. The mycelial culture was prepared by cultivating the fungus in 100 mL of potato dextrose agar (PDA) medium (Atlas 1997) for 1 month at room temperature. The mycelium was separated from the broth medium using a fine mesh screen and washed several times with distilled water. The washed mycelium was blotted with paper towel and 50 g were homogenised in 150 mL of distilled water using a blender to prepare the slurry mycelium.

The fungus was inoculated into the seedlings without transplanting. Each of the 10, 25 or 50 mL of spore suspension or 25 mL of slurry mycelium was added into a hole in the soil (6 cm deep) near the seedling stems. Six replications were prepared for each fungal inoculation treatment as well as a non-inoculated control. Thus, 30 pots were prepared in total. The experimental pots were placed in a completely randomised design on a wire screen above the ground in a greenhouse at the Faculty of Forestry, Kasetsart University. They were irrigated daily with drinking water

until draining to ensure watering to field capacity.

Growth measurement and plant harvest

The pots were cultured for 8 months after fungal inoculation. The height and basal stem diameter of the seedlings were measured during fungal inoculation and harvesting. After harvest, roots were washed in tap water to remove soil and sand particles. For each seedling, around 10% of the root was randomly collected to determine the rate of ECM formation under a dissecting microscope using the gridline intersection method (Brundrett et al. 1996). This method gave the proportion of root system that was colonised. At least 350 intersections per sample were examined to determine the rate of ECM formation. The total root and shoot materials were oven dried at 80 °C for 48 hours and their dry weights measured.

Observations of morphological characters

For cleaned ECM roots, the surface colour, texture, emanating hyphae, rhizomorphs and ectomycorrhizal branching pattern were described. Fruiting bodies found on the soil surface of pots were examined for morphological characteristics. Mature stage of basidiospores was observed using scanning electron microscope.

Identity of sclerotia

Five sclerotia were collected from ECM root system of *D. alatus* from each of the 10 pots (2 pots randomly chosen per treatment). The surface of the sclerotium was sterilised by soaking in 30% hydrogen peroxide for 20 s and then immediately rinsed several times with sterilised distilled water. The sclerotia were blotted on sterile filter paper and transferred onto PDA medium plates. After 1–2 weeks, the characteristics of growing hyphae such as colour, medium pigmentation, clamp connection and hyphal diameter were observed under the dissecting microscope and compared with those of the inoculated fungi. Fungi isolated from sclerotia were co-cultured with the inoculum fungus on the same PDA medium plate for somatic compatibility test.

Data analysis

An allometric equation (Madgwick & Satoo 1975) was applied to estimate the dry weights of the shoot and root materials at the time of fungal inoculation. The allometric equation was constructed from the basal stem diameter and height of the seedlings at harvested time.

$$y = ax^h$$

where y = shoot dry weight or root dry weight (g) at harvested time, x = square of the basal stem diameter D^2 (mm) × height H (cm) of the seedling at harvested time, a and h = constants.

The basal stem diameter and height at fungal inoculation were input into the equation to calculate the shoot dry weight and root dry weight at the time. Their increments were assessed by one-way analysis of variance and means were compared by Tukey's test ($p < 0.05$).

RESULTS

Morphological description of *A. odoratus* ectomycorrhiza

Seven months after fungal inoculation, fruiting bodies of *A. odoratus* were found in two pots of seedlings inoculated with 25 mL of spore suspension inoculum (Figure 1a). The peridium layer of the fruiting body was composed of several layers. The spores were globose, purplish chestnut and ornamented with moderately dense, rounded long, narrow and coalesced spines (Figure 1b). These characteristics were the same as those described for this fungal species by Phosri et al. (2004) and Petcharat (2005).

ECM roots of *D. alatus* inoculated with *A. odoratus* were pale to dark brown with smooth and shiny surface. The branching pattern was irregular with abundant branches. Around the ECM roots, many yellowish-brown rhizomorphs (100–150 µm in diameter) were

found as well as numerous sclerotia (1 mm in diameter) that were coloured reddish to blackish brown with smooth and shiny surface (Figure 2a). A cross-section of the ECM root showed a hyaline mantle surrounding the epidermal cells (Figure 2b). A Hartig net was found in the epidermal layer with thickness of 0.5–0.9 µm. ECM roots were also formed in three non-inoculated seedlings with the same morphological features but in lower rate compared with the inoculated seedlings (Figure 3a).

For fungal isolation from sclerotia, 20 cultures were obtained: 3 from SS10, 5 from SS25, 4 from SS50, 4 from PC25 and 4 from the control. All cultured mycelia had the same characteristics as those of inoculated fungus: yellowish brown, velvety, 3–4 µm diameter hyphae with clamp connections and producing dark brown pigment accumulating in the medium. Furthermore, 20 trials to culture the fungus from sclerotium with inocula fungus on the same PDA plates resulted in anastomoses between the two colonies. These results indicated that the ECM roots of *D. alatus* were colonised by the inoculated *A. odoratus* and the contaminated fungi originated from the inoculated fungus.

Growth of seedlings

ECM formed even in the control. Inoculated seedlings had significantly higher ECM rates (Figure 3a). The increments of shoot dry weight with fungal inoculations seemed to be higher than the control but the difference

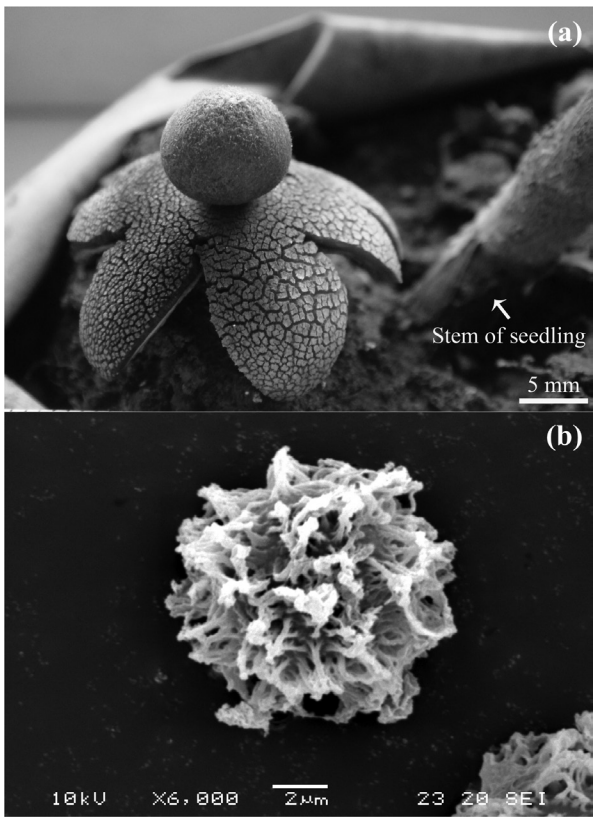

Figure 1 A fruiting body and a spore of *Astraeus odoratus* with a pot-cultured seedling of *Dipterocarpus alatus* after 7 months of fungal inoculation: (a) a mature fruiting body on soil surface, (b) scanning electron micrograph of a basidiospore with ornamentation

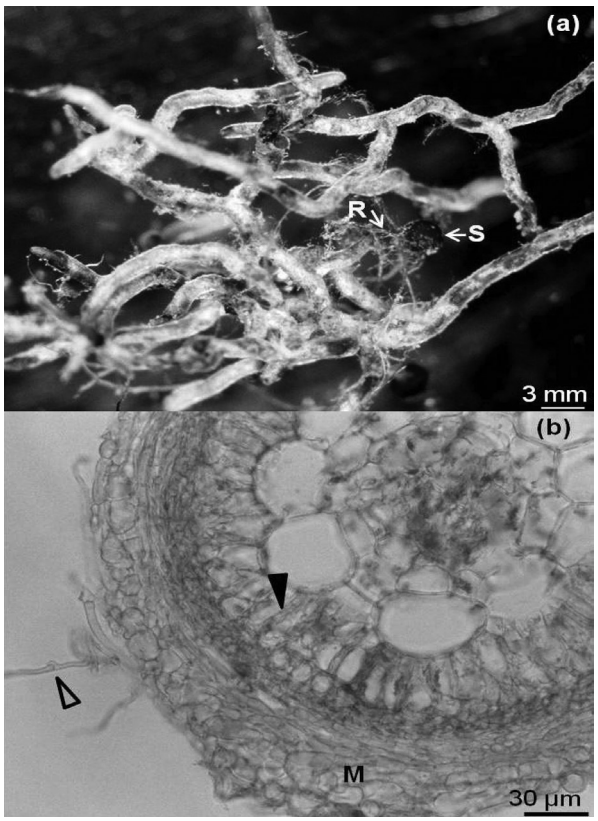

Figure 2 Ectomycorrhizal roots of *Dipterocarpus alatus* by *Astraeus odoratus* after 8 months of inoculation: (a) ECM roots, (b) cross-section of an ECM root; R = rhizomorph, S = sclerotium, M = mantle, outlined arrowhead = clamp connection, solid arrowhead = Hartig net

was not significant (Figure 3b). However, increment in the root dry weight of SS25 was significantly greater than the control (Figure 3c). The correlation between the ECM rate and increment of total dry weight of seedlings showed significant positive correlation (n = 30, r = 0.732, p < 0.001) (Figure 4).

DISCUSSION

Both inocula of *A. odoratus*, spore suspension and cultivated mycelium, successfully induced ECM formations on *D. alatus* seedling roots. The formation of ECM roots in non-inoculated seedlings was induced by cross contamination from inoculated seedling as the spores used as inocula could easily disperse. Such contaminations have been reported in other studies (Turjaman et al. 2005, Lee et al. 2008). The amount of inoculated spores may be too high. Reducing the amount could be a desirable way in this kind of experiment. The positive significant correlation between ECM formation and seedling growth suggested that the ECM formation on roots of *D. alatus* could enhance the growth of dipterocarp seedlings under the pot culture condition. Many studies also reported growth enhancement of dipterocarp seedlings by ECM fungi (Turjaman et al. 2005, Brearley 2006, Ogawa 2006, Lee et al. 2008, Turjaman et al. 2011). This is the first study to demonstrate the growth enhancement of dipterocarp seedlings by *Astraeus* species. Since there was no significant difference in the ECM formation rate between the two different inoculum types (spore suspension and cultivated mycelium), both methods could be equally effective. When *A. odoratus* fruit bodies are available, the preparation of the ECM inoculum from spores is comparatively easy. Meanwhile, the pure hyphae of this fungus can be maintained on culture media in a laboratory and thus be a practical inoculum to produce ECM dipterocarp seedlings at anytime.

The fruiting bodies of *A. odoratus* found in some inoculated pots indicated the high potential of this fungus to produce fruiting bodies in *D. alatus* plantations established with *A. odoratus* ECM seedlings. This edible mushroom may provide benefits to both plants and people.

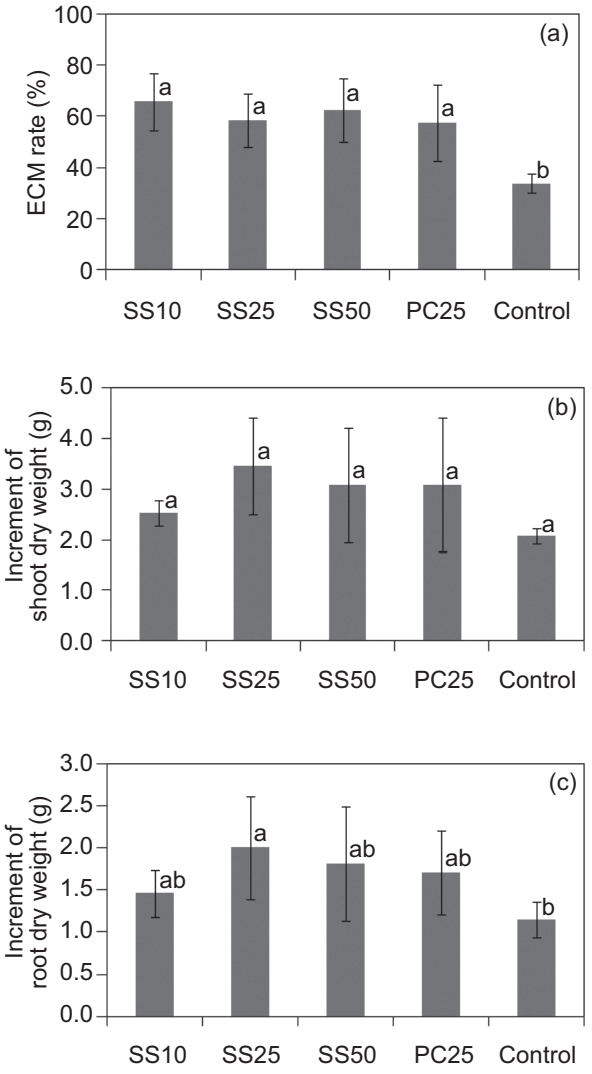

Figure 3 Ectomycorrhizal (ECM) formation rate and growth increment of *Dipterocarpus alatus* seedlings inoculated with ECM fungus *Astraeus odoratus*: (a) ECM rate, (b) increment of shoot dry weight, (c) increment of root dry weight; SS10, SS25, SS50 = inoculation with spore suspension at 10, 25 and 50 mL/seedling respectively, PC25 = inoculation with cultured mycelium at 25 mL/seedling; vertical lines on bars show standard deviations, the same letter shows bars are not significantly different (Tukey's test, n = 30, p < 0.05)

ACKNOWLEDGEMENTS

This study was supported by the Global Center of Excellence Program, 'Advanced Utilization of Fungus/Mushroom Resources

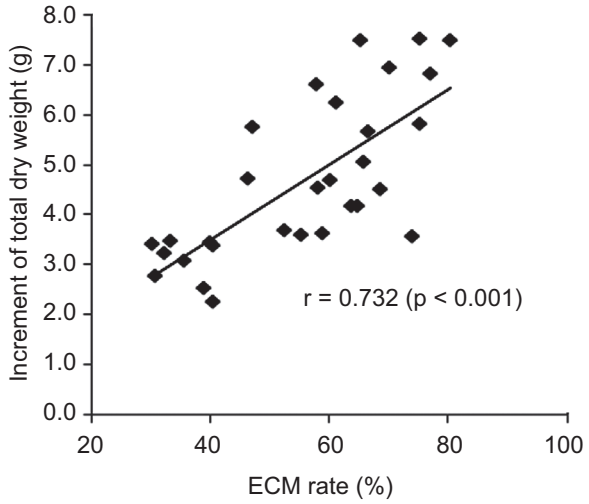

Figure 4 Correlation between ectomycorrhizal (ECM) formation rate and increment of total dry weight of seedlings

for Sustainable Society in Harmony with Nature', from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

Atlas RM. 1997. *Handbook of Microbiological Media.* Second edition. CRC Press, New York.

Boontawee B. 1999. *Dipterocarpus alatus* plantation. Pp 285–297 in *Dipterocarpus alatus and Dipterocarps. Volume III: Miscellaneous on Dipterocarps*. The Agricultural Cooperatives Publishing of Thailand Ltd, Bangkok.

Boontawee B. 2001. The status of *in situ* and *ex situ* conservation of *Dipterocarpus alatus* Roxb. in Thailand. Pp 171–181 in *Proceedings of the International Conference on Ex situ and In situ Conservation of Commercial Tropical Trees.* 11–13 June 2001, Yogyakarta.

Brearley FQ. 2006. Differences in the growth and ectomycorrhizal community of *Dryobalanops lanceolata* (Dipterocarpaceae) seedlings grown in ultramafic and non-ultramafic soils. *Soil Biology and Biochemistry* 38: 3407–3410.

Brundrett M, Bougher N, Dell B, Grove G & Malajczuk N. 1996. *Working with Mycorrhizas in Forestry and Agriculture*. ACIAR Monograph 32. ACIAR, Canberra.

De Roman M, Claveria V & De Miguel AM. 2005. A revision of the descriptions of ectomycorrhizas published since 1961. *Mycological Research* 109: 1063–1104.

Fangfuk W, Okada K, Petchang R, To-anun C, Fukuda M & Yamada A. 2010. *In vitro* mycorrhization of edible *Astraeus* mushrooms and their morphological characterization. *Mycoscience* 51: 234–241.

Lee SS & Lim KL. 1989. Mycorrhizal infection and foliar phosphorus content of seedlings of three dipterocarp species growing in a selectively logged forest and a forest plantation. *Plant and Soil* 117: 237–241.

Lee SS, Patahayah M, Chong WS & Lapeyrie F. 2008. Successful ectomycorrhizal inoculation of two dipterocarp species with a locally isolated fungus in Peninsular Malaysia. *Journal of Tropical Forest Science* 20: 237–247.

Lee SS, Watling R & Sikin YN. 2002. Ectomycorrhizal basidiomata fruiting in lowland rain forests of Peninsular Malaysia. *Bois et Forêts des Tropiques* 274: 33–43.

Madgwick HAI & Satoo T. 1975. On estimating the aboveground weights of tree stands. *Ecology* 56: 1446–1450.

Ogawa M. 2006. Inoculation method of *Scleroderma columnare* onto Dipterocarps. Pp 185–197 in Suzuki K et al. (eds) *Plantation Technology in Tropical Forest Science*. Springer-Verlag, Tokyo.

Petcharat V. 2005. Edible *Astraeus* (Basidiomycota) from Thailand. *Nordic Journal of Botany* 23: 499–503.

Phengklai J & Niyomdham C.1999. The Dipterocarpaceae of Thailand. Pp 5–206 in *Yang-na and Trees in Dipterocarpaceae*. Kasetsart University, Bangkok.

Phosri C, Martín MP, Sinhanonth P, Whalley AJS & Watling R. 2007. Molecular study of the genus *Astraeus*. *Mycological Research* 3: 275–286.

Phosri C, Watling R, Martín MP & Whalley AJS. 2004. The genus *Astraeus* in Thailand. *Mycotaxon* 89: 453–463.

Sanmee R, Dell B, Lumyong P, Izumorid K & Lumyong S. 2003. Nutritive value of popular wild edible mushrooms from northern Thailand. *Food Chemistry* 82: 527–532.

Sangwanit U & Sangthian T. 1991. Ectomycorrhizae of *Dipterocarpus alatus* Roxb. Pp 45–57 in *Proceedings of the Pre-workshop of BIO-REFOR*. 26–28 March 1991, Bogor.

Sangwanit U & Sangthian T. 1993. Ability of some ectomycorrhizal fungi in forming ectomycorrhizae with *Dipterocarpus alatus* Roxb. seedlings. Pp 16–24 in *Proceedings of the Tsukuba Workshop of BIO-REFOR*. 19–21 May 1992, Tsukuba.

Smith SE & Read DJ. 2008. *Mycorrhizal Symbiosis*. Third edition. Academic Press, San Diego.

Turjaman M, Santoso E, Susanto A, Gaman S, Limin SH, Tamai Y, Osaki M & Tawaraya K. 2011. Ectomycorrhizal fungi promote growth of *Shorea balangeran* in degraded peat swamp forests. *Wetlands Ecology and Management* 9: 331–339.

Turjaman M, Tamai Y, Segah H, Limin SH, Cha JY, Osaki M & Tawaraya K. 2005. Inoculation with the ectomycorrhizal fungi *Pisolithus arhizus* and *Scleroderma* sp. improves early growth of *Shorea pinanga* nursery seedlings. *New Forests* 30: 67–73.

Yuwa-Amornpitak T, Vichitsoonthonkul T, Tanticharoen M, Cheevadhanarak S & Ratchadawong S. 2006. Diversity of ectomycorrhizal fungi on dipterocarpaceae in Thailand. *Journal of Biological Sciences* 6: 1059–1064.