

Growth Response of *Dipterocarpus tuberculatus* and *Shorea roxburghii* Seedlings to *Astraeus odoratus*

Tharnrat Kaewgrajang^{1,2*}, Baramee Sakolrak³ and Uthaiwan Sangwanit¹

¹Department of Forest Biology, Faculty of Forestry, Kasetsart University, 50 Ngamwongwan Rd, Lat Yao, Chatuchak, Bangkok 10900, Thailand

²Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Chatuchak, Bangkok 10900, Thailand

³Department of National Parks, Wildlife and Plant Conservation, 61 Phahonyothin Rd, Lat Yao, Chatuchak, Bangkok 10900, Thailand

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* Corresponding author:

E-mail: ffortrk@ku.ac.th

ABSTRACT

In this study, we investigated the effects of the highly prized ectomycorrhizal (ECM) mushroom, *Astraeus odoratus*, on the growth of *Dipterocarpus tuberculatus* Roxb. and *Shorea roxburghii* G. Don seedlings. The two seedling species were inoculated using two methods: 1) spore suspension of 25 mL/seedling; and 2) hyphal suspension of 25 mL/seedling. On harvesting, it was found that 30-60% of the roots formed by the inoculated seedlings were ectomycorrhizal roots. Seedling development was influenced by the inoculation method and the host plant species. A higher growth response was observed in the *S. roxburghii* seedlings inoculated with *A. odoratus* compared to *D. tuberculatus* seedlings. Moreover, the non-inoculated *S. roxburghii* seedlings had a lower growth response than *D. tuberculatus* seedlings, but their growth response was significantly higher than *D. tuberculatus* seedlings inoculated with a spore or hyphal suspension of *A. odoratus*. However, there was no difference between the effects of the spore and hyphal suspension treatments. Therefore, both the inoculation methods can be used to enhance the growth of seedlings, especially that of *S. roxburghii*.

1. INTRODUCTION

Dipterocarpaceae is one of the most important tree families in the lowland forests of Southeast Asia and also has a major ecological and economic significance. They provide high quality timber and are a source of oils and resin in the area (Brearley, 2011). Owing to a high demand for good quality wood, deforestation has continued at a rapid pace during the years 2000 to 2010, with an annual net loss of forest cover of more than 0.41 million hectares (FAO, 2011). As a result of the high loss of dipterocarp forests in Southeast Asia, there has been an increased interest in establishing plantations of such forest trees and promoting restoration strategies (Kettle, 2010). Success of such strategies consequently depends on mass production of dipterocarp seedlings, which have a good growth rate.

Mycorrhiza is a symbiotic association between fungi and plant roots. Host plants provide photosynthetically fixed carbon and habitat to the fungi, while the mycobionts provide dissolved and

organically bound nutrients, particularly nitrogen and phosphorus, to their hosts (Smith and Read, 2008). Nearly 90% of the terrestrial plants have an associated mycorrhizal fungi (Brundrett, 2009). However, ectomycorrhizal (ECM) fungi are the major colonizers found in the roots dipterocarps (Lee, 2006; Hoang and Tuan, 2008). ECM fungi have been used to enhance the growth of many dipterocarp seedlings species, including *Dipterocarpus alatus* (Kaewgrajang et al., 2013; Kaewgrajang et al., 2014), *Hopea odoratus* (Lee et al., 2008) and *Shorea* spp. (Turjaman et al., 2005; Turjaman et al., 2006; Turjaman et al., 2011; Lee et al., 2008). The number of ECM fungal species has been estimated to be around 20,000-25,000 (Rinaldi et al., 2008; Tedersoo et al., 2010). In dipterocarps, the most frequently found ECM fungi are members of the Thelephoraceae and Russulaceae family, followed by the Cortinariaceae, Sebacinaceae, Clavulinaceae, Boletaceae and Inocybaceae families (Sirikantaramas et al., 2003; Yuwa-Amornpitak et al., 2006; Riviere et al., 2007; Peay et al., 2010; Tedersoo and Nara, 2010;

Phosri et al., 2012). However, only a few species of ECM fungi have successfully produced inoculum and been maintained in pure culture in Southeast Asia (Brearley, 2012). In Malaysia, *Pisolithus* spp. and a member of the Thelephorales are popularly used as an ECM fungal inocula (Lee et al., 2008), while *Scleroderma* spp. are preferred in Indonesia (Turjaman et al., 2005; Turjaman et al., 2006; Turjaman et al., 2011). In Thailand, *Astraeus* spp. have been widely used for producing ECM fungal inoculum because they can produce inoculum for large-scale inoculation and can be maintained on culture media (Kaewgrajang et al., 2013). Moreover, *Astraeus* spp. have multiple benefits which include 1) a high market price of edible mushrooms in Asia (Mortimer et al., 2012; Pavithra et al., 2015); 2) potential as a antitubercular and anticancer agent (Arpha et al., 2012); and 3) potential to improve the growth of seedlings as ectomycorrhiza (Aggangan et al., 2012; Kaewgrajang et al., 2013). There have been several reports on the association of ECM fungi with various tree species in the Dipterocarpaceae, (Phosri et al., 2004), although only the growth of two dipterocarp species-*Anisoptera thurifera* (Aggangan et al., 2012) and *Dipterocarpus alatus* (Kaewgrajang et al. 2013) has been successfully promoted by *Astraeus* spp. Recently, *Astraeus* spp. have been used not only to improve the establishment of dipterocarp seedlings, but have also been used to establish dipterocarp plantations by producing fruiting bodies on the plantation floor. The current study aimed to examine the effects of two different ECM fungal inoculum types of *A. odoratus* spore suspension and pure cultured mycelium on the formation of ECM in *Dipterocarpus tuberculatus* and *Shorea roxburghii* seedlings, as this analysis is yet to be comprehensively reported. We also quantitatively evaluated the effect of the type of inoculation and host plant species on the growth and development of the seedling.

2. METHODOLOGY

2.1 Seed preparation

Mature seeds of two dipterocarp species *D. tuberculatus* and *S. roxburghii* were collected from the Phang-Nga province, in southern Thailand (8°46'5" N, 98°16'7" E, Figure 1). After cutting off the seed wings, the seeds were washed several times with tap water and soaked in water for 24 h. All the seeds were transplanted into a plastic tray filled with

sterilized sand and were then incubated in a sealed polyethylene bag in a nursery. After one week, the germinated seeds, with a root length of 4-5 cm, were selected for fungal inoculation.

2.2 ECM inoculum production

Astraeus odoratus was collected from a natural dipterocarp forest mixed with *Pinus* spp., at the Watchan Royal Project, Kalaya Niwattana district, Chiang Mai province, Thailand (19°05'15" N, 98°19'57" E, Figure 1). Both the spore suspension and mycelial culture of *A. odoratus* were used as inocula, which were prepared by following the method of Kaewgrajang et al. (2013). The spore suspension was prepared by homogenizing air-dried mature spores in distilled water (1:10 v/v), using a blender, for 1 min. The final spore concentration was 1.58×10^8 spores/mL. After the surface sterilization of fruiting bodies with 95% alcohol, the inner tissue of the fungus was initially isolated on a potato dextrose agar (PDA) medium and grown at room temperature for a month. After that, each mycelial disk (1 cm in diameter) was transferred to the PDA, which had been covered with cellophane paper. After a month, the mycelial cultures were taken and homogenized in distilled water (1:3 v/v) using a blender. The final mycelial concentration was 1.48×10^8 mycelial fragments/mL.

2.3 Fungal inoculation

Each germinated seed was dipped into either 25 mL of a spore or hyphal suspension, and the seed was then transplanted into a pot (15 cm × 20 cm), filled with soil fumigated with methyl bromide. The soil (0-10 cm depth) was collected from the Phang-Nga Research Station, Takuapa district, Phang-Nga province, Thailand (8°46'5" N, 98°16'7" E, Figure 1). Its texture was sandy clay loam with a pH ranging between 5.01 and 5.11. Its chemical composition was 6.08% organic matter, 0.19 % nitrogen (N), 3.85 mg/kg phosphorus (P), 39.27 mg/kg potassium (K), 221.75 mg/kg calcium (Ca), and 27.39 mg/kg magnesium (Mg). Each treatment had three replications and each replication had four seedlings. Therefore, a total of 36 seedlings/plant species were examined. After inoculation, the pots were placed on raised benches in a screen-sided greenhouse. The seedlings were irrigated daily with the same amount of water.

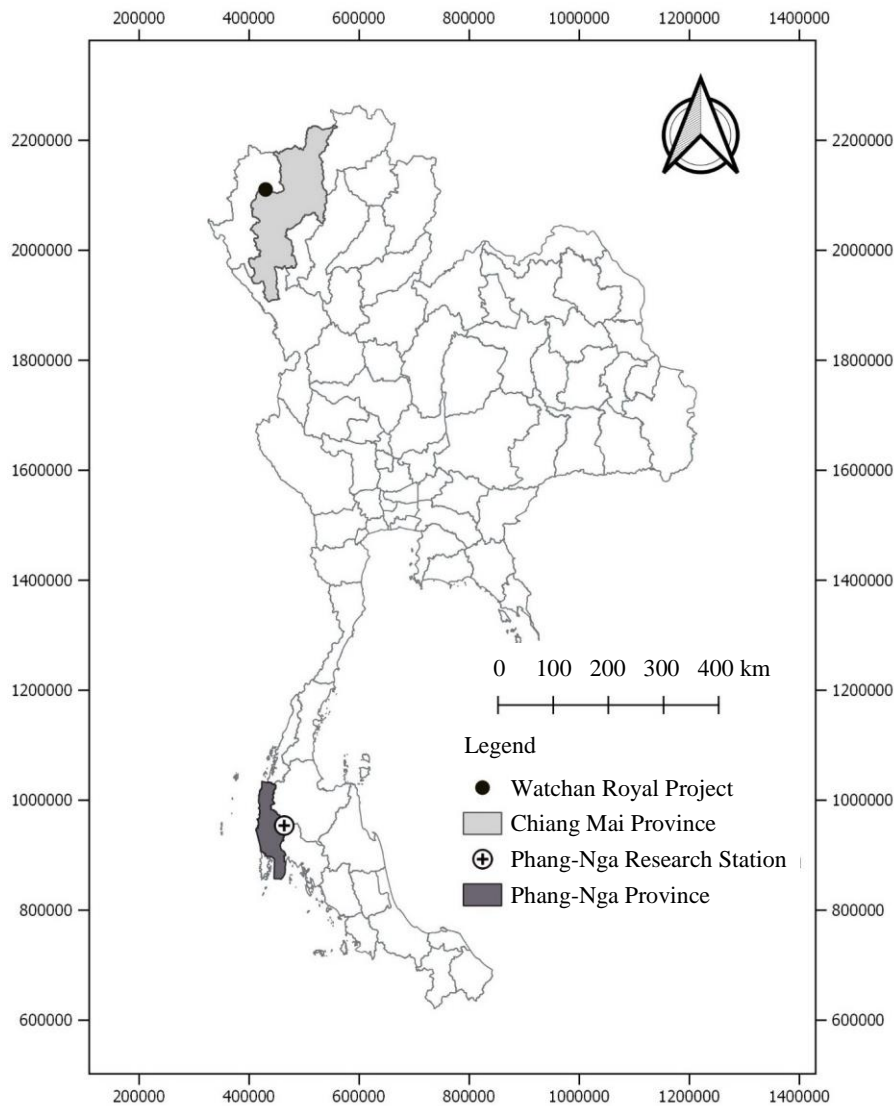


Figure 1. Seeds and soil sampling site at the Phang-Nga Research Station, Takuapa district, Phang-Nga province and *Astraeus odoratus* collection site at the Watchan Royal Project, Kalaya Niwattana district, Chiang Mai province.

2.4 Parameters measurement

All the seedlings were measured once a month for their total height and diameter at the root collar. Eight months after inoculation, each seedling replicate (3 seedlings/treatment) was randomly selected and soil samples were collected using a soil core (2 cm in diameter, 15 cm in depth). Three soil cores were taken from each seedling pot and mixed together to make one soil sample. Each soil sample was washed thoroughly with tap water to remove the soil particles. Cleaned roots were characterized for ECM root morphotypes based on morphological and anatomical characteristics (branching pattern, color, mantle textures, and rhizomorph) according to Agerer (1987-2012). The roots were then cut into lengths of 1 cm and evenly spread on a Petri dish to

determine the rate of ECM colonization by using the gridline intersection method proposed by Brundrett et al. (1996). The remaining roots in the pot were washed thoroughly with tap water to remove any soil debris. The shoot and roots of each seedling were dried in an oven at 60-70 °C for 48 h to measure their dry weight. The concentration of N, P, and K was determined in the shoot of each seedling.

2.5 DNA analysis

Two root tips were selected from each morphotype. Each tip was placed in a 0.2 mL tube containing 25 µL of Dream Tag Green PCR Master Mix (Thermo Scientific, California, USA), 12.5 µL distilled water, 7.0 µL BSA, 2.0 µL DMSO, 1.5 µL ITS1F, and 1.0 µL ITS4. The tube was placed in a

PC-818S Program Temperature Control System (Astec, Fukuoka, Japan) for a polymerase chain reaction (PCR). The PCR reaction involved an initial denaturation step at 94 °C for 5 mins, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 mins, and a final extension step at 72 °C for 10 mins. The amplified PCR samples were purified using a GeneJET PCR Purification Kit (Thermo Scientific, California, USA). The purified PCR products were submitted to Marcogen (Marcogen, Korea) for sequencing. The obtained sequences were analyzed to infer the putative taxa of the fungal group using the basic local alignment search tool (BLAST) (Altschul et al., 1997).

2.6 Statistical analysis

The stem diameter at root collar (SDC), stem height (SH), root dry weight (RDW), shoot dry weight (SDW), total dry weight (TDW), concentration of N, P, and K in the seedlings, and the ECM colonization rate were determined using a two-way analysis of variance. The obtained treatment means, at a significant F-value ($p < 0.05$), were compared using the least significant difference (LSD). To reduce the high variations in ECM colonization, the means were transformed using the arcsin function prior to the statistical analysis. All the statistical analyses were performed using the R Statistical software (Version R386.3.5.1).

3. RESULTS AND DISCUSSION

3.1 ECM formation

In total, over 330 root tips of *D. tuberculatus* and *S. roxburghii*, inoculated with *A. odoratus*, were observed. Two morphotypes were distinguished through the morphological diversity of the inspected fine roots (Figure 2): 1) light orange brown, smooth and shiny, monopodial-pinnate ramification (Figure 2A, 2E); and 2) orange brown to dark brown, smooth and shiny, irregular pinnate (Figure 2C). Both morphotypes had numerous brownish rhizomorphs and sclerotia (Figure 2C). The ECM roots differed from those of the non-inoculated seedlings, with the latter being simple in shape, thin, smooth, brownish in color with less developed branching and mantle with the Hartig net absent. All morphotypes of ECM roots had a well-developed mantle, were 20-30 μm across, consisting of two layers of hyphae and a Hartig net between the epidermal cells (Figure 2).

The molecular extraction of extracted root samples revealed a morphotype-taxon inconsistency in both the morphotypes. A DNA analysis confirmed the presence of *A. odoratus* in the ECM tips with a 97-99% identification rate/confidence (Table 1), in all the morphotypes. This study revealed that *A. odoratus* can produce sclerotia when associated with dipterocarp trees (Figure 2C). These findings are similar to those of Kaewgrajang et al. (2013), who found sclerotia surrounding the roots of *D. alatus* seedlings associated with *A. odoratus*. Sclerotium is a fungal structure which is important for the dispersal and survival under stressful conditions, such as freezing temperatures, desiccation, microbial attack, or the long-term absence of a host. Recently, 11 species of ECM fungi which can form sclerotia, were found (Smith et al., 2015), however *A. odoratus* was absent.

3.2 ECM colonization rates

The inoculated seedlings of the two dipterocarp species had an ECM colonization rate in the range of 32-60%. The type of inoculation method significantly affected the ECM colonization rate (Table 2). No ECM colonization was found in the non-inoculated seedlings. It was observed that the inoculated seedlings (with spore and hyphal suspension) had a significantly higher ECM colonization rate than the controls, however there was no significant difference in ECM colonization rate between the two inoculation methods (Figure 3). Additionally, the ECM colonization rate of *D. tuberculatus* seedlings (32.14%) was not significantly different from that of the *S. roxburghii* seedlings (29.23%) (Table 2). The ECM colonization rate reported in this study was less than that of a previous study (Kaewgrajang et al., 2013), which found that *D. alatus* seedlings had an ECM colonization rate of around 60%. Moreover, *Astraeus*, isolated from the dipterocarp forest, resulted in a higher percentage of root colonization in the roots of *A. thurifera* seedlings (Aggangan et al., 2012). The error bars (indicating standard deviation) on ECM colonization rates were high variation, although the same amount of inoculum suspension was used for each seedling. This could be caused by a non-regular spore viability and the delay in ECM formation in some mycelium fragments (Repáč, 2011).

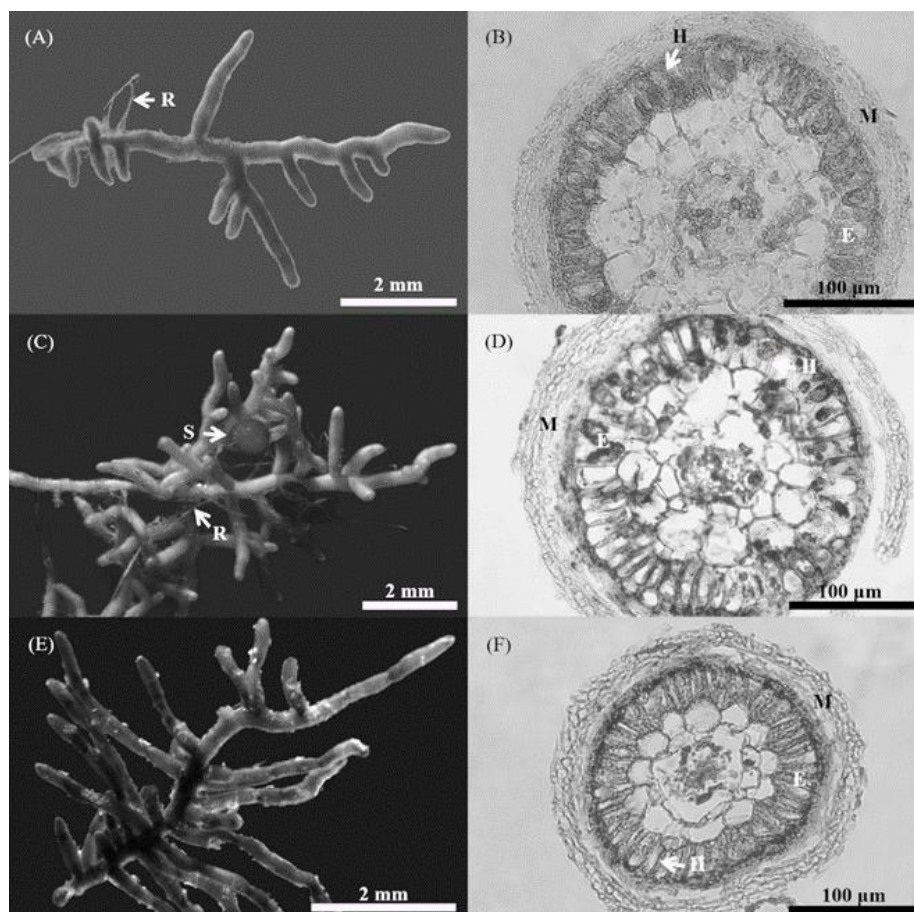


Figure 2. Morphological features and cross-sectioned of ECM roots of *Shorea roxburghii* (A-D) and *Dipterocarpus tuberculatus* (E-F) seedlings associated with *Astraeus odoratus* (R=rhizomorph, S=sclerotium, M=mantle, H=Hartig net, E=epidermal cell).

Table 1. ECM fungal species identified from ECM root tips of *Dipterocarpus tuberculatus* and *Shorea roxburghii* seedlings, and their similarity with those listed in the GenBank database.

Plant species	Inoculation method	Accession No.	Morphotype	BLAST match	Identity (%)
<i>D. tuberculatus</i>	Spore suspension	LC307148	Irregular pinnate	<i>Astraeus odoratus</i> (JQ292818)	97
		LC307151	Irregular pinnate	<i>Astraeus odoratus</i> (JQ292818)	98
		LC307149	Irregular pinnate	<i>Astraeus odoratus</i> (AJ629882)	98
		LC307150	Irregular pinnate	<i>Astraeus odoratus</i> (AJ629411)	99
	Hyphal suspension	LC307144	Irregular pinnate	<i>Astraeus odoratus</i> (AJ629878)	99
		LC307145	Irregular pinnate	<i>Astraeus odoratus</i> (JQ292818)	97
		LC307146	Irregular pinnate	<i>Astraeus odoratus</i> (AJ629878)	99
		LC307147	Irregular pinnate	<i>Astraeus odoratus</i> (AJ629411)	98
<i>S. roxburghii</i>	Spore suspension	LC307156	Monopodial pinnate	<i>Astraeus odoratus</i> (AJ629882)	98
		LC307157	Monopodial pinnate	<i>Astraeus odoratus</i> (AJ629882)	97
		LC307158	Monopodial pinnate	<i>Astraeus odoratus</i> (AJ629882)	99
		LC307159	Monopodial pinnate	<i>Astraeus odoratus</i> (AJ629882)	99
		LC307160	Irregular pinnate	<i>Astraeus odoratus</i> (AJ629411)	99
		LC307161	Irregular pinnate	<i>Astraeus odoratus</i> (JQ292818)	97
	Hyphal suspension	LC307153	Irregular pinnate	<i>Astraeus odoratus</i> (AJ629882)	99
		LC307154	Irregular pinnate	<i>Astraeus odoratus</i> (AJ629411)	99
		LC307155	Irregular pinnate	<i>Astraeus odoratus</i> (AJ629878)	99

Table 2. Result from a two-way analysis of variance (F- and P- values) related to the effects of plant species and ECM inoculation methods on the growth parameters and ECM colonization.

Variable	Plant species		Inoculation		Plant species x inoculation	
	F	P	F	P	F	P
Stem diameter at root collar	1.095	0.316	0.476	0.632	6.850	*
Stem height	17.076	**	3.237	0.075	5.158	*
Shoot dry weight	6.860	0.424	3.111	***	3.390	0.719
Root dry weight	2.819	0.119	1.157	0.347	7.964	**
Total dry weight	6.565	*	3.003	0.088	4.997	*
N	15.099	**	5.768	*	0.417	0.668
P	8.379	*	2.209	0.153	0.204	0.818
K	25.869	***	3.032	0.086	0.404	0.676
ECM colonization rate	0.067	0.801	7.381	**	1.452	0.272

*, **, *** Effect significant at a level of $p < 0.05$, $p < 0.01$, and $p < 0.001$ of significance, respectively.

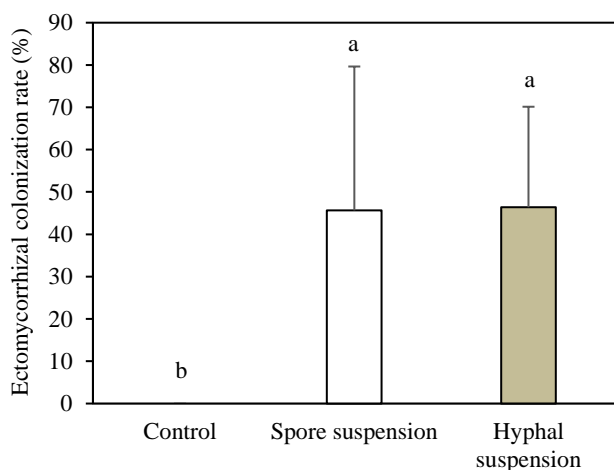


Figure 3. Mean percentage of ECM colonization rates in both the host plants. Columns marked with different letters differ significantly according to least significant difference at $p < 0.01$, with the error bars indicating standard deviation.

3.3 Growth response of seedlings

A significant interaction was detected between the plant species and type of ECM inoculation method, with the stem diameter at root collar (SDC), stem height (SH), root dry weight (RDW), and total dry weight (TDW) (Table 2). The inoculated *D. tuberculatus* seedlings (with spore or hyphal suspension) had no significant differences in SDC, SH, RDW, and TDW when compared with the control treatment. On the other hand, the inoculated *S. roxburghii* seedlings with either methods, had a significantly higher SDC, SH, RDW, and TDW than the control (Figure 4). This was especially the case for RDW and TDW, which were over two times higher than that of the control seedlings (2.13, 2.13-

2.66, respectively). Although the non-inoculated *S. roxburghii* seedlings had the lowest SDC, SH, RDW, and TDW among various treatments, the ECM inoculated *S. roxburghii* seedling had the highest levels of these growth variables. It means that the ECM inoculation by *A. odoratus* affected the growth of the *S. roxburghii* more than the *D. tuberculatus* seedlings.

Our results show that the plant species were significantly different in other growth variables, except SDC, RDW, and TDW (Table 2). Almost all of the significant growth variables indicated that the *S. roxburghii* seedlings had a significantly higher SH (17.380 ± 4.772 cm), N concentration (2.413 ± 0.288 %), and P concentration (0.558 ± 0.120 mg/kg) than the *D. tuberculatus* seedlings (12.168 ± 1.729 cm, 2.012 ± 0.250 %, 0.408 ± 0.104 , mg/kg respectively).

Several nursery experiments have shown that the ECM fungi, such as *Anisoptera thurifera*, can improve the growth and nutrient uptake of dipterocarp seedlings (Aggangan et al., 2012), *Dipterocarpus* spp. (Kaewgrajang et al., 2013; Kaewgrajang et al., 2014; Tapwal et al., 2016), *Dryobalanops lanceolata* (Brearley, 2006), *Hopea odoratus* (Lee et al., 2008), and *Shorea* spp. (Turjaman et al., 2005; Turjaman et al., 2006; Turjaman et al., 2011; Lee et al., 2008). It has been shown in some previous studies that *Astraeus* has been able to promote the growth in some dipterocarp seedlings (Aggangan et al., 2012; Kaewgrajang et al., 2013). Aggangan et al. (2012) reported that inoculation by *Astraeus*, isolated from a dipterocarp forest, resulted in a higher percentage of

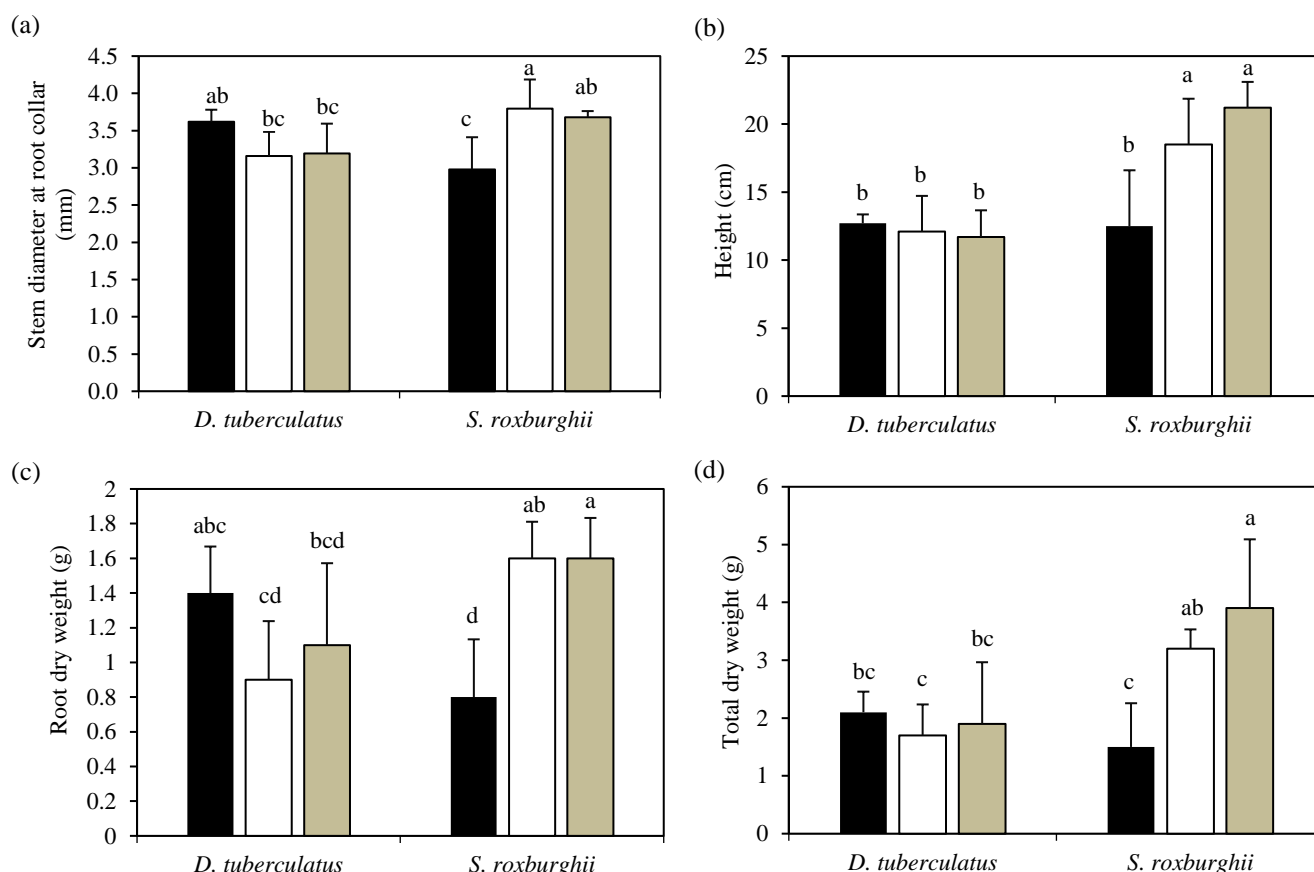


Figure 4. (a) Stem diameter at root collar (SDC); (b) height (SH); (c) root dry weight (RDW); and (d) total dry weight (TDW) of the *Dipterocarpus tuberculatus* and *Shorea roxburghii* seedlings, inoculated with different methods. Columns marked with different letters indicate significantly different values between the plant species and ECM inoculation method combinations ($p < 0.05$), with the error bars indicating the standard deviation.

root colonization in *A. thurifera* seedlings, while at the same time increasing the height increment and P uptake. Additionally, the results of Kaewgrajang et al. (2013) revealed that the *A. odoratus* not only had the potential to promote the growth of *Dipterocarpus alatus* but could also produce sporocarps in the experimental pots. Nevertheless, the growth response of *D. tuberculatus* and *S. roxburghii* seedlings, inoculated by *A. odoratus*, is yet to be comprehensively reported. In this study, it was observed that the error bars (indicating the standard deviation) in all growth variables were high. This could be due to genetic variations in seeds. However, we observed a significant interaction between the plant species and the type of ECM inoculation method. It can be concluded that the seedling development was affected by both factors, therefore making it impossible to examine the effect of the inoculation method on the growth alone. The ECM inoculation by *A. odoratus* positively affected the growth of *S. roxburghii* seedlings only. This

conclusion is supported by an enhanced SDC, SH, RDW, and TDW. The results showed that the non-inoculated *S. roxburghii* seedlings had a lower growth response than *D. tuberculatus* seedlings, but their growth response was significantly higher than *D. tuberculatus* seedlings inoculated with a spore or hyphal suspension of *A. odoratus*. It could be that the inoculation with ECM fungi may not always have a positive effect on the growth of the host plant. A similar finding was reported by Holuša et al. (2015), who observed that some plots of inoculated and control oak seedlings had no significant difference in the root dry weight, total dry weight, and maximum root length. Seedling response to inoculation can be affected by several factors, including the type of inoculum, the inoculation pattern, interspecific and intraspecific host-fungus variation, environmental conditions, and seedling production practices (Repáč, 2011). Therefore, the screening of fungal species and inoculum type are important for a successful production of ECM

seedlings in the nursery. Given a high potential of promoting the growth of *S. roxburghii* seedling inoculated with *A. odoratus*, it should be recommended to produce faster growth of seedlings before transplanting in the field conditions. Further studies should focus on screening other potential ECM fungi, as well as investigating the suitable inoculation types to promote the growth of *D. tuberculatus*.

4. CONCLUSIONS

Our results reveal that the growth and development of seedlings was affected by the inoculation method and the species of the host plant. The inoculated *S. roxburghii* seedlings experienced a higher growth compared to the inoculated *D. tuberculatus* seedlings. Moreover, the *S. roxburghii* seedlings, inoculated with either method (spores and hyphal suspension), had a significantly higher SDC, SH, RDW, and TDW than the control (non-inoculated seedlings). This was especially the case with RDW and TDW of the inoculated *S. roxburghii* seedlings, which were over two times higher than the control seedlings. Therefore, both the *A. odoratus* inoculation methods can be used to produce *S. roxburghii* seedlings for promoting faster growth of the seedlings. At the same time, other suitable ECM fungi, which can improve the growth of *D. tuberculatus* seedlings, should also be examined.

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