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Inoculation Methods and Mycorrhizal Formation on Dipterocarp Seedlings under Greenhouse Conditions

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Abstract

Dipterocarps are one of the most important families of timber trees in Thailand and Southeast Asia. They form ectomycorrhizal (EcM) symbiotic root-inhabiting fungal association with hundreds of EcM fungal species and rely on them for their nutrient acquisition. In this present study several inoculation experiments were applied to dipterocarp seedlings in greenhouse. After 6 months growth performance including roots infection were measured. Generally it found that EcM colonization significantly increased stem height, diameter and biomass. The internal transcribed spacer (ITS) region of the ribosomal DNA was analyzed for EcM infection in plant roots.

Keywords: Dipterocarp, Ectomycorrhizas, Tropical trees, ITS

Introduction

Dipterocarps are one of the most important families of timber trees in Thailand and Southeast Asia. Similarly to many other tropical countries not only degradation of forest cover but also a demand for a large quantity of timber for construction and industrial uses is rising. There is therefore an interest in reforestation programme to maintain sustainable wood supplied products and forest area. Although many reforestation programmes have focused on fast growing exotic trees but to provide the highest quality timber, reforestation should also have to focus on the Dipterocarpaceae (Brearley 2011). Member of Dipterocarpaceae form ectomycorrhizal (EcM) symbiotic root-inhabiting fungal association with hundreds of EcM fungal species and rely on them for their nutrient acquisition. Several studies based on fruiting body survey revealed that the most common fungal species found in dipterocarp forests are the families of Amanitaceae, Boletaceae and Russulaceae (Watling & Lee 1995, 1998, 2007, Lee et al. 2002, 2003). More recent studies using molecular techniques have revealed that member of the Thelephoraceae are the most common ectomycorrhizal fungi found in association with dipterocarp followed by Russulaceae and Sclerodermataceae (Sirikantaramas et al. 2003, Yuwa-Amornpitak et al. 2006, Phosri et al. 2012).

During a visit to a royal reforestation initiative project in Nong Aueng subdistrict, Yasothon province, Thailand in 2011, roots of 3-year olds *Dipterocarp alatus* trees were

observed to be heavily colonized by whitish and brownish fungi with fruiting bodies of *Astraeus odoratus* were found adjacent to the trees. An examination under stereo- and compound microscopes shown that roots were well infected by ectomycorrhizas. In present study several inoculation experiments are established to test a potential use of different inoculation methods in enhancing the growth of dipterocarp seedlings in the forest nursery.

Experimental and Methods

Isolation and culture of fungi

Fungal isolates were initially obtained from fresh basidiomes. *Astraeus odoratus* were collected from the forest plantation plots in Nong Aeung area. The cultures were routinely maintained on modified Norkran's "C" agar medium (MNC) as described by (Yamada & Katsuya 1995) at 30°C with subculturing every 1-2 months. A 6 mm cork borer was used to cut plugs of inoculum from the edge of 2-3 week-old fungal colonies. These fungal plugs were used to start mass culture in MNC liquid media for inoculation. (Yamada & Katsuya 1995)

Seeds preparation

Dipterocarpus alatus selected as hosts for testing because of their potential use in reforestation and commercial planting in Thailand. Seeds were stored at 4°C. Healthy, uniformly sized seeds were selected for use and then stratified for 24 hours at 4°C in distilled water to break dormancy. Seeds floated on the surface of water were discarded since it has shown that seeds behaving in this way are difficult to germinate (Royal Forest Department) whilst seeds which sank in water were retained for surface sterilization. To surface sterilized, seeds were pretreated with 70% ethanol for 3-5 minutes followed by soaking in 30% hydrogen peroxide for 30 before rinsing in 3 changes of sterile water. Seeds were germinated on sterile, moist sand for 2 weeks.

Inoculation preparation

Vermiculite and peat moss (ratio of 1:1) were used as substrate (pH 5.4). They were autoclaved at 121°C for 20 min and left overnight. The mass cultures were grown on vermiculite-peat moss soaked with MNC solution in Erlenmeyer flask. The flasks and their contents were autoclaved for 20 minutes and each flask was inoculated with fungal culture grown in liquid as previously described. The flasks were incubated at 28-30 °C for 2-3 months. The method for preparation soil inoculum and spore inoculum were described by Marx & Bryan (1975) and Kaewkrajang (2008), respectively.

Inoculation of seedlings

One hundred ml, 50 ml and 20 ml of pure mycelium culture, spore suspension and soil inoculum, respectively, were used for inoculation for each *Dipterocarpus alatus* seedlings. There were 10 replicate per treatment including control. A sterilized sand and top soil mixture (ratio 2:1 v/v) were used as substrate. Control seedlings had no inoculum. All seedlings were grown for 6 months and roots were examined for ectomycorrhizal formation (Phosri et al. 2012). After 6 months seedlings were removed from each pot and measured for growth performance i.e. root collar diameter, height increasing, dry weight.

EcM root segments were stored in CTAB buffer (1% cetyltrimethylammonium-bromide, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, and 20 mM EDTA) for DNA extraction. Molecular analyses was followed Phosri et al. (2012) with the universal primers ITSOF-T/LB-W as following 5' acttggtcatttagaggaagt3' and 3'ctttcatctttccctcacgg5' (Tedersoo et al. 2008).

Results and Discussions

Seedling growth

Root collar diameter

Root collar diameters of seedlings inoculated with fungal inocula were higher than control seedlings in all treatments. It was greatest for spore suspension followed by vermiculite+peat moss inoculum, soil inoculums and the control, respectively. Analysis of variance showed no significant differences between spore suspension and vermiculite+peat moss inoculum, but there were significant differences when compared with control seedlings with the exceptional of soil inoculums and control (Fig. 1).

Seedling height

No visual differences in seedling growth were obvious between treatments in the greenhouse. Seedling height was greatest with spore suspension, followed by soil inoculum, vermiculite+ peat moss inoculums and control, respectively. Analysis of variance for seedling height showed no significant differences between any inoculation treatments and the control (Fig. 1).

Shoot dry weight

Inoculation with spore suspension resulted in the highest shoot dry weight followed by soil inoculum, vermiculite+ peat moss inoculum, and control, respectively (Fig. 1). Analysis of variance, however, showed that shoot dry weights of seedling inoculated with spore suspension was significantly different compared to other control seedlings (Fig. 1).

Root dry weight

Inoculation seedlings with soil inoculum resulted in the highest root dry weight followed by spore suspension, vermiculite +peat moss inoculums and control respectively. Nevertheless, analysis of variance revealed that root dry weights of seedlings were not significantly different compared to control seedlings (Fig. 1).

Ectomycorrhizal infection

Dipterocarpus alatus seedlings were harvested after 6 months. Short roots were randomly examined under the microscope. Generally it was found that monopodial and Y-shaped short roots could be detected (Fig. 3a & b). This is thought to be characteristic of ectomycorrhizas. Mantle was obviously formed. Many of these root tips were associated with hyphae, suggesting that the fungi were beginning to encase the root tips. The outer mantle region of ectomycorrhizas showed intertwining hyphae with clamp connections. For vermiculite+peat moss and spore suspension, ectomycorrhizas were monopodial, unbranched, lateral roots were encased with fungal mantle, umber to brown in colour, straight tip and the length of system varied between 3-5 mm. (Figs. 3b). The outer mantle region of ectomycorrhizas showed intertwining hyphae with numerous clamp connections

(Fig. 3g & h). The ectomycorrhizas formed in seedlings by soil inoculums were recognized morphologically by their blunt-ended shape and the distinctive white colour (Fig. 3a). Lateral roots were colonized along their entire length with dense hyphae. The length of the system ranged from 6-10 mm. (Fig. 3a). The outer mantle region of ectomycorrhizas showed intertwining hyphae with clamp connections (Fig. 3i). Emanating hyphae were also easily seen (Fig. 3c).

Seedling lateral roots inoculated with spore suspension showed the highest colonization (87%). However, the percent of colonized roots were not significantly different ($p = 0.05$) between spore suspension, vermiculite+peat moss inoculum and soil inoculum. There were significant differences between those three treatments and the control (Fig. 2).

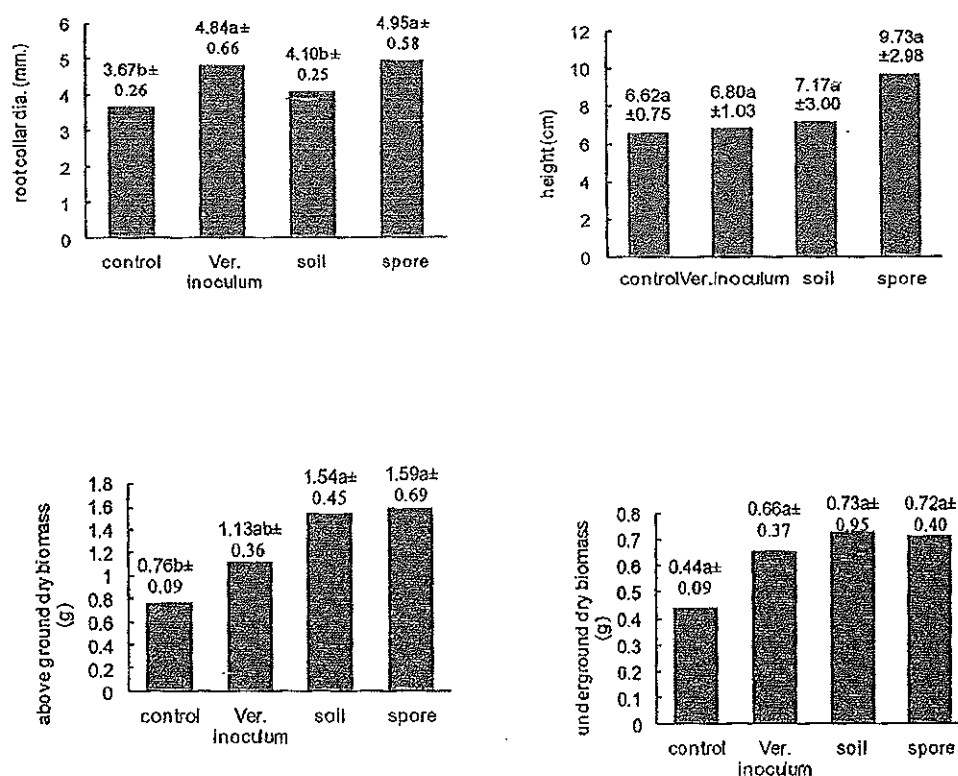


Fig.1 Growth performance of seedlings after 6 months . Number within column not sharing a common letter differ significantly ($p=0.05$) by Duncan's multiple ranges test comparison (DMRT).

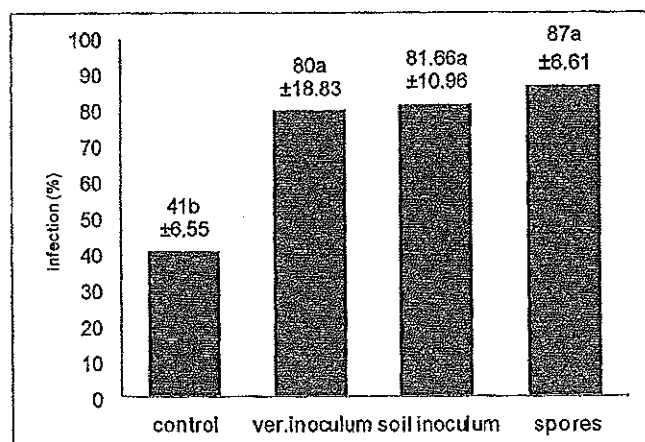


Fig.2 Percentage of ectomycorrhizal infection in seedlings root tips after harvesting. Number within column not sharing a common letter differ significantly ($p = 0.05$) by Duncan's multiple ranges test comparison.

Molecular detection

ITS sequences of ECM root tips were amplified using primer ITSOF-T/LB-W. The total length of the ITS amplified with these primer was 800-1,000 bp. Although Ectomycorrhizal tips were observed in seedling after inoculation with spore inoculum, however, we did not obtain ambiguous sequences from spore inoculums. The results of BLAST comparison ranged from 88-99% similarity (data not shown). *Russula* sp. was found to be colonized in control seedling's root tips with 97-99% similarity (DQ778002.1 & DQ422001.1). This could be a result from cross-contamination. Root tips from soil inoculums showed 88-90% similarity to uncultured ectomycorrhizas (JN969399.1 and FM999497.1). The species with the highest blast similarity of 91% in the NCBI database for vermiculite+peat moss inoculum was uncultured *Tomentella* sp. (FR852161.1). Our study has similar result to several works, that member of the Thelephoraceae are the most common ectomycorrhizal fungi found in association with dipterocarp followed by Russulaceae and Sclerodermataceae (Sirikantaramas et al. 2003, Yuwa-Arnornpitak et al. 2006). In more recent study in Thailand, Phosri et al. (2012) found Russulales and Thelephorales to be the most important taxa.

In the present study, all inoculation methods improved the growth of seedlings although there was some EcM colonization of the controls. Percentage of EcM colonization was higher when seedling were inoculated with spores than with mycelium and soil inoculum. This is in agreement with Turjaman et al. (2006). They found that EcM colonization was ranges from 61-65% when seedlings were inoculated with spore whereas EcM colonization was lower, ranges from 35-37% when seedlings were inoculated with mycelium. It is apparently that there are significant challenges in conducting field experiment (Tata et al. 2010). The main one being that it is very difficult to create truly

non-mycorrhizal controls especially where EcM inoculum is already present in the soil. Pure mycelium inoculums may not remain viable if they are poorly adapted to the biotic or abiotic environments of the greenhouse or forest soils (Change et al. 1994, 1995). Therefore our pure culture inoculum could not be established with tree host seedlings.

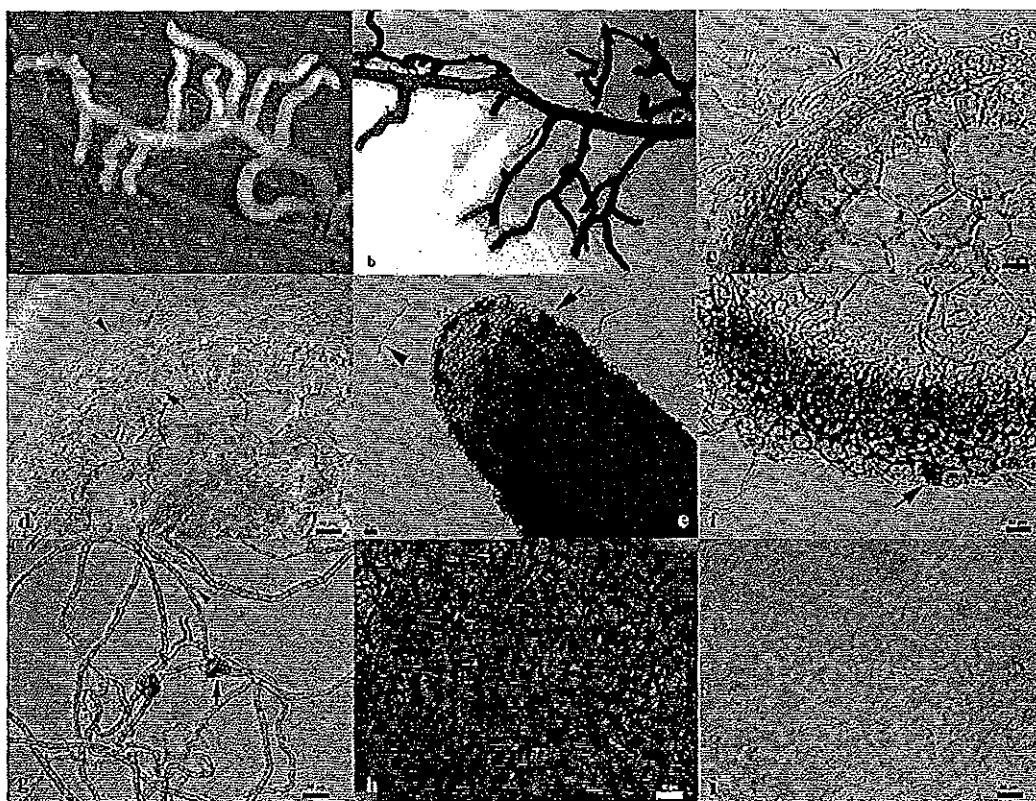


Fig.3 Establishment of Ectomycorrhizas in dipterocarp seedlings. (a) EcM morphotype from soil inoculum, (b) EcM morphotype from spore suspension, (c-d) root tip cross section demonstrated mantle (arrowhead) and hartig net (arrow) inoculated with soil inoculum, bar=10 μ m (e) root tip in plain view exhibited EcM colonization with mantle (arrowhead) and external hyphae (arrow) inoculated with spore suspension, bar = 10 μ m, (f) root tip in cross section showed thick mantle (arrowhead) inoculated with spore suspension, bar = 10 μ m, (g) external hyphae showed clamp connection (arrowhead) and collapsed spore (arrow) inoculated with spore inoculum, bar = 10 μ m, (h) plectenchymatous mantle inoculated with spore inoculum, bar = 10 μ m, (i) plectenchymatous with loose hyphae inoculated with soil inoculum, bar = 10 μ m.

Conclusions

In the present study ectomycorrhizal formation by several inoculation methods with *Dipterocarp* seedling were demonstrated. Experiment approach to producing seedling colonized by EcM fungi for nursery bed with practically method could be achieved. With the molecular technique, EcM colonization in *D. alatus* seedlings can be identified as a member of the Thelephoraceae and most likely a species of *Tomentella*. Our results further confirm the occurrence of the thelephoroid fungi forming EcM on the root of the Dipterocarpaceae.

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