Inoculation of Dipterocarps *Anisoptera thurifera* and *Shorea guiso* with Ectomycorrhizal Fungi in Philippine Red Soil

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Dipterocarpaceae is the most important tree family in the tropical forests of Southeast Asia and the Philippine dipterocarp forests are famous for their high diversity and for the dominance of this family in its lowland forests. Unfortunately some of the species in the country are already considered as endangered. This experiment aimed to develop protocol in the production of quality rooted cuttings of *Anisoptera thurifera* (Blanco) Blume and *Shorea guiso* (Blanco) Blume for plantation experiments in red soil of Caliraya, Laguna, Philippines using controlled inoculation with ectomycorrhizal (ECM) fungi. Rooted cuttings of *A. thurifera* and *S. guiso* were prepared and inoculated with mycelial beads containing vegetative mycelia of ECM fungi: *Pisolithus* sp.1 (from *Acacia* coded internationally as PTG), *Pisolithus* sp.2 (from *Eucalyptus* coded internationally as H6394) or *Astraeus* sp. (from dipterocarps) and grown under nursery conditions. After four months, *Astraeus* sp. and *Pisolithus* sp.2 gave the highest levels of root colonization and height increments in *A. thurifera*. *Pisolithus* sp.1 promoted the highest fine root P concentration (1.37 mg/g) and P uptake (0.273 mg/root) while *Astraeus* sp. promoted the highest (0.199 mg/root) coarse root P uptake. In *S. guiso*, *Pisolithus* sp.2 promoted better growth and root P uptake than *Pisolithus* sp.1. *Pisolithus* sp.2 increased height increment (116%), root (26%), shoot (36%), and total (54%) plant dry weight over the control treatment. This fungus increased root P concentration and uptake by 15% and 153%, respectively, relative to the uninoculated control counterpart. Uninoculated cuttings had the lowest height increment, dry weight and root P concentration and uptake. In conclusion, *Pisolithus* sp.2 and *Astraeus* can be used to inoculate *A. thurifera* and *Pisolithus* sp.2 for *S. guiso* rooted cuttings in order to have quality planting materials for plantation establishment in red soil in Caliraya, Laguna and other reforestation sites in the Philippines with similar soil conditions.

Key Words: *Astraeus*, cuttings, dipterocarps, ectomycorrhiza, *Pisolithus*

INTRODUCTION

The Dipterocarpaceae is the most important tree family in the tropical forests of South-east Asia (Brearley 2011). The Philippines has a total land area of 30 million hectares which was by nature covered with tropical rain forest dominated by dipterocarps (Garrity et al. 1992). The Philippine dipterocarp forest was famous for their high diversity (Newman et al. 1996) and for the dominance of this family in its lowland forests, where dipterocarps contributed 94% of the timber volume (Soerianegara & Lemmens 1994). Nowadays, only few remnants of
dipterocarp forests are left in the Philippine archipelago. As of 1999, the forest cover of the Philippines was estimated at 18.3% of total land area, and the extent of primary forest at 2.7% (ESSC 1999). Most dipterocarp forests in the Philippines vanished due to logging, shifting cultivation and transformation into settlements or agricultural fields (Langenberger 2006). Philippine dipterocarps such as *Anisopterathurifera* (Blanco) Blume and *Shorea guiso* (Blanco) Blume are now considered as endangered species. Fast exploitation was due to their good wood and fiber qualities for a range of products.

Establishment of dipterocarp plantations or enrichment planting in the existing forest plantations with dipterocarps, is one of the top priorities of the Philippine government. This will ensure an adequate and sustained supply of the best quality timber and other wood products for local and export market, and to conserve endangered species of Philippine dipterocarps (Aggangan et al. 1997). Field plantings have been unsuccessful due to soil acidity. Mycorrhizal fungi work well in marginal soil with acidic pH (Harley & Smith 1983).

Dipterocarps form symbiosis with ectomycorrhizal (ECM) fungi (Pampolina et al. 1994; Hoang and Tuan 2008). ECM fungi have shown a pivotal role in the growth and survival of seedlings in degraded sites (De la Cruz et al. 1988). ECM fungi mobilize plant water and nutrient uptake via hyphae and increase plant resistance to environmental stresses (Harley & Smith 1983; Mejstrick 1989; De la Cruz & Aggangan 1990; Smith & Read 2008). Production of dipterocarp planting materials could be from wildlings, seeds or from cuttings. Collected wildlings are possibly infected with indigenous mycorrhizal fungi but the effectiveness in terms of growth promotion when outplanted in the field is not certain. Planting materials from seeds or from cuttings are of advantage where controlled inoculation with preferred plant growth promoting species or strains of mycorrhizal fungi can be done. Macropropagation by cuttings is usually done to produce planting materials in spite of the danger of genetic erosion. The growth of dipterocarp cuttings is normally slower than from seeds and this requires longer rearing period in the nursery before they can be outplanted in the field. Controlled inoculation with mycorrhizal fungi will foster growth of nursery grown rooted cuttings and field planting can be done earlier thus shortening the growing period in the nursery and at the same time attaining the required height of planting materials (Malajczuk et al. 1994; Aggangan 1996; Turjaman et al. 2006).

ECM inoculants could be rhizosphere soil from dipterocarp forests, spores or vegetative mycelia. Soil from dipterocarp forests can be used to inoculate seedlings or cuttings grown in the nursery but it may contain both beneficial (e.g. mycorrhizal fungi) and harmful (pathogenic fungi) microorganisms. The use of spores or vegetative mycelia of ECM fungi is the most convenient and practical technique. Spores are extracted from mature fruit bodies which usually appear during the onset of the rainy season. The National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB) process the spores into tablet form with brand name “MYCOGROE”. The drawback is the supply of spores which is seasonal and dependent on the appearance of fruit bodies during the onset of the rainy season. Normally, our group go out in forest plantations during the onset of the rainy season (usually July to August) to collect fruit bodies. These days, however, rainy season is unpredictable. For example, for the past few years, rainfall started as early as March but there were no fruit bodies. Moreover, some ECM fungi belonging to the genera *Scleroderma*, *Suillus*, *Russula*, *Astraeus*, etc. are edible at young stage, thus, the indigenous people (upland communities) collect these mushrooms and puffballs for food or as additional income. Mature fruit bodies are needed for the production of Mycogroe tablets. Sustainable supply of Mycogroe is not assured. Vegetative mycelia can be taken from young fruit bodies and can be produced in aseptic culture. ECM fungi such as *Scleroderma*, *Suillus*, *Astraeus*, and *Pisolithus* can be grown and mass produced in aseptic culture thus offering continuous supply of ECM inoculum. Mycelia are macerated and can be applied as slurry or can be encapsulated in alginate beads.

Many soils in the Philippines are acidic (pH 3-5), with low nutrient availability especially nitrogen (N) and phosphorus (P) and lack other essential nutrients (Maglinao 1988). These soils geographically occur throughout the country (Atienza 1989). Caliraya, Lumban, Laguna is one of the problem sites for reforestation because the soil is very acidic (pH between 4 to 5) and contains high concentration of iron (around 50-100ppm) making the color red due to iron oxides. Planted fast growing reforestation species such as mahogany (*Swietenia macrophylla*) and *Acacia mangium* and even the native plant species in the area exhibit poor and stunted growth with yellow leaves indicating either deficiency of essential nutrients such as phosphorus or toxicity symptoms. Due to acidity, applied inorganic fertilizers could be readily fixed making it unavailable for plant use (Brady & Well 1999). At present, the Caliraya-Lumot watershed dam which is generating 40,000 horsepower hydroelectric power for Manila and Southern Luzon (personal communication with Greg Paredes, NAPOCOR, Lumban, Laguna), is barely surrounded with forests. Thus, there is an urgent need to develop techniques that will ensure high survival and fast growth of reforestation species including dipterocarps specifically for Caliraya and for degraded lands similar to that of Caliraya red soil. Example of places with red soil are in Tanay and Antipolo in the province of Rizal, Novaliches and in Guimaras.
The main goal of this research was to develop protocol in the production of quality rooted cuttings of *A. thurifera* and *S. guiso* for plantation establishment or enrichment planting in red soil of Caliraya, Laguna, Philippines. Specifically, it aimed to determine the effect of three ECM fungi on the early growth and development of the two aforementioned dipterocarp species using controlled inoculation technique, to quantify P levels present in the mycorrhizal and non-mycorrhizal plant system and to select ECM fungi that will facilitate early growth and nutrient accumulation in *A. thurifera* and *S. guiso* rooted cuttings.

**MATERIALS AND METHODS**

**Experimental design:** Two separate experiments (*A. thurifera* and *S. guiso*) were conducted concurrently using three strains of ECM fungi in a screenhouse at the College of Forestry and Natural Resources (CFNR) and at the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines at Los Baños, College, Laguna, following a Randomized Complete Block Design (RCBD) with 20 replicates. The inoculation treatments were: uninoculated (Control), inoculated with *Pisolithus* sp.1 (PTG), inoculated with *Pisolithus* sp.2 (H6394) and *Astraeus* sp. (99-4). Three fungi were used in *A. thurifera* and only two (*Pisolithus* spp.) in *S. guiso* due to unavailability of *Astraeus* sp. during the time of inoculation.

**Soil collection and preparation:** The soil (0 – 15 cm depth) used was collected in a grassland area in Caliraya, Lumban, Laguna and was brought to BIOTECH, UPLB. Caliraya soil was chosen because it is one of the problem soils in the Philippines for reforestation. The soil is characterized as very acidic (pH between 4 to 5) and contains high concentration of iron (around 50-100ppm) thus giving to the soil, a bright red color due to iron oxides. The texture is clay loam with an acidic pH ranging from 4.2 to 5.0 (1:2 soil: 0.005 M CaCl₂) (Table 1). The chemical analysis are as follows: 1.12% organic matter (Walkey-Black Method by Walkey & Black 1947), and 0.03% N (Modified Kjeldahl Method by Black 1965), and 3.51 ppm available P (Bray No. 2 by Dewis & Freitas 1970), Exchangeable cation are (me/100 g soil): 0.32±0.6 K, 0.09±0.01 Mg, 3.26±0.2 Ca, 62.4±10 Al and 50±27 Fe. Cation exchange capacity is 219±40 meq/kg.

The soil for experimentation was air-dried, pulverized and passed through a 5 mm screen and oven-dried for 3 days at 100°C. Two hundred fifty grams of dry soil were dispensed into 4" x 8” PE bags.

**Ectomycorrhizal inoculants:** The species of ECM fungi were *Astraeus* sp., and two strains of *Pisolithus* (*Pisolithus* sp.1 coded internationally as H6394). These isolates are collections in the Mycorrhiza Laboratory, BIOTECH, UPLB, College, Laguna. *Pisolithus* sp.1 was collected under *Acacia mangium* stand in Malaysia, *Pisolithus* sp. 2 from *Eucalyptus* trees growing in a nickel site in New Caledonia and *Astraeus* sp. from dipterocarp forest in Mt. Makiling, Philippines. *Astraeus* sp. produces dark colored hyphae while *Pisolithus* spp. produce golden yellow mycelia on Modified Melin Norkrans medium (MMN, Marx 1969). Three ECM fungi were used in *A. thurifera* while only the two *Pisolithus* were used in *S. guiso* due to insufficient *Astraeus* sp. cultures.

ECM fungi were initially grown on MMN agar medium. After three weeks, mycelial disks (1 cm diameter) were taken from the actively growing portion of the culture and were transferred in MMN liquid medium. Fungi were grown in MMN solution at 28°C before being fragmented in a blender and subsequently entrapped in calcium alginate. Mycelial beads were produced from macerated four-week old culture. Inocula were prepared by immobilization of mycelium in 4% sodium alginate following the 8:10 ratio (alginate and mycelium suspension) technique of Rodriguez et al. (1999). This ratio gave well-formed beads and highest viability for ECM fungi *Paxillus involutus* and for *Pisolithus tinctorius* (Rodriguez et al. 1999). Broth culture of mycelium was mixed with alginate soluble in hot water and cooled to 30°C. Then the solution containing mycelium and alginate was dropped into a sterile solution of 0.7 M CaCl₂. The beads formed were washed in sterile distilled water prior to inoculation to rooted cuttings.

**Dipterocarp species and propagation:** *Anisoptera thurifera* (common name: palosapis) and *S. guiso* (common name: guiho) were chosen because of their high commercial value. Stem cuttings were obtained from selected parent representatives maintained at the College of Forestry and Natural Resources, UPLB, College, Laguna. The cuttings were cut into 2 nodal length (approximately 2-4 inches), rinsed with water, the leaves were cut into halves to reduce respiration which can facilitate faster rooting and recovery, treated with rooting hormone (100 ppm IAA) and grown in trays containing sterilized river sand. The trays were placed inside plastic bags to maintain humidity that favors rooting.

**Ectomycorrhizal inoculation:** Inoculation was done during transplanting of rooted cuttings (two months) into 4”x 8” PE bags filled with red soil. The rooted cuttings were inoculated with vegetative mycelia of *Pisolithus* and *Astraeus* sp., entrapped in alginate bead at a rate of one bead per seedling. The seedlings were watered to field capacity. The inoculants were placed approximately 4 cm depth, beneath the root system of the plant. The inoculated rooted cuttings were placed inside plastic bags to maintain...
relative humidity and reduce desiccation, which can foster faster seedling recovery. One plastic bag contained cuttings with similar fungus to reduce cross contamination. After 2 weeks, the plastic bags were partially opened and were gradually exposed from low (25-50%) to higher light intensity (75-85%) for another 2 weeks.

**Parameters measured**

1. **Height and Height increment.** Initial height of the sprouted shoot (not the original planting stock) was measured during transplanting. Height of the sprout was measured from the node where the sprout originated up to the apical shoot tip. Measurement was done once a month for 4 months. Height increment was calculated as the difference between the monthly height measurement and the initial height.

2. **Diameter.** Initial diameter was measured during transplanting and the final diameter was measured after four months. Diameter increment was calculated similar to that of height increment. Diameter of the sprout was taken 1 cm above the node where it originated.

3. **Plant Biomass.** The dipterocarps were harvested at the end of the fourth month. The sprouted shoot was cut at the base of the node of the original parent material while the roots were also cut where they originated. The fine roots (diameter less than 0.5 mm) and the coarse roots were separated. Fine roots, coarse roots and the shoots were wrapped separately with tissue paper, oven-dried at 70°C for two days and dry weights were obtained.

4. **Mycorrhizal infection.** Fine root samples were chopped into 2-3 mm length and evenly spread in Petri dishes to assess mycorrhizal colonization under a dissecting microscope. Mycorrhizal colonization was assessed using the gridline intersection method (Giovannetti & Mosse 1980).

5. **Phosphorus concentration and uptake.** Powdered (0.5 g) dried fine roots and shoots from each treatment was digested with concentrated H₂SO₄ and 30% H₂O₂. P concentration was obtained by colorimetric method (Cunniff 1995). Phosphorus uptake was computed as the product of plant weight (in g/plant) and P concentration (in mg/g sample).

**Root sectioning and micrography:** The microtechnique work was done at the Electron Microscopy Laboratory, BIOTECH, UPLB to verify degree of colonization by ECM fungi. Root samples underwent gluteraldehyde fixation, buffer washing, series of dehydration and infiltration and flat embedded in LR (London Resin) white. Semi-thin sections were obtained using Latta and Hartmann triangular glass knives after which cut sections were stained with toluidine blue or methylene blue. The samples were observed and photographed under a light microscope.

**Statistical analysis:** Data on height increment, diameter, P concentration, P uptake, mycorrhizal colonization and root, fine root, coarse root and total dry weight were analyzed using analysis of variance (ANOVA) of raw data and treatment means were compared using Duncan’s new Multiple Range Test (DMRT) and Least Significant Difference (LSD) at p<0.05 (Duncan 1955). The relationship between plant growth and plant P concentration and uptake with mycorrhizal infection was evaluated using regression analysis. Statistical analyses were done using MSTATC statistical computer program (MSU 1989).

**RESULTS**

Mycorrhizal infection: Roots colonized by *Pisolithus* were bright golden yellow (Figs. 1a-c). On the other hand, roots colonized by *Astraeus* were dark brown with numerous external mycelia prominently ramifying in the soil (Fig. 1d). The color of colonized roots was similar to the color of the mycelia in aseptic culture. The white mycorrhizas in Fig. 1e were infected with *Scleroderma* sp. while the other white mycorrhizas in Fig. 1f were infected with an unidentified ECM fungus. The visual difference of roots infected by *Scleroderma* and the unidentified ECM fungus was that root system (root tips and old roots) colonized by the former was heavily covered with mycelia or hyphae. By contrast, the unidentified ECM fungi colonized only the root tips and hyphae from the entire root tips appeared as rootlets. The contaminant ECM fungi (*Scleroderma* and unidentified fungus) did not infect the roots *A. thurifera* inoculated with the introduced *Pisolithus* and *Astraeus*.

Roots of both dipterocarp species colonized by any of the ECM fungi tested could be readily seen by the naked eyes. The inoculated plants had fine root tips fully covered with thick interwoven hyphae (Fig. 2). The mycorrhiza-colonized roots (Figs. 2a-c) were very distinct from those of the non-mycorrhizal roots. Ultra thin sections revealed a dense layer of hyphae forming a thick fungal mantle and a well-defined Hartig net (Figs. 2a-c). Few (2-3%) of the uninoculated plants became mycorrhizal with *Scleroderma* sp. (Fig. 1e) or with an unidentified ectomycorrhizal fungus forming white mycorrhizas, suspected to belong to the genus *Riziella* (Fig. 1f). *Riziella* colonized root tips had thin mantle in small patches on the epidermis (Fig. 2d). Root tips of most of the uninoculated plants not colonized by either by *Scleroderma* or *Riziella* (contaminant ECM fungi) had exposed epidermis.

Mycorrhizal infection in *A. thurifera* was significantly affected by the inoculation treatment. The three strains of ectomycorrhizal fungi colonized 23% to 38% of the root tips of the inoculated seedlings (Table 1). *Astraeus* sp.
Figure 1. Root colonization in *Shorea guiso* (a) and in *Anisoptera thurifera* (b-f). a = roots of *S. guiso* colonized by *Pisolithus* sp.1 (PTG), b and c = Micrograph of *Pisolithus* sp.1 in *A. thurifera*, d = Numerous mycelia of *Astraeus* sp. in *A. thurifera*, e = *Scleroderma* sp. in *A. thurifera*, f = colonized by an unidentified ECM fungus suspected to be *Rizija*. RT = colonized root tips, M = mycelia.

Figure 2. Sectioned roots of *Anisoptera thurifera* (a and b) and *Shorea guiso* (c and d). Longitudinal sections of *A. thurifera* inoculated with *Pisolithus* (a) and *Astraeus* (b) stained with methylene blue and toluidine blue, respectively. Comparative cross sections of inoculated and uninoculated guiho roots. * = Fungal mantle, ↓ = epidermal cells, ▼ = Hartig net, ↔ = cortical cells and S = stele.
gave the highest percent root colonization and *Pisolithus* sp.1 (PTG) the strain from *A. mangium* had the lowest percent mycorrhizal infection. These levels of root colonization by the introduced ECM fungi were higher \((p<0.01)\) than the root colonization by the indigenous (6%) contaminant fungi. In *S. guiso*, *Pisolithus* sp. 2 gave a higher (35%) root colonization than *Pisolithus* sp.1 (25%) (Table 2). Five percent of the root system of the uninoculated control plants became mycorrhizal with an unidentified fungi.

### Growth and dry matter: *Anisoptera thurifera* rooted cuttings responded positively to inoculation with the two strains of *Pisolithus* and the *Astraeus* isolate used in this study. Inoculated *A. thurifera* had relatively broader leaves and more vigorous growth (Fig. 3a) than the uninoculated control plants in spite of being colonized by *Scleroderma* and an unidentified ECM fungus. *Astraeus* sp. consistently promoted, the highest height increment starting from the second month up to the fourth month (Fig. 3b). *Pisolithus* sp.2 (H6394) gave the lowest height increment, one month

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**Table 1.** ANOVA results for growth, mycorrhizal infection, dry weight and P concentration and uptake in *Anisoptera thurifera* grown in Philippine red soil (Caliraya soil). \(n=20, n=4\) for P analysis.

<table>
<thead>
<tr>
<th>Source of variation/Parameter</th>
<th>Level of significance</th>
<th>Uninoc</th>
<th><em>Pisolithus</em> sp.1 (PTG)</th>
<th><em>Pisolithus</em> sp.2 (H6394)</th>
<th><em>Astraeus</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anisoptera thurifera</em> (Palosapis)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mycorrhizal infection (%)</td>
<td>**</td>
<td>6 c</td>
<td>23 b</td>
<td>33 ab</td>
<td>38 a</td>
</tr>
<tr>
<td>Height increment (cm)</td>
<td>**</td>
<td>3.04 b</td>
<td>4.63 ab</td>
<td>5.29 a</td>
<td>5.64 a</td>
</tr>
<tr>
<td>Diameter (cm)</td>
<td>NS</td>
<td>0.227 a</td>
<td>0.236 a</td>
<td>0.208 a</td>
<td>0.232 a</td>
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<tr>
<td>Fine root dry weight (mg/plant)</td>
<td>NS</td>
<td>26.4 a</td>
<td>34.2 a</td>
<td>39.8 a</td>
<td>39.8 a</td>
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<tr>
<td>Coarse root dry weight (g/plant)</td>
<td>NS</td>
<td>0.144 a</td>
<td>0.159 a</td>
<td>0.154 a</td>
<td>0.181 a</td>
</tr>
<tr>
<td>Shoot dry weight (g/plant)</td>
<td>NS</td>
<td>1.032 a</td>
<td>1.149 a</td>
<td>1.127 a</td>
<td>1.472 a</td>
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<tr>
<td>Total plant dry weight (g/plant)</td>
<td>NS</td>
<td>1.21 a</td>
<td>1.307 a</td>
<td>1.348 a</td>
<td>1.693 a</td>
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<td>Fine root P conc (mg/g sample)</td>
<td>***</td>
<td>1.193 c</td>
<td>1.370 a</td>
<td>1.157 c</td>
<td>1.31 b</td>
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<tr>
<td>Coarse root P conc (mg/g sample)</td>
<td>NS</td>
<td>0.508 a</td>
<td>0.450 a</td>
<td>0.440 a</td>
<td>0.440 a</td>
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<tr>
<td>Shoot P conc (mg/g sample)</td>
<td>NS</td>
<td>0.645 a</td>
<td>0.666 a</td>
<td>0.580 a</td>
<td>0.620 a</td>
</tr>
<tr>
<td>Fine root P uptake (mg/root)</td>
<td>***</td>
<td>0.204 c</td>
<td>0.273 a</td>
<td>0.152 d</td>
<td>0.261 b</td>
</tr>
<tr>
<td>Coarse root P uptake (mg/root)</td>
<td>*</td>
<td>0.184 b</td>
<td>0.177 c</td>
<td>0.171 c</td>
<td>0.199 a</td>
</tr>
<tr>
<td>Shoot P uptake (mg/shoot)</td>
<td>NS</td>
<td>0.645 a</td>
<td>0.829 a</td>
<td>0.659 a</td>
<td>0.910 a</td>
</tr>
</tbody>
</table>

NS = not significant, *, ** and *** = significant at 5, 1 and 0.1% confidence level, respectively
Means with the same letters are not significantly different from each other using DMRT at \(p<0.05\)

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**Table 2.** ANOVA results for growth, mycorrhizal infection, dry weight and P concentration and uptake in *Shorea guiso* grown in Philippine red soil (Caliraya soil). \(n=20, n=4\) for P analysis.

<table>
<thead>
<tr>
<th>Source of variation/Parameter</th>
<th>Level of significance</th>
<th>Uninoc</th>
<th><em>Pisolithus</em> sp.1 (PTG)</th>
<th><em>Pisolithus</em> sp.2 (H6394)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Shorea guiso</em> (Guiho)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Height increment (cm)</td>
<td>*</td>
<td>1.00 b</td>
<td>1.54 ab</td>
<td>2.16 a</td>
</tr>
<tr>
<td>Mycorrhizal infection (%)</td>
<td>**</td>
<td>5 c</td>
<td>25 b</td>
<td>35 a</td>
</tr>
<tr>
<td>Diameter (cm)</td>
<td>NS</td>
<td>0.109 a</td>
<td>0.116 a</td>
<td>0.120 a</td>
</tr>
<tr>
<td>Shoot dry weight (g/plant)</td>
<td>**</td>
<td>0.182 c</td>
<td>0.169 b</td>
<td>0.247 a</td>
</tr>
<tr>
<td>Root dry weight (g/plant)</td>
<td>**</td>
<td>0.094 c</td>
<td>0.124 b</td>
<td>0.182 a</td>
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<tr>
<td>Total plant dry weight (g/plant)</td>
<td>**</td>
<td>0.273 c</td>
<td>0.295 b</td>
<td>0.420 a</td>
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<td>Root P conc (mg/g sample)</td>
<td>***</td>
<td>0.920 c</td>
<td>1.269 a</td>
<td>1.056 b</td>
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<tr>
<td>Shoot P conc (mg/g sample)</td>
<td>NS</td>
<td>0.645 a</td>
<td>0.666 a</td>
<td>0.580 a</td>
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<tr>
<td>Root P uptake (mg/root)</td>
<td>***</td>
<td>0.086 c</td>
<td>0.157 b</td>
<td>0.192 a</td>
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<tr>
<td>Shoot P uptake (mg/shoot)</td>
<td>NS</td>
<td>0.117 a</td>
<td>0.112 a</td>
<td>0.143 a</td>
</tr>
</tbody>
</table>

NS = not significant, *, ** and *** = significant at 5, 1 and 0.1% confidence level, respectively
Means with the same letters are not significantly different from each other using DMRT at \(p<0.05\)
to three months after inoculation. However, at fourth month, height increment due to inoculation with this _Pisolithus H6394_ ranked second to that of _Astraeus_ (Fig. 4b). At fourth month, _Astraeus_ significantly increased height increment of _A. thurifera_ by 86% relative to the uninoculated (1.0 cm) counterpart (Table 1). _Pisolithus H6394_ gave an increase of 74% and _Pisolithus_ sp.1 (PTG) gave an increase of 52% (Table 1). Diameter growth and plant biomass were not affected by inoculation.

_Shorea guiso_ grew slower than _A. thurifera_. In spite of the slower growth rate, _S. guiso_ showed positive growth response to inoculation with the two strains of _Pisolithus_ (Table 2). Similar to _A. thurifera_, inoculated cuttings had broader, greener and longer leaves and grew more vigorously than the uninoculated plants (Fig. 4a). _Pisolithus sp.2_ (H6394) consistently gave higher height increment than the uninoculated treatment (Fig. 4b) and this was evident throughout the four months observation period. By contrast, _Pisolithus sp.1_ (PTG) was consistently had the lowest height increment from one month until the third month. During the first three months, the uninoculated plants had higher height increment than the _Pisolithus_ sp.1 (PTG) inoculated _S. guiso_ (Fig. 4b). At fourth month, plants inoculated with _Pisolithus_ sp.1 (PTG) outgrew the uninoculated ones where it promoted height growth by 54% (Table 2). At this stage, _Pisolithus H6394_ increased height increment by 116% relative to the control plants. Diameter growth was not affected by inoculation with _Pisolithus_ (Table 2).

Shoot, root and total dry weight of _S. guiso_ were significantly affected by inoculation with the two strain of _Pisolithus_ tested (Table 2). _Pisolithus H6394_ consistently promoted the highest root (94%), shoot (34%) and total dry (54%) weight of _S. guiso_ (Table 2). _Pisolithus H6394_ promoted (p<0.01) greater plant biomass than by _Pisolithus sp.1_ (PTG).

**Phosphorus concentration and uptake:** Phosphorus concentrations measured in the fine roots of _A. thurifera_ was affected (p<0.01) by inoculation with ECM fungi (Table 1). Fine roots of _Pisolithus sp.1_ (PTG) _A. thurifera_ inoculated plants had the highest (1.37 mg/g) P concentration which was 15% higher than that of the uninoculated (1.19 mg/g) counterpart (Table 1). _Astraeus_ inoculated plants had fine root P concentration of 10% higher than the uninoculated counterpart. _Pisolithus sp.2_ (H6394) inoculated and uninoculated plants had similar fine root P concentration (1.16 and 1.19 mg/g, respectively).

**Figure 3.** Root colonization by the three ectomycorrhizal fungi in _Anisoptera thurifera_ rooted cuttings. Bar represents LSD value at p<0.05. n=5.

**Figure 4.** Comparative growth of _Anisoptera thurifera_ at four month (a) and periodic height increment (b) due to inoculation with _Pisolithus sp.1_ (PTG from _Acacia mangium_) and _Pisolithus sp.2_ (H6394 from _Eucalyptus_) and an _Astraeus_ sp. (from dipterocarps) as compared with the uninoculated control plants. Bars indicate SE of the treatment means. n=20.
Fine and coarse root P uptake were affected by ECM inoculation (Table 1). The highest (0.273 mg/root) uptake was obtained in the fine roots of *Pisolithus* sp.1 (PTG) inoculated plants followed by those inoculated with *Astraeus* sp. *Astraeus* sp. promoted the highest (0.199 mg/root) coarse root P uptake. *Pisolithus* sp.1 (PTG) increased fine root P uptake by 35% while *Astraeus* sp. increased coarse root P uptake by 8% over the control (0.204 mg/root and 0.184 mg/root, respectively). Coarse root P uptake of plants inoculated with PTG (0.177 mg/root) and H6394 (0.177 mg/root) were comparable and significantly lower than the control (0.184 mg/root).
In *S. guiso*, differences in P concentration and uptake were significant only in the roots (Table 2). Root P concentration was highest (1.269 mg/g) in plants inoculated with *Pisolithus* sp.1 (PTG) while the highest (0.192 mg/root) root P uptake was obtained from plants inoculated with *Pisolithus* sp.2 (H6394). The lowest root P uptakes were obtained from the uninoculated plants.

Correlation between mycorrhizal formation vs. total plant dry weight and P uptake: There was a positive relationship between mycorrhizal infection and total plant dry weight and shoot P uptake with a correlation coefficient \( r^2 \) of 0.71 and 0.68, respectively. There was a weak correlation \( r^2 = 0.27 \) between mycorrhizal root tips and fine root P uptake.

**DISCUSSION**

**Effect of mycorrhizal infection on plant growth**

There are numerous experiments showing that ECM can improve plant growth (Smith & Read 2008). In this study, the three ECM fungi showed differential effectiveness in promoting growth and P status of *A. thurifera* and *S. guiso*. It could also mean that the two dipterocarps express preferences towards ECM isolates. The ECM fungi studied colonized fully the root tips of *A. thurifera* and *S. guiso* and formed symbiotic association, promoted plant growth and development. Although *Astraeus* gave the highest percent root colonization, and coarse root P uptake, the three ECM fungi studied comparatively promoted height growth of *A. thurifera*. *Pisolithus* sp.2 and *Astraeus* gave similar effect on growth of *A. thurifera* and *S. guiso*. This suggests that in this study, there is no effect of origin of these ECM fungi in terms of promoting growth and development on *A. thurifera* and *S. guiso*. *Pisolithus* sp.2 originated in New Caledonia where the usual Ni concentration is 2-5% and it could reach up to 10-15% in scattered deposits of green garnierite (Proctor 1992, Proctor & Nagy 1991, en.wikipedia.org). *Astraeus* sp. was collected from a dipterocarp stand growing in a marginal and slightly acidic (pH 5-6) soil around the vicinity of BIOTECH and at the Botanic Garden, UPLB. These fungi produced numerous external mycelia in the soil, which potentially served as extension of the nutrient absorbing roots. The results of this study also suggest that inoculums from different origins have similar effect on the growth of dipterocarp cuttings. The two *Pisolithus* strains also colonized the roots of *A. thurifera* and *S. guiso* which implies compatibility of the two partners but the plant growth promoting effect of *Pisolithus* sp.2 was more prominent than that by *Pisolithus* sp.1.

Mycorrhiza infected plants had significantly faster growth rates than uninfected plants as exhibited by the positive growth response of *A. thurifera* to *Pisolithus* and *Astraeus*, and *S. guiso* to *Pisolithus*. *A. thurifera* cuttings showed favorable and excellent growth. Broader leaves were observed in inoculated plants particularly those inoculated with *Astraeus*. The introduced ECM fungi promoted significant height increment (52-86%) in *A. thurifera* within four months of growth period. *Astraeus* and *Pisolithus* sp.2 promoted the highest height increments.

*Shorea guiso* showed a positive growth response, having broader, longer and greener leaves than that of the uninoculated ones. Inoculation also showed a significant effect on height increment (54-100%) where *Pisolithus* sp.2 (H6394) promoted the highest height increment.

These findings are similar to those of Suhardi (1996) who compared the average height increase of inoculated and non-inoculated dipterocarp species *Dryobalanops* in the nursery. He observed that the inoculated plants had higher height increase than the uninoculated ones. Other studies also show that *Scleroderema*, promoted growth of dipterocarps namely, *S. acuminata*, *S. parvifolia*, *S. leprosula*, *S. macropera* and *Parashorea lucida* (Kikuchi 1996).

*Pisolithus* has been shown to have great potential in reforestation programmes because of its wide host range. Although *Pisolithus* is known for its broad host range, different isolates of this genus showed variation in colonizing the roots and in promoting the growth of associated hosts (Burgess et al. 1994). It is reported that the fruit bodies of *Pisolithus tinctorius* are common under *Eucalyptus spp.* (Aggangan 1996). Studies also show that application of *Pisolithus* to dipterocarp cuttings greatly enhanced growth (Pollisco 1991). The contaminant ECM fungi were not observed in the inoculated plants. This suggests that the introduced ECM strains were able to overcome the indigenous species (Garbaye & Bowen 1987).

Dipterocarpaceae plants which are the dominant tree species of tropical forests in Southeast Asia are known to form ectomycorrhiza on their roots. The ectomycorrhizal formation has been shown to increase the growth of dipterocarp species such as *Shorea macropera* (Turner et al. 1993), *Hopea odorata* and *Hopea halferi* (Yazid et al. 1994). Dipterocarps are obligatory mycorrhizal, without mycorrhiza, they show signs of serious deficiencies: growth is stunted, leaves become very chlorotic and they eventually die (Pollisco 1991). By contrast, under most favorable conditions, inoculation with ECM fungi is not an absolute requirement for survival and growth of dipterocarp seedlings. Inoculation may however be necessary when seedlings are to be grown in areas deficient in or totally devoid of suitable inoculum, for example in cases of rehabilitation of degraded areas (Lee 1991). The presence of ectomycorrhizas in the
dipterocarps has led to much speculation on the role played by these mycorrhizas in dipterocarp regeneration and ecology. The promotion of seedling growth of different dipterocarp species by inoculation with mycorrhizal fungi has been studied (Kikuchi 1996, De la Cruz 1991). In the Philippines, De la Cruz (1991) pointed out that mycorrhiza is indispensable for the good growth of dipterocarp seedlings. In Indonesia, Hadi et al. (1996) also stressed the need to inoculate dipterocarps with ECM fungi for better growth and development and consequently successful dipterocarp plantings.

Ectomycorrhizal inoculation is assumed to greatly enhance growth of dipterocarp species like *A. thurifera* and *S. guiso*. It has been shown that ectomycorrhizas can play a pivotal role in the growth and survival of dipterocarp seedlings in degraded sites by mobilizing plant water and nutrient uptake via hyphae and increasing resistance to environmental stresses (Pampolina et al. 1994)

**Effect of mycorrhizal infection on phosphorus nutrition**

The improved growth due to ECM inoculation can be explained by the improved nutrient status of the mycorrhizal plants. Lee & Lim (1989) correlated percentage ECM colonization with foliar P in dipterocarps seedlings they studied. In another study, Lee & Alexander (1994) and Yazid et al. (1994) clearly showed increased P concentration in response to ECM colonization in two species of *Hopea* studied. Hadi & Santoso (1989) also reported ECM fungi increased foliar concentration of N, P, K, Ca, and Mg of five dipterocarp species.

Many dipterocarps grow on soils deficient in all plant nutrients most particularly in phosphorus (Heinrich & Patrick 1985). Several adaptive mechanisms for growth in phosphorus-deficient conditions have been identified in *Eucalyptus* seedlings and mature trees (Heinrich & Patrick 1985). These adaptations can be divided into those, which increase the efficiency of phosphorus usage and features which increase the efficiency of phosphorus extraction from the root environment (Heinrich & Patrick 1985).

Phosphate is the heart of nutrient exchange in mycorrhizas. By far, the most striking feature of absorption by ectomycorrhizas is the accumulation of nutrients in the fungal sheath (Harley 1978). Major effect of mycorrhizal infection may be an increase in absorption of mineral nutrients from the soil, particularly relative immobile ones such as phosphates. Only a fraction of the phosphorus (P-total) in the soil occurs and is readily available for absorption by the ectomycorrhizal fungi. Continual absorption of P-total requires P to be replenished in order to offset possible phosphorus deficiency. Roots and mycorrhizal hyphae excrete several types of organic acids (e.g. citrate and oxalate) in response to phosphorus deficiency. These acids improve phosphorus availability either by acting as an exchangeable liquid with iron- or aluminum-bound phosphorus, or by complexing metals in solution metals in solution and thereby reducing precipitation of metal phosphates (Pfeffer et al. 2001). In the process, polyphosphates is synthesized from P-total and comprise much of the mobile phosphorus present in the ectomycorrhizal fungal tissue (Suhardjo 1996).

The phosphorus concentrations in *A. thurifera* were greatly affected by the dilution effect. Plants with low P concentration levels were those with high root and shoot biomass. Plants with high root and shoot biomass had lower P concentration, suggesting that P was diluted in a larger plant biomass. The uninoculated plants gave the highest root and shoot P concentration (0.508mg/g and 0.64mg/g, respectively) and correspondingly had the lowest root and shoot biomass. On the other hand, inoculated plants had lower levels of P concentrations (0.44mg/g to 0.45mg/g in the roots and 0.58mg/g to 0.67mg/g in shoot P concentrations) than the uninoculated counterpart.

Polyphosphates are stored in the sheath (or fungal mantle) of mycorrhizas during phosphate uptake, and represents up to 90% of plant P content (Harley 1978). The significantly higher (p<0.01) values for fine root P concentrations in mycorrhizal plants can therefore be interpreted as indication of effective P uptake. Plants inoculated with *Pisolithus* sp.1 (PTG) obtained the highest (0.273mg/root) fine root P uptake value. For *Pisolithus* sp.1 and *Astraeus*, these values were 34% and 28% higher than the one observed from uninoculated plants. Similarly, the fine root phosphorus concentration obtained with *Pisolithus* sp.1 (PTG) and *Astraeus* were respectively 15% and 10% higher than the one observed for uninoculated plants. Higher amount of phosphates absorbed by the host occurred where higher ECM infection was present as denoted by the positive relationship between the mycorrhizal infection and shoot P uptake ($r^2=0.68$). By contrast, the correlation coefficient between mycorrhizal infection and fine root P uptake is $r^2=0.27$ which indicates a very weak relationship. This may imply that total P absorbed in the roots is readily transported into the shoot as polyP, since polyP is a mobile and low molecular weight element (Harley & Smith 1983).

**Effectivity of ECM mycelia entombed in alginate beads**

The results obtained in this study suggest that ECM mycelia entombed in alginate beads were effective in promoting growth and P uptake of dipterocarps *A. thurifera* and *S. guiso*. Supriyanto (1999) also reported the effectiveness of six species of ECM fungi *Scleroderma columnare*, *S. dictyosporum*, *Laccaria laccata*, *Rhizopogon luteolus*, *Rhizopogon luteolus*, *Astraeus*, and *Pisolithus* sp.2 (PTG) inoculated dipterocarps seedlings.
Amanita umbronata and Descomyces sp. entrapped in alginate beads in promoting the growth of four species of dipterocarp seedlings (Shorea pinanga, S. leprosula, S. ovalis and Hopea odorata). Root colonization by ECM fungi ranged from 46 to 75%. Turjaman et al. (2006) reported a lower root colonization (35-37%) in seven month old Shorea seminis inoculated with mycelium slurry (not entrapped in alginate beads) of Pisolithus arhizus and Scleroderma columbare.

Mycelial inoculum (also known as vegetative inoculum) is produced from fungal pure cultures and is the most recommended type of inoculum. This is because, it allows the selection of the isolates before their application in nurseries and it assures continuous supply of inoculum since these can be cultured in the laboratory unlike spores wherein supply is uncertain. Techniques recently developed employ submerged cultivation procedures followed by immobilization in calcium alginate gel. Inocula produced by these techniques are efficient in colonizing and promoting the growth of different plant species (Mauperin et al. 1987; Kuek et al. 1992). Immobilized mycelium can survive longer in the soil, is easily stored, and shows greater viability than non-immobilized inoculum (Kuek et al. 1992). However, the technique still has limited application due to constraints related to the cultivation in submerged conditions such as in fermenters or in bioreactors, which may compromise the quality of the inocula in large scale production system.

In conclusion, the beneficial effect of ECM inoculation was demonstrated in the aforementioned nursery experiments. Mycelia of Astraeus (from dipterocarps) and Pisolithus sp.2 (from eucalypts) entrapped in alginate beads can be used to inoculate A. thurifera rooted cuttings. Pisolithus sp.2 also entrapped in alginate beads can be used to inoculate S. guiso rooted cuttings. In A. thurifera, Astraeus sp. Pisolithus sp.1 and Pisolithus sp.2 gave comparable root colonization and height increments. Pisolithus sp.1 promoted the highest fine root P concentration while Astraeus sp. promoted the highest coarse root P uptake. In S. guiso, Pisolithus sp.2 promoted better growth and P uptake than Pisolithus sp.1. Pisolithus sp.2 increased height increment, fine root, coarse root, shoot and total dry weight of over the control treatment. The uninoculated cuttings had the lowest height increment, dry weight and P uptake. Thus, inoculation of dipterocarp rooted cuttings with growth promoting ECM fungi mycelia entrapped in alginate beads may ensure the production of high quality planting materials for plantation establishment in red soil such as in Caliraya, Lumban, Laguna and in other reforestation sites in the Philippines with acidic and red soil conditions.

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