

**EFFECTS OF SOIL STERILIZATION ON THE FORMATION AND  
FUNCTION OF TWO STRAINS OF *PISOLITHUS TINCTORIUS*  
ON *EUCALYPTUS UROPHYLLA*\*)**

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**ABSTRACT**

To examine the effects of soil microbial population on mycorrhizal development and function, *Eucalyptus urophylla* seedlings were inoculated with two *Pisolithus tinctorius* isolates and grown in sterile, partly sterile and non-sterile soil. The two isolates of *Pisolithus* were an effective isolate (H445) collected from under eucalypts in Australia and an isolate (H615) collected from under eucalypts in the Philippines. Soils used were infertile acid soils collected from field sites in Pangasinan, Luzon and Surigao, Mindanao.

In both soils, the Australian *Pisolithus* H445 improved the growth of *E. urophylla* seedlings more than Philippine isolate H615. The uninoculated seedlings exhibited stunted growth typical of P deficiency. Height at 8 weeks was significantly taller in sterile than in non-sterile soil. A significant interaction effect of inoculation and soil sterilization on height at harvest was observed only in Surigao soil.

Soil sterilization had a varied effect on mycorrhizal formation. In Pangasinan soil, root colonization by H445 was significantly greater in non-sterile soil than in sterile soil. Whereas in Surigao soil, root colonization was significantly reduced by 54% from partly sterile to non-sterile soil. On the other hand, H615 showed significant mycorrhizal colonization in non-sterile soil compared from those in partly sterile and sterile soils. The degree of infection did not necessarily correspond to growth promotion in *E. urophylla* seedlings.

These results indicate that the performance of the H445 was markedly affected by the microbial flora of the two soils. Thus, its potential use in the Philippines needs to be thoroughly tested in the field before its widespread use in any inoculation program.

**Key words:** Mycorrhizas/Soil sterilization/*Pisot/ws tinctorius/Eucalyptus urophylla*.

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## INTRODUCTION

Eucalypts are important reforestation species grown in the Philippines to provide raw materials for the pulp and lumber industries. Their fast growth and adaptability to low nutrient soils might be dependent on the presence of symbiotic mycorrhizal associations. For example, previous inoculation work carried out in the Philippines on *Pisolithus* and eucalypts indicate growth enhancement with a *Pisolithus* isolate collected from under *Pinus* in the Philippines (De la Cruz *et al.* 1990, 1991).

*Pisolithus* species have been shown to increase survival and growth of a wide range of tree species such as oaks, pines and eucalypts. In the tropics, Lee Su See *et al.* (1994) found that a *Pisolithus* isolate colonized the roots and subsequently improved the growth of two dipterocarp seedlings grown in the nursery. Recent work conducted by Burgess *et al.* (1994a) found that growth stimulation of *E. grandis* seedlings varied greatly among 20 isolates of *Pisolithus*. An isolate (H445) collected from under eucalypts was one of the best growth promoters. This same isolate has also been reported to dramatically increase the growth of *E. globulus* and *E. diversi-color* in other studies (Burgess *et al.* 1993). However, not all isolates are beneficial to plant growth. For example, Tonkin *et al.* (1988) observed severe root reduction and retarded shoot growth of *E. marginata* when inoculated with certain *Pisolithus* isolates in aseptic condition. One factor influencing the response of eucalypts to inoculation with *Pisolithus* is the host from which the isolate was isolated. Pine isolates of *Pisolithus*, for example, have been shown to form poor associations with eucalypts (Malajczuk *et al.* 1982, 1990; Burgess *et al.* 1994a).

In the development of an inoculation program for nurseries in the Philippines, it is essential to introduce compatible ectomycorrhizal isolates that can improve the growth of eucalypts. Isolate H445 presents a possible candidate for such introduction. Introduction of *Pisolithus* isolates into the Philippines from Australia requires a thorough testing of the behaviour of these fungi on roots of eucalypt seedlings growing in soil collected from field sites in the Philippines. In addition, it is necessary to compare the performance of introduced Australian isolates with local Philippine *Pisolithus* isolates.

The glasshouse screening experiments conducted, so far, for *Pisolithus* and eucalypts have used pasteurized soil (Burgess *et al.* 1993, 1994a). It is important to understand factors that affect inoculum survival and persistence of fungi on eucalypt roots in the field. Predicting the effectiveness and competitiveness of introduced ectomycorrhizal fungi in the field, and comparison with previous glasshouse experiments, requires preliminary testing of the fungi in the presence or in the absence of native soil microflora. In a sterile condition, all the native microorganisms

are eliminated by sterilization, thus leaving the introduced mycorrhizal fungi to function freely without interference from other microbes. In non-sterile soil, on the other hand, the introduced fungi must compete with other microorganisms in order to survive and function. This would represent conditions similar to those in the field. It is also possible that partial sterilization such as incorporation of a small amount of non-sterile soil to a fumigated one, can stimulate an isolate to develop and absorb more nutrients.

In this paper, the effectiveness of an Australian (H445) and a Philippine *Pisolithus* isolate (H615) on the formation of ectomycorrhizas and growth stimulation of *E. urophylla* seedlings was compared. Plants were grown in soils collected in the Philippines and subjected to three sterilization treatments (non-sterile, partly sterile and sterile soil).

## MATERIALS AND METHODS

### Seed Germination and Mycorrhizal Synthesis

Seeds of *E. urophylla* (seedlot # 18094 from Mt. Egon, Flores Island, Indonesia), obtained from CSIRO Australian Tree Seed Centre (Canberra, Australia), were aseptically germinated on agar plates. Seeds were washed with 70% ethanol containing Tween 20 for one minute, surface sterilized with 10% sodium hypochlorite for 5 minutes and washed three times with sterile water.

Two isolates of *Pisolithus* were selected: a) H445 - an isolate provided by CSIRO Division of Forestry, collected under *E. marginata* in Western Australia and which has been shown to stimulate the growth of eucalypts in field trials, and b) H615 - collected under *E. camaldulensis* in the Philippines but probably associated with *Pinus* (Burgess *et al.* 1994b).

Ectomycorrhizas were synthesized aseptically in petri dishes by transferring 7 day-old germinants onto the surface of 10-day old hyphal mats growing on Modified Melin Norkrans medium (Marx 1969) with low glucose concentration (1.75 g/L) for two weeks (Malajczuk *et al.* 1990). Plates were incubated in a growth room set at 12 hrs photoperiod and temperature of 25°C. At 10 days they were transplanted into undrained pots containing 2 kg soil. The surface of each pot was covered with aluminium foil with four holes where the seedlings were inserted. The foil reduced water loss from the soil and minimized spore contamination. After four weeks, the seedlings were thinned to two plants per pot.

## **Soil Treatments**

Soils (0- 15cm depth) were collected at sites in: a) Labrador, Pangasinan, Luzon and b) in Bislig, Surigao Sur, Mindanao, referred to in figures as Pangasinan and Surigao soil, respectively. Both sites are grasslands which previously were monsoon forests dominated with dipterocarps. The Pangasinan soil has a pH of 4.1 (1:1 soil, 0.005M CaCl<sub>2</sub>), 2.02% organic matter (Walkey-Black Method), 0.11% Total N (Modified Kjeldahl Method), and 0.84 ppm available P (Bray No. 2) while the Surigao soil has a pH of 5.9, 2.48% organic matter, 0.06% Total N and 0.21 ppm available P. Textural class of the two soils is silt loam. The soils were air dried, pulverized and passed through a 2 mm screen. Two kg dry soil were dispensed into plastic pots with polyethylene bag liners.

Soil treatments were: sterile, partly sterile and non-sterile. Pots designated as sterile and partly sterile were fumigated with methyl bromide (one canister per m<sup>3</sup> soil) in a fumigation chamber for 3 days. The non-sterile designated pots were left unfumigated. The partly sterile soil treatment was prepared by mixing 1% unfumigated soil with 99% methyl bromide, fumigated soil.

## **Addition of Fertilizer**

One week after fumigation, all pots received the following basal nutrients (per kg soil): 16 mg Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub>.H<sub>2</sub>O, 270 mg NH<sub>4</sub>NO<sub>3</sub>, 233 mg K<sub>2</sub>SO<sub>4</sub>, 71.3 mg CaCl<sub>2</sub>, 21.4 mg Mg SO<sub>4</sub>.7H<sub>2</sub>O, 10 mg ZnSO<sub>4</sub>.7H<sub>2</sub>O, 5 mg CuSO<sub>4</sub>.5H<sub>2</sub>O, 0.36 mg CoSO<sub>4</sub>.7H<sub>2</sub>O, 0.7 mg H<sub>3</sub>BO<sub>3</sub> and 1.62 mg Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O. Manganese and iron were not included because the soils had high concentration of these elements (Dell, unpublished). The nutrient solutions were added evenly on the soil surface after sterilization. The soil was mixed thoroughly and watered to 80% field capacity and incubated in benches inside a screenhouse for five days prior to planting.

## **Seedling Maintenance and Growth Monitoring**

Two weeks after transplanting, 2.5 ml nitrogen solution (54g NH<sub>4</sub>NO<sub>3</sub>/L H<sub>2</sub>O) pot<sup>-1</sup> week<sup>-1</sup> was added. The same rate was added for the next two weeks after which the rate of nitrogen was increased to 5 ml pot<sup>-1</sup> week<sup>-1</sup> for a period of 7 weeks. All pots were watered to field capacity by weight when necessary. Height was monitored twice a month.

### **Harvest and Mycorrhizal Assessment**

After 12 weeks, the seedlings were harvested. Shoots were cut 1 cm above the soil surface and the root systems were gently washed under running water. Fine roots (diameter less than 0.5 mm) were separated from the coarse roots, blotted dry in between paper towels and cut into 1 cm lengths. Fine root samples of 0.2 g fresh weight were further chopped into 1-2 mm lengths and were fixed in 70% ethanol for counting mycorrhizal root infection. Fine roots were cleared and stained as described by Phillips and Hayman (1970). Stained roots were spread evenly over a petri dish and were examined under a stereomicroscope. Ten randomly selected fields of view were chosen and all roots crossing a hair-line were examined for the presence or absence of infection. Fully colonized root tips were scored as mycorrhizal.

### **Experimental Design and Statistical Analysis**

The experiment was conducted in a screenhouse at BIOTECH, UP Los Banos, Philippines following a two factor (fungi and soil treatment) factorial in Randomized Complete Block Design (RCBD) with three replicates. Pots were rearranged in benches once a week. Within each soil type, all data collected were analyzed statistically using two way ANOVA. Treatment means were compared using Duncan's Multiple Range Test at  $P < 0.05$ .

## **RESULTS**

### **Plant Growth**

In Pangasinan soil, inoculation (as Factor A) significantly ( $P < 0.01$ ) affected heights at 6, 8, 10 and 12 weeks (at harvest) after planting, and dry matter yields (shoot, root, fine root, coarse root and total biomass). Generally, the Australian *Pisolithus* H445 was more effective in increasing height growth of *E. urophylla* seedlings from 6 weeks to 12 weeks after planting, dry matter yields and phosphorus uptake (data not shown) than the Philippine isolate H615. Inoculation with the Australian isolate increased ( $P < 0.05$ ) height at 6 weeks until 12 weeks (harvest) after planting. H615 significantly increased seedling heights from 6 weeks to 10 weeks after planting compared with the uninoculated plants but significantly shorter than those inoculated with H445. At harvest (12 weeks) however, height of seedlings inoculated with the Philippine isolate was not significant compared with the uninoculated counterpart. Likewise, soil sterilization (as Factor B) significantly affected

height at 6 weeks ( $P < 0.01$ ) and at 8 weeks ( $P < 0.05$ ) and fine root dry matter yield ( $P < 0.05$ ). Height at 6 and 8 weeks after planting and fine root dry weight obtained in sterile soil were significantly higher than in non-sterile soil. Heights and fine root dry weight in partly sterile soil were intermediate and these were not significant compared with those in sterile soil as well as those in non-sterile soil. There was no significant interaction effects of inoculation and soil sterilization on any of the periodic height measurements (Fig. 1A). Height at harvest due to inoculation with H445 was increased by 4x, 5x and 8x in sterile, partly sterile and in non-sterile soil, respectively, relative to the uninoculated seedlings. On the other hand, H615 increased seedling heights by 1.2x, 2x and 3x in the same soil treatment order. Although the mean height of H445 plants were two times taller than those of H615, there was no significant differences among treatments. The uninoculated seedlings had the shortest height in all the three soil treatments. Height of uninoculated plants was reduced by 2 folds while total dry weight (data not presented) by 10 folds from sterile to non-sterile soils. Whereas, the inoculated seedlings did not differ much irrespective of soil treatment. Shoot dry weight (data not presented) showed similar trends to plant height. A significant interaction effect was observed only on fine root dry weight. Fine root dry weight was dramatically increased ( $P < 0.05$ ) by H445 in sterile (Fig. 2A) compared with H615 and uninoculated treatments. Fine root dry weight was greater ( $P < 0.05$ ) in sterile than in non-sterile soil.

In Surigao soil, inoculation (as Factor A) significantly affected height at 6 to 12 weeks after planting, coarse root dry weight, root P concentration and uptake and shoot P uptake. H445 and H615 inoculated seedlings were of similar height which was significant compared with height of the uninoculated ones from 6 to 10 weeks after planting. At harvest, H445 inoculated plants were significantly taller than those inoculated with H615 and the uninoculated ones. Although, height at harvest, coarse root dry weight and root P concentration obtained by H615 inoculated plants were twice those of uninoculated counterpart, the differences were not statistically significant. Soil sterilization (Factor B) significantly affected height at 8 weeks and shoot P concentration. Height in sterile soil was significantly taller than in partly sterile and non-sterile. The latter soil treatments (partly sterile and non-sterile soil) yielded similar height growth response. Shoot P concentration in partly sterile soil was significantly higher than in non-sterile soil. Unlike in Pangasinan soil, there was a significant interaction effect of inoculation and soil treatment. In sterile, H445 was consistent in promoting a significant increase in height ( $P < 0.01$ ) from 6 weeks until harvest (Fig. 1B) compared with the uninoculated seedlings. Two way analysis revealed that height growth obtained by seedlings inoculated with H445 in sterile soil was significant compared with those inoculated with H615 and the uninoculated ones. In partly sterile soil, height obtained by H445

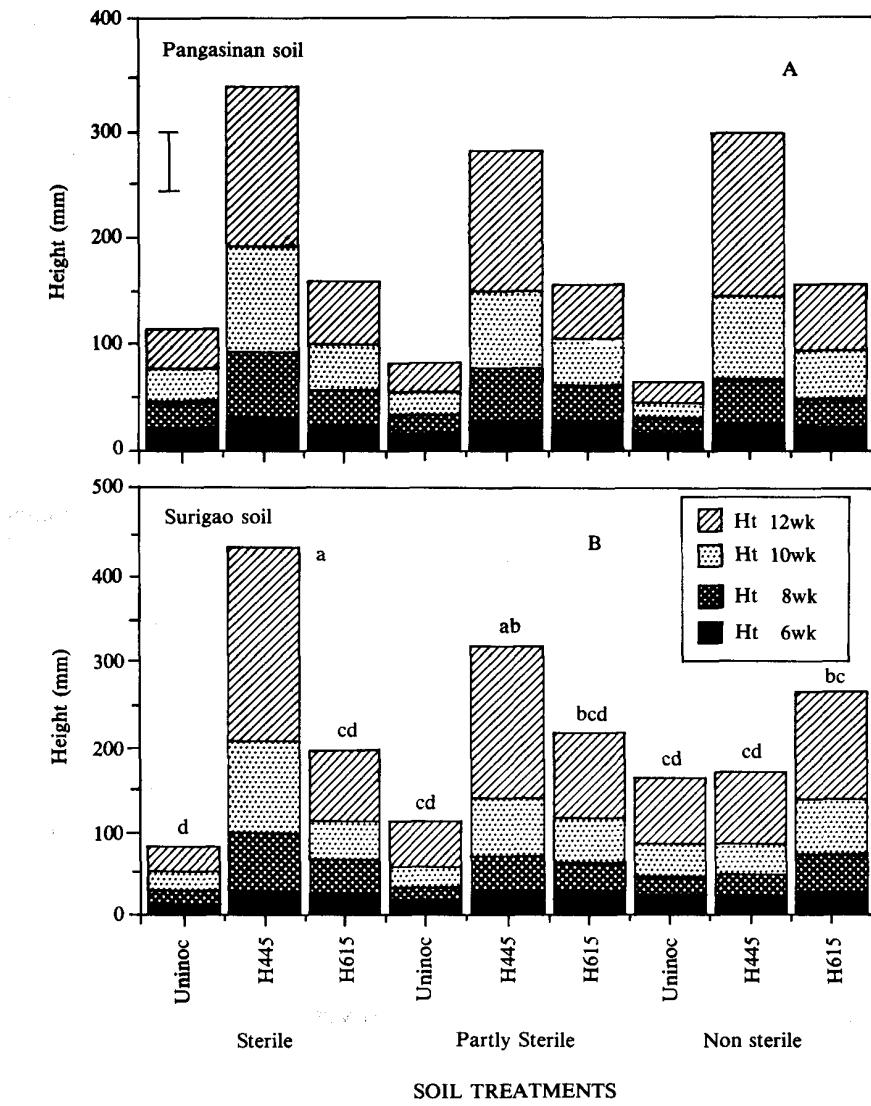


Figure 1. Effects of mycorrhizal inoculation on the accumulated height growth of *E. urophylla* seedlings grown in Pangasinan (A) and Surigao (B) soils subjected to three sterilization treatments. Letters represent results of Duncan's Multiple Range Test after two way ANOVA on height at 12 weeks on each soil treatment. LSD bar = 56.

inoculated seedlings was significantly taller than the uninoculated seedlings but not significant as compared with those inoculated with H615. In non-sterile soil, height differences between the inoculated and uninoculated seedlings was not significant. H615 inoculation gave intermediate height and shoot dry weight in sterile and in partly sterile soil which were not statistically different from the uninoculated seedlings. Unlike seedlings grown in Pangasinan soil, there was no significant interaction effect of inoculation and soil sterilization on fine root dry weight (Fig. 2B). However, plants had much larger shoot and root systems in the Surigao than in the Pangasinan soil.

### **Mycorrhizal Development**

In Pangasinan soil, there was a significant difference between the three inoculation treatments and soil sterilization significantly affected mycorrhizal development. Mycorrhizal infection by H445 was significantly higher (41%) than H615 (21%). The uninoculated seedlings had 7% infection. Mycorrhizal infection in non-sterile soil was significantly higher (30%) than in sterile soil (18%). Root colonization in partly sterile soil was intermediate which was not significant compared with those obtained in sterile or in non-sterile soils. Two way analysis indicates that in sterile and non-sterile soils, H445 and H615 had similar infection levels (Fig. 2C). However, in partly sterile soil, H445 had greater ( $P<0.01$ ) root colonization than H615. Root colonization by H445 was significantly higher in non-sterile than in sterile soil but not significant compared with those in sterile and in non-sterile soils. Uninoculated seedlings had low levels (not more than 11 %) of infection in the two soils (Fig. 2C).

In Surigao soil, mycorrhizal development formed by H445 and H615 (as Factor A) was not significantly different from each other but significant compared with the uninoculated treatment. Root colonization was significantly higher in non-sterile and in partly sterile soils than in sterile soil. There was a significant interaction effect of inoculation and soil sterilization treatments. In non-sterile soil, H615 formed higher ( $P<0.05$ ) infection compared with H445 (Fig. 2D). Greatest mycorrhizal formation by H445 was observed in partly sterile soil which was reduced ( $P<0.05$ ) in sterile and in non-sterile soils. Percent mycorrhizal infection by H445 in non-sterile soil was similar to that of uninoculated plants grown in either of the three soil treatments. Root colonization by H615 was higher ( $P<0.01$ ) in non-sterile (65%) than in partly sterile soil (34%). Infection in sterile soil was intermediate and not significantly different to percent mycorrhizal infections observed in non-sterile and in partly sterile soils. Correlation analyses revealed a low relationship ( $r^2 = 0.6$ ) between percent mycorrhizal infection and height or shoot and fine root dry weight.

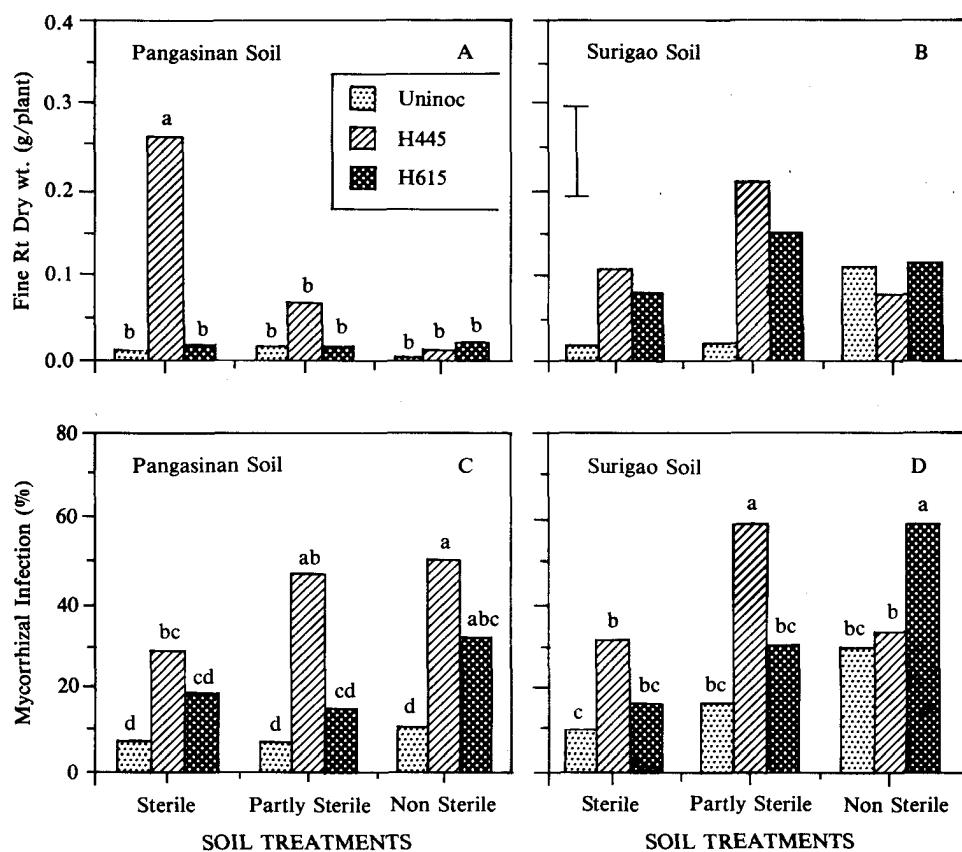


Figure 2. Fine root dry matter weight (A and B) and mycorrhizal infection (C and D) of *E. urophylla* seedlings inoculated with two *Pisolithus* isolates grown on sterile, partly sterile and non-sterile acid soils. Letters represent results of Duncan's Multiple Range Test after two way ANOVA on each soil. LSD bar = 0.116.

## DISCUSSION

Growth of *E. urophylla* seedlings in the two Philippine soils was stimulated by inoculation with two isolates of *Pisolithus*. Growth of plants inoculated with the Australian eucalypt isolate, H445, was enhanced more than those inoculated with the Philippine isolate, H615. Previously, H615 was classified as a pine isolate (Burgess *et al.* 1994b) on the basis of polypeptide patterns identified by ID SDS-PAGE.

The effectiveness of a pine *Pisolithus* isolate in promoting growth of eucalypts has been demonstrated in field trials (De La Cruz *et al.* 1990, 1991). Potentially, the introduction of Australian isolates in any nursery inoculation programs in the Philippines would provide an advantage for growth promotion of eucalypts. It remains to be determined whether these specific eucalypt isolates would persist and outperform the indigenous pine *Pisolithus* isolates in the field. It is interesting to note that Garbaye *et al.* (1988) observed the replacement of an introduced pine isolate (Marx strain 270), by an indigenous *Scleroderma* species within 2 years after outplanting.

In this experiment, mycorrhizal root development was markedly affected by sterilization treatment in one Philippine soil (Surigao). Mycorrhizal infection by H445 was reduced by 54% in non-sterile soil. In contrast, H615, was unaffected by soil sterilization treatments. This would suggest a possible role of soil microflora in affecting the infection process. Several studies have suggested that soil microflora play an important role in influencing the inoculation process of seedling roots by mycorrhizal fungi (Garbaye and Bowen 1987; Marx *et al.* 1982, 1984). Such effect may be inhibitory or stimulatory to mycorrhizal formation (Bowen and Theodorou 1979; Garbaye and Bowen 1989; Fitter and Garbaye 1994).

It would appear that the introduction of new strains of ectomycorrhizal fungi from Australia and screening of endemic strains requires a thorough testing of the performance in a range of soils. It is anticipated that no single species would be appropriate for all soil types in the Philippines and the introduction should include a wide range of physiologically distinct species. Introduced ectomycorrhizal fungi should be able to survive and tolerate ecological variation within a site and progressively colonize the new roots after outplanting (Garbaye 1982). Most importantly, the introduced fungi must enhance host performance to a greater degree than the native fungi (Danielson 1988). Competitiveness or persistence of the association would depend on three factors: the host, mycorrhizal fungus and soil microbes wherein each factor is subject to environmental influences. Further work is required to examine the influence of the composition of the microbial population on ectomycorrhizal formation on a range of Philippines soil.

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