

# Changes during early ectomycorrhiza formation by *Pisolithus tinctorius* on *Acacia mangium*, and their impact on nodule formation by *Bradyrhizobium* sp.

P. Jayakumar<sup>1,2\*</sup> and T.K. Tan<sup>1</sup>

<sup>1</sup>Mycology and Plant Pathology Laboratory, Department of Biological Sciences, National University of Singapore, Singapore 117 543;

<sup>2</sup>Semiarid Prairie Agricultural Research Centre, Agriculture and Agri-Food Canada, P.O. Box 1030, Airport Road, Swift Current, Saskatchewan, Canada S9H 3X2, Tel. +1-306-778-3128, Fax. +1-306-778-3188, Email. jayakumar@agr.gc.ca

(Received September 24, 2005; Accepted November 21, 2005)

## Abstract

Changes in the root morphology and rhizosphere pH during ectomycorrhiza formation have been reported for various plants. These changes are mediated by root-growth-regulating compounds and organic acids secreted into the rhizosphere by the ectomycorrhizal fungus. Plate culture experiments were conducted to study the effects of early stages of ectomycorrhiza formation by *Pisolithus tinctorius* on the root morphology, rhizosphere pH, and nodulation of *Acacia mangium* seedlings. Also, experiments were conducted to find out the effects of putative compounds produced by *P. tinctorius* on nodule formation by *Bradyrhizobium* in *A. mangium*. In the presence of *P. tinctorius*, the root morphology of *A. mangium* seedlings was significantly altered (length of the tap root decreased, number of lateral roots cm<sup>-1</sup> increased, and length of the lateral roots decreased), the pH of the rhizosphere region was reduced, and nodule formation by *Bradyrhizobium* was inhibited. The role of indole-3-acetic acid (IAA) produced by *P. tinctorius* in root morphogenesis was confirmed by using different concentrations of IAA and auxin transport inhibitor, 2,3,5-triiodobenzoic acid (TIBA). However, there is no evidence for the involvement of fungal IAA in nodule inhibition. Concentrations of IAA that induced changes in root morphology also induced nodule formation by *Bradyrhizobium*. Aminoethoxyvinylglycine (AVG), inhibitor of ethylene biosynthesis, did not reverse the effect of *P. tinctorius* on nodule formation indicating that inhibition of nodules was not due to ethylene-mediated mechanism. Low rhizosphere pH, probably due to production of organic acids, can be one of the factors responsible for nodule inhibition during ectomycorrhiza formation.

**Keywords:** *Acacia mangium*, *Pisolithus tinctorius*, *Bradyrhizobium*, ectomycorrhization, nodulation

## 1. Introduction

Ectomycorrhiza formation involves morphological, physiological and biochemical changes of the roots and the plant (Martin and Hilbert, 1991). Changes in the root morphology as a result of ectomycorrhiza formation have been widely reported for various plants such as *Pinus sylvestris* (Slankis, 1950), *Eucalyptus* spp. (Béguiristain and Lapeyrie, 1997), *Picea abies* (Karabaghli-Degron et al., 1998; Regvar and Gogala, 1996) and *Quercus* spp. (Oh et al., 1995). Barker et al. (1998) reviewed the events taking place during ectomycorrhiza formation and suggested that

the entire process can be classified into early (pre-contact) and late steps (post-contact). According to them, early steps include the secretion of fungal compounds into the rhizosphere followed by changes in the root morphology. Based on comparative root morphology and root ramification following IAA application or fungal colonization, Gogala (1991) suggested that fungal auxins play a key role in root development during ectomycorrhiza formation. This was later confirmed by Karabaghli-Degron et al. (1998) in *Picea abies* seedlings, through the use of IAA transport inhibitor.

Ectomycorrhizal fungi also secrete organic acids, especially oxalic acid and citric acid, which significantly reduce the pH of the rhizosphere soil (Arocena and Glowa, 2000; Wallander, 2000; Griffiths et al., 1994; Lapeyrie et al., 1991). Although gross concentrations of organic anions

\*The author to whom correspondence should be sent.

in the soil solution may appear insufficient to cause a significant reduction in the pH, higher concentrations are likely to be present in microenvironments surrounding fungal hyphae (Drever and Stillings, 1997). It has been reported that individual organic acids in the soil solution exceeds millimolar concentrations (Fox and Comerford, 1990; Stevenson, 1967), with extremely high concentrations in the vicinity of certain plants and fungal hyphae (Gardener et al., 1983; Cromack et al., 1979). Moreover, some reports indicate that ethylene production in the host plant is stimulated during ectomycorrhiza formation (Rupp et al., 1989), and there is a positive relationship between auxin and ethylene production (Scagel and Linderman, 1998). Earlier, Graham and Linderman (1981) have suggested that the presence of ethylene in the root zone may have a different effect on root infection by mycorrhizal fungi and other pathogenic fungi. However, the effects of various compounds produced by the ectomycorrhizal fungi on the nodulating bacteria has not been reported.

*Acacia mangium* is a fast-growing leguminous tree that can form symbiotic association with both N<sub>2</sub>-fixing *Bradyrhizobium* sp. and P-solubilizing ectomycorrhizal *Pisolithus tinctorius* (Pers) Coker and Couch. Individual inoculations with *Bradyrhizobium* (Fremont et al., 1999; Prin et al., 2003) or *P. tinctorius* (Jayakumar and Tan, 2005) have been reported to enhance the growth of *A. mangium*. However, Bâ et al. (1994) found that inoculation of *P. tinctorius* to *A. holosericea* seedlings prior to inoculation with *Bradyrhizobium* resulted in the inhibition of nodule formation. There is no information available on the direct interactions between *Bradyrhizobium* and *P. tinctorius* in the rhizosphere and their processes in the host system. One of the objectives of the present study was to find out the effects of early stages of ectomycorrhiza formation by *Pisolithus tinctorius* on the root morphology, rhizosphere pH, and nodulation of *Acacia mangium* seedlings. Also, experiments were designed to detect the fungal compounds involved in the root morphogenesis during early stages of ectomycorrhiza formation. Another objective was to study the effects of putative compounds produced by *P. tinctorius* on nodule formation by *Bradyrhizobium* in *A. mangium*.

## 2. Materials and Methods

### *Culture maintenance and inoculum production*

*Bradyrhizobium* (WAS 9) was stored on modified Yeast-extract-mannitol (YM) agar plates at 4°C and used as the stock culture. Ten-day-old cultures on YM agar plates incubated at 27°C were used as mother cultures. *Bradyrhizobium* cultures on plates were transferred to sterile distilled water with 0.5% glucose and adjusted to a final concentration of 10<sup>9</sup> cells ml<sup>-1</sup> by measuring the optical

density of the suspension at 650 nm as described by Cooper (1979) and Hoben and Somasegaran (1982).

*Pisolithus tinctorius* (P53 and E3418) were cultured on modified Melin-Nokran's (MMN) (Marx, 1969) agar plates, stored at 4°C, and sub-cultured every 2 months. Twenty-day-old colonies on MMN agar plates incubated at 27°C were used as mother cultures. The *P. tinctorius* fungal card was prepared by placing a piece of sterile filter paper (40 mm × 40 mm) on a standard petri plate (100 mm × 15 mm) containing 20 ml of sterile MMN agar and inoculated with the fungus (8 mm diameter mycelial disc) at six points. The plates were sealed with parafilm, inverted and incubated in the dark at 27°C until the filter papers were evenly covered with mycelium (3 weeks).

### *Effects of compounds produced by P. tinctorius on root development, rhizosphere pH, and nodule formation*

To study the effects of compounds produced by *P. tinctorius* on the root development of *A. mangium*, the original 'paper-sandwich' technique (Chilvers et al., 1986) was modified according to Béguiristain and Lapeyrie (1997) where the fungal card and the roots were separated by a sterile cellophane membrane.

*Acacia mangium* seeds were surface sterilized in 95% sulphuric acid for 30 min, rinsed with sterile distilled water, and germinated on 1% water agar at 25°C in the dark. One 5-d-old seedling free of bacterial contamination was aseptically transferred to a standard petri plate containing 20 ml of sterile modified low sugar Pachlewski's medium (Béguiristain and Lapeyrie, 1997). A sterile cellophane (50 mm × 50 mm) was placed over the root system over which the fungal card was placed with the fungal side in contact with the cellophane.

For control seedlings, filter papers (40 mm × 40 mm) without fungal mycelium were used. The plates were sealed with parafilm along 90% of their circumference and stacked in rows in plastic trays at an angle of 70°. Black plastic sheets were inserted in between the plates to shade the roots. The plates were incubated in 16 h day-illuminated environment cabinets at 27°C for 15 d. The fungal card/sterile filter paper was lifted after 15 d and the root morphology (tap root length, number of lateral roots and length of lateral roots) was assessed. No fungal growth was observed when the roots were transferred to fresh MMN agar plates indicating that the fungal hyphae did not come in contact with the root surface. The rhizosphere pH was measured by placing a drop of bromothymol blue (pH indicator) in the rhizosphere region. The colour change (yellow, bluish green and green) was observed, and the seedlings were classified into 3 groups: pH <6.1, 6.1 to 6.7 and >6.7.

The same 'paper-sandwich' technique was used to study the effects of compounds produced by *P. tinctorius* on nodule formation by *Bradyrhizobium* in *A. mangium*. To facilitate nodulation, the concentration of diammonium



tartarate in the medium was reduced by 50% and pH was adjusted to 6.8 with KOH. The roots were treated with 0.5 ml of *Bradyrhizobium* in solution ( $10^9$  cells of bacteria  $\text{ml}^{-1}$ ) at the time of transplantation. Sterile cellophane (50 mm  $\times$  50 mm) was placed over the root system over which the fungal card was placed with the fungal side in contact with the cellophane. Control seedlings received the *Bradyrhizobium* solution but, instead of fungal card, filter papers without fungal mycelium were used. The fungal card/sterile filter paper was lifted after 21 d and nodulation (number of nodules and nodule distribution) was assessed.

#### *Effects of different concentrations of IAA on root development*

To study the effects of different concentrations of IAA on root development, one 5-d-old seedling was aseptically transferred to 20 ml of culture medium supplemented with filter sterilized aqueous IAA (Sigma-Aldrich Co.) to give final concentrations of 0.1, 1, 10, 100  $\mu\text{M}$ . A sterile filter paper was placed over the root system. The culture medium of the control seedlings was not supplemented with IAA. The root morphology was assessed after 15 d.

#### *Effect of auxin transport inhibitor on P. tinctorius-mediated changes in root morphology*

The following treatments were used to establish whether auxin was involved in root morphogenesis during ectomycorrhiza formation: (1) Control – Seedlings not grown in the presence of *P. tinctorius*, and culture medium not supplemented with 2,3,5-triiodobenzoic acid (TIBA), an auxin transport inhibitor, (2) Control + TIBA – Seedlings not grown in the presence of *P. tinctorius* but supplemented with TIBA in culture medium, and (3) P53 + TIBA – Seedlings grown in the presence of *P. tinctorius* and culture medium supplemented with TIBA. The compound (TIBA, Sigma-Aldrich Co.) was dissolved in absolute ethanol and added to culture medium to give a final concentration of 10  $\mu\text{M}$ . The culture medium of control seedlings received the same volume of ethanol without any TIBA.

One 5-d-old seedling was aseptically transferred to 20 ml of culture medium with or without TIBA as mentioned above. Fungal card or sterile filter paper without fungal mycelium was placed over the root system according to the treatmental condition. The seedlings roots and the fungal card/sterile filter paper were separated by a sterile cellophane membrane. The root morphology was assessed after 15 d.

#### *Effects of different concentrations of organic acids on root development*

To study the effect of different concentrations of organic acids (oxalic acid and citric acid) on root development, one 5-d-old seedling was aseptically transferred to 20 ml of culture medium supplemented with different concentrations

(0.1, 1, 2.5 mM) of oxalic acid or citric acid. The pH of growth medium containing different concentration of organic acids was measured using a pH meter (MeterLab PHM210, Radiometer, Copenhagen). A sterile filter paper was placed over the root system. The culture medium of controls did not contain oxalic or citric acid. Changes in root morphology were measured after 15 d.

#### *Effects of different concentrations of IAA on nodule development*

To study the effects of different concentrations of IAA on nodule development, one 5-d-old seedling was aseptically transferred to 20 ml of culture medium supplemented with filter sterilized aqueous IAA to give final concentrations of 0.1, 1, 10, 100  $\mu\text{M}$ . The nitrogen concentration in the culture medium was reduced by 50% and the pH adjusted to 6.8, to facilitate nodulation. The culture medium of the control seedlings was not supplemented with IAA. All the seedlings were inoculated with 0.5 ml of *Bradyrhizobium* in solution ( $10^9$  cells of bacteria  $\text{ml}^{-1}$ ) at the time of transplantation. A sterile filter paper was placed over the root system. Number of nodules was measured after 21 d.

#### *Effect of inhibitor of ethylene biosynthesis on P. tinctorius-mediated inhibition of nodules*

The following treatments were used to establish whether ethylene was involved in nodule inhibition during ectomycorrhiza formation: (1) Control – Seedlings not grown in the presence of *P. tinctorius* and culture medium not supplemented with aminoethoxyvinylglycine (AVG), inhibitor of ethylene biosynthesis, (2) Control + AVG – Seedlings not grown in the presence of *P. tinctorius* but culture medium supplemented with AVG, (3) P53 – Seedlings grown in the presence of *P. tinctorius* but culture medium not supplemented with AVG, and (4) P53 + AVG – Seedlings grown in the presence of *P. tinctorius* and culture medium supplemented with AVG. Aminoethoxyvinylglycine (Sigma-Aldrich Co.) was dissolved in absolute ethanol and added to culture medium to give a final concentration of 10  $\mu\text{M}$ . The culture medium of control seedlings received the same volume of ethanol without any AVG.

One 5-d-old seedling was aseptically transferred to 20 ml of culture medium with or without AVG as mentioned above. The nitrogen concentration in the culture medium was reduced by 50% and the pH adjusted to 6.8, to facilitate nodulation. All the seedlings were inoculated with 0.5 ml of *Bradyrhizobium* in solution ( $10^9$  cells of bacteria  $\text{ml}^{-1}$ ) at the time of transplantation. Fungal card or sterile filter paper without fungal mycelium was placed over the root system according to the treatmental condition. The seedlings roots and the fungal card/sterile filter paper were separated by a sterile cellophane membrane. The nodule formation was assessed after 21 d.

### *Effects of different concentrations of organic acids on nodule development*

To study the effect of different concentrations of organic acids (oxalic acid and citric acid) on nodule development, one 5-d-old seedling was aseptically transferred to 20 ml of culture medium supplemented with different concentrations (0.1, 1, 2.5 mM) of oxalic acid or citric acid. The nitrogen concentration in the culture medium was reduced by 50% to facilitate nodulation, but the pH was not adjusted. The pH of growth medium containing different concentration of organic acids was measured using a pH meter (MeterLab PHM210, Radiometer, Copenhagen). A sterile filter paper was placed over the root system. The culture medium of controls did not contain oxalic or citric acid. All the seedlings were inoculated with 0.5 ml of *Bradyrhizobium* in solution ( $10^9$  cells of bacteria  $\text{ml}^{-1}$ ) at the time of transplantation. To confirm whether the effect on nodule development was due to the changes in pH, two additional treatments with 2.5 mM of oxalic or citric acid in culture medium and pH adjusted to 6.8 using KOH were maintained. Number of nodules was measured after 21 d.

### *Statistical analysis*

Data are means of three independent sets of experiments. Each set of experiment contained 30 replicate treatments. The data set was analyzed using SPSS program. A single factor analysis of variance (one way ANOVA) was used to test for significant differences in treatments.

## 3. Results

### *Changes in root morphology and rhizosphere pH of A. mangium grown in the presence of P. tinctorius*

The compounds produced by both P53 and E3418 isolates of *P. tinctorius* significantly reduced the length of the tap root (Fig. 1a). The number of lateral roots  $\text{cm}^{-1}$  was significantly increased by both the isolates of *P. tinctorius* (Fig. 1b). Distribution of lateral roots based on length showed that 40 to 54% of the lateral roots produced by seedlings grown in the presence of *P. tinctorius* were less than 2 mm in size (Fig. 1c). On the other hand, in control seedlings only 12% of the lateral roots were less than 2 mm in size. The majority of the lateral roots (58%) in control seedlings were 2–5 mm long, while 37 to 48% of the lateral roots in seedlings grown in the presence of *P. tinctorius* were of that size.

Presence of *P. tinctorius* reduced the pH of the rhizosphere region in *A. mangium* (Fig. 1d). A reduction in rhizosphere pH from 6.8 to less than 6.1 was noticed in 42 to 56% of the seedlings grown in the presence of *P. tinctorius*, while none of the control seedlings reduced the pH below 6.1. While about 25% of the control seedlings

showed a  $\text{pH} > 6.7$ , none of the seedlings grown in the presence of *P. tinctorius* showed such a high pH.

### *Confirmation of early role of IAA in root morphogenesis during ectomycorrhiza formation by P. tinctorius on A. mangium*

Different concentrations of IAA (0.1, 1, 10 and 100  $\mu\text{M}$ ) significantly affected root development in *A. mangium*. The length of the tap root significantly decreased with increasing concentrations of IAA, while the number of lateral roots  $\text{cm}^{-1}$  increased with increasing concentrations (Table 1). The size of the lateral roots decreased when IAA concentration was increased. Very high concentration of IAA (100  $\mu\text{M}$ ) totally inhibited root growth. The compounds produced by *P. tinctorius* did not have any effect on length of the tap root, number of lateral roots  $\text{cm}^{-1}$  and size of the lateral root when auxin transport inhibitor (TIBA) was added to the culture medium. Both control seedlings and seedlings grown in the presence of *P. tinctorius* had similar root morphology when TIBA was added to the growth medium (data not shown). Also, different concentrations of organic acids and the corresponding lower pH did not have any significant effect on the root morphology of *A. mangium* seedlings (data not shown).

### *Effects of compounds produced by P. tinctorius on nodulation in A. mangium*

Nodule formation was significantly inhibited by compounds produced by *P. tinctorius*. The control seedlings produced 34 to 53% more nodules than the seedlings grown in the presence of *P. tinctorius* (Fig. 2a). Between the two strains of *P. tinctorius*, P53 severely inhibited nodulation. Distribution of seedlings based on nodulation pattern showed that majority of the seedlings grown in the presence of *P. tinctorius* did not produce nodules (about 44%) or the nodules were distributed within top 1 cm of the root (39 to 42%) (Fig. 2b). Among the control seedlings, 22.2% of the seedlings did not produce nodules, while none of the seedlings showed nodules distributed within top 1 cm of the root.

### *Factors involved in nodule inhibition during ectomycorrhiza formation by P. tinctorius on A. mangium*

The compounds produced by *P. tinctorius* affected nodule formation even in the presence of AVG, inhibitor of ethylene biosynthesis (Fig. 3). Seedlings exposed to both AVG and compounds produced by *P. tinctorius* had significantly lower number of nodules when compared to control seedlings, and seedlings exposed to AVG alone. Different concentrations of IAA significantly influenced nodule formation by *Bradyrhizobium* in *A. mangium* (Fig. 4). IAA concentrations at 1 and 10  $\mu\text{M}$  increased the



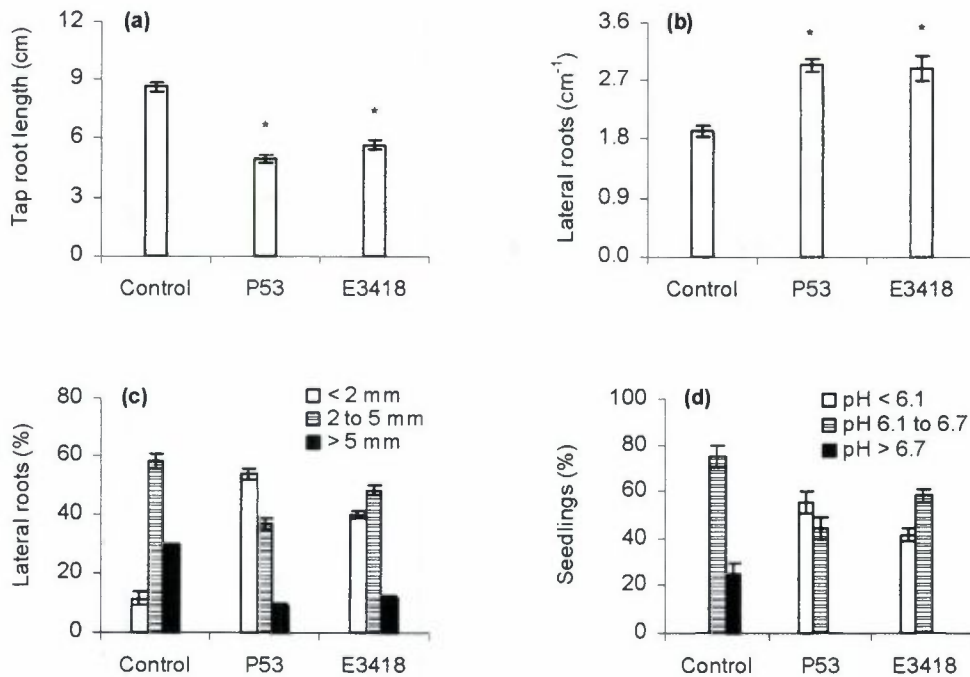


Figure 1. Changes in the root morphology and rhizosphere pH of 15-d-old *A. mangium* following contact with *P. tinctorius* through a cellophane membrane. Tap root length (a), number of lateral roots  $\text{cm}^{-1}$  (b), distribution of lateral roots based on length (c) and distribution of seedlings based on the rhizosphere pH (d). Values are means of three independent set ( $n=30$ ) of experiments, bars indicate standard error of the mean. \*Significantly different from control at  $P \leq 0.05$ . Statistical analyses were not performed for c and d. P53, E3418: *P. tinctorius* strains.

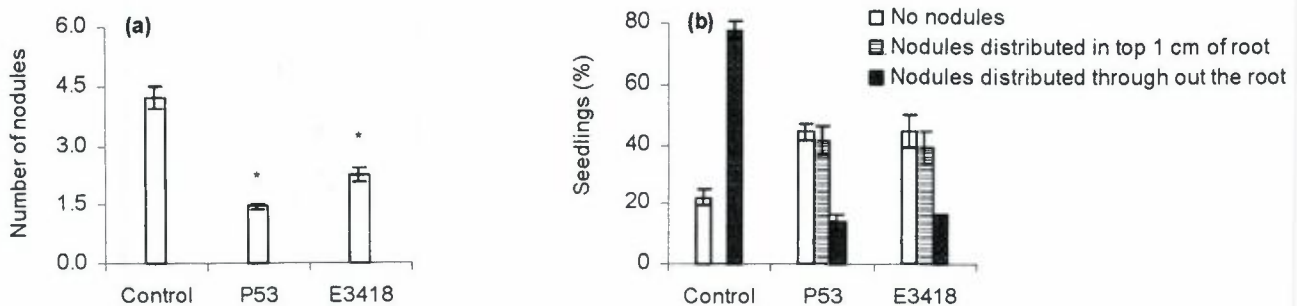


Figure 2. Number of nodules (a) and nodule distribution (b) in 21-d-old *A. mangium* grown in the presence of *P. tinctorius* separated by a cellophane membrane. Values are means of three independent set ( $n=30$ ) of experiments, bars indicate standard error of the mean. \*Significantly different from control at  $P \leq 0.05$ . Statistical analysis was not performed for b. P53, E3418: *P. tinctorius* strains.

Table 1. Effects of different concentrations of indole-3-acetic acid (IAA) on the root morphology of 15-d-old *A. mangium*. Values are means of three independent set ( $n=30$ ) of experiments  $\pm$  standard error of the mean.

Concentrations in the growth medium	Length of tap root (cm)	Number of lateral roots ( $\text{cm}^{-1}$ )	Distribution of seedlings based on length of lateral roots (%) <sup>a</sup>		
			<2 mm	2-5 mm	>5 mm
Control	9.0 $\pm$ 0.1	1.5 $\pm$ 0.1	6.0 $\pm$ 0.4	59.0 $\pm$ 1.6	35.1 $\pm$ 1.2
IAA (0.1 $\mu\text{M}$ )	8.6 $\pm$ 0.1	2.2 $\pm$ 0.2*	10.1 $\pm$ 1.4	49.2 $\pm$ 2.0	40.7 $\pm$ 2.0
IAA (1 $\mu\text{M}$ )	7.0 $\pm$ 0.2*	2.4 $\pm$ 0.2*	16.2 $\pm$ 1.6	61.4 $\pm$ 1.3	22.4 $\pm$ 0.9
IAA (10 $\mu\text{M}$ )	5.9 $\pm$ 0.1*	2.9 $\pm$ 0.2*	40.4 $\pm$ 1.8	46.9 $\pm$ 1.0	12.7 $\pm$ 0.7
IAA (100 $\mu\text{M}$ )	4.7 $\pm$ 0.2*	1.3 $\pm$ 0.1	83.1 $\pm$ 2.8	16.9 $\pm$ 2.8	0.0 $\pm$ 0.0

\*Significantly different from control at  $P \leq 0.05$ . <sup>a</sup>Statistical significance was not analyzed.

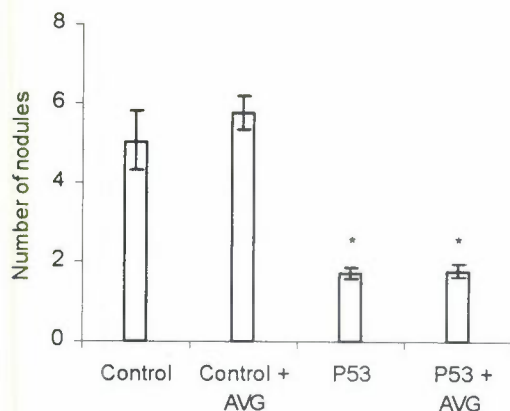


Figure 3. *Acacia mangium* nodule development following contact with *P. tinctorius* through a cellophane membrane in the presence of aminoethoxyvinylglycine (AVG), inhibitor of ethylene biosynthesis. Values are means of three independent set ( $n=30$ ) of experiments, bars indicate standard error of the mean. \* Significantly different from control at  $P \leq 0.05$ . P53: *P. tinctorius*.

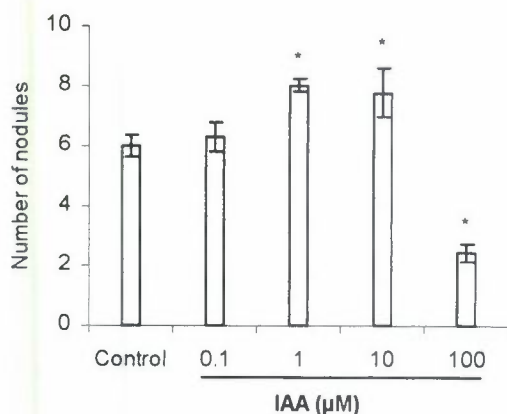


Figure 4. Effects of different concentrations of indole-3-acetic acid (IAA) on nodule formation in 21-d-old *A. mangium*. Values are means of three independent set ( $n = 30$ ) of experiments, bars indicate standard error of the mean. \*Significantly different from control at  $P \leq 0.05$ .

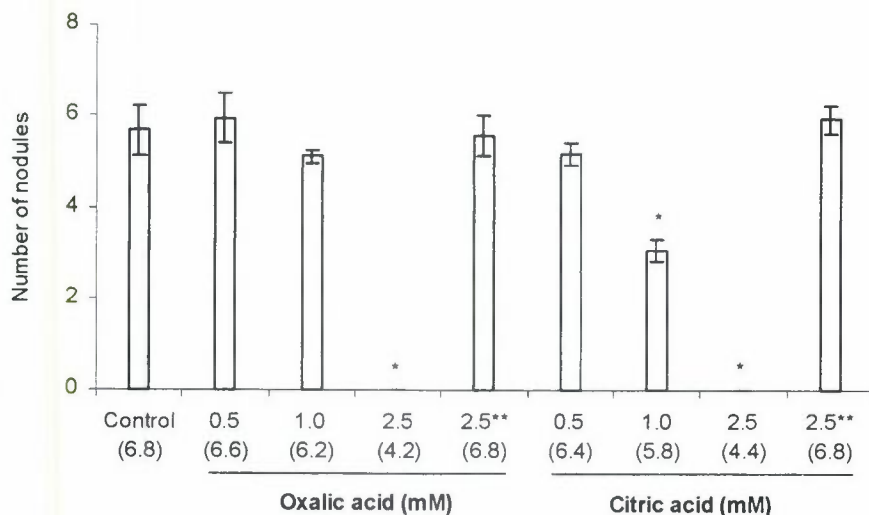


Figure 5. Effects of different concentrations of organic acids and the corresponding pH on nodule formation in 21-d-old *A. mangium*. Numbers in parentheses indicate pH of the growth medium. Values are means of three independent set ( $n=30$ ) of experiments, bars indicate standard error of the mean. \*Significantly different from control at  $P \leq 0.05$ . \*\*pH adjusted to 6.8 using KOH solution.

number of nodules when compared to the control seedlings. On the other hand, nodulation was inhibited when higher concentrations of organic acids (2.5 mM oxalic acid; 1 and 2.5 mM citric acid) were used in the growth medium (Fig. 5). Almost 100% of the seedlings did not produce nodules when grown in the presence of 2.5 mM oxalic acid or 2.5 mM citric acid. However, when the pH was adjusted to 6.8 the seedlings produced nodules.

#### 4. Discussion

*Changes during ectomycorrhiza formation by P. tinctorius on A. mangium*

In the present study the compounds produced by *P. tinctorius* significantly altered the root morphology of 15-d-old *A. mangium*. The compounds produced by *P. tinctorius* decreased the length of the tap root, increased the number of lateral roots  $\text{cm}^{-1}$  and decreased the length of lateral roots. Similar result in terms of tap root length was obtained by Slankis (1973) in pine roots, and lateral root length and distribution was obtained by Béguiristain and Lapeyrie (1997) in *Eucalyptus* roots. When synthetic auxin (IAA) was supplied exogenously to *A. mangium* seedlings, the root morphology of seedlings exposed to 1 and 10  $\mu\text{M}$  concentrations of IAA resembled ectomycorrhiza-like roots. When an auxin transport inhibitor (TIBA) was used in the growth medium, the root morphology of seedlings grown in the presence of *P. tinctorius* resembled that of the control seedlings.

These results suggest that fungal auxin is responsible for root morphogenesis during ectomycorrhiza formation. Similarly, Slankis (1973) reported that root structures resembling ectomycorrhizas could be induced in pine roots either by fungal exudates or by synthetic auxins. Likewise, Gay et al. (1994) showed that IAA over producer mutants of *Hebeloma cylindrosporium* formed 3 to 5 times more

mycorrhizas *in vitro* than the wild type, and the phenotype was positively correlated with the quantity of IAA detected in the culture media of different mycelia. Karabaghli-Degron et al. (1998) reported that fungal effect mimicked the effect of exogenous IAA on the root morphogenesis; however, in the presence of auxin transport inhibitor (TIBA) the fungal effect was reversed. They suggested that active transport of IAA from the external medium towards the central cylinder is responsible for these changes in root morphology, which was also confirmed by Rincon et al. (2001).

The rhizosphere pH of seedlings grown in the presence of *P. tinctorius* was lower when compared to control seedlings. Secretion of organic acids and significant reduction in the rhizosphere pH (Arocena and Glowa, 2000; Wallander, 2000; Griffiths et al., 1994; Lapeyrie et al., 1991) have been reported earlier suggesting that the pH reduction in the present case is probably due to the production of organic acids. It has been reported that with increasing soil acidification the root penetration into the subsoil is inhibited leading to a more shallow root system (Murach and Matzner, 1987). Furthermore, Marschner (1990) has reported that leguminous plants respond to soil acidification by changes in root morphology. On the contrary to the earlier reports, higher concentrations of organic acids, and correspondingly lower pH did not have any influence on the root morphology of *A. mangium* seedlings suggesting that the eco-type used in the present study might have higher tissue tolerance for acidic pH.

#### *Factors involved in nodule inhibition during ectomycorrhiza formation*

From the present study, it is clear that nodulation in *A. mangium* is inhibited in the presence of compounds produced by *P. tinctorius*. Similarly, depressive effect on the rhizosphere bacterial population by ectomycorrhizal roots (Meyer and Linderman, 1986; Ames et al., 1984), and post-contact inhibition of nodulation of *A. holosericea* by *P. tinctorius* (Bâ et al., 1994) have been reported earlier. In the present study, it was found that higher concentrations of exogenous IAA (except for 100 µM) did not inhibit nodule formation. On the contrary, concentrations of IAA that induced changes in root morphology also induced nodule formation by *Bradyrhizobium*. Mathesius et al. (1998) have reported that nodule formation required high auxin levels for initiation of cell divisions in the cortical region and establishment of the nodule primordium.

A drastic increase in the number of nodules formed in *Medicago truncatula* ethylene insensitive mutant showed that ethylene is involved in a local regulation of infection (Penmetsa and Cook, 1997). Ligerio et al. (1991) treated nitrate-grown, *Rhizobium*-inoculated alfalfa roots with AVG and found that the plants nodulated. Similarly, a pea mutant which usually nodulates poorly at 20°C had a greater number of infections and produced more nitrogen fixing nodules when treated with AVG (Fearn and LaRue,

1991; Guinel and LaRue, 1991). From these studies it is evident that ethylene plays a major role in the regulation of nodule formation in the host plant. However, in the present study, the presence of AVG in the growth medium did not restore nodulation in *A. mangium* seedlings, suggesting that nodule inhibition in the present case is not due to ethylene-mediated mechanism.

The role of organic acids in the reduction of rhizosphere pH during ectomycorrhiza formation has been well established. In this study, higher concentrations of organic acid (1 and 2.5 mM citric acid, 2.5 mM oxalic acid) and the corresponding lower pH significantly affected nodulation in *A. mangium* seedlings. However, when the pH was maintained at 6.8, higher concentrations of citrate and oxalate did not affect nodulation suggesting that nodule inhibition was mainly due to low pH. Negative effect of acidic pH on the nodulation process has been widely reported for a variety of legumes (Richardson et al., 1988; Whelan and Alexander, 1986; Wood et al., 1984; Lie, 1969). Inhibition of rhizosphere microorganisms by oxalic acid secreted by the ectomycorrhizal fungus *Paxillus involutus* (Duchesne et al., 1989) and the pathogenic fungus *Sclerotium delphini* (Ghaffar, 1976) has been reported earlier. These studies indicate that production of organic acids by ectomycorrhizal fungus *P. tinctorius* could be one of the factors responsible for inhibition of nodulation.

#### 5. Conclusion

From the present study, it is evident that changes in the root morphology of *A. mangium* seedlings during ectomycorrhiza formation by *P. tinctorius* are controlled by IAA produced by the fungus. There is no evidence for the involvement of IAA or ethylene in nodule inhibition. However, the regulation of IAA concentrations in different root tissues during mycorrhizal ontogeny, and their impact on nodule formation needs to be investigated. Low rhizosphere pH, probably due to production of organic acids, can be one of the factors responsible for nodule inhibition during ectomycorrhiza formation.

#### Acknowledgement

The authors are grateful to National University of Singapore, Singapore for providing a postgraduate research scholarship to P. Jayakumar and to Laboratoire de Biotechnologie des Symbioses Forestières Tropicales (ORSTOM-CIRAD/Foret), Nogent-sur-Marne, France for providing the fungal cultures.

#### REFERENCES

- Ames, R.N., Reid, C.P.P., and Ingham, E.R. 1984. Rhizosphere bacterial population responses to root



- colonization by vesicular arbuscular mycorrhizal fungus. *New Phytologist* **96**: 555–563.
- Arocena, J.M. and Glowa, K.R. 2000. Mineral weathering in ectomycorrhizosphere of subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) as revealed by soil solution composition. *Forest Ecology and Management* **133**: 61–70.
- Bâ, A.M., Balaji, B., and Piché, Y. 1994. Effect of time of inoculation on in vitro ectomycorrhizal colonization and nodule initiation in *Acacia holosericea* seedlings. *Mycorrhiza* **4**: 109–119.
- Barker, S.J., Tagu, D., and Delp, G. 1998. Regulation of root and fungal morphogenesis in mycorrhizal symbiosis. *Plant Physiology* **116**: 1201–1207.
- Béguiristain, T. and Lapeyrie, F. 1997. Host plant stimulates hypaphorine accumulation in *Pisolithus tinctorius* hyphae during ectomycorrhizal infection while the excreted fungal hypaphorine controls root hair development. *New Phytologist* **136**: 525–532.
- Chilvers, G.A., Douglass, P.A., and Lapeyrie, F.F. 1986. A paper sandwich technique for rapid synthesis of ectomycorrhizas. *New Phytologist* **103**: 397–402.
- Cooper, J.E. 1979. Rapid method for counting antibiotic-resistance *Rhizobium* in soils. *Soil Biology and Biochemistry* **11**: 433–435.
- Cromack, K., Sollins, P., Graustein, W.C., Speidel, K., Todd, A.W., Spycher, G., Li, C.Y., and Todd, R.L. 1979. Calcium oxalate accumulation and soil weathering in mats of hypogeous fungus, *Hysterangium crassum*. *Soil Biology and Biochemistry* **11**: 463–468.
- Drever, J.I. and Stillings, L.L. 1997. The role of organic acids in mineral weathering. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **120**: 167–181.
- Duchesne, L.C., Ellis, B.E., and Peterson, R.L. 1989. Disease suppression by the ectomycorrhizal fungus *Paxillus involutus*: contribution of oxalic acid. *Canadian Journal of Botany* **67**: 2726–2730.
- Fearn, J.C. and LaRue, T.A. 1991. Ethylene inhibitors restore nodulation to *sym5* mutants of *Pisum sativum* L. cv Sparkle. *Plant Physiology* **96**: 239–244.
- Fox, T.R. and Comerford, N.B. 1990. Low-molecular-organic acids in selected forest soils of the south eastern USA. *Soil Science Society of America Journal* **54**: 1139–1144.
- Fremont, M., Prin, Y., Chauvière, M., Diem, H.G., Pwee, K.H., and Tan, T.K. 1999. A comparison of *Bradyrhizobium* strains using molecular, cultural and field studies. *Plant Science* **141**: 81–91.
- Gardener, W.K., Barber, D.A., and Parbery, D.G. 1983. The acquisition of phosphorus by *Lupinus albus* L.: III. The probable mechanism by which phosphorus movement in the soil/root interface is enhanced. *Plant and Soil* **70**: 107–204.
- Gay, G., Normand, L., Marmeisse, R., Sotta, B., and Debaud, J.C. 1994. Auxin overproducer mutants of *Hebeloma cylindrosporum* Romagnesi have increased mycorrhizal activity. *New Phytologist* **128**: 645–657.
- Ghaffar, A. 1976. Inhibition of fungi as affected by oxalic acid production by *Sclerotium delphinii*. *Pakistan Journal of Botany* **8**: 69–73.
- Gogala, N. 1991. Regulation of mycorrhizal infection by hormonal factors produced by hosts and fungi. *Experientia* **47**: 331–340.
- Graham, J.H. and Linderman, R.G. 1981. Effect of ethylene on root growth, ectomycorrhiza formation and *Fusarium* infection of Douglas-fir *Pseudotsuga Menziesii*. *Canadian Journal of Botany* **59**: 149–155.
- Griffiths, R.P., Baham, J.E., and Caldwell, B.A. 1994. Soil solution chemistry of ectomycorrhizal mats in forest soil. *Soil Biology and Biochemistry* **26**: 331–337.
- Guinel, F.C. and LaRue T.A., 1991. Light microscopy study of nodule initiation in *Pisum sativum* L. cv Sparkle and its low nodulation mutant E2 (*sym5*). *Plant Physiology* **97**: 1206–1211.
- Hoben, H.J. and Somasegaran, P. 1982. Comparison of the pour, spread and drop plate methods for enumeration of *Rhizobium* sp. in inoculants made from pre-sterilized peat. *Applied and Environmental Microbiology* **44**: 1246–1247.
- Jayakumar, P. and Tan, T.K. 2005. Phosphorus solubilization by ectomycorrhizal *Pisolithus tinctorius* in pure culture and in association with *Acacia mangium*. *Symbiosis* **39**: 125–130.
- Karabaghli-Degron, C., Sotta, B., Bonnet, M., Gay, G., and Le Tacou, F. 1998. The auxin transport inhibitor 2,3,5 triiodobenzoic acid (TIBA) inhibits the stimulation of in vitro lateral root formation and the colonization of the tap root cortex of Norway spruce (*Picea abies*) seedlings by the ectomycorrhizal fungus *Laccaria bicolor*. *New Phytologist* **140**: 723–733.
- Lapeyrie, F., Ranger, J., and Vairelles, D. 1991. Phosphate-solubilizing activity of ectomycorrhizal fungi *in vitro*. *Canadian Journal of Botany* **69**: 342–346.
- Lie, T.A. 1969. The effect of low pH on different phases of nodule formation in pea plants. *Plant and Soil* **31**: 391–406.
- Ligero, F., Caba, J.M., Lluch, C., and Olivares, J. 1991. Nitrate inhibition of nodulation can be overcome by the ethylene inhibitor aminoethoxyvinylglycine. *Plant Physiology* **97**: 1221–1225.
- Marschner, H. 1990. Mechanisms of adaptation of plants to acid soils. *Plant and Soil* **134**: 1–20.
- Martin, F.M. and Hilbert, J.L., 1991. Morphological, biochemical and molecular changes during ectomycorrhiza development. *Experientia* **47**: 321–331.
- Marx, D.H. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* **59**: 153–163.
- Mathesius, U., Schlaman, H.R.M., Spaink, H.P., Sautter, C., Rolfe, B.G., and Djordjevic, M.A. 1998. Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. *Plant Journal* **14**: 23–34.
- Meyer, J.R. and Linderman, R.G. 1986. Selective influence of populations of rhizosphere bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. *Soil Biology and Biochemistry* **18**: 191–196.
- Murach, D. and Matzner, E. 1987. The influence of soil acidification on the root growth of Norway spruce (*Picea abies* Karst.) and European beech (*Fagus sylvatica* L.). In: *IUFRO Workshop on Woody Plant Growth in a Changing Chemical and Physical Environment*. Vancouver, BC, Canada, 27–31 July 1987.
- Oh, K.I., Melville, L.H., and Peterson, R.L. 1995. Comparative structural study of *Quercus serrata* and *Quercus acutissima* formed by *Pisolithus tinctorius* and *Hebeloma cylindrosporum*. *Trees* **9**: 171–179.
- Penmetza, R.N. and Cook, D.R. 1997. A legume ethylene-insensitive mutant hyper infected by its rhizobial symbiont. *Science* **275**: 527–530.
- Prin, Y., Galiana, A., Le Roux, C., Méléard, B., Razafimaharo, V., Ducouso, M., and Chaix, G. 2003. Molecular tracing of *Bradyrhizobium* strains helps to correctly interpret *Acacia mangium* response to inoculation in a reforestation experiment in Madagascar. *Biology and Fertility of Soils*



- 37: 64–69.
- Regvar, M. and Gogala, N. 1996. Changes in root growth patterns of (*Picea abies*) spruce roots by inoculation with an ectomycorrhizal fungus *Pisolithus tinctorius* and jasmonic acid treatment. *Trees* **10**: 410–414.
- Richardson, A.E., Simpson, R.J., Djordjevic, M.A., and Rolfe, B.G. 1988. Expression of nodulation genes in *Rhizobium leguminosarum* biovar *trifolii* is affected by low pH and by Ca and Al ions. *Applied and Environmental Microbiology* **54**: 2541–2548.
- Rincón, A., Gérard, J., Dexheimer, J., and Le Tacon, F. 2001. Effect of an auxin transport inhibitor on aggregation and attachment processes during ectomycorrhiza formation between *Laccaria bicolor* S238N and *Picea abies*. *Canadian Journal of Botany* **79**: 1152–1160.
- Rupp, L.A., Mudge, K.W., and Negm, F.B. 1989. Involvement of ethylene in ectomycorrhiza formation and dichotomous branching of roots of mugo pine seedlings. *Canadian Journal of Botany* **67**: 477–482.
- Scagel, C.F. and Linderman, R.G. 1998. Relationships between differential *in vitro* indole-acetic acid or ethylene production capacity by ectomycorrhizal fungi and conifer seedling responses in symbiosis. *Symbiosis* **24**: 13–34.
- Slankis, V. 1973. Hormonal relationship in mycorrhizal development. In: *Ectomycorrhizae*. Marks, G.C., and Koslowski, T.T., eds. Academic Press, London and NY, pp. 231–298.
- Slankis, V. 1950. Effect of naphtalene acetic acid on dichotomous branching of isolated roots of *Pinus sylvestris*. *Physiologia Plantarum* **3**: 40–44.
- Stevenson, F.J. 1967. Organic acids in soil. In: *Soil Biochemistry* Vol 1. McLaren, A.D., and Peterson, G.H., eds. Marcel Dekker, NY, pp. 119–146.
- Wallander, H. 2000. Uptake of P from apatite by *Pinus sylvestris* seedlings colonized by different ectomycorrhizal fungi. *Plant and Soil* **218**: 249–256.
- Whelan, A.M. and Alexander, M. 1986. Effects of low pH and high Al, Mn, and Fe levels on the survival of *Rhizobium trifolii* and the nodulation of subterranean clover. *Plant and Soil* **92**: 363–371.
- Wood, M., Cooper, J.E., and Holdings, J.A. 1984. Soil acidity factors and nodulation of *Trifolium repens*. *Plant and Soil* **78**: 367–379.