Biol Fertil Soils (1997) 25:7–12

ORIGINAL PAPER

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A new approach to enhance growth and nodulation of *Acacia mangium* through aeroponic culture

Received: 18 July 1996

Abstract This work was designed to determine whether a plant culture method on non-solid media could be used as an alternative for inoculation of *Acacia mangium* with selected strains of *Bradyrhizobium* spp. *A. mangium* seedlings were grown and inoculated with *Bradyrhizobium* strain Aust13c and strain Tel2 in hydroponics, aeroponics and sand. Aeroponics was found to be the best system of the three, allowing the production of tree saplings 1 m in height after only 4 months in culture. Moreover, compared to plants grown in liquid or sand media, aeroponically grown saplings inoculated with *Bradyrhizobium* spp. developed a very high number of small nodules distributed all along the root system, resulting in an increase in nitrogen and chlorophyll content in plant tissues. We propose aeroponics as an alternative method to classical soil inoculation procedures for the production of hypernodulated legume tree saplings.

Key words Aeroponic culture · N_2-fixation · *Acacia mangium* · *Bradyrhizobium* spp. · Hypernodulation · Tree saplings · *Imperata cylindrica*

Introduction

There are more than 1000 described species of *Acacia*, of which 650 occur in Australia, and the rest in Africa and tropical America (Bolland et al. 1984). One, *Acacia mangium* Willd., has gained increasing popularity in the last 2 decades for reforestation of degraded lands in the humid tropics, particularly in Sabah, Malaysia, where it competes well with noxious grasses such as *Imperata cylindrica* (National Academy of Sciences 1983; Turnbull 1986). Thanks to its natural nitrogen-fixing ability, *A. mangium* possesses the remarkable growth potential of pioneer tree legumes and will grow well even on a very acid and infertile soil. Its introduction is, however, not always successful due to growth limitations induced by detrimental factors in the soil of the site of introduction. Indeed, it needs to establish an association with symbiotic soil organisms including rhizobia and/or mycorrhiza to survive and grow in natural forest ecosystems (De la Cruz and Garcia 1991). *A. mangium* is specifically associated with slow-growing rhizobium strains belonging to the *Bradyrhizobium* group (Dreyfus and Dommergues 1981; Souvannavong and Galiana 1991). However, many strains naturally present in the soil have a low nitrogen fixation efficiency (Dart et al. 1991). In order to improve the growth of *A. mangium*, the seedlings should be inoculated with the appropriate *Bradyrhizobium* strain, which is effective in fixing atmospheric nitrogen and aggressive enough to compete with the less efficient strains present naturally in the soils. The technique commonly used to produce nodulated seedlings is soil inoculation with large amounts of rhizobial inoculant. Technologies for the mass production of rhizobial inoculants are available from a number of sources worldwide (Streeter 1994; Van Elsas and Heijnen 1990; Diem et al. 1988). Investment in such large quantities of inoculant is, however, relatively costly and diminishes economic returns. There is a need to improve the current inoculation technique to minimize the input of rhizobial inoculant and optimize the nodulation rate of the seedlings.

For this purpose non-solid culture media are a good alternative. Several approaches in which plants are grown in non-solid media have been used in studies on nodulation. Hydroponics has been employed in evaluating the effectiveness of plant nitrogen-fixing symbiosis (Alvarez-Solis and Leon-Martinez 1990). The inability to accurately

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study symbiotic relationships in hydroponics led to the first significant research with aeroponics (Zobel et al. 1976). Aeroponics is defined as the culture of whole plants with their roots fed by an air/water nutriment fog. Unlike water culture, plants grown aeroponically always show good root hair development due to the highly aerated environment surrounding the root system. This is an extremely important consideration in nodulation studies (Weathers and Zobel 1991). To our knowledge, no attempt has been made to use an aeroponic system for producing legume tree saplings inoculated with the appropriate strain of *Bradyrhizobium.*

The objective of this work was to investigate the potential of the aeroponic culture system as an effective alternative for growing and inoculating *A. mangium* saplings with *Bradyrhizobium.*

Materials and methods

Seed germination

Acacia mangium seeds were collected from one tree planted in Lua Song Forestry Center (Sabah, East Malaysia) chosen for its superior growth and provided by the CIRAD-Foret/Inoprise Corporation, Tawau, Sabah, Malaysia (seed lot PNG 94). Seeds were scarified by immersion for 2 min in boiling deionized water and germinated for 1 week on moist tissue paper at room temperature. They were then carefully transferred into sponge plugs, which had been previously soaked in water and grown for another week but at greenhouse temperatures (27–32 °C). Fourteen-day-old seedlings were simultaneously transferred into sand, hydroponic and aeroponic systems.

Design of the experiment

The seedlings were grown in three different systems, aeroponics, hydroponics and sand. Within each system, plants were either (1) inoculated with *Bradyrhizobium* strain Aust 13c, (2) inoculated with *Bradyrhizobium* strain Tel 2 or (3) not inoculated (control). For each treatment, 48 plants were grown in the aeroponic system, 24 plants were grown in the hydroponic system and 24 plants were grown in sand. After 3 months, 12 plants were harvested to measure shoot and root fresh and dry weights, number and weight of nodules, total shoot nitrogen content, leaf area, photosynthetic rate and chlorophyll content. At the end of the experiment, aeroponically inoculated acacia saplings were transferred to soil in plastic planting bags and subsequently transplanted onto degraded land for further growth and observations.

Sand, hydroponic and aeroponic systems

Seedlings were planted in 50 × 30-cm plastic trays in washed sand and watered with modified Hoagland solution buffered at pH 5.5 (Hoagland and Arnon 1950) once a week. The hydroponic and aeroponic systems used in this study were previously described by Lee (1993). Briefly, the hydroponic system comprises a plastic trough (0.50 × 0.30 × 0.15 m) and an aquarium pump. The aeroponic system comprises a trough (2.20 × 1.50 × 0.50 m) constructed with polystyrofoam. The nutrient mist is supplied to the plants by a mechanical pump of 1.12 kw via a pipe and a series of nozzles placed horizontally at the base of the trough. The frequency and duration of the misting is regulated by a microprocessor. Seedlings were grown in the aeroponic system with a misting schedule of 15 s at 1-min intervals with modified Hoagland solution. The nozzles used provided a very fine mist fully covering the root system. The droplet size ranged from 300 to 500 μm (Lee et al. 1994). The system used a recirculating, filtered nutrient solution and the root environment temperature was maintained at 27–30 °C. Acacias were grown in the hydroponic system using the same nutrient solution and under the same conditions.

Inoculum production

Bradyrhizobium, strains Aust 13c and Tel 2, was recovered from root nodules of *A. mangium* growing in a clay-sand soil (Daintree, Australia) (Galiana et al. 1990) and a clay soil (Sabah, East Malaysia), respectively. Aust 13c has been previously studied for its nitrogen fixation ability in association with *Acacia* sp. (Galiana et al. 1994; Lesueur et al. 1993). The two strains were cultured for 1 week in liquid YM medium (mannitol 1%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.2%, NaCl 0.01%, yeast extract 0.05%, pH 6.8) at 30 °C with agitation. The concentration of the bacteria inoculant was adjusted to 10^9 bacteria ml^{-1} according to the relationship between the number of bacteria and the optical density at 650 nm determined by a series of counting of bacterial populations as described by Cooper (1979) and Hoben and Somasegaran (1982).

Inoculation method

Two weeks after transplanting, the seedlings were inoculated with either *Bradyrhizobium* strains Aust 13c or Tel 2.

Inoculation of seedlings grown in hydroponics. Fifty milliliters of bacterial inoculant was added directly to the nutrient solution (10 l). The nutrient solution with the bacteria was replaced with fresh nutrient solution without bacteria after 1 week. The solution was renewed each week.

Inoculation of seedlings grown in aeroponics. The aeroponic system was stopped overnight and the roots were inoculated by manually spraying approximately 5 ml bacterial inoculant per plant. After inoculation, plants were fed with nutrient solution containing 41 ppm nitrogen (i.e., 20% of the initial concentration of nitrogen). The mineral composition of the nutrient solution was checked daily by pH and conductivity measurements and adjusted.

Leaf area determination

For each treatment, three leaves per plant from six different plants were harvested and the area of leaves was measured with an Area Measurement System (Delta-T Devices Ltd., England).

Nitrogen content

Nitrogen content was determined by the Kjeldahl digestion of 0.2 g dry weight leaves in concentrated sulphuric acid using a Kjeltec auto 1030 analyser (Allen 1989). For each treatment, there were three replicates.

Measurement of photosynthetic oxygen exchange and quantum yield

Maximum photosynthetic rates from acacia leaves were determined with a Hansatech leaf disc O_2 electrode (King's Lynn, UK) at 25°C and in saturating CO_2 conditions (1% CO_2 from a 1 *M* carbonate/bicarbonate buffer, pH 9) according to Ball et al. (1987). For each treatment, three replicates were used.

Chlorophyll content determination

Four leaf discs 1 cm in diameter each were cut from different leaves and ground using a mortar and pestle in 2 ml 80% acetone solution. The supernatant was collected, adjusted to 10 ml with 80% acetone

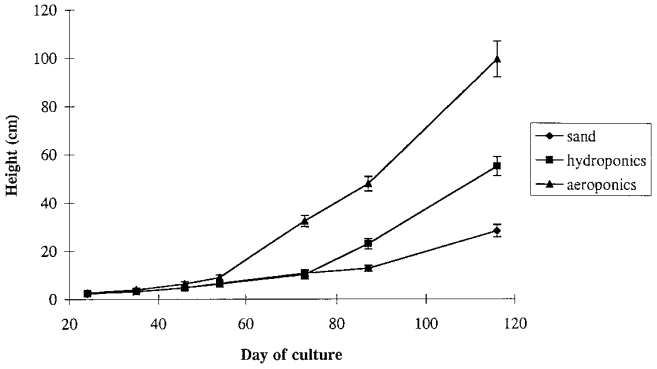

Fig. 1 Comparison of growth of *A. mangium* in different culture systems over a 4-month period. Growth is expressed as mean height ± SE (in cm)

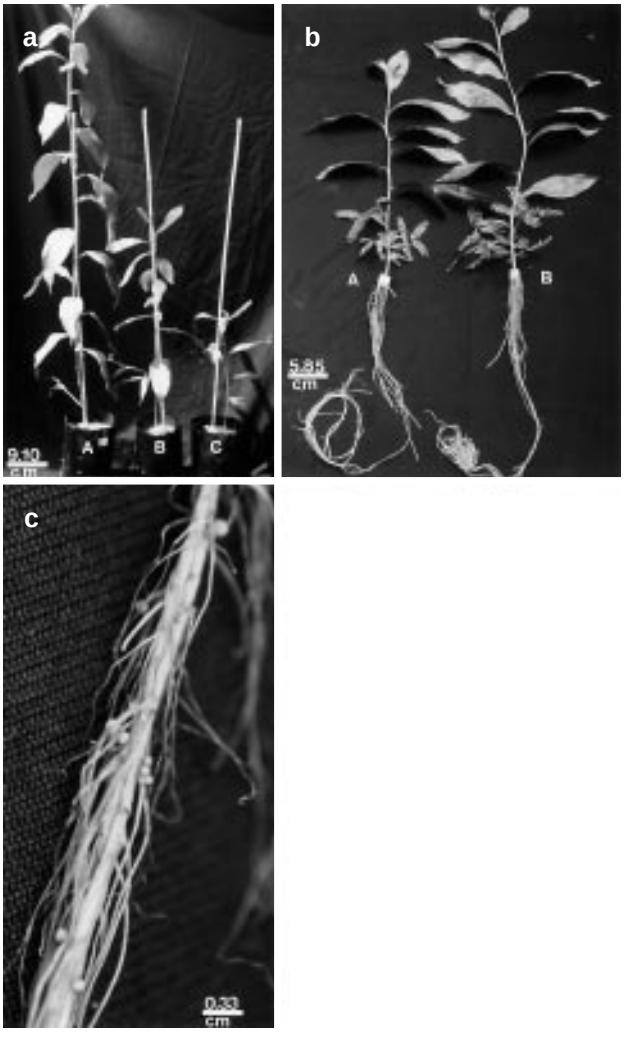

Fig. 2 **a** Twenty-week-old *A. mangium* grown for 17 weeks in aeroponics (*A*), hydroponics (*B*) and sand (*C*), respectively, from the left to right, and then transferred to soil in plastic planting bags. Plants were inoculated with *Bradyrhizobium* strain Aust 13c. **b** Ten-week-old *A. mangium* grown in aeroponics, non-inoculated (*A*) or inoculated (*B*) with the *Bradyrhizobium* strain Aust 13c. **c** Distribution of nodules on the roots of aeroponically grown *A. mangium*

Table 1 Comparison of different growth parameters of 10-week-old *A. mangium* grown in hydroponic and aeroponic systems

	Hydroponic	Aeroponic
Mean height ± SE (cm)	12.9 ± 2.2	37.8 ± 1.7*
Mean shoot dry weight ± SE (g plant^{-1})	0.459 ± 0.085	1.842 ± 0.374*
Mean root dry weight ± SE (g plant^{-1})	0.175 ± 0.031	0.563 ± 0.086*
Mean leaf area ± SE (cm^2)	61.7 ± 3.4	112.3 ± 3.7*

* Statistically significant difference between plants grown in hydroponic and aeroponic systems at $P < 0.05$

and centrifuged for 10 min at 4000 rpm. The clear supernatant was decanted into a 3-ml glass cuvette and the absorbance was recorded at 663 and 645 nm using a UV visible recording spectrometer UV-160A (model Shimadzu, Japan). Chlorophyll content was calculated according to the method of Arnon (1949) and expressed as micromoles per unit of leaf area (μmol chl m^{-2}). There were three replicates for each treatment.

Statistical analysis

The data set was statistically analysed by using the MINITAB programme (Ryan et al. 1985). A single factor analysis of variance (ANOVA) was used to test for significant differences in treatment. Data from each of the experimental treatments were then compared with the control treatment using Dunnett's procedure at $P < 0.05$.

Results

Effect of culture system on *A. mangium* growth and development

During the first 54 days of culture, the growth of *A. mangium* seedlings expressed by plant height was not significantly affected by the culture system used (Fig. 1). In contrast, plant development expressed by the formation of full phyllodes on shoots was greatly influenced, suggesting that plants grown aeroponically had already reached the mature stage. Seedlings grown in hydroponics and sand had developed only juvenile intermediary and compound leaves, respectively. Plants growing in aeroponics, hydroponics and sand took 8, 10 and 12 weeks, respectively, to reach the mature leaf morphology stage. Once plants had developed mature leaf morphology, i.e., after 87 days in culture, their growth was dramatically influenced by the culture system (Fig. 1). Plants grown aeroponically were twice as high as those in hydroponics and 4 times taller than those grown in sand. The mean heights of plants in aeroponics, hydroponics and sand at this stage were 47.8 ± 3.0 cm, 22.9 ± 2.2 cm and 12.7 ± 1.2 cm, respectively. Aeroponically grown plants also had longer internode lengths than those grown in hydroponics and sand.

Statistically significant effects of the culture system on other parameters of *A. mangium* growth were also clearly observed by comparing hydroponic and aeroponic plants (Table 1). Plants grown aeroponically showed not only greater height but greater root and shoot dry weights, as

Table 2 Effect of different culture systems on nodulation and nitrogen content of *A. mangium* inoculated with *Bradyrhizobium* strain Aust 13c after 3 months of culture

System	Hydroponics		Aeroponics	
Treatment	Control	Aust 13c	Control	Aust 13c
Mean number of nodules plant^{-1} ± SE	0	7 ± 2	0	32 ± 12
Mean weight of nodules plant^{-1} ± SE (mg plant^{-1})	0	9.7 ± 3.3	0	15.7 ± 5.6
Mean nitrogen content ± SE (g N^{-1} g plant dry weight)	0.397 ± 0.028	0.435 ± 0.056	0.424 ± 0.019	0.526 ± 0.012*

* Statistically significant difference between control and inoculated plants at $P < 0.05$

Table 3 Effects of inoculation with *Bradyrhizobium* strains Aust 13c and Tel 2 on 3-month-old aeroponically grown *A. mangium*

Treatment	Control	Aust 13c	Tel 2
Mean number of nodules plant^{-1} ± SE	0	32 ±12	25 ±6
Mean weight of nodules plant^{-1} ± SE (mg plant^{-1})	0	15.7 ±5.6	14.1 ±3.9
Mean nitrogen content ± SE (g N^{-1} g plant dry weight)	0.424±0.019	0.526±0.012*	0.515±0.021*
Mean height ± SE (cm)	37.8 ±1	45.2 ±3.7*	47.5 ±2.5*
Mean shoot dry weight ± SE (g plant^{-1})	1.842±0.374	2.612±0.316*	2.575±0.305*
Mean root dry weight ± SE (g plant^{-1})	0.563±0.086	0.656±0.095	0.675±0.085
Mean leaf area ± SE (cm^2)	112.3 ±3.7	153.2 ±6.7*	156.3 ±5.7*
Mean photosynthetic rate ± SE (μmol O_2 m^2 s^{-1})	15.3 ±0.3	20.4 ±1.4*	19.5 ±1.4*
Mean chlorophyll content ± SE (μmol m^2)	460.8 ±9.5	562.3 ±12.9*	554.8 ±8.4*

* Statistically significant difference between control and inoculated plants at $P < 0.05$

well as leaf area, compared to the plants grown hydroponically. Our results show that the aeroponic system is clearly superior to the other systems for growth and development of *A. mangium* (Fig. 2a).

Effect of the culture system on the nodulation and N_2-fixing ability of *A. mangium* inoculated with *Bradyrhizobium* strain Aust 13c

From our results, it was observed that hydroponic and aeroponic systems were superior in enhancing growth of *A. mangium* as compared to sand (Figs. 1, 2a). Plants grown in sand were only 27 cm in height after 3.5 months. Nodulation experiments were thus conducted predominantly with plants grown in hydroponic and aeroponic systems to assess further enhancement of growth.

Hydroponically and aeroponically grown plants inoculated with *Bradyrhizobium* strain Aust 13c exhibited significantly different rates of nodulation. Interestingly the number of nodules in plants grown aeroponically was fourfold higher than that of plants grown in the hydroponic system (Table 2). The nodules were spherical, healthy and small in size and were well distributed along the root system (Fig. 2c). The diameter and weight of the nodules were also influenced by the culture system used. Although the number of nodules on plants grown hydroponically were fewer than those grown aeroponically, the nodules were larger in size. The mean diameters of the nodules ± standard error (SE) were 0.113 ± 0.015 mm and 0.059 ± 0.008 mm for the hydroponic and aeroponic systems, respectively. The weight of individual nodules on hydroponically grown plants was also greater than those on aeroponically grown plants.

In hydroponic conditions, nodulation effect of inoculated plants was negligible as shown by nitrogen content in shoots, which was similar to that of non-inoculated plants (Table 2). This is probably due to the very low number of nodules present in inoculated plants grown in liquid medium (plants in sand inoculated with bacteria also produced extremely low numbers of nodules; mean number of nodules per plant was 5 ± 2). By contrast, the nodules were so numerous on the root system of the inoculated aeroponically grown plants that they significantly increased the nitrogen content of these plants as compared to that of the controls grown in the same conditions (Fig. 2b).

Nodulation and N_2-fixation ability of *A. mangium* grown in the aeroponic system and inoculated with *Bradyrhizobium* strains Aust 13c and Tel2

The two *Bradyrhizobium* strains isolated from different sites nodulated *A. mangium* with the same intensity in the aeroponic system (Table 3). The mean numbers of nodules produced for plants inoculated with Aust 13c and Tel2 were 32 ± 12 and 25 ± 6 plant^{-1}, respectively; this corresponded to mean nodule weights of 15.7 ± 5.6 mg and 14.1 ± 3.9 mg, representing approximately 2% of the total root dry weight in both cases.

As expected, inoculation of *A. mangium* in the aeroponic system with both strains, Aust13c and Tel2, resulted in significantly better growth. The internodes of nodulated plants were longer than those of the control plants, with nodulated plants being in general 20–25% taller than controls. Both Aust 13c and Tel2 nodulated plants showed a significant increase in height, root and shoot dry weights and leaf area as compared to the controls. The nitrogen contents of the nodulated plants were also notably different from those of the controls. Compared to that in the controls, nitrogen content in nodulated plants increased by 22%. This additional amount of nitrogen was derived from nitrogen fixation of the nodules formed in the root system of *A. mangium* after inoculation with Aust 13c or with Tel 2. The photosynthetic rate and the chlorophyll content of the nodulated plants were also significantly higher than in the control plants. Since the nodulation process is stimulated by an air-rich environment around the roots, it is suggested that the aeroponic system allowed the plants to fully maximize their growth potential.

Discussion

In the last few decades, several groups have studied nitrogen-fixing symbioses by growing the host plant, i.e. leguminous and actinorhizal plants in aeroponic conditions (Newcomb 1976; Newcomb et al. 1977; Weisz and Sinclair 1988a, b). However, it has not been used extensively because of incomplete knowledge about the operational parameters and the difficulty in maintaining the operating system (Bowes et al. 1977; Callaham and Torrey 1977; Newcomb et al. 1977). Recently the operational system has been simplified to allow the application of aeroponics to produce, on a commercial scale, temperate vegetables in tropical regions. This aeroponic system was also used to accelerate rooting of hardwood cuttings which are difficult to root (Lee 1993). This paper reports for the first time that the aeroponic system can be used successfully to enhance development, growth and nodulation of *A. mangium*.

The development of the aerial part of *A. mangium* was strongly affected by the culture system used. Aeroponically grown plants reached the full phyllode stage that characterizes the mature condition earlier than hydroponically grown plants or plants grown in sand, the latter two reaching only the intermediary and compound leaf stages, respectively. *A. mangium* is known to show salient differences in leaf morphology which are associated with its ontogeny (Doorenbos 1965). Although plants grown in the three culture systems were at the same age chronologically, they exhibited great differences in leaf morphology, suggesting that they were not at the same stage as far as ontogenesis of the shoot is concerned. The aeroponic culture system was able to accelerate the ontogenetic process of *A. mangium*, demonstrating that environmental factors can be manipulated to allow for the full expression of its genetic potential.

Four-month-old plants in aeroponics were more than 1 m in height and were approximately 2 times and 4 times taller than plants in hydroponics and sand. This tremendous increase in growth due to aeroponic culture is economically important because it increases the already remarkable potential of *A. mangium* as a fast-growing tree species. *A. mangium* commonly reaches 20–25 m in height within 10–15 years in Sabah, with a wood production averaging 25–30 $m^3\ ha^{-1}\ year^{-1}$ (Sim 1986). Compared to growth results obtained in the nursery and field, the simple use of aeroponics can cut the time required to establish a plantation by 1–2 years, and perhaps more if the effect of rapid growth is sustained after transfer to the field.

Acacia mangium has already been used for screening the effectiveness of *Bradyrhizobium* strains (Souvannavong and Galiana 1991). Two *Bradyrhizobium* spp. strains, Aust 13c from Australia and Tel2 from Malaysia, were found to be highly effective and competitive compared to indigenous strains of *Bradyrhizobium* spp. in Africa (Galiana et al. 1994). In our present research, Aust 13c and Tel 2 were used to evaluate the effect of the culture system on nodulation of *A. mangium*. Our results clearly demonstrate that with a relatively small quantity of inoculum, plants grown aeroponically nodulated more profusely than plants grown in liquid or sand media. The high number of nodules observed along the root system of aeroponically grown *A. mangium* and the increase in nitrogen content in shoots suggest that the establishment and the functioning of the N_2-fixation symbiosis are facilitated in aeroponics. Aeroponics has been shown to be able to modify root architecture (Lee et al. 1994). The development of numerous root hairs and secondary roots of aeroponically grown *A. mangium* can facilitate the initial interaction between the host plant and the microsymbiont, characterized by the attachment of the rhizobia to root hairs all over the roots (Bhuvaneswari et al. 1980), by increasing the number of infection sites. Rhizobia enter the host by the root hair tip and, depending on the host, root hair deformation takes place 6–18 h after inoculation (Smit et al. 1987; Vesper and Bauer 1986). In our present study, the aeroponic system was switched off immediately after inoculation with rhizobia for a 16-h period, and this appeared to be sufficient for contact to be made between the two symbiotic partners. *A. mangium* grown in aeroponics and inoculated with the Australian *Bradyrhizobium* strain Aust 13c or Malaysian *Bradyrhizobium* strain Tel2 showed a significant improvement in nitrogen content and growth. Indeed, the chlorophyll content and the photosynthetic rate were significantly increased certainly due to the high nitrogen content in the shoot.

Aeroponics significantly increased both growth and nodulation of *A. mangium*, confirming the idea that aeroponics can be used as a good method to produce in a short time hypernodulated legume tree saplings for reforestation of degraded land in the humid tropics. The quantity of bacteria inoculum required for nodulating a large number of trees can be kept relatively low. The investment in aeroponic culture is cost effective taking into account the number of trees that can be grown in a limited area. We will be conducting further experiments using the aeroponic

culture system to root and nodulate *A. mangium* from stem cuttings of elite mature trees.

Acknowledgements We gratefully acknowledge the French Ministry of Foreign Affairs and the French Embassy in Singapore, CIRAD (France) and NTU-NIE (Singapore) for providing financial support. We would like to thank Dr. Shirley Lim for helpful advice on statistical analysis. Many thanks are due to Ho Wah Chai and Siti Alimah Retno for their technical assistance in the greenhouse.

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