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Variations in the responses of *Acacia mangium* to inoculation with different strains of *Bradyrhizobium* sp. under nursery conditions

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Abstract

Acacia mangium Willd. is a leguminous tree that can form symbiotic association with N_2-fixing *Bradyrhizobium* sp. A nursery experiment was conducted to study the variations in the responses of *A. mangium* seedlings to inoculation with different strains of *Bradyrhizobium* in terms of dry matter production and allocation to underground parts, P uptake, and nodulation and N_2 fixation. *Bradyrhizobium* strains Aust 11c, Aust 13c, BuT 2, Nlu 27, Tel 2 and Was 9 were used in the present study. Inoculated seedlings were also compared with N-fed seedlings. All the inoculated seedlings allocated more biomass for undergrowth and had higher P contents when compared to N-fed seedlings. However, N-fed seedlings had higher N contents. The shoot N:P ratio was significantly higher in N-fed seedling when compared to *Bradyrhizobium*-inoculated seedlings indicating imbalances in nutrient uptake. Among the different strains of *Bradyrhizobium*, Was-9-inoculated seedlings allocated more biomass for root and nodule growth, and had higher N and P contents when compared to seedlings inoculated with other strains. Only inoculation with Was 9 had a significant positive effect on the growth of *A. mangium* when compared to N application. The differences in underground production in *A. mangium* seedlings inoculated with different strains of *Bradyrhizobium* can be attributed to the differences in nodule carbon consumption. Strains that efficiently utilize the resources allocated for N_2 fixation can stimulate underground growth without affecting aboveground production, and such strains are more suitable for inoculation to nursery seedlings used in reforestation programme.

Keywords: *Acacia mangium*, *Bradyrhizobium*, nodulation, N_2 fixation, P uptake

1. Introduction

Acacia mangium Willd. (Pedley, 1987) is a leguminous tree that can form symbiotic association with both N_2-fixing *Bradyrhizobium* sp. and mycorrhizal fungi. Out of the 1,000 described species of Acacia (Bolland et al., 1984), *A. mangium* has gained popularity in the last two decades for reforestation of degraded lands (National Academy of Sciences, 1983; Turnbull, 1986), because of its ability to grow on degraded soils. *Acacia mangium* is the most widely planted *Acacia* species in Asia (Lee and Nguyen, 1991) and Africa (Kessy, 1991). Large-scale plantations have been successfully introduced in Malaysia and Indonesia where most of the trees were planted on poor soils (Galiana et al., 1998). *Acacia mangium* trees can reach a height of 20–25 m in 10–15 years with wood production averaging 25–30 m^3 ha^{-1} $year^{-1}$ (Sim, 1986). Apart from being used as a fallow species in agroforestry and as fuel, the wood is good for pulp and paper. The fast development of *A. mangium* and its ability to colonize degraded lands is attributed only to its symbiotic association with rhizobia and mycorrhizal fungi (de la Cruz and Garcia, 1991).

Inoculation with certain strains of *Bradyrhizobium* has been reported to enhance the growth of *A. mangium* seedlings under *in vitro* and nursery conditions (Galiana et al., 1990; Galiana et al., 1998; Fremont et al., 1999; Prin et al. 2003). However, in most of the studies, the strain efficiencies were assessed mainly based on aboveground production. Nodules produced by leguminous plants during their symbiotic association with rhizobia require carbon for their growth and development, and N_2 fixation, assimilation and transportation (Schubert and Wolk, 1982; Mahon, 1983). Nitrogen fixation (reduction of N_2 to NH_3 by nitrogenase) is the most energy-intensive process which consumes 60% to 80% of the nodule ATP (Heytler and Hardy, 1984; Rainbird et al., 1984). The estimates of total carbon costs of N_2 fixation vary with host species,

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rhizobial strain and stage of plant development (Schubert and Wolk, 1982; Atkins, 1984; Rainbird et al., 1984; Salsac et al., 1984). Root and nodule growth depends upon carbon use within nodulated roots, which in turn depends upon the respiratory carbon costs induced by roots and nodules (Voisin et al., 2003). According to Schulze et al. (1999), *Vicia faba* L. plants were able to compensate for the carbon loss during N_2 fixation by higher photosynthesis, while *Pisum sativum* L. plants did not show any change in their photosynthetic activity. In the later case, the carbon requirement for N_2 fixation was met at the expense of root growth (Schulze et al., 1999; Voisin et al., 2003).

Seedlings of trees used in reforestation programme must be able to establish themselves in nutrient poor soils because such soils are unfavourable for growth from the start. *Bradyrhizobium* strains that efficiently utilize the resources allocated for N_2 fixation can stimulate underground growth without affecting aboveground production and such strains are more suitable for inoculation to nursery seedlings used in reforestation programme. The present experiment was designed to study the variations in the responses of *A. mangium* seedlings to inoculation with different strains of *Bradyrhizobium* in terms of dry matter production and allocation to underground parts, P uptake, and nodulation and N_2 fixation. In addition, a comparison was made between seedlings inoculated with *Bradyrhizobium* and those treated with mineral N to understand the responses of *A. mangium* to available N in the soil.

2. Materials and Methods

Origin and source of Bradyrhizobium *strains*

Six *Bradyrhizobium* strains namely, Aust 11c, Aust 13c, BuT 2, Nlu 27, Tel 2 and Was 9 were used in the present study. Strains Aust 11c and Aust 13c were originally isolated from Australia (Galiana et al., 1990; Galiana et al., 1994). The strains Nlu 27, Was 9 and Tel 2 originated from Sabah, Malaysia, while the strain BuT 2 originated from Singapore (Fremont et al., 1999). The Australian strains were obtained from Laboratoire de biotechnologie des Symbioses Forestiéres Tropicales ORSTOM/CIRAD-Forét Nogent, France. All the Malaysian and Singaporean strains belonged to the Mycology and Plant Pathology Laboratory, Department of Biological Sciences, National University of Singapore.

Culture maintenance and inoculum production

All the *Bradyrhizobium* strains were stored in modified Yeast-extract-mannitol (YM) agar plates at 4°C and sub-cultured every 2 months. Ten-day-old cultures on YM agar plates incubated at 27°C were used as mother culture. The bacterial culture on agar plates were transferred to sterile distilled water with 0.5% glucose to attain a final concentration of 10^9 cells ml^{-1}. This was estimated using the relationship between the number of bacteria and its optical density at 650 nm as described by Cooper (1979) and Hoben and Somasegaran (1982).

Seedling inoculation experiment

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 14-d-old healthy seedling free of contamination was aseptically transferred into a plastic cup containing 300 g of sterile, water-washed river sand. The seedling was then inoculated with 2 ml suspension of *Bradyrhizobium* (10^9 cells ml^{-1}). Control seedlings did not receive any form of N and were not inoculated with *Bradyrhizobium*. Nitrogen-treated seedlings were supplied with 20.4 mg of NH_4NO_3 in solution at 15 d intervals. A total of 25 replicate plants were maintained for each treatment and control. All the plants were grown in a net house and supplied with 30 ml of N-free nutrient solution, pH 6.7 (Broughton and Dilworth, 1971) once in 15 d. The seedlings were watered with sterile distilled water as required to maintain moisture conducive to seedling growth.

After 6 months of growth, the seedlings were harvested. The number of nodules $plant^{-1}$ was counted. The shoot, root and nodules were separated and dried in an oven with air circulation at 60°C for 72 h, and the dry weights were recorded. Percentage dry weight allocated to underground growth was calculated from the plant dry weight. The dried shoot, root and nodule samples were homogenized and digested separately (Novozamsky et al., 1983). The N content in the digested plant samples was analyzed by indophenol blue method (Novozamsky et al., 1974) and the P content analyzed by stannous chloride reaction method (Allen, 1989).

Statistical analysis

The data was analyzed using the SPSS program. A single factor analysis of variance (one way ANOVA) was used to test for significant differences in treatments. A multiple range analysis was used to test for significant differences between treatments using Duncan's procedure at $P \leq 0.05$.

3. Results

Dry matter production and allocation

The total, shoot, root and nodule dry matter of *A. mangium* seedlings inoculated with different strains of *Bradyrhizobium* are shown in Table 1. Both *Bradyrhizobium* inoculation and N application significantly enhanced dry matter production by *A. mangium.* Seedlings inoculated with *Bradyrhizobium* had about 2.5 times higher dry matter when compared to control seedlings. Among the *Bradyrhizobium* strains, seedlings inoculated with Was 9 produced significantly higher dry matter when compared to seedlings inoculated with Aust 13c and Tel 2. Seedlings inoculated with Was 9 also produced significantly higher dry matter when compared to seedlings treated with N. The dry matters produced by seedlings inoculated with other strains of *Bradyrhizobium* were similar to those produced by seedlings treated with N.

The shoot production was significantly higher in *A. mangium* seedlings inoculated with *Bradyrhizobium* when compared to control seedlings. On an average, inoculated seedlings produced 2.4 times higher shoot dry matter when compared to control seedlings. But inoculation with *Bradyrhizobium* did not have a significantly positive effect on the shoot growth of *A. mangium* when compared to N application. Although not statistically different, Was-9-inoculated seedlings produced higher shoot dry matter when compared to N-treated seedlings. Strains of *Bradyrhizobium* did not vary in their potential to increase shoot production in *A. mangium.* However, the strains varied significantly in their ability to enhance underground production in *A. mangium.*

Seedlings inoculated with Was 9 strain of *Bradyrhizobium* had the highest root dry weight followed by Nlu-27-inoculated seedlings. Inoculation with these two strains of *Bradyrhizobium* resulted in significantly higher root dry matter in the seedlings when compared to inoculation with other strains, except Aust 11c. Inoculation with Was 9 and Nlu 27 strains also had a significantly positive effect on the root dry weight of *A. mangium* seedlings when compared to N application. Control seedlings produced significantly lower root dry matter when compared to all other seedlings.

Seedlings inoculated with *Bradyrhizobium* had significantly higher nodule dry matter when compared to control and N-treated seedlings. Among the seedlings inoculated with different strains of *Bradyrhizobium*, Was-9-inoculated seedlings produced significantly higher nodule dry matter that was 1.6 to 2.1 times higher when compared to seedlings inoculated with other strains of *Bradyrhizobium.* Seedlings inoculated with the strain Aust 13c had the lowest nodule dry weight.

The percentage dry weight, calculated as percentage of total plant dry weight, allocated for underground production was highest in control seedlings and lowest in N-treated seedlings (Fig. 1). Seedlings inoculated with *Bradyrhizobium* allocated significantly lower dry matter for underground growth when compared to control seedlings, but allocated higher dry matter when compared to N-treated seedlings. Among the *Bradyrhizobium* strains, inoculation with Was 9 and Nlu 27 significantly enhanced dry matter

Table 1. Dry matter production and nodule formation in 6-month-old *A. mangium* inoculated with different strains of *Bradyrhizobium* or treated with N. Control: Uninoculated and not treated with N. Means (n=25) within columns followed by same letter are not significantly different at $P \leq 0.05$ according to Duncan's Multiple Range Test.

Treatments	Total dry weight (g)	Shoot dry weight (g)	Root dry weight (g)	Nodule dry weight (g)	Number of nodules
Control	0.685a	0.563a	0.094a	0.028a	15a
Mineral N	1.623b	1.392b	0.222bc	0.009b	12a
Aust 11c	1.790bc	1.423b	0.270cd	0.096cd	51b
Aust 13c	1.607b	1.319b	0.204b	0.083c	48b
BuT 2	1.716bc	1.406b	0.215bc	0.094cd	52b
Nlu 27	1.746bc	1.348b	0.294d	0.104d	81c
Tel 2	1.576b	1.274b	0.210b	0.091cd	55b
Was 9	1.951c	1.498b	0.301d	0.152e	60b

Table 2. Nitrogen and phosphorus contents in 6-month-old *A. mangium* inoculated with different strains of *Bradyrhizobium* or treated with N. Control: Uninoculated and not treated with N. Means (n=25) within columns followed by same letter are not significantly different at $P \leq 0.05$ according to Duncan's Multiple Range Test.

Treatments	Total N content (mg)	Shoot N concentration (%)	Total P content (mg)	Shoot P concentration (%)	Shoot N:P ratio
Control	17.15a	1.66a	0.191a	0.031a	57.05a
Mineral N	51.15c	3.33c	0.462b	0.029a	139.37c
Aust 11c	44.51b	2.55b	0.669cd	0.037ab	79.26ab
Aust 13c	43.34b	2.68b	0.599bc	0.036ab	88.30b
BuT 2	40.98b	2.43b	0.552bc	0.030a	90.86b
Nlu 27	43.09b	2.55b	0.640cd	0.037ab	81.69ab
Tel 2	40.71b	2.58b	0.554bc	0.035ab	87.46b
Was 9	46.70bc	2.40b	0.785d	0.041b	67.94ab

allocation for underground growth of *A. mangium* when compared to inoculation with other strains, except Aust 11c.

Nodule number

Control and N-treated seedlings produced significantly lower number of nodules when compared to *Bradyrhizobium*-inoculated seedlings (Table 1). Among *Bradyrhizobium*-inoculated seedlings, seedlings inoculated with Nlu 27 produced significantly higher number of nodules when compared to seedlings inoculated with other strains.

N content and concentration

Seedlings inoculated with *Bradyrhizobium* or treated with N had significantly higher N contents when compared to control seedlings (Table 2). Seedlings inoculated with different *Bradyrhizobium* strains had an average of 2.5 times higher N content when compared to control seedlings. However, inoculated seedlings had significantly lower N contents when compared to N-treated seedlings. Seedlings inoculated with different strains of *Bradyrhizobium* did not vary significantly in their N contents. However, Was-9-inoculated seedlings had the highest N content among *Bradyrhizobium*-inoculated seedlings.

The shoot N concentration was the highest in N-treated seedlings, which was significantly higher when compared to inoculated and control seedlings (Table 2). Among the *Bradyrhizobium*-inoculated seedlings, the shoot N concentration did not vary significantly, and was comparable with control seedlings.

P content and concentration

Seedlings inoculated with *Bradyrhizobium* had higher P contents when compared to control and N-treated seedlings (Table 2). However, the P uptake was significantly different from N-fed seedlings only when the seedlings were inoculated with Was 9, Nlu 27 and Aust 11c strains of *Bradyrhizobium*. Seedlings inoculated with Was 9 had 1.7 times higher P contents followed by Nlu-27- and Aust-11c-inoculated seedlings, both of which showed 1.4 times higher P uptake, when compared to N-treated seedlings. However, only Was-9-inoculated seedlings had significantly higher P contents when compared to seedlings inoculated with Aust 13c, Tel 2 and BuT 2.

All the *Bradyrhizobium*-inoculated seedlings had higher P concentration in the shoots when compared to N-treated seedlings (Table 2). Among the inoculated seedlings, Was-9-inoculated seedlings had the highest shoot P concentration. However, the shoot P concentration in Was-9-inoculated seedlings was not significantly different from those of other inoculated seedlings, except BuT-2-inoculated seedlings.

Shoot N:P ratio

Seedlings treated with N had significantly higher shoot N:P ratio when compared to control and *Bradyrhizobium*-inoculated seedlings (Table 2). Seedlings inoculated with different strains of *Bradyrhizobium* did not vary significantly in their shoot N:P ratio, although Was-9-inoculated seedlings had the lowest value.

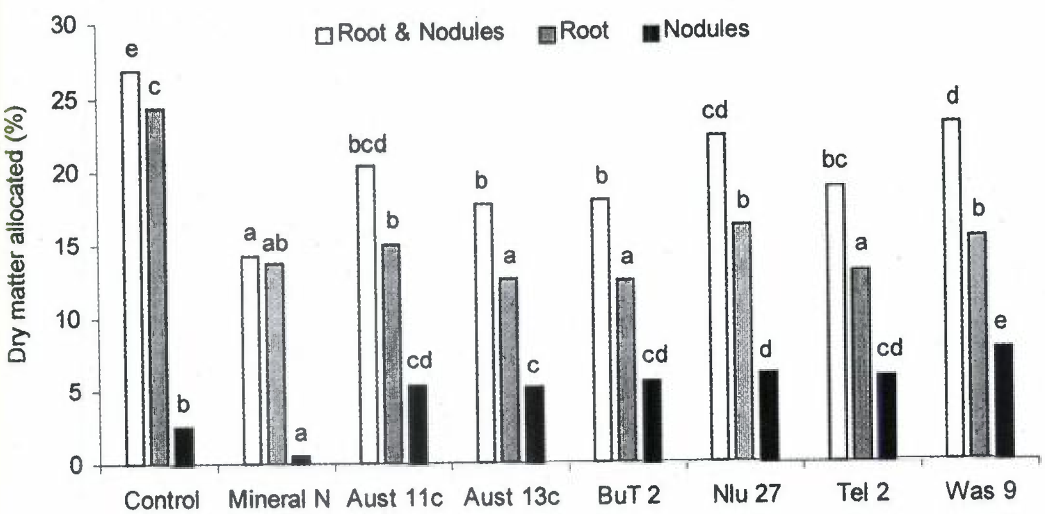

Figure 1. Percentage of total dry matter allocated to underground parts in 6-month-old *A. mangium* inoculated with different strains of *Bradyrhizobium* or treated with N. Control: Uninoculated and not treated with N. Data are means of 25 replicates per treatment. Different letters mean significant differences ($P \leq 0.05$) according to Duncan's Multiple Range Test.

4. Discussion

The ultimate structure of a natural root system is determined largely by the environment during the early stages of development (Eis, 1974). Nutrition and especially the N form that a plant species utilizes have a profound effect on its physiological and morphological characteristics (Bedell et al., 1999). When grown on N-infertile soils, N_2-fixing legumes exhibit N stress during the early stages of growth before appreciable amounts of N are fixed (Farrington et al., 1977). It has been well established that plants react to N deprivation by allocating more dry matter to the root system (van der Werf and Nagel, 1996; Ameziane et al., 1997). Tan and Hogan (1997) studied the physiological and morphological responses to N limitation in jack pine seedlings and reported that low N seedlings yielded a higher root/shoot ratio. Higher biomass allocation to underground parts in the inoculated seedlings may be due to the initial time taken for nodulation and N_2 fixation, and non-availability of N, as compared to the seedlings that received N in the available form.

In the present study, inoculated *A. mangium* seedlings showed a higher P uptake (about 1.4 times) when compared to seedlings treated with N. Similar results were earlier obtained by Israel (1987). The author investigated the role of P in symbiotic N_2 fixation and reported that P uptake and whole plant P concentration of N_2-fixing plants were equal to or greater than those of N-fed plants, across the entire range of external P concentration. A positive relationship between dry matter allocation to underground parts and P uptake in this study complements the earlier statement made by Binkley et al. (2000), who suggested that higher rates of P uptake by N_2-fixing *Albizia* is due to higher allocations of carbohydrate to underground production. In the present study, application of N to *A. mangium* did not result in significantly higher biomass production as compared to inoculation with *Bradyrhizobium*, despite higher N content and higher shoot N concentration of the seedlings. In fact, Was-9-inoculated seedlings produced significantly higher biomass when compared to N-fed seedlings. Schulze et al. (1994) have earlier reported that nodulated leguminous plants often reach the same growth rates as N-fed plants by means of a more intensive photosynthesis. The N:P ratio of the seedlings treated with N or inoculated with *Bradyrhizobium* explains the imbalances in nutrient uptake by *A. mangium* seedlings when supplied with N. Such imbalances in N and P uptake can affect the dark reactions of photosynthesis resulting in reduced production of assimilate required for growth (Ben Brahim et al., 1996).

Intraspecific variation in the efficiency of *Bradyrhizobium* strains to enhance shoot height (Galiana et al., 1994; Galiana et al., 1998; Fremont et al., 1999; Martin-Laurent et al., 1999) and nodulation and nodule efficiency (Galiana et al., 1990) have been reported earlier. Differences in P uptake by *A. mangium* inoculated with different strains of *Bradyrhizobium* have also been reported by Martin-Laurent et al. (1999). For instance, inoculation with *Bradyrhizobium* Lu 4 strain doubled the foliar P concentration of 20-month-old *A. mangium* when compared to inoculation with *Bradyrhizobium* Aust 13c strain. The present study demonstrates that the differences in P uptake by *A. mangium* inoculated with different strains of *Bradyrhizobium* are due to the variations in underground production. It has been reported that plants respond to carbon loss due to N_2 fixation with higher photosynthetic rate. In the absence of more intensive photosynthesis, carbon loss due to N_2 fixation was met at the expense of root growth (Schulze et al., 1999; Voisin et al., 2003). The differences in the underground production in seedlings inoculated with different strains of *Bradyrhizobium* may be due to the differences in nodule carbon consumption. Truelsen and Wyndaele (1984) reported that *Rhizobium leguminosarum* strains varied in their ability to recycle the energy lost during N_2 fixation due to variations in their hydrogenase uptake activity. Similar studies may help to further understand the differences among *Bradyrhizobium* strains reported in this study.

On transplanting to reforestation sites, seedling growth will be affected by low intensity of light as a result of shading (Lorimer et al., 1994) and/or low availability of nutrients in the soil (Helmisaari, 1990; Attiwill and Adams, 1993). While relatively higher shoot growth helps to capture light and higher root growth helps the seedlings to explore more area and absorb nutrients efficiently from the soil, higher nodule growth helps in better N_2 fixation. *Bradyrhizobium* strains that enhance both aerial and underground growth can contribute to the establishment of seedlings upon transplantation to degraded sites. The reported variations among *Bradyrhizobium* strains in stimulating underground production suggest that, in future, screening of *Bradyrhizobium* strains for inoculation to *A. mangium* should be based on their ability to enhance both above and belowground production. Based on the strains used in the present study, *Bradyrhizobium* strain Was 9 seem to be suitable for inoculation to nursery seedlings used in reforestation programme as they enhance the shoot, root and nodule growth of *A. mangium* seedlings when compared to other strains. Moreover, the higher N and P contents in the seedlings inoculated with Was 9, prior to transplantation, will also enhance their survival rates in soils with low N and P.

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