Ann. For. Sci. 60 (2003) 379–384
© INRA, EDP Sciences, 2003
DOI: 10.1051/forest:2003029

Original article

Optimisation of inoculation of *Leucaena leucocephala* and *Acacia mangium* with rhizobium under greenhouse conditions

Diégane DIOUF[a], Sylvain FORESTIER[b], Marc NEYRA[c] and Didier LESUEUR[b]*

[a] Université C.A. Diop, Département de Biologie Végétale, Laboratoire de Microbiologie des Sols, IRD, BP 1386, Dakar, Sénégal
[b] Programme Arbres et Plantations du CIRAD-Forêt, Laboratoire de Microbiologie des Sols, IRD, BP 1386, Dakar, Sénégal
[c] Unité de Recherche "Symbioses Tropicales et Méditerranéennes" de l'IRD, Laboratoire de Microbiologie des Sols, IRD, BP 1386, Dakar, Sénégal

(Received 10 December 2001; accepted 13 August 2002)

Abstract – Our work concerned the optimization of inoculation of two agroforestry species of woody leguminous plants: *Leucaena leucocephala* and *Acacia mangium* with various strains of rhizobium. First, we showed that the physiological stage of the bacterial culture had no effect on nodulation and growth of the seedlings of *Acacia mangium* inoculated and cultivated in vitro for four months. For *Leucaena leucocephala*, the number of nodules was significantly higher when the seedlings were inoculated with a bacterial culture in stationary phase. On the other hand, whatever the species, no significant difference was noted with regards to the dry weight of the shoots. The effect of the size of inoculum on the nodulation and growth of the seedlings was studied in *L. leucocephala* after five months in a greenhouse. Our result show that an inoculation with bacterial cultures containing 10^9 to 10^{10} bacteria per milliliter are the optimal conditions to have a maximum nodulation and growth of the seedlings. The two legume plant species showed significant differences with regard to the effect of the method of inoculation on nodulation and growth of the seedlings. For *Acacia mangium*, inoculation with a liquid culture one week after sowing was more favourable for the growth of the seedlings. On the other hand, for *Leucaena leucocephala*, this method of inoculation and the coating of seeds with a bacterial culture mixed with arabic gum improved significantly the growth of the seedlings. Results obtained in our study can be useful for the partners from developing countries involved in the large scale production of tree seedlings.

***Acacia mangium* / *Leucaena leucocephala* / rhizobium / size of inoculum / symbiosis**

Résumé – Optimisation de l'inoculation de *Leucaena leucocephala* et de *Acacia mangium* avec rhizobium en conditions de serre. Nos travaux ont porté sur l'optimisation de l'inoculation de 2 espèces agroforestières de légumineuses ligneuses : *Leucaena leucocephala* et *Acacia mangium* avec différentes souches de rhizobium. Tout d'abord, nous avons montré que le stade physiologique de la culture bactérienne n'a aucune influence sur la nodulation et la croissance des plants d'*Acacia mangium* inoculés et cultivés in vitro pendant 4 mois. Pour *Leucaena leucocephala*, le nombre de nodules est significativement supérieur pour les plants inoculés avec une culture bactérienne en phase plateau. En revanche, quelle que soit l'espèce, aucune différence significative n'est notée en ce qui concerne le poids sec des parties aériennes. L'effet de la taille de l'inoculum sur la nodulation et la croissance des plants de *L. leucocephala* a été étudié après 5 mois de culture en serre. Nos résultats ont montré qu'une inoculation avec des cutures bactériennes contenant 10^{10} à 10^9 bactéries par millilitre sont les conditions optimales pour avoir une nodulation et une croissance maximale des plants. Les 2 espèces de légumineuses montrent des différences significatives en ce qui concerne l'effet de la méthode d'inoculation sur la nodulation et la croissance des plants. Pour *Acacia mangium*, l'inoculation avec une culture liquide une semaine après les semis est plus favorable pour la croissance de la plante. En revanche, pour *Leucaena leucocephala*, cette méthode classique d'inoculation de même que l'enrobage des semis avec une culture bactérienne mélangée avec de la gomme arabique améliorent significativement la croissance de la plante. Nos travaux pourront servir aux partenaires du développement pour la production en régie de plants forestiers de ces 2 espèces.

***Acacia mangium* / *Leucaena leucocephala* / rhizobium / taille de l'inoculum / symbiose**

1. INTRODUCTION

Trees that can fix nitrogen, particularly forest leguminous ones, are more and more used to improve agricultural and forest outputs [1] The ability of these trees to associate with soil bacteria called rhizobium allow them to be able to fix atmospheric nitrogen and grow quickly on soils poor in nitrogen. These properties enable them to be amongst the first species considered for the rehabilitation of degraded soils and the production of both fodder and woody biomass [8]. Among these species, *Acacia mangium* and *Leucaena leucocephala* have shown great ability to grow quickly in marginal lands [6, 11, 27]. However, in order to ensure optimal exploitation of their economic and agricultural potential, it is necessary to go for inoculation

* Correspondence and reprints
Tel.: (221) 849 33 20; fax: (221) 832 16 75; e-mail: Didier.Lesueur@cirad.fr

Table I. Methodologies already published and used for the inoculation of the tested species of woody legumes.

For *Acacia mangium*

Strain used	Methodology of inoculation	Size of inoculum	Type of experience	Time of inoculation	References
Aust. 13c	Liquid inoculum	10^9 cells/plant	In vitro	2 weeks after sowing	[11]
Aust. 13c, Tel 2	Liquid inoculum	5×10^9 cells/plant	In vitro	2 weeks after sowing	[22]
Aust. 13c, Tal 72, PBG3, AG3, RMBY	Inoculum included in alginate beads dissolved in phosphate buffer 0.1 M	10^8 cells/plant	Planting site	One week after sowing	[12]
Aust. 13c, CB756	Inoculum included in alginate beads dissolved in phosphate buffer 0.1 M	10^8 cells/plant	Planting site	One week after sowing	[13]

For *Leucaena leucocephala*: according to [10].

Strain used	Methodology of inoculation	Size of inoculum	Type of experience	Time of inoculation	References
Tal82, Tal582 Tal1145 = CB3060	Coated seed	10^5 to 10^7 cells/seed	Planting site	Just after sowing	[31]
Irc 1045, Irc 1050	Coated seed	10^7 cells/seed	Planting site	Just after sowing	[26]
Tal 1145 = CB 3060	Coated seed	4×10^7 cells/seed	Planting site	Just after sowing	[15]
LdK4	Liquid inoculum	2×10^9 cells/seed	Nursey	One week after sowing	[16]
$CB8_1$ et NGR8	Coated seed	1.6×10^5 cells/seed	Planting site	Just after sowing	[23]

using effective symbionts [9]. This inoculation stage is particularly indispensable with soils rich in ineffective native symbionts [21]. However very often, the techniques of inoculation yield irregular results [3]. Currently the different inoculation techniques are used within the framework of many experiments [25, 33]. However, until now these techniques were used without including several factors that can optimise inoculation. Experiments that were carried out with the help of many leguminous species did not take into account the effect of the method of inoculation, the size of the inoculum, the period of inoculation or the effects of the physiological stage of the bacterial strains on nodulation and biomass production [5]. Some of the key studies carried out on the inoculation of *Acacia mangium* and *Leucaena leucocephala* are summarised in Table I. It shows the large diversity of inoculation methods used and the size of the inoculum. The inoculum employed must be easy to use, available at a reasonable cost and allow for a great number of rhizobia to survive [20, 29].The paper reports an approach to optimise the inoculation of these two agroforestry species and proposes for each species a simple and efficient technology of inoculation. The study evaluated the effect of the physiological stage of the bacterial culture on nodulation and growth of inoculated seedlings and investigated whether the methodology followed during the inoculation process could have significant effects on nodulation and growth of the seedlings. The work was directed at determining the types of inoculum and the best inoculum formulation to ensure optimal growth.

2. MATERIALS AND METHODS

2.1. Plant material

The Oxford Forestry Institute (UK) provided the seeds of *L. leucocephala* and the *A. mangium* seeds were provided by ICSB/ Innoprise Sabah (Sabah-Malaysia). In order to ensure quick and homogenous germination of the seeds, both the *A. mangium* and *L. leucocephala* seeds were scarified by soaking them for 60 min and 30 min respectively in 95% sulphuric acid. After rinsing thoroughly with distilled water within an aseptic environment, the seeds were disinfected by soaking them for two to three minutes in $HgCl_2$ (0.1%; p/v). After rinsing for the last time, they were soaked in distilled water for six hours for *L. leucocephala* and for one night for *A. mangium*. Afterwards they were arranged in Petri dishes containing sterile agar-agar (0.8%; p/v). The Petri dishes were then sealed with parafilm and kept at 30 °C for 48 hours.

2.2. Conditions of culture

The pre-germinated seedlings were cultivated in vitro or in nurseries as described below. For the in vitro culture, the seedlings were transplanted under sterile conditions in a Gibson tube [14] containing a culture medium composed of Jensen sterile agar water [34]. Nitrogen-free agar is poured (approximately 30 mL) into tubes (150 × 20 mm), slanted and allowed to solidify in such a way that the slope of the agar reaches the top of the tube. The top of each tube is covered by three circles of aluminium foil and held in place with a rubber band. A small aperture is made on the foil and fitted with plastic wool, which acts as an inlet replenishing nutrient solution in tubes. The watering hole is used for pouring in sterile liquid medium and for inoculating seedlings. In all these procedures, the usual sterility control must be maintained to prevent contamination. Aseptically grown seedlings are placed in a second hole (opposite to hole carrying the plastic plug). In such a way the root system lies on the agar slope and the shoot system comes out of the tube. The tubes are now covered with a piece of cotton wetted with sterile water to prevent desiccation of seedlings. After 24 hours in enclosed environment, the teguments of the seeds were taken off. The seedlings were then kept in a culture environment for four months with a photoperiod of 16 hours (under daylight) and eight hours (night), temperature of 30 ± 1 °C (night), relative humidity of 70 ± 5% and a photosynthetically active radiation (PAR) of 120 µmol/m^2/s.

As for the seedlings grown in nursery, they were individually transplanted in plastic bags (17 cm × 9.5 cm) containing a substrate of variable nature depending on the type of experiment. The bags were kept in greenhouse and laid on cement boards uplifted in such a way as to limit the risk of contamination.

2.3. Bacterial material

The strain of *Bradyrhizobium* Aust 13 c from Australia [11] was used for the inoculation of *A. mangium*. The seedlings of *L. leucocephala* were inoculated with LdK4 *Rhizobium* strain from Kenya [16, 19]. The bacterial strains were cultivated on a YEM medium (yeast extract mannitol) [34] and the culture was incubated at 30 °C under high orbital turbulence.

2.4. Description of the experiments

2.4.1. The effect of the physiological stage of the bacteria

The experiment aiming at determining the effect of the physiological stage of the bacteria growth on nodulation and growth of seedlings was carried out using seedlings cultivated in vitro. The seedlings were inoculated one week after their transplantation with 100 µL of liquid culture containing approximately 10^9 bacteria per milliliter. This experiment included two treatments: T1 where inoculation was practiced with bacterial culture in exponential phase and T2 where inoculation was practiced with bacterial culture in stationary phase. Each treatment was repeated 20 times. After four months of culture, the seedlings were harvested in order to determinate the level of infection (which was measured counting the number of obtained nodules) both in terms of biomass content of the nodules, shoots and roots.

2.4.2. The effect of the size of the inoculum

The effect of the size of the inoculum on nodulation and biomass production was studied using seedlings cultivated in nurseries with a mixture of vermiculite and sterilised peat (9/1, v/v) with a pH = 6.5. The young seedlings were inoculated one week after their transplantation with one milliliter from a bacterial suspension for each seedling. Six series of dilutions were carried out and each of them contained: 10^2; 10^4; 10^6; 10^8; 10^9 and 10^{10} bacteria per milliliter. For each series, ten seedlings per specie were selected. The pots were arranged to form random beds and watered on a daily basis with a N-free nutritive solution [2] according to the field capacity in water. After four months of growth under greenhouse conditions, plants were harvested. The following parameters were studied: the number and dry biomass of the nodules formed, the dry weights and the percentage of total nitrogen in shoots.

2.4.3. The effect of the type of inoculation

The effect of the type of inoculation on nodulation and biomass production was studied under greenhouse conditions and focussed on seedlings cultivated in a mixture composed of polystyrene beads and soil from Sangalkam (North West Senegal). Characteristics of Sangalkam soil were: pH (H_2O) 5.7, C 0.25%, N 0.21, P 5.2 mg/kg (Olsen) and organic matter 0.43%. Five different inoculum formulation were tested:

- M1: Inoculation with 20 mg of non-dissolved alginate beads [7] containing a culture of LdK4 strain (*L. leucocephala*) or Aust 13c (*A. mangium*).
- M2: Inoculation with a pure liquid culture of the LdK4 or Aust 13c strain one week after sowing at the surface of the soil, around the root system of plant (5 mL of inoculum).
- M3: Coating seeds with arabic gum and then of pure liquid culture of the LdK4 or Aust 13c strain.
- M4: Mix arabic gum and pure liquid culture of the LdK4 or Aust 13c strain. Coating *A. mangium* and *L. leucocephala* seedlings with the mix.
- M5: Inoculation at the level of plant collar (5 mL) of the seedlings with a liquid pure culture of the LdK4 or Aust 13c at planting in the plastic bags.
- Control: The seedlings that were not inoculated and were used as control.

Each inoculation treatment comprised 12 replicates. Plants were grown for six months (December to May 2000) in the greenhouse.

After this period, plants were harvested and several parameters were measured: number and dry weight of nodules, shoot and root dry weight and shoot total nitrogen content.

Data were subjected to a three-way analysis of variance using the Super Anova Computer program, and means were compared with the Fisher multiple range test [4].

3. RESULTS AND DISCUSSION

3.1. Effect of the physiological stage of the bacterial culture on nodulation and growth of seedlings

Data on nodulation and growth of seedlings of the two species are presented in Table II. The physiological stage of the bacterial culture Aust 13c did not have significant effects on the level of infection of *A. mangium* (measured by the number of nodules formed). In contrast, inoculation of *L. leucocephala* seedlings with Ldk4 culture in stationary phase significantly improved nodulation. The number of nodules increased by 36% compared to seedlings inoculated with a culture in exponential growth phase. However, whatever species may be involved, the physiological stage of the bacterial culture did not have significant effects on the nodule dry weight. It seemed that with *L. leucocephala*, culture in stationary phase enabled the formation of a great number of small nodules. Indeed, despite the increase in the number of nodules, their content in biomass was similar for both phases. However, for the two species no significant difference was noted as far as the biomass of the shoots and root are concerned. The high variability noticed with *A. mangium* seedlings could be related to the intraspecific genetic diversity demonstrated by this species [30].

3.2. Effect of the size of the inoculum on nodulation and the production of biomass by seedlings of *L. leucocephala*

The level of infection of the strains was variable and depended on the size of the inoculum as shown in Table III. Our results show that a great number of nodules appeared at low dilutions. Globally, we note that the size of the inoculum does not seem to have significant effects on the rate of infection of the strains LdK 4. However, the optimum value for nodulation was reached with a dilution containing 10^9 bacteria per millliliter that allowed to obtain 70 nodules per seedling.

If we consider the dry weight of the nodules, no correlation was found between the size of the inoculum and this parameter. The optimal dry weight of the nodules was more or less the same no matter how diluted the medium. It was showed that *L. leucocephala* is able to use a mechanism of control which indicates that the seedling compensates the decrease in the number of rhizobia present in the soil by an increase in the size of the nodules formed [28]. Our results do not confirm this hypothesis but need to be confirmed through further experiments. Regarding the growth of plants of *L. leucocephala*, our results show that the optimal growth was obtained with a dilution containing 10^9 bacteria per milliliter. It is in accordance with

Table II. Effect of the physiological stage of the bacterial culture of the *Rhizobium* strain LdK4 and the *Bradyrhizobium* strain Aust 13c on nodulation (nodule number and nodule dry weight) and growth of *L. leucocephala* and *A. mangium* seedlings respectively after 4 months of growth in culture chamber (Gibson tube).

Parameters measured	Species	Physiological stage of the bacterial culture	
		T1	T2
Number of nodules per plant			
	L. leucocephala	14a	19b
	A. mangium	47a	46a
Nodule dry weight (g/plant)			
	L. leucocephala	0.017a	0.016a
	A. mangium	0.026a	0.029a
Shoot dry weight (g/plant)			
	L. leucocephala	0.196a	0.202a
	A. mangium	0.287a	0.313a
Root dry weight (g/plant)			
	L. leucocephala	0.182a	0.200a
	A. mangium	0.051a	0.056a

For each parameter measured and for each tested specie, the values (average of 20 repetitions) on the same line followed by the same letter are not significantly different according to the Newman et Keuls test ($P < 0.05$).

Table III. Effect of the size of the rhizobial inoculum on nodulation (number of nodules and dry weight of nodules) and the growth (shoot and root dry weight) of plants of *L. leucocephala* cultivated during 4 months under greenhouse conditions.

Parameters measured	Dilutions	Values measured
Number of nodules per plant		
	10^2	58ab
	10^4	45a
	10^6	56ab
	10^8	51ab
	10^9	70b
	10^{10}	69ab
Nodule dry weight (g/plant)		
	10^2	0.149ab
	10^4	0.117a
	10^6	0.169c
	10^8	0.158bc
	10^9	0.147b
	10^{10}	0.112a
Shoot dry weight (g/plant)		
	10^2	1.56a
	10^4	1.67a
	10^6	1.88ab
	10^8	1.90ab
	10^9	2.10b
	10^{10}	1.74a
Root dry weight (g/plant)		
	10^2	1.91a
	10^4	1.72a
	10^6	1.93a
	10^8	2.04a
	10^9	1.93a
	10^{10}	1.81a

For each parameter measured, the values (average of 10 repetitions) in the same column followed by the same letter are not significantly different according to the Newman et Keuls test ($P < 0.05$).

good results obtained by others authors who improved significantly under greenhouse conditions the growth of *L. leucocephala* by using an inoculum containing 10^9 cells per milliliter [16]. Similar results were obtained with *Calliandra calothyrsus*, another woody legumes species cultivated under greenhouse conditions [17, 24].

3.3. Effect of the mode of inoculation on nodulation, growth and shoot total nitrogen of plants of *L. leucocephala* and *A. mangium*

All the results obtained are presented in Table IV. We showed that for both leguminous species, the seedlings that were not inoculated developed an important number of nodules. The dry weight of the nodules seen on the control seedlings was higher than for inoculated ones if we consider all the modes of inoculation. This situation could be explained by the presence of an important number of native bacteria in the soil living in association with these host seedlings.

For *L. leucocephala* the dressing of the pre-germinated seeds with arabic gum mixed with the bacterial suspension (M4 technique) enhanced the growth of the shoots and roots. This technique was also more favourable for the nodulation of inoculated seedlings. Our results confirmed already published data [32], and could be explained by an early fixation of bacteria on the root system. Micro-organisms would thus migrate towards the roots following the development of the roots and colonise root hair before autochthonous bacteria. In a soil containing a low population of native rhizobia able to nodulate *L. leucocephala*, it was showed that plants inoculated with non-dissolved alginate beads were more significantly developed and more nodulated than plants inoculated with the other methods [10]. It is not the case in our present study. These difference could be explained by the fact that in soil with a large amount of native rhizobia, selected rhizobia contained in alginate beads, which are released progressively in soil are available in to limited amount for occupying a large number of nodules. Usually, in these soils, nodules formed are essentially occupied by native rhizobia which could be ineffective as in Sangalkam's soils.

Table IV. Effect of the methodologies used for the inoculation practiced with the *Rhizobium* strain LdK4 or the *Bradyrhizobium* strain Aust 13c on nodulation and growth of respectively *L. leucocephala* and *A. mangium* cultivated during 6 months under greenhouse conditions.

Parameters measured	Methodologies used for the inoculation	*L. leucocephala*	*A. mangium*
Nodules dry weight (g/plant)			
	M1*	0.119b	0.110a
	M2	0.116b	0.123ab
	M3	0.096a	0.149bc
	M4	0.101ab	0.136abc
	M5	0.091a	0.14bc
	Control	0.154c	0.167c
Root dry weight (g/plant)			
	M1	4.11b	1.79a
	M2	3.69ab	2.17bc
	M3	4.08ab	1.70a
	M4	4.14b	2.11bc
	M5	3.90ab	1.93ab
	Control	3.39a	2.41c
Shoot dry weight (g/plant)			
	M1	4.31d	4.36abc
	M2	3.65bc	4.93cd
	M3	4.07cd	4.76bcd
	M4	4.04cd	5.15d
	M5	3.14b	4.11ab
	Control	2.39a	3.54a
Shoot total nitrogen content (%)			
	M1	1.97bc	1.68b
	M2	1.82ab	1.45a
	M3	1.96ab	1.51ab
	M4	2.14c	1.45a
	M5	1.92ab	1.63b
	Control	1.79a	1.55ab

For each parameter measured and for each tested specie of woody legumes, values (means of 10 repetitions) placed in the same column and followed by the same letter are not significantly different according to Newman and Keuls test ($P < 0.05$).
*M1: Inoculation with 20 mg of nondissolved alginate beads containing a culture of LdK4 strain (*L. leucocephala*) or Aust 13c (*A. mangium*); M2: Inoculation with a pure liquid culture of the LdK4 or Aust 13c strain one week after sowing at the surface of the soil, around the root system of plant (1 mL of inoculum); M3: Coating of *L. leucocephala* seedlings with arabic gum and then of pure liquid culture of the LdK4 or Aust 13c strain; M4: Mix gum arabic and pure liquid culture of the LdK4 or Aust 13c strain then put in contact with the *A. mangium* and *L. leucocephala* seedlings; M5: Inoculation at the level of plant collar (1 mL) of the seedlings with a liquid pure culture of the LdK4 or Aust 13c in the same time that seedlings is planted in the plastic bags; Control: the seedlings that were not inoculated were used as control.

For *A. mangium*, the M4 technique also improved significantly the growth of the inoculated seedlings compared to control seedlings. The inoculation of the seedlings with 5 milliliters of liquid suspension poured at the root of the collar one week after transplantation (M2 technique), increased the dry weight of the shoots by 45% compared to control seedlings. This observation could be correlated with the morphology of the root system of *A. mangium*. With a root system which is ramose and superficial, the maintenance of the inoculum in the soil upper horizons allowed for the optimisation of inoculation. Bringing in the inoculum under liquid form one-week after the replanting of the young seedlings enabled the rhizobia to be in direct contact with an early ramification process. The methods "M2" and "M3" were also favourable for the growth of the seedlings. However, it must be pointed out that the inoculation method used with this species seemed to inhibit the growth of the roots, particularly when the inoculum is brought to the seedlings under the form of a liquid suspension poured at the root of the collar during transplantation by pots. Similar results were reported on *A. mangium* seedlings inoculated with different strains and cultivated in greenhouse for 96 days [18].

It is interesting to compare our results with those obtained with *C. calothyrsus* in the same soil [24]. These authors showed that the inoculation practiced with a liquid suspension poured directly at the root of the seedlings is more favourable for the growth of *C. calothyrsus* both in terms of biomass content of the shoots and dry weight of the root. This beneficial effect of inoculation was all the more important that the inoculum was provided one week after transplantation, that is to say after the apparition of secondary roots. All these results confirm the importance to know exactly how to practice the inoculation of woody legumes in order to optimise the treatment and to improve significantly the growth of the host plant.

As a whole, inoculation conducted with the help of non-dissolved alginate beads placed at the lower part of the collar was less favourable to the growth of the seedlings than the other methods. This lack of effect of the non-dissolved alginate beads could be linked to the fact that in the framework of our experiments unlike in another work [12], the beads were not made soluble in a phosphate buffer solution. This choice was motivated by the fact that we intend in the future to work with an inoculum (rhizobium and mycorrhizae) kept in fresh alginate beads.

4. CONCLUSION

Our results propose reliable protocols for the inoculation of these two species in nurseries. All the results obtained showed that the improvement of the growth of *L. leucocephala* and *A. mangium* by inoculation in nurseries with efficient rhizobium strains was very significantly dependent on the mode of inoculation.

For *L. leucocephala* the dressing of the pre-germinated seeds with arabic gum mixed with the bacterial suspension favoured nodulation and enabled optimal growth of the shoots. For *A. mangium*, inoculation of the seedlings with 5 milliliters of liquid suspension poured at the lower part of the collar one-week after transplantation significantly improved their growth.

Our results clearly indicated that, whatever the species concerned, the physiological stage of the bacterial culture did not have significant effects on the growth of inoculated seedlings. The Ldk4 strain in stationary phase reached a higher infection level with *L. leucocephala*. On the other hand, the level of infection of the strains Aust 13c was not significantly affected by the stage of the bacterial culture.

Nodulation and growth of inoculated seedlings was more or less variable depending on the size of the inoculum. The greatest number of nodules were recorded on *A. mangium* for a dilution containing 10^{10} bacteria per milliliter. On the other hand for *L. leucocephala*, an inoculum containing 10^9 bacteria per milliliter corresponds to the optimal dilution. If we consider the dry weight of the nodules, whatever the specie concerned, the dilution containing 10^6 bacteria per milliliter improved significantly the growth of *A. mangium* seedlings. For *L. leucocephala* optimal growth was obtained with a medium containing 10^9 bacteria per milliliter.

Acknowledgements: The authors are grateful to Mr Jacques Biagui, Leon Biagui and Lamine Ba for their technical assistance in the nursey. We thank Dr. Alan Pottinger for correcting the English grammar in the original text. This work was supported for the most part by the CBFR Grant No. 2000/6.

REFERENCES

[1] Blair G.J., Catchpoole D., Horne P., Forage tree legumes. Their management and contribution to the nitrogen economy of wet and humid tropical environments, Adv. Agron. 44 (1990) 27–54.

[2] Broughton W.J., Dilworth M.J., Control of leghaemoglobin synthesis in snake beans, Biochem. J. 125 (1971) 1075–1080.

[3] Brunck F., Colonna J.P., Dommergues Y.R., Ducousso M., Galiana A., Prin Y., Roederer Y., Sougoufara B., La maîtrise de l'inoculation des arbres avec leurs symbioses racinaires : Synthèse d'une sélection d'essais au champ en zone tropicale, Bois et Forêts des Tropiques, 223 (1990) 24–42.

[4] Dagnélie P., Théories et méthodes statistiques : applications agronomiques. Les Presses Agronomiques de Gembloux, Gembloux, Belgium, 1973.

[5] Danso S.K.A., Kapuya J., Hardarson G., Nitrogen fixation and growth of soybean as influenced by varying the methods of inoculation with *Bradyrhizobium japonicum*, Plant Soil 125 (1990) 81–86.

[6] Date R.A., Inoculated legumes in cropping systems of the tropics, Field Crops Research 65 (2000) 123–126.

[7] Diem H.G., Ben Kalifa S., Neyra M., Dommergues Y.R., Recent advances in the inoculant technology with special emphasis on plant microorganisms, in: Leone U., Riadli G., Vanoré Genova R. (Eds.), Advanced technologies for increased agricultural production (International Workshop Santa Margherita Ligure Italy 25–29 Sept 1988), Università degli studi di Genova, Roma: Consiglio Nazionale delle Ricerche, 1989, pp. 196–209.

[8] Diouf D., Sougoufara B., Neyra M., Lesueur D., Le reboisement au Sénégal/Bilan des réalisations de 1993 à 1998 – Dakar: IRD/ Laboratoire de Microbiologie des Sols, 2000, 49 p.

[9] Dommergues Y.R., Nitrogen fixation by trees in relation to soil nitrogen economy, Fert. Res. 42 (1995) 215–230.

[10] Forestier S., Alvarado G., Badjel Badjel S., Lesueur D., Effect of *Rhizobium* inoculation methodologies on nodulation and growth of *Leucaena leucocephala*, W.J. Microbiol. Biotech. 17 (2001) 359–362.

[11] Galiana A., Chaumont J., Diem H.G., Dommergues Y.R., Nitrogen-fixing potential of *Acacia mangium* and *Acacia auriculiformis* seedlings inoculated with *Bradyrhizobium* and *Rhizobium* spp., Biol. Fertil. Soils 9 (1990) 261–267.

[12] Galiana A., Prin Y., Mallet B., Gnahoua G.M., Poitel M., Diem H.G., Inoculation of *Acacia mangium* with alginate beads containing selected *Bradyrhizobium* strains under fields conditions: Long-term effect of plant growth and persistence of the strains in soil, Appl. Environ. Microbiol. 60 (1994) 3974–3980.

[13] Galiana A., Gnahoua G.M., Chaumont J., Lesueur D., Prin Y., Mallet B., Improvement of nitrogen fixation in *Acacia mangium* through inoculation with rhizobia, Agr. Syst. 40 (1998) 297–307.

[14] Gibson A.H., Physical environment and symbiotic nitrogen fixation. I. The effect of temperature on recently nodulated *Trifolium subterraneum* (L.) plants, Aust. J. Biol. Sci. 16 (1963) 28–42.

[15] Homchan J., Date R.A., Roughley R.J., Responses to inoculation with root-nodule bacteria by *Leucaena leucocephala* in soils of N, Trop. Grass. 23 (1989) 92–97.

[16] Lemkine G., Lesueur D., Assessment of growth, nodulation and nitrogen fixation of the know *Leucaena* species inoculated with different rhizobium strains in geenhouse conditions, in: Shelton H.M., Gutteridge R.C., Mullen B.F., Bray R.A. (Eds.) Proceedings of the Workshop on *Leucaena* adaptation, quality and farming systems, ACIAR Publication No. 86, Canberra, 1998, 168–171.

[17] Lesueur D., Tassin J., Enilorac M.P., Sarrailh J.M., Peltier R., Study of the *Calliandra calothursus – Rhizobium* nitrogen fixing symbiosis, in: Evans D.O. (Ed.) Proceedings of the International Workshop on the genus *Calliandra*, Forest, Farm, and Community Tree Research Reports, Special Issue (1996) 62–76.

[18] Lesueur D., Diem H.G., The requirement of iron for nodulation and growth of *Acacia mangium*, Can. J. For. Res. 27 (1997) 686–692.

[19] Lesueur D., Date R.A., Mullen B.F., *Rhizobium* specificity in *Leucaena*, in: Shelton H.M., Gutteridge R.C., Mullen B.F., Bray R.A. (Eds.), Proceedings of the Workshop on *Leucaena* adaptation, quality and farming systems, ACIAR Publication No. 86, Canberra, 1998, pp. 86–95.

[20] Lupwayi N.Z., Olsen P.E., Sande E.S., Keyser H.H., Collins M.M., Singleton P.W., Rice W.A., Inoculant quality and its evaluation, Field Crops Res. 65 (2000) 259–270.

[21] Martensson A.M., Competitiveness of inoculants strains of *Rhizobium leguminosaum* bv. *trifolii* in red clover using repeated inoculation and increased inoculum levels, Can. J. Microbiol. 36 (1990) 136–139.

[22] Martin-Laurent F., Lee S-K., Tham J. He, Diem H.G., Durand P., A new approch to enhance growth and nodulation of *Acacia mangium* through aeroponic culture, Biol. Fertil. Soil 25 (1997) 7–12.

[23] Norris D.O., Seed pelleting to improve nodulation of tropical and subtropical legumes. The contrasting response of lime pelleting of two *Rhizobium* strains on *Leucaena leucocephala*, Aust. J. Exp. Agric. An. 13 (1973) 98–101.

[24] Odee D.W., Indieka S.A., Lesueur D., Inoculation of *Calliandra calothyrsus* in sterile and unsterile (soil) conditions: effect of rhizobial inoculum size and method of inoculation, Biol. Fertil. Soils 36 (2002) 124–128.

[25] Olsen P.E., Rice W.A., Bordeleau L.M., Bierberdeck V.O., Analysis and regulation of legums inoculants in Canada: the need for increase in standards, Plant Soil 161 (1994) 127–134.

[26] Sanginga N., Mulongoy K., Ayanaba A., Effectivity of indigenous rhizobia for nodulation and early nitrogen fixation with *Leucaena leucocephala* grown in Negerian soils, Soil Biol. Biochem. 21 (1989) 231–235.

[27] Sanginga N., Danso S.K.A., Mulongoy K., Ojeifo A.A., Persisence and recovry of introduced *Rhizobium* ten years after inoculation on *Leucaena leucocephala* grown on an Alfisol in Southwestern of Nigeria, Plant Soil 159 (1994) 199–204.

[28] Singleton P.W., Tavarez J.W., Inoculation response of legumes in relation to the number and effectiveness of indigenous rhizobium populations, Appl. Environ. Microbiol. 51 (1986) 1013–1018.

[29] Stephens J.H.G., Rask H.M., Inoculant production and formulation, Field Crops Research 65 (2000) 249–258.

[30] Sun J.S., Sands R., Simpson R.J., Genetopic variation in growth and nodulation by seedlings of *Acacia* species, For. Ecol. Manag. 55 (1992) 209–223.

[31] Thies J.E., Singleton P.W., Bohlool B.B., Influence of the size of indegenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field –grown legumes. Niftal project, Appl. Environ. Microbiol. 57 (1991) 19–28.

[32] Thies J.E., Singleton P.W., Bohlool B.B., Modeling symbiotic performance of introduced rhizobia in the field by use of indices of indigenous population size and nitrogen status of the soil, Appl. Environ. Microbiol. 57 (1991) 29–37.

[33] Thompson J.A., Legume inoculant production and quality control, in: Thompson J.A. (Ed.), Report on the expert consultation on Legume inoculant production and quality control, FAO, Rome, United Nations, 1991, pp. 15–32.

[34] Vincent J.M., A manual for the practical study of root-nodule bacteria. International Biological Programme handbook No. 15, Blackwell Scientific Publications, Oxford, England, 1970.