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Final Summary Report

The first objective of the project was to establish a collection of microsymbionts (rhizobium and Arbuscular Mycorrizae or AM) of *Calliandra calothyrsus*. For this reason, all the tropical partners harvested samples of soils and nodules in the place where the future field experiments with *C. calothyrsus* will be set up by the project. OFI carried out a long trip in Central America in order to harvest soil and nodules samples from Mexico (3 sites), Honduras (2 sites), Guatemala (4 sites), Nicaragua (3 sites) and Costa Rica (3 sites). OFI was assisted in this trip by the help of CONSEFORH. In Cameroun, IRAD harvested soils samples in 3 sites but nodules in only 2 sites. Regarding New Caledonia, samples were harvested in 5 sites. In Kenya, soils samples were harvested in 8 sites in Kenya located in the Western and Central highlands. One part of all the soils samples were sent to CEH/ITE for the mycorrhizal work, and the rest of soils and nodules samples were sent to CIRAD-Forêt in Senegal for rhizobia work. The isolation of rhizobia from nodules harvested in Kenya was done in the KEFRI Microbiology lab in Nairobi.

A total of 293 strain of rhizobia were isolated . By using a semi-automated restriction fragment length polymorphism (RFLP) of 16S rDNA, the biodiversity of strains collected from root nodules was evaluated. Representatives (30) of defined *Rhizobium*, *Agrobacterium*, *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium* species were included in the study. Major genotypes: **A**, **B**, **C**, **D**, **E** and **F** with 116, 63, 40, 24, 14 and 12 strains and minor genotypes: **G**, **H**, **I**, **J**, **K**, **L**, **M**, **N** and **O** with 8, 6, 4, 1, 1, 1, 1, 1 strains, respectively were used to construct the UPGMA dendrogram. Genotypes **A**, **C**, **E**, **G**, **H** and **I** were clustered with established rhizobial species and genotypes **B**, **D**, **F** remained unclustered. Representative of genotype **A** was initially clustered with *R. tropici*. *Bradyrhizobium* like isolates represented mainly genotype **B**. Inoculant strains used in field test (CCNC26, CCCR15, CCCR1, CCC22, KWN35, KCC6) represented genotypes **A**, **C** and **D**. Biodiversity of isolates was greater in Central America and New Caledonia than in Kenya and Cameroon.

Concerning AM, ITE/CEH has established "trap" cultures using *C. calothyrsus*, sorghum, millet and cowpea as host plants to "bait out" the AM fungi present in the soils. Thirty-five singles of species isolations were set up in a controlled environment cabinet using AM spores extracted from the "trap" culture soils. It was observed that soils from Honduras (H2), Guatemala (G2 and G4), Cameroun (C2) and Kenya (K7 and K9) contained most spores compare with the other soils. AM fungal species that occurred most frequently included *Gigaspora albida*, *Glomus etunicatum*, *Scutellospora heterogama*, *Scutellospora verrucosa* and *Acaulospora scrobiculata*.

Following the objectives of the project which planed the production and the distribution of effective rhizobial inoculants, an operational UPIL production system was installed in Cameroon. Inoculants for *C. calothyrsus* can now be produced locally on a large scale. The UPIL should be helpfull for producing inoculants for legume crops, woody legumes and shrubs widely used in agroforestry. This facility, located near Yaounde, can also be used more efficiently by encouraging collaboration between the various local scientific institutions and IRAD. In Kenya, existing inoculant production system using filtermud as a carrier have successfully initiated the production of *C. calothyrsus* rhizobial inoculants for local and the East and Central African region.

All the rhizobia and arbuscular mycorrhiza (AM) from the collection were systematically screened for symbiotic effectiveness on provenances of *Calliandra calothyrsus*. Most were effective in experiments conducted in sterile conditions. We selected the best seven rhizobia; CCK13, KCC6, KCC17, KCC39 and KWN35 from Kenya; CCCR1 and CCCR15 from Costa Rica; CCN26 from New Caledonia, and CCC22 from Cameroon. Similarly, we selected the best five AM isolates; *Gigaspora albida* (types GA1b and GA2)

and *Scutellospora verrucosa* (SV2c) from Kenya, *S. calospora* (SC2) from Guatemala, and *Glomus etunicatum* (GE1) from Honduras. Evaluation of selected rhizobia and AM on *C. calothyrsus* provenances in unsterile soil substrates showed that inoculation responses were more pronounced in Senegal than in Kenya, and none at all in Costa Rica, reflecting the variations in indigenous microsymbiont populations and fertility of the soils used. In addition, inoculation responses varied with regard to parameter, provenance, inoculant and substrate, thus prompting the use of a multi-strain inoculant comprising a mixture of the best strains.

In Senegal and in Kenya, nursery pot experiments were conducted using rhizobium and arbuscular mycorrhizal inoculants singly and in combination. Growth responses of *Calliandra calothyrsus* seedlings were examined in unsterilised local soils using Flores and San Ramon provenances. The experiments also examined the effects of using single and multi-isolate inoculants and soil solarisation on seedling growth. Results showed that dual inoculation with rhizobium and mycorrhizal inoculants gave better results than single inoculation with either microsymbiont, and that rhizobium and mycorrhizal inoculants were more effective in promoting seedling growth than microsymbionts occurring naturally in the soils. Responses varied according to the fertility of the soils. Rhizobium and mycorrhizal inoculation tended to benefit different aspects of plant growth, with rhizobium inoculation increasing leaf dry mass and mycorrhizal inoculation increasing stem and root dry mass. Solarisation of two Kenyan soils produced contrasting effects on seedling growth, which may have been related to differences in the activity of naturally occurring microsymbionts and soil pathogens. For both rhizobium and mycorrhiza, single isolate inoculants performed better than mixed isolate inoculants.

In conclusion, we can say that the technical information obtained in this project is applicable to other future legume-microsymbiont research and development, and should be repackaged for wider adoption by the smallholder farmers.

Consolidated Scientific Report

I. Objectives.

The main objectives of the project was to optimize the forage production of *C. calothyrsus* in field conditions by inoculation with highly efficient strains of rhizobia and/or mycorrhiza strains selected in laboratory, greenhouse and in field conditions. However, in order to achieve this objective, it was necessary to investigate the main symbiotic characteristics of *C. calothyrsus*, evaluate a range of potential inocula, and to be able to produce a suitable microbial inoculum for inoculating plants in field conditions. Consequently, research activities are concentrated in two main areas :

(i) Collection of microsymbionts of *C. calothyrsus*. The project established a large collection of rhizobia and mycorrhiza isolated respectively from nodules and roots of this species harvested in its native range (Honduras, Costa Rica and Mexico), and in humid countries where it has been introduced with success (Cameroon, Kenya and New Caledonia). After evaluation of the biodiversity within the collection of microsymbionts, a symbiotic screening will be carried out in laboratory and in greenhouse in order to select the most efficient strains for inoculation in field conditions.

(ii) Optimisation of the inoculation of *C. calothyrsus* for preparing further field trials. Existing methodologies for producing rhizobial inoculum and inoculating plants produced in nursery planted under field conditions after three months of growth were developped during the project.

II. Activities.

II.1. Collection work : Central America, New Caledonia, Kenya and Cameroon.

II.1.1. In Central America

The collection of soil and root nodule samples for culturing and subsequent evaluation with *Calliandra calothyrsus* was undertaken in Honduras, Nicaragua, Costa Rica, Guatemala and Mexico. The collections were made in collaboration with CONSEFORH (Conservation and Silviculture of Honduran Dry Zone Forest Species Project) in Honduras, ICTA (Instituto de Ciencas Tecnicas Agricolas) in Guatemala, UNAM (Universidad Nacional Autonoma de Mexico), the Instituto de Ecología, Jalapa, and the British Council in Mexico (collecting permit No. DOO750-1803/98).

In general, our success in finding fresh, white to pink root nodules that should contain live *Rhizobia* was varied, but in three sites no nodules were found (Ocoman, Honduras; Zihuatenjo, Mexico; Playa Azul, Mexico). Old nodules (mid- to dark brown in colour) were found in the majority of the sites, and seemed to be characteristic of the drier sites that receive less rainfall. A small sample of soil (~100 g) was collected from each site and was sent with the nodules to Didier Lesueur at CIRAD-Forêt, IRD-Bel Air, Dakar, Senegal.

Soil samples were collected from within the first 20 cm depth of soil, and within a radius of 50 cm from the main stem of the tree. Soil samples were taken from 8-12 trees scattered across the population and the samples bulked. At least one kilogramme of soil was sampled, usually more, and placed in a sealable plastic bag. Both the soil and nodule samples were kept in a cool box during transit. The nodules were kept dry and maintained at a temperature of 15-20 ?C, and the soil was kept moist and maintained at the same temperature.

The soil samples were sent, along with a kilo of sorghum seed from Honduras, for mycorrhiza culturing to CEH/ITE, Scotland, UK.

Details of the site locations, mean annual rainfalls, soil types, vegetation cover, etc., were copied from the OFI seed collection manual and were included with each soil and nodule sample. All these informations are presented in the individual part of CNRD.

II.1.2. In Cameroon, Kenya and New Caledonia.

In Cameroon, soil samples were collected in three different locations (Yaoundé, Ebolowa and Bamenda)[see IRAD part]. From each site, several trowels of soil were collected from the top 20 cm, coarse surface litter was removed but half decomposed organic material and plant roots were retained. These different spot samples were mixed together to have one composite soil sample per location. About 100 g of soil per sample was then sealed in a polythene bag , labelled and mailed respectively to CEH/ITE (Edinburg) for myccorhizae and to Dakar (Senegal) for rhizobium trapping, identification and testing. Climatic as well as geo-referenced information of the locations were also recorded. The similar method was used in New Caledonia for harvesting nodules and soil samples in 5 locations : La coulée, Col Pirogue, Ile de Maré, Champs de Bataille, Port Laguerre.

In Kenya, Eight sites located in the Western and Central highlands of Kenya were surveyed. Seven of these sites had history of *Calliandra calothyrsus* cultivation ranging from 1 to over 15 years. The sites were : Muguga Centre (KEFRI Headquarters), Muguga Centre (KEFRI Nderi Campus), Embu Agroforestry Centre, Mama Elizabeth Farm (near Embu centre), Mama Wachira Farm (near Embu Centre), Maseno Agroforestry Centre and Nyabeda Farm (near Maseno Centre). The eighth site, Owila farm (near Maseno Centre), had no previous history of *C. calothyrsus* cultivation. The site has been fallow for the last 5 years with *Cymbopogon nardus* (L.) Rendle (Gramineae) and isolated shrubs of *Psidium guajava.* For mycorrhizal work, several trowels of soil were collected from the top 20 cm, removing course surface litter, but retaining half decomposed organic material and plant roots. For *Calliandra* cultivated sites, trowels of soil were collected about 1 metre away from *C. calothyrsus* plants. At each site, the trowels of soil were pooled, mixed thoroughly and a sub-sample of about 500 g packed into a sealable poly bag. Care was taken to prevent cross-contamination between soils from different sites. Soils from Muguga Centre (KEFRI Headquarters), Muguga Centre (Nderi campus), Embu Centre, Maseno Centre and Nyabeda Farm were sampled in two ways: Soils from directly under *Calliandra* saplings/trees and 1-3 m. Soils for MPN were kept in a cool box during sampling and transportation back to the laboratory. Where available, *Calliandra* root nodules were collected and stored in vials containing silica gel until isolation work in the laboratory. Fine roots of *C. calothyrsus* were also collected and preserved in vials containing 60 % alcohol for mycorrhizal assessment.

II.2. Establishment of a collection of microsymbiont : rhizobia and mycorrhiza (AM)

II.2.1. For rhizobia.

For the establishment of this collection, it was necessary to carry out a trapping experiment in a greenhouse in order to obtain fresh nodules from which it was possible to isolate rhizobia.

The four provenances are of *C. calothyrsus* under study were Flores (OFI references 16/96 and 10/91) from Guatemala and San Ramon (OFI references 110/94 and 11/91) from Nicaragua. The Oxford Forestry Institute (OFI), U.K, supplied seeds that were scarified mechanically. A small nick was made in the outer coat, halfway round the horseshoe shaped

as described by Macqueen (1993). The scarified seeds were germinated on sand under greenhouse conditions.

Eight-day-old seedlings were transplanted into 12 x 8 cm plastic bags filled with sand. N-free nutrient solution (Broughton and Dilworth 1971) and distilled water were alternately added. The nodules of *C. calothyrsus* harvested in fields in several tropical countries were crushed in distilled water and the solution obtained was put on the root collar of plants. There were 5 replicates per place of harvest and per *C. calothyrsus* provenance's.

After two months of culture, the plants were cut and the fresh young nodules were harvested for the isolation of rhizobia in laboratory. Nodules were sterilised superficially in Ethanol 95% during 30s and rinsed with sterile distilled water. After that, nodules were placed in solution of $HgCl_2$ (0.1 %) during 3 minutes for a second sterilisation. After several rinsings in sterile distilled water, each nodule was crushed on a sterile glass slide and streaked onto YEM agar plates. When the culture obtained were pure, all the strains were maintained in YEM medium containing 20% (vol/vol) glycerol at 80°C.

In order to distinguish the *Rhizobium* strains from the *Bradyrhizobium* strains, all the strains of the collection were streaked onto YEM agar plates supplemented with bromothymol blue, which is a pH indicator. After 5 days of culture at 28°C, we observed whether the rhizobia had acidified the medium (yellow colour around the colonies like for the *Rhizobium*) or alkalised it (blue colour; like for the *Bradyrhizobium*).

In Kenya, silica gel-dried nodules were re-hydrated in sterile distilled water. Nodules (fresh and silica gel-dried) were surface sterilized in 1 % NaOCl for 6 min, rinsed in several changes of sterile water, and then crushed with a flame-sterilized blunt-tipped pair of forceps. A loopful of the crushed nodule was then streaked across the surface of a petri-dish containing yeast mannitol agar (Vincent, 1970). Typical well-isolated colonies were re-isolated on two diagnostic media containing congo red dye and bromothymol blue pH reaction indicator (Vincent, 1970). Growth characteristics in these two media were recorded after 7 days incubation at 28 °C. Rhizobial isolates were store at -80 °C in 15 % glycerol yeast mannitol broth.

II.2.2 For AM.

In all, 33 different soils were collected from 8 countries. Methods used processing the soil samples and the use of 'trap' cultures in the development of pot cultures is summarised in the manual produced for the training course.

After thorough mixing of the sample, spores were extracted from 50 g portions of each soil, so that the original numbers and species of AM spores present in the soils could be determined. Permanent reference slides of dominant spore types that occurred in the soil samples were prepared.

Within 1-3 days of receipt, 'trap' cultures were set up in the glasshouse. Pots were set up as described for the preliminary tests and sown with seed of *C. calothyrsus* (seedlot 6/97 ex. Flores, Guatemala), *P. typhoides* or *Sorghum* sp. As root growth and rooting density of *C. calothyrsus* had been much less than that of the crop species in the preliminary tests, smaller pots (150 ml) were used for *C. calothyrsus* this time. The intention was to increase the rooting density of *C. calothyrsus* over the 4 month growth period, and therefore obtain more effective 'trapping' of AM fungi present in the soils. At the end of the 4 month growth period, these replicates were utilised for testing further amendments to the loam/sand mixture in an effort to improve aeration and optimise AM spore production. Full details of all 'trap' cultures set up are given in the manual mentioned above.

The examination of each 'trap' culture was completed by the end of September 1998. After 4 months growth, soil cores (approximatively 25-35 g soil in total) were removed from each culture using a 2 cm diameter cork borer. Spores were extracted from the soil and

populations of spores assessed. Full details of spore numbers and species composition of the AM fungi present in the 'trap' cultures are given in CEH/ITE part of report.

II.3. Evaluation of the biodiversity of rhizobia of *C. calothyrsus.*

The biodiversity was assessed by using PCR reactions. For that, all strains were grown in 250 µl yeast-mannitol extract for 2 days at 28°C in 2,2 ml 96 Deep Well Plates (ABgene®House, UK). After 2 days of cultivation, all 250 µl of culture was carefully washed two times with 250 µl of distilled, autoclaved water. Finally, washed cells were suspended in 10 µl water and stored at -80°C until use.

PCR reactions were carried out in 0,2 ml Skirted Termo-Fast®96 Tube Plates (ABgene®House, UK) in 50 µl volume containing 15 pmol of rD1 and fD1 primers (Weisburg *et al.,* 1991), 200 µM each of dATP, dCTP, dGTP, dTTP, 2U of Dynazyme polymerase, 1 ml buffer supplied with polymerase and 1 µl of previously prepared intact cells as a template. The parameters of the cycles included an initial denaturation at 95°C for 5 min (to destroy the cells), followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min with slow ramp to 72°C, extension at 72°C for 3 min and finally 72°C for 10 min. Aliquots of 5 µl of each amplified DNA (96 samples at a time) were examined by horizontal electrophoresis in 1% agarose using Maxicell® Primo Submarine Gel System (TermoQuest, U.S.A).

For RFLP analysis aliquots (8 to 15 µl) of PCR products were digested with three four-base recognizing restriction endonucleases (*Hae I, Mbo I, Msp I*) as described by Laguerre *et al.* (1994). Restricted DNA was analyzed by horizontal electrophoresis in 3% agarose gel (96 samples at a time) and stained with ethidium bromide. To facilitate the analysis, different main genotypes were chosen from previously analyzed 293 strains and run again with RFLP genotypes obtained from 30 reference strains representing different *Rhizobium, Agrobacterium, Sinorhizobium, Mesorhizobium* and *Bradyrhizobium* species. In order to facilitate comparisons, molecular size standard, pGEM (Promega, U.S.A.) was loaded every 8 samples. Electrophoresis was carried out at 100 V for 1 h, followed by 200V for 1 h. The gels were photographed by using KODAK DC290 Zoom Digital Camera and images were stored in TIFF files. Stored restriction genotypes were analyzed with GelCompar 4.1 (Applied Maths, Belgium). To create one file, combined-gels option was used when analyzing RFLP genotypes obtained by using three restriction endonucleases. Following conversion and normalization, the levels of similarity between all RFLP genotypes were calculated by using Dice similarity coefficient, S_D, equal to the ratio of twice the number of common bands in two compared patterns, to the sum of all bands in both patterns. Finally, unweighted pair group method with arithmetic averages (UPGMA) was used to cluster genotypes obtained from representative and reference strains. To verify, if the dendrogram faithfully represents the calculated similarity matrix, a cophenetic correlation value (CC) was calculated.

II.4 Production of rhizobial inoculum for the inoculation of *C. calothyrsus.*

The key functions of a solid carrier are multiplication and protection of microorganisms. The carrier becomes the principal element of the product and provides a convenient base to facilitate the application and the use of the inoculum (Smith, 1992). Characteristics of an excellent solid carrier would include, good physical and chemical characteristics without toxic elements, high water-holding capacity, naturally near neutral or easily adjustable pH, sterility before addition of liquid culture, close connection between pH, organic matter and survival (Beunard et *al.*, 1990).

Many mineral and organic materials have been evaluated as inoculant carriers. These are grouped in different categories: peat soils, charcoal, compost made from bagasse, filtermud, plant compost, inert materials, rock phosphate, polyacrylamide gel.

During the project, some organic materials have been tested for suitability as inoculant carriers during this project : *Senna spectabilis*, *Chromoleana odoratum*, *Tithonia diversifolia*, and an unknown vegetable powder from Honduras

In order to identify a suitable local substitute of peat, we tested the survival of selected *Rhizobium* strains in several organic materials. The tests were carried out in two different laboratories: IRD Bel Air, Senegal, and IRAD, Nkolbisson, Cameroon. In Bel Air, each carrrier was inoculated with *Rhizobium* strain CCK13 at a concentration of 10^{10} g^{-1}. At each assessment, the number of rhizobia was determined by dilution techniques on YEMA media. Other carrier were used in KEFRI. In Kenya, filtermud is the principal carrier for rhizobial inoculants. Filtermud, a waste product of the sugarcane mills, is a fine deposit obtained during the filtration and clarification processes of crushed cane juice. Muhoroni filtermud, the type used routinely for inoculant production at KEFRI, is neutral (around pH 6.4), and therefore requires no additional treatment.

II.5 Selection of multi-origin *C. calothyrsus* microsymbionts (rhizobia and arbuscular mycorrhiza) for field inoculation trials

II.5.1. Screening rhizobia for N_2-fixation potential Rhizobia isolated from Central America, New Caledonia and Cameroon.

A total of 259 rhizobial isolates from Honduras, Mexico, Nicaragua, Costa Rica, New Caledonia, Cameroon were screened for N_2-fixation effectiveness with *C. calothyrsus* under glasshouse conditions in Bel Air Centre, Dakar, Senegal. *Rhizobium* strain CCK13 was included as a reference strain because Lesueur *et al.* (1996) had demonstrated that it was very effective with *C. calothyrsus* under greenhouse conditions. Seeds of *C. calothyrsus* provenance Flores (OFI seedlot number 16/96) were mechanically scarified as described by Macqueen (1993) and germinated in water agar (8 g l^{-1} w/v) petri plates at 28 °C for 48 h. Seedlings were cultivated in sand for 3 months. After this period, they were transferred to sterilized soil from Sangalkam (50 m from Dakar, Senegal). Plants were harvested after 6 months of growth and several parameters were measured: number of nodules, and shoot, root and nodule dry wt. The shoot total N nitrogen content was determined by the Kjeldahl method (Bremner and Mulvaney, 1982).

For Kenya, a subset of 17 indigenous *Rhizobium* strains was selected from the Kenyan rhizobial collection for this study. The selection was based on visual observation of shoot colour and biomass production during trapping experiments and/or authentication experiments with *C. calothyrsus*. They were tested on 2 provenances: San Ramón and Flores. Strains TAL 1145 from NifTAL and MUN 24 from Moi University were included for comparisons. In total, there were 19 inoculation treatments, a plus N control with no inoculation, and non-inoculated control with no N. *Rhizobium* cultures were grown to late exponential phase in yeast mannitol broth prior to inoculation of seedlings in Leonard jars (Vincent, 1970). Growth conditions, duration and assessments were as described above for evaluation of symbiotic N_2-fixation potential. The experimental design was a randomized complete block design with 8 replications.

II.5.2. Screening of AM

Mycorrhizal inocula were screened in a single experiment, which tested all inocula under identical growing conditions. The experiment was set up in a glasshouse. Three pre-

germinated seeds (San Ramón 11/91) were transferred to 1-l pots filled with a sterilized sand/Terragreen mixture (1:1 v/v). Approximately, 20 g of chopped roots/soil/spores inoculum was added to the pots at about 1 cm below the soil surface. Control treatment plants were inoculated with 20 g of a sterilized mixture of all 19 inoculants. All seedlings were also inoculated with 1 ml of *Rhizobium* suspension containing 1.7×10^5 cells ml^{-1}. After 1 week, seedlings were thinned to one per pot. Treatments were laid out on benches in 8 randomized blocks with each treatment represented once within each block.

Chemical analysis of the substrate showed unexpectedly high levels of P were available (NPK: 24, 10, 305 mg kg-1) and no additional nutrients were supplied during the course of the experiment. Plants were harvested after 12 weeks and measurements made of shoot and root growth, AM infection and nodulation, and nutrient allocation to shoots.

II.5.3. Screening effective strains of rhizobia in potted field soils

Testing selected rhizobia in Senegalese substrates

Rhizobia strains used in this experiment were CCCR22 from Cameroon, CCCR1 and CCCR15 from Costa Rica, and, CCK13, KCC6 and KWN35 from Kenya. They were grown in yeast extract mannitol medium (YEM) at 28 °C for 15 - 20 h before use. The experiment was set up in a greenhouse placed at Bel Air Research station, Dakar, Senegal. Plants were cultivated in two types of substrates: Sangalkam soil (see properties in Table 1) and a peat : vermiculite (1:19 v/v) mixture. Two-day old seedlings were planted in 15 × 5 cm plastic bags containing substrate. Inoculation was done 7 days after planting with cultures of rhizobia. Each plant received 10 ml of liquid culture applied around the root collar area. For each soil, there were 20 replicates per inoculation treatment (6 strains and uninoculated control). Plants cultivated in peat : vermiculite substrate received N-free nutrient solution (Broughton and Dilworth, 1971) twice a week. After 4 months of culture, plants were harvested, and shoot, root and nodule dry wt were measured for each plant after drying at 50 °C for 5 d. Shoot N content (%) was analysed by the Kjeldahl method (Bremner and Mulvaney, 1982). Data were subjected to a three-way ANOVA using the Super Anova Computer Program, and means were compared with the Fisher's multiple range test.

Testing selected rhizobia in Kenyan substrates

The strains used were KCC6, KCC17, CCK13, KCC39 and KWN35 from Kenya, CCCR1 and CCCR15 from Costa Rica, CCNC26 from New Caledonia, and, CCC22 from Cameroon. Two types of soils were used: Muguga nursery and Maseno field soils. They were sieved through < 5 mm mesh. Black polyethylene bags (size 6 × 9 cm flat dimensions) with drainage holes at the bottom were filled with 1 kg of respective soil per bag. Seeds were appropriately scarified, surface sterilized and pre-germinated. Two seedlings were planted per bag. Within 2 days after planting, each plant received a 1 ml of late log phase culture (about 10^9) culture grown in yeast mannitol broth. The experiment was a complete randomized block design consisting of 2 soil types, 2 provenances, 9 *Rhizobium* strains inoculation treatments plus non-inoculated control treatment. All treatments were replicated 20 times. The experiment was carried out over a period of 24 weeks (6 months) under glasshouse conditions with watering of pots maintained near field capacity. At harvest, nodules were detached from roots and counted, and shoot and root separated at the soil level. Shoot, root and nodule dry wt were determined after oven drying.

Testing selected rhizobia and assessment of natural mycorrhizal root infection in Costa Rican substrates

Rhizobium strains CCNC26, CCK13, KCC6, CCCR1 and CCCR15 were used as a mixed culture. They were evaluated in 5 soils namely; Quebradas, General Viejo, San Carlos,

Puriscal, and Cañas. Soil cores were collected using metal tube and transferred to PVC tubes according to the methodology described by Sylvester-Bradley *et al.* (1983). The soils were weighed and their moisture holding capacity estimated. Twenty five cores of each soil were filled and the moisture was maintained by weight at 25 - 35 %. The cores were fertilised as follows: 141 mg $CaCO_3$, 180 mg $Ca(H_2PO_4)_2.H_2O$, 59 mg K_2SO_4, 0.89 mg $Na_2MoO_4.2H_2O$ and 59 mg MgO, all calculated on the basis of surface area of 88.3 cm^3 to give the equivalent of 50 kg P, 96 kg Ca, 30 kg K, 0.4 kg Mo and 40 kg Mg ha^{-1}; Cu, Zn and B were also added to give the equivalent of 1, 5 and 0.5 kg ha^{-1}, respectively. Germinated seeds (seedlot number 1/99, provenance San Ramón) were planted in each core. Subsequently they were thinned to one plant $core^{-1}$. Fully-grown *Rhizobium* cultures on yeast mannitol agar plates were suspended in sterile water, mixed and 0.5 ml was used to inoculate each seedling. The experimental design consisted of one provenance, 5 soils, inoculated and uninoculated treatments; each replicated 10 times. The experiment was carried out over a period of 19 weeks. Plant heights and stem diameters were measured, and nodules were detached from roots and counted, and shoot and root separated at the soil level. Natural mycorrhizal root infection was also assessed using the methods described by Ingleby and Mason (1999). Shoot, root and nodule dry wt were determined after drying.

II.5.4. Evaluation of effectiveness of AM cultures on C. calothyrsus provenances in potted field soils

Mycorrhizal evaluation in Senegal

Five most effective AM cultures namely *Glomus etunicatum* (GE1), *Gigaspora albida* (GA1b), *Gi. albida* (GA2), *Scutellospora verrucosa* (SV2c) and *S. calospora* (SC2) were used. They were bulked up on *Sorghum bicolor* (millet) grown in sterilized Sangalkam soil. The experiment was set up in a greenhouse at the IRD Bel-Air Centre in Dakar, Senegal. It consisted of 2 provenances (San Ramón and Flores), 6 mycorrhizal inoculation (5 cultures and one uninoculated control) and 3 soils/substrates (Bel Air, Sangalkam and peat : vermiculite mixture). Each treatment was replicated 10 times, except 6 for peat.: vermiculite-grown plants.

Two-day- old seedlings were planted in 15 × 5 cm plastic bags containing sterilised soil that had received 20 g of mycorrhizal culture. All mycorrhizal inoculated plants also received 5 ml of the mixed rhizobial culture (strains CCC22, CCCR1, CCCR15, CCNC26, KCC6, KWN35 and CCK13) containing 10^9 cells ml^{-1} around the root collar area 4 d later. Seedlings were harvested after 4 months. The soil was gently washed from the roots, nodules were collected and aerial parts were separated from the rest of the plant. A sub-sample of roots was taken for clearing and staining as described by Ingleby and Mason (1999), and the mycorrhizal infection was assessed by the gridline intersect method. Nodules, shoot and roots were dried at 60°C and weighed. Shoot N content was measured by the Kjeldahl method (Bremner and Mulvaney 1982). Shoot K and P were determined by atomic absorption spectrophotometry after digesting plant material in a mixture of concentrated hydrochloric and fluoric acids. Data were subjected to a three-way analysis of variance using the Super Anova Computer program, and means were compared with the Fisher's multiple range test.

Mycorrhizal evaluation in Kenya

Mycorrhizal cultures were as used in Senegal (above). Two soils collected from farms in Nyabeda (near Maseno Agroforestry Centre) and Mama Wachira (near Embu Agroforestry Centre) were used. Soils were sieved (<5 mm) and thoroughly mixed with sterile river sand at the ratio of 3:1 and 1:1 (soil : sand) for Mama Wachira and Nyabeda, respectively. White PVC pots of 3.5 l capacity and 15 cm diameter were lined with polythene bags and filled with approximately 2 kg of one soil type (mixture) per pot. Each pot received a mycorrhizal

inoculum of 20 g consisting of root, spores and soil from established *Sorghum* cultures (6 months) above. Pre-germinated seedlings were transplanted in pairs per pot. Every pot was inoculated with 1 ml of a mixture of fully-grown rhizobial cultures (10^9 ml^{-1}) 3 d after seedling transplanting. The rhizobial mixture consisted of 9 strains namely KCC6, KCC17, KCC39, CCCR1, CCCR15, CCNC26, CCK13, KWN35 and CCC22. Number of replicates per treatment, experimental conditions and duration were as for rhizobial strain evaluation in potted soils described above (section: screening in Kenyan substrates).

II.5.5. In field conditions

Testing genetic compatibility of rhizobial strain CCK13 with C. calothyrsus provenances under on-station field conditions at Bel Air Centre, Senegal

Provenances used were San Ramón, Bonampak and Georgesville. Germinated seedlings in plastic bags containing soil from Bel Air station were inoculated with liquid culture of strain CCK13 (10 ml $seedling^{-1}$) one week after planting. The experimental layout consisted of 3 provenances and 2 inoculation treatments (inoculated and uninoculated seedlings). Each treatment was replicated 15 times. Seedlings were transplanted to the station's plot after 3 months of growth in the nursery conditions. Leaf and stem biomass was assessed at 7, 11, 15 and 19 months after transplanting (MAP). At each each assessment, trees were pruned to a height of one metre from the ground, and leaves and stems dried separately before weighing. Leaves were also sampled appropriately to determine N content (%) and ^{15}N natural abundance (^{15}N ‰). The ^{15}N natural abundance and N contents were determined by a Finnigan Mat mass spectrophotometer (Delta S type, Bremen, Germany) coupled to an elemental analyzer manufactured by the Service Central d'Analyse of C.N.R.S (Vernaison, France). The precision of measurements was 0.3 ‰. The proportion of N derived from atmospheric N_2 (Ndfa %) was calculated according to the following equation (Amarger *et al.*, 1977, Bardin *et al.*, 1977):

$$Ndfa\ \% = (^{15}N_{nf} - {}^{15}N_f/{}^{15}N_{nf} - {}^{15}N_a) - 100$$

Where $^{15}N_{nf}$ is the natural isotopic abundance of the non-fixing reference plant (*Eucalyptus camaldulensis*); $^{15}N_f$, the isotopic abundance of *C. calothyrsus*, and $^{15}N_a$, the ^{15}N measured in leaves of nonnodulated *C. calothyrsus* plants growing in N-free medium (it could be considered as 0 if not already determined). Data were statistically analysed and means separated by the Newman and Keuls test ($P < 0.05$).

II.6. Effects of the dual inoculation with selected rhizobium and arbuscular mycorrhizal isolates on growth of *C. calothyrsus* cultivated in unsterile soils.

Nursery pot experiments were conducted in Senegal and Kenya. Seedlings of *C. calothyrsus* were inoculated with *Rhizobium* and AM inoculants, either singly or in combination and with appropriate uninoculated control treatments. In Senegal, comparisons were made between inoculants consisting of single isolates and mixtures of isolates of both microsymbionts. In Kenya, only one inoculant, consisting of a cocktail of isolates, was used for each microsymbiont and provenance/soil interactions were also examined. Experiments in both countries were set up using unsterilised local soils.

Soils of varying fertility and pH were selected for use in the experiments. In Senegal, a single experiment compared two contrasting soils: Bel Air a fertile alkaline soil and Sangalkam a more acidic infertile soil. In Kenya, experiments were set up at 2 different nurseries (Embu and Maseno). Local soils were collected from the sites where the field

experiments were to be established. Of these two soils, Maseno was the more acidic and less fertile, although both soils can be considered infertile on the basis of P and N present. Unsterilised soils were used in all experiments but, in the Kenyan experiments, an additional solarisation treatment was included in order to reduce natural populations of microsymbionts and harmful organisms and therefore try to monitor effects attributable to inoculation. Both soils were solarised in the KEFRI glasshouses in Nairobi. For solarisation, soils were spread between clear polythene sheeting and left exposed to natural sunlight for 1 month.

Regarding inoculums, in Senegal, the inoculation of rhizobia, KWN35 and CCK13 isolates were used singly, in combination and with a cocktail of these 2 isolates plus isolates CCC22, CCCR1, CCCR15 and KCC6. For inoculation of AM, G.etu.1 and Gi.alb.1b isolates were used singly, in combination and with a cocktail of these 2 isolates plus isolates Gi.alb.2, S.cal.2 and S.ver.2c. This resulted in 22 different inoculation treatments. In Kenya: For both microsymbionts, a cocktail of rhizobium (CCC22, CCCR15, CCNC26, KCC6, KWN35, CCK13, KCC39) and AM (G.etu.1, Gi.alb.1b, Gi.alb.2, S.cal.2) isolates were used as inoculants. These inoculants were applied singly (+R-M or +M-R), in combination (+R+M) or with an uninoculated control (-R-M), resulting in 4 inoculation treatments.

Both provenances (San Ramon and Flores) were included in the Kenyan experiments, whereas only one provenance (Flores) was used in the Senegal experiments so that a large number of inoculation treatments could be accommodated.

Experiments were set up on free-draining benches in glasshouses at the IRD Bel-Air Centre (Dakar, Senegal), and KEFRI (Muguga and Maseno nurseries, Kenya). AM inoculum was incorporated into the pots prior to sowing. Seeds were scarified, soaked in water and germinated on water agar before being transferred into the pots. Rhizobial strains were cultured in YEM and 5 ml of culture was added to the seedlings 5 days after germination (Lesueur et al. 2001). In Senegal, treatments were replicated in 12 randomised blocks, and in both Kenyan experiments treatments were laid out in 10 randomised blocks.

In Senegal, experiments were harvested after 28 weeks and assessments made of shoot, root and nodule dry mass. Experiments in Kenya were harvested after 16 weeks, and measurements were made of leaf, stem, root and nodule dry mass, stem diameter, leaf area, nodule number, AM infection and leaf nutrient content. AM infection was determined on sub-samples of roots as described in Ingleby et al. (2001).

Data from the experiments in Kenya were examined for normality (EDF tests; Stephens, 1974)) and transformed where necessary to ensure homogeneity of variances (Bartlett's test; Sokal and Rohlf, 1995), before performing 3-way analysis of variance (ANOVA) using inoculation, soil and provenance as treatment factors. Means were compared using Fisher's LSD test when the F-test from ANOVA was significant at $P<0.05$. Similar statistical analyses were conducted in Senegal.

III. Results achieved.

III.1. Collection work : Central America, New Caledonia, Kenya and Cameroon.

During this work, it appeared that a large majority of plants of *C. calothyrsus* were naturally nodulated by native rhizobia. By this way, it was possible to collect both fresh and partially dried nodules from them, trapping experiments could be done in Senegal. Regarding soils samples, each of them were sent to CEH/ITE in order to extract the natural arbuscular endomycorrhiza.

III.2. Establishment of a collection of microsymbiont : rhizobia and mycorrhiza (AM)

III.2.1. For rhizobia.

From nodules collected under the fields, 242 rhizobia strains were isolated from the several countries : 22 from Mexico, 26 from Honduras, 50 from New Caledonia, 52 from Guatemala, 36 from Nicaragua, 30 from Costa Rica and 26 from Cameroon. A large majority of them belong to the *Rhizobium* genus (fast growing rhizobia). Usually, *C. calothyrsus* is described as being a species of woody legumes capable to nodulate essentially with *Rhizobium* rather than with *Bradyrhizobium* genus (Turk and Keyser, 1992; Lesueur et al., 1996). However, Peoples et al. (1989) showed that some *Bradyrhizobium* strain can nodulate and form an effective nitrogen-fixing symbiosis with *C. calothyrsus*. Our results show that *C. calothyrsus* can nodulate naturally with *Bradyrhizobium* in Central America, Mexico and New Caledonia. In return, no *Bradyrhizobium* strains were isolated in Cameroon. Now we will have to verify if nodules formed are able to fix nitrogen in symbiosis with *C. calothyrsus*.

III.2.2 For AM.

In soil samples, spores were extracted from the test soils using the sucrose centrifugation method (Walker *et al.*, 1982). In each case, sporulation of AM fungi in the loam/sand substrate was better (at least twice the number of spores) than in the fibre/sand substrate (Table 1), although growth of the three plant species was not noticeably different in either substrate. Some differences in spore numbers and species composition are suggested between the three host species and the two substrates, however the lack of replication and/or the heterogeneity of soil samples may account for these differences. It was also observed that root growth and rooting density of *C. calothyrsus* was much less than that of the crop species in both substrates. Further amendments to the loam/sand substrate are planned which might optimise production of AM spores.

In general, soils with most spores in the original soil produced most spores after culturing, with soils from sites in Honduras (H2), Guatemala (G2, G4), Cameroun (C2) and Kenya (K7, K9) being among the most productive. AM fungal species that occurred most frequently included *Gigaspora albida*, *Glomus etunicatum*, *Scutellospora heterogama*, *Scutellospora verrucosa* and *Acaulospora scrobiculata.*

The results also indicated that *C. calothyrsus* and the crop plants (sorghum and millet) were equally effective when used as host plants in the 'trap' cultures. In future, crop plants may only need to be used in the final phase of inoculum production, when large quantities of infected (and non-nodulated) roots are required.

Successful cultures (i.e. those where large numbers of fresh spores had been produced) were either potted-on or re-sown. Cultures baited with crop species were dried, the plant shoots removed, fresh sterilised potting mixture added and the pots re-sown with the same host species used in the first 'trap' culture cycle. Repeat cycling of 'trap' cultures is recommended as it has been shown to 'bait out' AM fungal species that were dormant during the first cycle (Morton, 1995) and generally increases the activity of the AM fungi present in the cultures.

Successful cultures baited with *C. calothyrsus* seedlings were potted on into 1 litre pots filled with fresh sterilised potting mixture. Amendments to the loam/sand mixture were tested using *C. calothyrsus* seedlings grown in 4 of the test soils (H2, G2, G4 and M3). These seedlings were potted on into mixtures of either loam/sand/Terragreen (granules of compressed clay obtained from Oil-Dri, UK Ltd) or loam/sand/rockwool (granules of artificial fibre obtained from LBS, UK Ltd) mixed in equal proportions (1:1:1 by volume). Better growth of *C. calothyrsus* was observed in the loam/sand/terragreen mixture, which suggests that it should be adopted as the standard potting mixture. The effect of these mixtures on AM spore production has still to be investigated.

In all, thirty-five single species isolations (13 different species) were set up in the growth cabinet (Table 2). Permanent slides were prepared as reference specimens of each spore type used in these isolations.

III.3. Evaluation of the biodiversity of rhizobia of *C. calothyrsus.*

RFLP analysis of amplified 16 S rDNA and genetic relationships between obtained genotypes

Universal primers fD1 and rD1 were used to amplify 16S rDNA gene from intact cells without isolating DNA. All strains produced a single band of about 1500 bp, corresponding to the expected size of 16S rDNA gene (data not shown), (Laguerre *et al.* 1994). For RFLP analysis, 16S rDNA PCR products obtained from 293 strains were digested with three endonucleases (*Hae I, Mbo I, Msp I*). Restricted samples were loaded into 3 different gels: *Hae I, Mbo I, Msp I* digestions separately. Fifteen different combinations of patterns, representing 15 different 16S rDNA genotypes (**A, B, C, D, E, F, G, H, I, J, K, M, N** and **O**) were detected (Figure 1). Major genotypes with the highest amount of strains were represented by genotypes as follows: **A** (116 strains), **B** (63 strains), **C** (40 strains), **D** (24 strains), **E** (14 strains) and **F** (12 strains). Minor groups with the low amount of strains were represented by following genotypes: **G** (8 strains), **H** (6 strains), **I** (4 strains) and **J, K, L, M, N, O** (1 strain). One representative from each obtained genotype and genotypes obtained from 30 reference strains were compared pairwise. Finally, similarity matrix was calculated and UPGMA dendrogram was constructed. The cophenetic correlation value (CC) was calculated for constructed dendrogram. The CC value is the product-moment correlation between all original matrix similarities and all corresponding similarity values derived from the dendrogram. If CC value is high (more than 90%), similarity matrix can be represented faithfully by an UPGMA dendrogram. The CC value for created dendrogram was 95%, indicating a good quality of the cluster analysis.

Tentative identification showed that using three restriction endonucleases, 188 strains from 293 examined in this study (genotypes: **A, C, E, G, H, I**) grouped together with different strains of *Agrobacterium, Mesorhizobium, Sinorhizobium* and *Rhizobium* species. This indicated the genetic similarities with known rhizobial species. The representative of genotype **E** (CCN6) with 14 isolates had exactly the same 16S rDNA-RFLP genotype as *A. radiobacter, A. tumefaciens, M. tianshanense*. The representative of genotype **C** (CCC19) with 40 isolates showed 100% similarity with *S. medicae* and *S. meliloti*. The biggest group was represented by CCN1 (**A** genotype with 116 isolates). The genotype **A** included almost 40% of analyzed 293 bacterial isolates (Figure 2). *A. rhizogenes, R. tropici* and *R. hainanense*, which clustered together with the representative of genotype **A** were not distinguished in our work. This was in agreement with the work of Young and Haukka (1996) and Nick (1998). *R. tropici/R. leguminosarum*-type strains have been also reported as the most effective in N_2 fixation with host legume *C. calothyrsus* tree (Bala *et al.* 2001a). Possibly, the most of 116 strains in genotype **A** belongs to *R. tropici* species. The representative of genotype **G** with 8 isolates (CCNC12) showed 100% similarity with *S. saheli*. Genotype **I** with 4 isolates (CCG52/2) was identical with genotype of *R. etli* (Figure 1).

Surprisingly, representatives of genotypes **B** (63 strains), **D** (24 strains), **F** (12 strains), *Mesorhizobium amorphae* and *Sinorhizobium fredii* and *Sinorhizobium. arboris* stayed unclustered. The three large groups of bacteria: **B** with 63 strains, **D** with 24 strains and **F** with 12 strains were not assigned to any known rhizobial species. It can suggest new group of rhizobia or intraspecies variability arising from geographical origins. Several rhizobial strains formed genotypes **J, K, L, M, N** and **O** with only one isolate as a representative. They do not

cluster with defined rhizobial species. They have been, however, reported as being able to nodulate *C. calothyrsus* tree (Lesueur *et al.* 2001).

Msp I restriction endonuclease has been found to be the best enzyme to start the identification of unknown rhizobia, because it has given the largest number of different patterns (genotypes) among rhizobia (Nick 1998). Using 3 enzymes *Hae I, Mbo I, Msp I,* has been established to have big discriminative power in clustering the unknown strains with defined *Rhizobium, Sinorhizobium* and *Mesorhizobium* strains (Nick 1998). Laguerre (1994) has, however, found a minimum of four enzymes to be necessary to resolve the rhizobial strains into species and cluster new isolates. Using the forth enzyme *Alu I* would divide obtained clusters into more detailed and possibly create separate *Agrobacterium* branch (Nick 1998). The *Alu I* restriction endonuclease will be probably applied to differentiate strains within main genotypes. This data may be useful before sequencing of representatives of each obtained 16S rDNA genotype. Nevertheless, several different representatives of each genotype should be chosen for sequencing, because all presented here groups might be heterogeneous.

Generally, rhizobia have been divided into fast growing rhizobia and slow growing bradyrhizobia (*Bradyrhizobium* sp). The majority of strains were fast growers, only 23 isolates were identified as *Bradyrhizobium* like rhizobia (Lesueur *et al.* 2001). Traditionally, *Bradyrhizobium* species have been differentiated from fast growers, because they grow slowly (slow-growing rhizobia) and they alkalize YEM agar plates supplemented with pH indicator, bromothymol blue. Isolated strains, previously marked as bradyrhizobia (23 strains from different geographical origins), were analyzed in order to obtain RFLP genotypes. Except three strains representing genotypes **A, L** and **M**, most of examined *Bradyrhizobium* like strains, belonged to genotype **B**, reflecting genetic homogeneity of this group of isolates (Table2). Nevertheless, genotype **B** with 63 strains, probably consisted alkali producing bacteria (*Bradyrhizobium* like with 23 species) as well as acid producing (fast growing rhizobia with 40 species).

Most of the isolated strains have formed nitrogen-fixing nodules with *C. calothyrsus*. Lesueur *et al.* (2001) have reported that only 9 strains were not able to form nodules. In our study, 9 strains CCCR33, CCG44, CCG1, CCG9, CCN10, CCNC73, CCNC97, CCH31 and CCC40 belonged to different genotypes **H, A, H, B, D, E, B, G,** and **B,** respectively. This indicates, that there can be significant genetical differences (nitrogen fixation ability and disability) between strains belonging to each genotype.

The six effective inoculant strains originated from New Caledonia (CCNC26), Costa Rica (CCCR15, CCCR1), Cameroon (CCC22) and Kenya (KWN35, KCC6) have been chosen as being very effective at nodulating the host plant and used in field tests (Lesueur *et al.* 2001). In our study, strain CCNC26 belonged to the unclustered big genotype **D** (24 strains). Strains CCCR1 and CCC22 belonged to the genotype **C** (40 isolates), which grouped with defined representatives of *Sinorhizobium* species. Others, CCCR15, KWN35, and KCC6 belonged to group **A** (116 isolates), which clustered with *R. tropici/R. leguminosarum, R. hainanense* and *A. rhizogenens* species. Rhizobial strains isolated from *C. calothyrsus, Leucaena leucocephala* and *Gliricida sepium* have been found to be able to effectively cross nodulate each others` hosts (Bala *et al.* 2001a). The strains most effective in nitrogen fixation with *C. calothyrsus* and *G. sepium* were tentatively identified as *R. tropici,* while those of *L leucocephala* belonged to the genera *Sinorhizobium* and *Agrobacterium. L leucocephala* is another tree widely used in the humid tropics but *C. calothyrsus* is regarded as better, because it tolerates more poor and acid soils.

Screening of isolates according to their 16S rDNA genotypes and geographical origins

There were differences between the rhizobial isolates from different geographical origins. It has been reported before that the most effective strains (the highest number of nodules and greater growth of host plant) have been isolated from Honduras, Mexico, Costa Rica and New Caledonia. The least effective strains (many small nodules and limited effect on the growth of host plant) have been harvested from Nicaragua and Cameroon (Lesueur *et al.* 2001). In our study, all obtained strains were divided according to the obtained 16S rDNA genotypes and geographical origins.

In our study, genotype **A** dominated in almost every country except Costa Rica and Cameroon. Representatives of genotype **B** were present in all countries except Kenya. Genotype **C** containing 40 strains were absent in Mexico, Honduras and Kenya but they dominated over all isolates in Cameroon. Representatives of genotype **D** were distributed in all countries except Cameroon. Genotype **E** was absent in Guatemala and Kenya. Representatives of unclustered genotype **F** did not occur in New Caledonia and Cameroon (Table 4).

New Caledonia, Cameroon and Kenya belong to countries where *C. calothyrsus* was introduced. In our study, distribution of different genotypes in New Caledonia (abundance and character) was comparable to proportions of different 16S rDNA-RFLP genotypes obtained from native areas in Mexico, Honduras, Guatemala, Nicaragua and Costa Rica. Cameroon and Kenya belonged to exceptional countries. Genotype **C** dominated in Cameroon (79% of all isolated strains). There were only three genotypes (**A**, **D** and **F**) present in Kenya. Generally, biodiversity of *C. calothyrsus* nodulating strains was greater in Central America and New Caledonia than in Kenya and Cameroon. It is, however, still open whether the nodulation of *C. calothyrsus* trees in Kenya, Cameroon and New Caledonia was induced by local indigenous rhizobia or rhizobia originating from native area of *C. calothyrsus*, Central America. Restriction patterns and sequence analysis of symbiotic genes *nodA* and *nifH* gene would give more information about origin of strains representing these genotypes (Haukka *et al.* 1998).

Generally, it is very difficult to conclude that genetic differences between rhizobial isolates are related to the country of origin. There can be clear shift in the structure (character) of genotypes compared to geographical origins arising simply from sampling. Competition between strains occupying nodules also exists. Less competitive and more numerous strains, forming many small nodules, can be easier sampled than more competitive and less abundant (Bala *et al.* 2001b). In our study, the number of the tested strains was possibly too small to draw sure conclusions of relationship between the geographical origin of the isolates and population structure.

Grouping of 293 strains presented here is still an initial measure of diversity of large group of bacteria. Until 16S rDNA partial or full length sequencing data is available, no conclusions on the degree of heterogeneity of isolates can be drawn. Sequencing data of 16S rDNA from numerous isolates of each genotype from different origins will be obtained. The big groups of isolates (**B**, **D**, and **F**), which stayed unclustered (no clear similarity to any defined rhizobia) are especially interesting. They may represent new group of rhizobia.

In conclusions, semi-automated PCR-RFLP method, used in this study, appeared to be rapid and reliable tool for the initial evaluation of the biodiversity of large number of isolates.

III.4 Production of rhizobial and AM inoculum for the inoculation of *C. calothyrsus*.

An UPIL fermentor is a modular system that is adaptable to local conditions (Saint Macary *et al.*, 1986). Selection and layout of the equipment depends on the specific requirements of the producer. An UPIL system was installed in the IRAD Laboratory,

Nkolbisson, Cameroon, by this project for local inoculant production. The system is operational and is expected to serve for several years to come.

Among organic materials which were tested for suitability as inoculant carriers during this project, it appeared that *Senna spectabilis* and *Chromoleana odoratum* powder cannot been used as carriers; they are too acidic and the liming does not significantly raise the pH to acceptable levels. Regarding the other materials, *Tithonia diversifolia* and vegetable powder did not require neutralization while it was necessary to neutralise peat with 1.5 g of lime for 20 g of peat.

Our Results showed that powder from Honduras and peat were comparable whereas survival in *T. diversifolia* was nil 1 h after inoculation. In contrast, tests carried out in Nkolbisson, Cameroon (Table 4) showed *T. diversifolia, Cajanus cajan* and *Arachis hypogea* supported survivals of $> 10^8$ g^{-1} of carrier a month after inoculation.

Survival of rhizobia in carriers may also depend on type of strain and storage temperature. Inoculants are generally cured at room temperature (20° to 25°C) for a period 2 - 3 weeks to facilitate attainment of maximum growth (Burton, 1967), afterwhich they can either be stored at room or 4 °C before use.

Regarding the effect of the temperature on the storage of inculum, we assessed the both growth and survival of rhizobia in two storage temperature conditions: 4°C or 28 °C using procedures described in earlier studies (Beunard *et al.*, 1990). We used strains CCK13 (*C. calothyrsus*) and Ldk4 (*Leucaena leucocephala*). Our results showed important differences between the strains carriers tested with respect to storage temperature conditions. The survival of strain CCK13 increased gradually at 4 and 28 °C in peat and Honduras powder, respectivley. In contrast, peat rhizobia stored at 28 °C and Honduras powder rhizobia stored at 4 °C showed contrasting survival trends. Survival in peat increased sharply to peak at 14 days and also dropped rapidly in the next 14 d at 28 °C, whereas in Honduras powder, there was a 14 days lag and rapid increase at 4 °C. With strain Ldk4, we observed increased survival levels in both carrriers irrespective of storage temperatures up to 14 days (Fig. 5). At one month, survival decreased to initial level except for Honduras powder stored at 28 °C, which continued to increase.

Regarding the inoculum production of AM during the course of the project, it was monitored pot culture quality through the examination of AM spore numbers and root infection levels, indicated that spore production in the loam/sand/Terragreen substrate was usually very high, whereas infection levels on the Sorghum roots were sometimes rather low. These quality checks also revealed that, with one exception, ingress by contaminant AM fungi had not occurred, and that use of a slow-release insecticide had eliminated mites and nematodes.

After despatch of the 5 selected inoculants to partners, subsequent nursery tests in Senegal have indicated that storage at 6°C has little or no detrimental effect on viability of the inoculum. This suggests that spores, rather than infected root fragments, are the most important component of the AM inoculants. An experiment conducted in Kenya, growing the 5 AM inoculants in contrasting local soils, has shown a differential response to the soils by the AM fungi in terms of infection and sporulation. This work shows that, in pot culturing, factors such as soil type, pH, nutrient regimes etc. need to be tailored to suit specific fungi.

Two Training workshops were organized during the project : the first managed by CIRAD-Forêt was held in Senegal and concerned Mr Sylvestre Badgel Badgel from IRAD and Mr Gaspar Alvarado from Conseforh who stayed from 23 January to 23 March 1999 in the Microbiology Laboratory of IRD/ Bel Air Center.

The objectives of this training course was to initiate the both technicians in basic methodologies on microbiology in order that they were able to produce the inoculum in their

country. After discussion with Pierre Beunard from CIRAD-Amis, several experiments were selected in preparation of the production :

- Preparation of culture media for rhizobia (liquid and solid)
- Isolation of pure cultures of rhizobia in Petri Dishes.
- Isolation of rhizobia from nodules harvested in the field.
- Enumeration of rhizobia in a liquid culture by using dilution method.
- Production of alginate beads containing rhizobia
- Manipulation of plants cultivated in tubes in sterile conditions for inoculation experiment.
- Inoculation experiment carried out with several methods

Each of them were realized by the both technicians, and in final, the results obtained were satisfactorily.

Pierre Beunard was in Cameroon from 21th November to 6th December 1999 for the installation of the UPIL System in IRAD laboratory. During the mission, Pierre concentrated his action on the demonstration of the specific technics of microbiology and the utilization of the fermentor for producing rhizobia inoculum. He insisted on the neccesity to follow and control all the production steps with attention to avoid contaminations . By this way, it should be possible to produce a rhizobium inoculum with a good quality. All the steps of multiplication were realized : first sample for inoculating fermentor, autoclaving of fermentor, multiplication of rhizobium, sterilization of carrier, enumeration of bacteria and distribution of liquid culture in plastic bags containing sterile carrier. In final, IRAD staff has the possibility to produce by themselves their rhizobia inoculum in good conditions. The second training course concerned AM organised by CEH/ITE in UK from 11 to 20 October 1999. The training course was attended by technicians from IRAD, CONSEFORH, and KEFRI. It was designed to provide each with the basic training on how to produce mycorrhizal inoculum in each of the partner countries. The first part aimed to familiarise participants with the general features of arbuscular mycorrhizas (AM) and the methods used to recognise and examine AM spores and infected roots. The second part dealt specifically with the nursery facilities and methods required by each partner in order to establish mycorrhizal cultures when they return to their own country.During the workshop, AM spore populations in field soils, 'trap' cultures and pure cultures were compared. This exercise not only familiarised the participants with the characteristics of the different inoculant fungi, but also clearly demonstrated the need for culture and isolation of mycorrhizal fungi from field soils. Participants saw that field soils contained relatively few spores, most of which were dead, and also contained fungal parasites, mites and nematodes. 'Trap' cultures made using the same field soils, and the resulting single species cultures, markedly increased numbers of 'live' spores and eliminated harmful organisms.

II.5 Selection of multi-origin *C. calothyrsus* microsymbionts (rhizobia and arbuscular mycorrhiza) for field inoculation trials

II.5.1. Screening rhizobia for N_2-fixation potential from rhizobia

Lesueur *et al.* (2001) have described in detail the combined average effectiveness of strains on the basis of country of origin. The results showed, in terms of shoot dry wt that strains from Costa Rica, New Caledonia and Honduras were best and those from Guatemala and Nicaragua were worst. Plants inoculated with the reference strain CCK13 were well nodulated (nodule number and biomass) and enhanced shoot growth, thus confirming the results of the earlier study (Lesueur *et al.*, 1996). However, interpretation of combined strain effects may mask the superior performance of individual strains. For example, strain CCC22

from the Cameroon was highly effective while the other 21 were ineffective (Lesueur *et al.*, 2001). We compared effectiveness of 4 new strains with the reference strain CCK13. Strains CCC22 (Cameroon), CCCR1 (Costa Rica), CCN26 (New Caledonia) and CCCR15 (Costa Rica) were globally more effective than the reference strain under glasshouse conditions. In Kenya, only 2 strains (KCC1 and KCC11) showed effectiveness indices ≤ 0, suggesting that either they were ineffective or saprophytic to the host plants (Table 4). Strain KCC6 from Mama Elizabeth's farm was the most effective with both provenances, and was consistent with the results obtained with 'whole soil inoculum' with respect to provenance San Ramón.

II.5.2. Screening of AM

Results from the screening experiment showed that mycorrhizal inoculation increased the shoot growth, nutrient allocation and nodulation of *C. calothyrsus* seedlings. Fourteen of the 19 inoculants significantly increased stem dry wt, 15 increased the proportion of P allocated to the shoot and 9 increased nodule dry wt, compared with uninoculated control treatment (Table 5). Mycorrhizal infection formed by the inoculants ranged from 22 - 26 %. On the basis of these results, 5 of the most effective isolates were selected for testing under nursery and field conditions. The experiment also showed that single species inoculants performed as well as mixed species inoculants, and that isolates originating from countries in Central America and Africa could be equally effective on *C. calothyrsus*, which is indigenous to Central America.

II.5.3. Screening effective strains of rhizobia in potted field soils

Testing selected rhizobia in Senegalese substrates

There was significant rhizobial strain effect ($P < 0.05$) for shoot and root dry wt, and shoot N in the peat : vermiculite substrate. There was no significant provenance effect from the combined rhizobial data. Provenance and rhizobia interaction effect was significant only for shoot N. Kenyan strain KWN35 gave the best inoculation response. Control plants nodulated in this substrate, indicating presence of indigenous rhizobia. A similar trend of inoculation response was obtained in Sangalkam soil, especially with shoot dry wt. In addition, there was significant ($P < 0.05$) provenance, rhizobia and interaction effects. These results indicated that the Kenyan strains appeared to be more effective in terms of shoot dry wt and N content. Strains KWN35, KCC6 and CCK13 are among the strains selected for further inoculation trials in nursery and field conditions. Results in Sangalkam soil also showed that provenance San Ramón significantly accumulated more biomass than Flores did.

Testing selected rhizobia in Kenyan substrates

In Muguga soil, there was significant ($P<0.01$) provenance effect on root and nodule dry wt. Generally, there was lack of rhizobial inoculation response in Muguga soil. This is attributed to the relatively high fertility and effective indigenous rhizobia. Strain KCC17 was originally isolated from Muguga soil (Odee *et al.*, unpublished data), and was among the selected strains. On the other hand, there was significant ($P<0.0001$) rhizobial strain inoculation response with several strains in number of nodules plant^{-1} in Maseno soil. Significant ($P<0.01$) strain effect was also observed in root dry wt. Significant ($P<0.05$) provenance effects were observed in nodule and total plant dry wt. There was also significant ($P<0.01$) interaction effect between strain and provenance in nodule numbers plant^{-1}. However, there were no consistencies in inoculation response in terms of rhizobial strain and provenance factors.

Testing selected rhizobia and assessment of natural mycorrhizal root infection in Costa Rican substrates

There was a marked significant effect of soil type on nodule number, but no response due to inoculation. The less fertile soils of General Viejo and Quebradas (Pérez Zeledón region) showed practically no nodulation, whereas in the more fertile soils of Cañas, Puriscal and San Carlos nodules were more abundant. Naturally growing *C. calothyrsus* has been found to have nodules in areas adjacent to General Viejo and Quebradas, which indicates presence of indigenous rhizobia. In addition, *C. calothyrsus* is native in all these regions except Cañas. Poor nodulation in General Viejo and Quebradas soils yet better plant growth implies that these seedlings were heavily dependent soil mineral N. Therefore the very poor nodulation in the 2 soils may be due to localised inhibition of nodule formation probably due to high mineral N or other nutrient deficiency or toxicity not analysed. Further studies are required to establish the cause of nodule inhibition. Unlike nodulation, natural mycorrhizal infections were extremely low (0 - 6 %) in all the soils and inoculation treatments, which may suggest very low indigenous populations of active AM propagules.

II.5.4. Evaluation of effectiveness of AM cultures on C. calothyrsus provenances in potted field soils

Mycorrhizal evaluation in Senegal

Mycorrhizal root infections were highly variable and appeared to be influenced differently by substrate, provenance and mycorrhizal type (Table 11). Plants grown in Sangalkam soil had higher root infections than those in Bel Air soil. The highest infection (100 %) was scored on Flores plants inoculated with *G. etunicatum* in Bel Air soil. Data for shoot, root and nodule dry wt were combined for both provenances San Ramón and Flores. Generally, mycorrhizal inoculation increased dry matter accumulation. However, we also noted some important differences due to substrate. For example, Sangalkam grown plants inoculated with *Gi. albida* (GA1b) had significantly ($P < 0.05$) higher shoot dry matter than those inoculated with other isolates. In Bel Air soil, the most effective isolates were *G. etunicatum* and *S. verrucosa*. In peat : vermiculite mixture, which was the least productive of the 3 substrates, inoculation with *S. verrucosa* produced better shoot dry matter than the other inoculation treatments.

Results of foliar nutrient content in Table 12 also show major differences between the soils. For example, K foliar content of Sangalkam grown plants were very low compared to those grown in Bel Air soil or peat : vermiculite mixture. Foliar N were greatest in Sangalkam and in peat : vermiculite inoculated with *S. verrucosa* and *G. etunicatum*, respectively. In Bel Air soil, foliar N was highest in uninoculated control plants. This is attributed to concentration effect of N in the leaves due to poor dry matter status. Similar results have also been reported for other woody legumes (Lesueur and Diem, 1997).

Mycorrhizal evaluation in Kenya

ANOVA results indicated significant differences ($P=0.0067$) due to mycorrhizal inoculation only for nodule numbers plant^{-1} in Mama Wachira soil (Tables 13 and 14). Plant height, number of nodules plant^{-1}, and nodule dry wt parameters were also significantly ($P<0.01$) different in both soils due to provenance effect. Significant provenance ($P<0.01$) effect was also observed for shoot and total plant dry wt in Mama Wachira soil and root dry wt in Nyabeda soil. Mycorrhizal inoculation response was highly variable, and depended on soil, provenance and parameter. In some parameters, uninoculated controls performed better than inoculated treatments. Results of mycorrhizal evaluation in similar but sterilized soils by Ingleby *et al.* (these proceedings) supports this interpretation. Greater dry matter was accumulated in Mama Wachira soil than Nyabeda soil, thus reflecting differences in soil

fertility. Flores appeared to produce more shoot and total plant dry wt than San Ramón in the more fertile Mama Wachira soil, whereas the reverse was true for root dry wt. Nodulation (number of nodules plant^{-1} and nodule dry wt) was also more prolific in Flores than San Ramón, and in both soils. However, it would be prudent to include root infection and tissue nutrient data once they are analyzed to make a more informed interpretation of this data.

II.5.5. In field conditions

Testing genetic compatibility of rhizobial strain CCK13 with C. calothyrsus provenances under on-station field conditions at Bel Air Centre, Senegal

It was important to evaluate genetic compatibility of *C. calothyrsus* provenances with the globally effective reference strain CCK13 under on-station field conditions while implementing the logical selection process of indigenous microsymbionts. Two new provenances, Georgesville and Bonampack, were tested alongside San Ramón. Results in Table 15 show variable response to inoculation with regard to parameters, and provenance and growth duration factors. There was inoculation response in all the provenances 7 and 15 MAP in terms of leaf dry matter. Inoculation differences observed 11 and 19 MAP after planting were not significant ($P < 0.05$). There was also significant provenance effect on leaf and stem dry matter 15 and 19 MAP. These results seem to indicate that choice of provenance could be important for optimizing the effect of inoculation and confirm the data obtained under greenhouse conditions in Senegal. Regarding leaf and stem N content, significant differences were observed only at 11 MAP (Table 16). Symbotically fixed N_2 also varied from 0 - 38.5 %, but significant differences for both provenance and inoculation factors were only observed 19 MAP. Detailed statistical inferences of these data are presented in Table 17.

II.6. Effects of the dual inoculation with selected rhizobium and arbuscular mycorrhizal isolates on growth of *C. calothyrsus* cultivated in unsterile soils.

In Senegal, seedlings inoculated with both rhizobium and AM had greater shoot dry mass (Table 2), root dry mass (Table 3) and nodule dry mass (Table 4)) than the other treatments. Seedlings receiving rhizobium alone tended to have greater shoot, root and nodule dry mass than those receiving AM alone or the uninoculated control treatment. Of the rhizobium inoculants, KWN35 was more effective than CCK13, and was also more effective when added singly rather than in combination with CCK13, or when added as part of the 'cocktail' of isolates. Of the AM inoculants, Gi.alb.1b was more effective than G.etu.1, and was also more effective when added singly rather than in combination with G.etu.1, or when added as part of the 'cocktail' of isolates.

Seedlings differed in their response to rhizobium and AM inoculation: rhizobium inoculation tended to increase shoot and nodule dry mass, but not root dry mass, whereas AM inoculation tended to increase root dry mass. Growth responses to inoculation also differed according to the soil: rhizobium inoculation producing larger responses in the fertile Bel Air soil, while AM inoculation produced larger responses in the infertile Sangalkam soil.

In Kenya, and more specially Muguga/Embu soil, our results showed significant effects of inoculation for most parameters of seedling growth, with plants receiving both microsymbionts having the greatest stem, leaf and root dry mass, and those receiving neither microsymbiont the least stem, leaf and root dry mass. A differential growth response was observed when microsymbionts were added separately: seedlings receiving rhizobium alone had significantly more leaves, with greater leaf dry mass and leaf area than seedlings receiving AM alone. In contrast, seedlings receiving AM alone had significantly greater root collar diameter, stem and root dry mass than seedlings receiving rhizobium alone, and had a

significantly greater root/shoot ratio than all other inoculation treatments. For most of the parameters measured, seedling growth was significantly greater in solarised Embu soil than in untreated soil. Seedlings of San Ramon provenance also grew significantly better than those of Flores provenance and tended to be more responsive to inoculation treatments. AM root infection and nodule dry mass did not differ between inoculation treatments, although seedlings inoculated with rhizobium had significantly more nodules.

In Maseno soil, significant effects of inoculation were found for most parameters of seedling growth, with plants receiving both microsymbionts showing the largest increases in stem and leaf growth, while seedlings receiving neither microsymbiont were the smallest. Unlike the Muguga experiment, no differential growth response was observed when the microsymbionts were added separately. In contrast to the Muguga experiment (Embu soil), seedling growth was significantly greater in untreated Maseno soil than in solarised soil. However, similar differences were observed between provenances, with growth of San Ramon seedlings being significantly better than that of Flores seedlings.

IV. Problems encountered.

As for CEH, the withdrawal of CONSEFORH from the project at the half of the project meant that the training course provided during two months in Dakar to Mr Gaspar Alvarado was not utilised. It was possible to allow to Ms Rosemary Bradley from SCA to incorporate the project. She is a specialist of rhizobia and she knows very well how produce the inoculum. Unfortunetaly, the lack of funds for Ms Bradley put a lot of problems for starting the activities in Costa Rica, especially during the third year of the project. Hopefully, situation was better during the last year, and Ms Bradley produced interesting results on the effect of inoculation with the both rhizobia and AM. No resuts were obtained in Cameroon regarding the effect of the inoculation with rhizobia whereas the UPIL system was set up in Nkolbisson station and the technician, Mr Badjel Badjel was trained during 2 months in Senegal and two others weeks in Cameroon with Mr Pierre Beunard. A mistake during the transfer of funds from France to Cameroon could explain one part of this lack of results. However, we could hope that the material will be used by others people from IRAD who are working on annual legumes and who was very interested when the UPIL system was installed.

V. Technology implementation plan.

There are no plan to commercially develop any of the both AM and rhizobia methodologies used and the both strains and isolates contained in the collection established during the project..

VI. Publications and papers.

Papers in refereed journals.

Forestier S, Alvarado G, Badjel Badjel S, Lesueur D (2001) Effect of inoculation methodologies with *Rhizobium* on nodulation and growth of *Leucaena leucocephala*. World J. Microbiol. Biotechnol. 17: 359-362.

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Lesueur D, Ingleby K, Odee, D, Chamberlain J, Wilson J, Tiki Manga T, Sarrailh JM, Pottinger A (2001) Improvement of forage production in *Calliandra calothyrsus* :

Methodology for the identification of an effective inoculum containing *Rhizobium* strains and arbuscular mycorrhizal isolates. J. Biotechnol. 91:269-282.

Odee DW, Indieka SA and Lesueur D Inoculation of *Calliandra calothyrsus* in sterile and unsterile (soil) conditions: effect of rhizobial inoculum size and method of inoculation. Article submitted to Biology and Fertility of Soils on 26th November 2001.

Odee, Ingleby, et al., Growth of arbuscular mycorrhizal fungi in pot cultures using different Kenyan soils and the implications for inoculum production. (in preparation)

Papers in no-refereed journals.

Muok, B.O., Gudu, S.O., Odee, D.W. 1998. A broad-range inoculant for legume trees in acid soils: fixing nitrogen in sub-Saharan Africa. Agroforestry Today, 10 (3) : 12-13.

Lesueur D (2000) Improving forage production of *Calliandra calothyrsus* through symbiotic association in Senegal (INCO/DXII Project). NFT News, 3 : 5-6.

Lesueur D (1999) Evaluation and utilization of the biodiversity of the microsybionts of *Calliandra calothyrsus*. Eur. Tropical For. Res. Network News. 29 : 50-51.

Training manual

Ingleby, K. & Mason, P.A. (1999). Production of arbuscular mycorrhizal inoculum in the glasshouse and nursery. Workshop at CEH Edinburgh, 11-20 October 1999. 20 pp

Production of books.

A proceedings will be produced with several articles describing the more important results obtained through the four year of the project. Alan Pottinger from the CNRD and Didier Lesueur will assume the scientific edition of the book.

Poster presentations.

Odee, D.W. 1998. Biodiversity and ecology of African tree rhizobia. *In* Dakora, F.D. (ed.), pp 193-194, *In* Dakora, F.D. (ed.), Proceedings of the Eighth Congress of African Association for Biological Nitrogen Fixation, November 23-27, Cape Town, South Africa, pp 93-94.

Odee, D.W., Njoroge, J., Esitubi, M., Oyoo, E., Ochieng, J., Mugwe, J., Niang, A. 1998. Microsymbiont status of *Calliandra calothyrsus* grown in the highlands of Kenya for soil fertility and fodder production. *In* Dakora, F.D. (ed.), Proceedings of the Eighth Congress of African Association for Biological Nitrogen Fixation, November 23-27, Cape Town, South Africa, pp196.

Lindström K., Dresler-Nurmi A., Räsänen L.A. and Terefework Z. (2001) Use of automated systems to study the biodiversity of rhizobia. 13th International Congress on Nitrogen Fixation, Hamilton, 2-7 juillet 2001.

Lesueur D, Diouf D (2001) Combined effects of the inoculation with rhizobia and origin of the host plant on the growth and nodulation in *Calliandra calothyrsus*. 13th International Congress on Nitrogen Fixation, Hamilton, 2-7 juillet 2001.

Diouf D, Forestier S, Neyra M, Lesueur D (2001) Optimisation of inoculation of *Calliandra calothyrsus*, *Leucaena leucocephala* and *Acacia mangium* with rhizobium. 13th International Congress on Nitrogen Fixation, Hamilton, 2-7 juillet 2001.

Production of pamphlets and posters.

Diouf D, Lesueur D (1999). Poster presentation of the forage tree legumes, *Calliandra calothyrsus*. CIRAD-Forêt, Programme Trees and Plantations.

Diouf D, Lesueur D (1999) Pamphlet on "*Calliandra calothyrsus* : Production, Inoculation and Plantation". CIRAD-Forêt, Programme Trees and Plantations.

VII. Conclusion.

The main objectives of the project were achieved. A collection of rhizobia and AM was established from nodules and soils collected in soils where *C. calothyrsus* was growing in its native and introduced areas. Regarding rhizobia, the biodiversity of all the collection was evaluated and the majority of AM occuring in the soil samples were indentified. Cultures of the both microsymbionts were produced and their efficiency with the host plant was determined under greenhouse conditions. Effective rhizobia strains and AM isolates were identified and copies/starter cultures were produced for project partners. Two training course were organized for partners from the South countries. One regarding the production of rhizobial inoculum which was held in Dakar (CIRAD-Forêt), and another one in CEH/ITE Office (UK) on the production and evaluation of AM inoculants. An UPIL system (Unit for rhizobial inoculum production) was installed in Cameroon, and a training course was organised by CIRAD for explaining the functionning of the system.

It is necessary to note that limited progress was made in the evaluation of inoculants under nursery and field conditions in Central America and Cameroon.

However, it is important to note that several papers were published in international journals. In the large majority, these papers associated partners from several countries (south and north countries). Results obtained through the project were the subject of several presentations (posters) to international conferences. Project was also the subject of papers in journals with a large diffusion through networks in order to distribute the available informations.

In conclusion, we can say that the technical information obtained in this project is applicable to other future legume-microsymbiont research and development, and should be repackaged for wider adoption by the smallholder farmers.

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Management Report

Organisation of the collaboration.

We have not important problem of collaboration between the several participants of the project. It is certain that between KEFRI, ITE/CEH and CIRAD-Foret, the exchanges were strong and several common experiments set up in Kenya and Senegal. It explains why three papers associating these partners were published or submitted for publication in international journals. It is important to note also that one article published in Journal of Biotechnology had associated a big majority of the participants involved in the project (except our colleagues from the University of Helsinki).

In contrast, the exchanges with the others partners from south countries were more limited. In Cameroon, activities were stopped during a long time because one part of the funds were transfered on another bank account from IRAD. It was really difficult to correct this mistake and AM cultures were totally contaminated. However, the UPIL system was set up in IRAD laboratory. But we have no information on the amount of liquid inoculum produced with it during the period of the project. In Central America, the fact that CONSEFORH from Honduras participated to the first half of the project, and SCA from Costa Rica was involved during the second half of the project induced did not facilitated the exchanges with the partners from Africa. CONSEFORH participated to the both training cessions but decided after that to leave the project. It was impossible to organise for SCA a second training cession. So it was possible for Rosemary Bradley from SCA to meet only David Odee in Edinburgh, during the third annual meeting. No contact were created between SCA and IRAD because Tiki Manga did not participated to this meeting. I am not certain that it was necessary to give an explanation to these few contacts between partners from South coutries. But my point of view is that when your are working with peoples from near countries as Kenya and Zimbabwe for exemple, or Mali, Niger and Burkina Faso, the exchanges are more easy. In contrast, when you have a partner from Central America, another one from West Africa and a last from East Africa, contacts are so difficult.

Meetings

Several meetings were organised during the project. The list is :

- 19 and 20 January 1998 : Start meeting in Dakar (Sénégal). Each partner was represented at this meeting except CONSEFORH from nobody was available at this period.
- 25 and 26 January 1999 : First annual meeting in Dakar (Sénégal). Each partner was represented at this meeting except the University of Helsinki because their activity within the period started only during the second year of the project.
- 10 and 11 April 2000 : Second annual meeting in Edinburgh (UK). Each partner was represented at this meeting except IRAD who waited for to receive funds.
- 12 and 13 November 2001 : Final meeting in Montpellier (France). Each partner was represented at this meeting except IRAD (Tiki Manga was in United States for starting a PhD), SCA (Budget of SCA was not able to support the cost of the travel in France) and CNRD (the contract of Alan

Pottinger in CNRD was finished one month ago before the organisation of the meeting).

The aim of these several meetings was to meet a maximum of partners involved in the project. It was also the opportunity for us to present our results and to compare with those obtained by the others partners. We had also the possibility to define together the protocoles of commun experiments set up in several countries (Kenya and Senegal for exemple). Several discussions concerned also the preparation of common articles as this one published in Journal of Biotechnology. Another important point concerns the idea submitted during the second annual meeting in Edinburgh regarding the preparation of a new INCO project. The result was the submission in September 2000 of the SAYSYS proposal which was selected for funding in January 2001. This project will involve several partners of the first project (KEFRI, CEH/ITE and CIRAD-Forêt).

During the final meeting, it was decided to prepare a Proceedings with the more interesting results obtained during the four years of the project. It was necessary to define the content of the proceedings, who would the leader for preparing the article, who will assume the scientific edition,... From the results of these discussions, a calendar was established and after the meeting, the preparation of the articles started. This meeting was also used for clarifying the preparation of the final report according to the EC recommandations. As we were together, it was more simple to describe that Brussels attemps from each participant. For partners who did not participated to the meeting, I sent them by fax a copy of the guidelines provided to me by EC.

Exchanges

Two training courses were organised during the project :

Training course in Dakar on the production of inoculum with rhizobia (23 January – 23 March 1999).

At the beginning of the Project, it was planed that the training course was held in Montpellier with Pierre Beunard (CIRAD-AMIS). But it was appeared that the final cost was so expensive for IRAD. For this reason, it was proposed to organize the training course in Dakar where accomodations are less expensive. Mr Sylvestre Badgel Badgel from IRAD and Mr Gaspar Alvarado from Conseforh were participated to the training course which started the 23 January to 23 March 1999 in the Microbiology Laboratory of IRD/ Bel Air Center.

The objectives of this training course was to initiate the both technicians in basic methodologies on microbiogy in order that they were able to produce the inoculum in their country. After discussion with Pierre Beunard, several experiments were selected in preparation of the production :

1. Preparation of culture media for rhizobia (liquid and solid)
2. Isolation of pure cultures of rhizobia in Petri Dishes.
3. Isolation of rhizobia from nodules harvested in the field.
4. Enumeration of rhizobia in a liquid culture by using dilution method.
5. Production of alginate beads containing rhizobia
6. Manipulation of plants cultivated in tubes in sterile conditions for inoculation experiment.
7. Inoculation experiment carried out with several methods

Each of them were realized by the both technicians, and in final, the results obtained were satisfactorily. It is important to note that an article was published in an International journal from experiments set up during this training course : Forestier S, Alvarado G, Badjel Badjel S, Lesueur D (2001) Effect of inoculation methodologies with *Rhizobium* on nodulation and growth of *Leucaena leucocephala.* World J. Microbiol. Biotechnol. 17: 359-362.

Mycorrhizal Workshop at ITE (11-20 October 1999).

The workshop was attended by technicians from IRAD, CONSEFORH, and KEFRI. It was designed to provide each with the basic training on how to produce mycorrhizal inoculum in each of the partner countries. The first part aimed to familiarise participants with the general features of arbuscular mycorrhizas (AM) and the methods used to recognise and examine AM spores and infected roots. The second part dealt specifically with the nursery facilities and methods required by each partner in order to establish mycorrhizal cultures when they return to their own country.

During the workshop, AM spore populations in field soils, trap cultures and pure cultures were examined and assessed. This exercise not only familiarised the participants with the characteristics of the different inoculant fungi that will be grown in culture, but also clearly demonstrated the need for culture and isolation of mycorrhizal fungi from field soils. Participants saw that field soils contained relatively few spores, most of which were dead and also contained fungal parasites, mites and nematodes. Trap cultures (3 of which were made using the same field soils) and resulting single species isolations (pure cultures) markedly increased numbers of live spores and eliminated harmful organisms.

An illustrated, 21-page manual was prepared to accompany and supplement the training provided in the workshop. The manual gives a brief introduction to AM associations and then describes in detail (with illustrations) the methods, which relate specifically to this phase of the projects work. The manual was appended to the second annual report.

Others less important training courses

- From 21th November to 4th December 1999, the UPIL system was set up by CIRAD in Cameroon, more specially Nkolbisson IRAD station, around Yaounde. During this period, Pierre Beunard trained technician from IRAD, Mr Sylvestre Badjel Badjel, in order that he was able to produce alone a good pure liquid inoculum of rhizobia.

- From 14th to 22 October 2001, Antoine Galiana from CIRAD-Forêt/Montpellier (Laboratoire des Symbioses Tropicales et Méditerranéennes) was in Senegal in order to set up the ELISA equipment in the Microbiology Laboratory in Dakar. The aim was to explain to studients and technicians how practice for doing the nodules analysis by using this equipment. However, before to be able to do these analysis, it will be necessary to test the antibodies and to define several parameters in order to optimise the system. This preliminary step was carried out by Antoine Galiana.

- From 10th to 30th November 2001, Amadou Sarr, a PhD student from the Microbiology Laboratory in Dakar, was in Montpellier for continuing with Antoine Galiana the preliminary work and for improving their knowledges on the ELISA techniques. By this way, we are now able to do several analysis of nodules by using ELISA.

Problems.

Two main problems were happened during the project :

- The decision from CONSEFORH to leave the project after two years of activities. It was a bad thing because Mr Gaspar Alavarado from CONSEFORH participated to the both training courses organised in Dakar and Edinburgh. Although he was able for working alone, he leaved the project. So SCA from Costa Rica who accepted to participate to the project did not have the opportunity to profit of these training course. It has also an incidence at the financial level, because SCA never received an advance of funds as CONSEFORH. During the first year of their involvement (third year of the project), CIRAD-Foret provided to SCA some funds from its own budget. So SCA has to compose with this amount of funds for starting their activities of research and buying the more important equipments.

- One major problem encountered in the whole project lifetime for IRAD was the late release and transfer of project funds. This created a lot of distortion for the activities planned in such a way that one of the major activities to be undertaken in Cameroon couldn't take place.

Individual Partner Final Report

Partner 1 : CIRAD-Foret

Didier Lesueur

A. Objectives.

The specific objectives of CIRAD-Forêt in the project were to :

8. Co-ordinate the current INCO project.
9. Establish a collection of rhizobial isolates from nodules and soil samples harvested in native areas and also in countries where *C. calothyrsus* was introduced with success
10. Conduct screening tests in greenhouse and nursery in Dakar to identify effective rhizobia isolates.
11. Produce several copies of the selected strains for use by project partners in their own country.
12. Provide an Inoculum Unit (UPIL System) and training technicians from Central America and West africa partners.
13. Optimize in nursery the inoculation of *C. calothyrsus* with *Rhizobium.*
14. Evaluate the effect of the inoculation with the both Rhizobium-AM on the growth of *C. calothyrsus* cultivated in nursery and under field conditions.

B. Activities.

B.1. Establishment of a collection of rhizobia from nodules of *Calliandra calothyrsus.*

For the establishment of this collection, it was necessary to carry out a trapping experiment in a greenhouse in order to obtain fresh nodules from which it was possible to isolate rhizobia.

The four provenances are of *C. calothyrsus* under study were Flores (OFI references 16/96 and 10/91) from Guatemala and San Ramon (OFI references 110/94 and 11/91) from Nicaragua. The Oxford Forestry Institute (OFI), U.K, supplied seeds that were scarified mechanically. A small nick was made in the outer coat, halfway round the horseshoe shaped as described by Macqueen (1993). The scarified seeds were germinated on sand under greenhouse conditions.

Eight-day-old seedlings were transplanted into 12 x 8 cm plastic bags filled with sand. N-free nutrient solution (Broughton and Dilworth 1971) and distilled water were alternately added. The nodules of *C. calothyrsus* harvested in fields in several tropical countries were crushed in distilled water and the solution obtained was put on the root collar of plants. There were 5 replicates per place of harvest and per *C. calothyrsus* provenance's.

After two months of culture, the plants were cut and the fresh young nodules were harvested for the isolation of rhizobia in laboratory. Nodules were sterilised superficially in

Ethanol 95% during 30s and rinsed with sterile distilled water. After that, nodules were placed in solution of $HgCl_2$ (0.1 %) during 3 minutes for a second sterilisation. After several rinsings in sterile distilled water, each nodule was crushed on a sterile glass slide and streaked onto YEM agar plates. When the culture obtained were pure, all the strains were maintained in YEM medium containing 20% (vol/vol) glycerol at 80°C.

In order to distinguish the *Rhizobium* strains from the *Bradyrhizobium* strains, all the strains of the collection were streaked onto YEM agar plates supplemented with bromothymol blue, which is a pH indicator. After 5 days of culture at 28°C, we observed whether the rhizobia had acidified the medium (yellow colour around the colonies like for the *Rhizobium*) or alkalised it (blue colour; like for the *Bradyrhizobium*).

B.2. Screening of rhizobia isolated from Central America, New Caledonia and Cameroon.

In greenhouse conditions, all the rhizobia strains isolated during the first year of the project were inoculated to seedlings of *C. calothyrsus* in order to identify among them the more efficient. The *Rhizobium* strain CCK13 was also tested because Lesueur et al. (1996) demonstrated that this strain was very efficient with *C. calothyrsus* in greenhouse conditions. Seedlings were planted in sand and stayed in this substract during 3 months. After this period, plants were transferred in sterilized soil of Sangalkam (Senegal). In final, six months old plants were harvested and several parameters were measured : Number of nodules, dry weight of nodules, shoot and root, and shoot total nitrogen content. Results presented in Table 1 were regrouped for country in order to facilitate the presentation.

B.3. Distribution of the selected rhizobia strains to partners from Kenya, Cameroon, Costa Rica and New Caledonia and training of two technicians from Cameroon and Honduras.

A copy of the selected rhizobia strains were provided to several partners : Kenya, Cameroon, Costa Rica and New Caledonia. Two partners needed to have a technician able to assume the production of rhizobia inoculum. For this reason, Mr Sylvestre Badgel Badgel from IRAD and Mr Gaspar Alvarado from Conseforh were participated to the training course which started the 23 January to 23 March 1999 in the Microbiology Laboratory of IRD/ Bel Air Center. No body from Kenya participated to this training course because Kefri has his own microbiology lab, with technicians to produce alone the inoculum.

The objectives of this training course was to initiate the both technicians in basic methodologies on microbiogy in order that they were able to produce the inoculum in their country. After discussion with Pierre Beunard, several experiments were selected in preparation of the production :

- Preparation of culture media for rhizobia (liquid and solid)
- Isolation of pure cultures of rhizobia in Petri Dishes.
- Isolation of rhizobia from nodules harvested in the field.
- Enumeration of rhizobia in a liquid culture by using dilution method.
- Production of alginate beads containing rhizobia
- Manipulation of plants cultivated in tubes in sterile conditions for inoculation experiment.
- Inoculation experiment carried out with several methods

Each of them were realized by the both technicians, and in final, the results obtained were satisfactorily.

Pierre Beunard was in Cameroon on 21th November to 6th December in order to set up an UPIL System in the Nkolbisson Research Station, near Yaounde. He trained specifically Mr Sylvestre Badjel Badjel on the utilization of the UPIL Systel, and to gether, they carried out several preliminaries experiments on carriers for the liquid inoculum. At the end of the training, Mr Badjel Badjel was able to produce alone several types of liquid inoculum and to mix them with sterilized carriers.

B.4. Optimisation of the inoculation of *C. calothyrsus* with rhizobia under greenhouse conditions.

B.4.1. Caracterisation of several carriers available in Cameroon and Central America.

Rhizobia are usually incorporated into a solid carrier which serves different functions: multiplication and protection of microorganisms. The carrier becomes the principal element of the product and provides a convenient base to facilitate the application and the use of the inoculum (Smith, 1992).

Characteristics of an excellent solid carrier would include : good physical and chemical characteristics without toxic elements, high water-holding capacity, nearly neutral pH or easily adjusted, sterility before the including of liquid culture, close connection between pH, organic matter and survival.

Peat has been the standard carrier around the world. It is considered as the most interesting carrier. However, many countries do not own peat, especially in tropical countries as the Cameroon. Many mineral and organic materials have been evaluated as inoculant carriers. These are grouped in different categories : soils-peat, charcoal, compost made from bagasse, filter-mud, plant compost, inert materials, rock phosphate, polyacrylamide gel. The form of the carrier depends on the intended method of application. The majority of inoculants have been in a powder form for seed application before planting. The choice of inoculant form depends on the general method of planting, equipment used for planting, and acceptable cost.

Each partners identified one or several vegetables carriers available in large amount in their country in order to not be limited in the futur. These several carriers will be characterized in terms of water retention, pH and possibility to neutralize them with addition of lime. The survival of rhizobia will be tested also in the more interesting carriers.

B.4.2. Effect of the size of the inoculum on the nodulation and the growth of *C. calothyrsus*.

Pre-treated seeds were pre-germinated on sterile water agar plates (8 g l^{-1}). Germinated seedlings were transplanted to plastic pots (9 _ 17 cm), one per pot, containing sterilized peat/vermiculite mixture (1:1) adjusted to pH 6.5. A culture of rhizobial strain CCK 13 was grown to late log phase in yeast extract mannitol (YEM) (Vincent 1970) and serially diluted to provide inoculum sizes of 10^2, 10^4, 10^6, 10^8, 10^9 and 10^{10} rhizobia ml^{-1}. These dilutions constituted the inoculated treatments. Each seedling was inoculated with one ml of the appropriate inoculum size. Strain CCK13 is an effective strain for *C. calothyrsus* originally isolated from root nodules of plants growing at Mombasa, Kenya (Lesueur et al. 1996).

B.4.3. Identification of the more performant methodology for the inoculation of *C. calothyrsus* in nursery.

Seedlings transplanted to potted Sangalkam soil. Two inoculant types were used; liquid broth and alginate bead each with rhizobial strain CCK 13. YEM was used to prepare the liquid culture and the alginate beads were prepared as described by Diem et al. (1989). There were 5 inoculation treatments and a control, designated as follows:
SM1- inoculation with 1 ml of liquid culture to pre-treated seed coated with gum arabic,
SM2- inoculation with 1 ml of liquid culture and gum arabic mixture (1:1) to pre-treated seed,
SM3- inoculation with 1 ml of liquid culture around root collar of seedlings immediately after transplanting,
SM4- inoculation with 1 ml of liquid culture around root collar of seedlings 1 week after transplanting,
SM5- inoculation with 20 mg of non-dissolved alginate beads containing rhizobia around root collar of seedlings, and
SM6- control - uninoculated seedlings.

Gum arabic, normally used to facilitate adhesion of inoculant to seed, was also used in the liquid inoculant treatment SM2 in order to allow direct comparison with treatment SM1. Rhizobial concentrations of liquid culture and alginate bead inoculant were approximately 10^9 ml^{-1} and 10^5 g^{-1} (dry weight), respectively. Plants were cultivated under greenhouse during 5 months.

B.5. Evaluation of the effect of the inoculation with the both *Rhizobium*-AM on the growth of *C. calothyrsus* cultivated in nursery and under field conditions.

B.5.1. Effect of individual inoculations with AM on growth of plants of *C. calothyrsus* inoculated with a cocktail of rhizobia.

Five AM cultures namely *Glomus etunicatum*, *Gigaspora albida* (type I), *Gi. albida* (type II), *Scutellospora verrucosa* and *S. calospora* were used. These cultures originated from isolates that had been selected for high root infections (Lesueur *et al.*, 2001). They were bulked up on *Sorghum bicolor* (millet) grown in sterilized Sangalkam soil. The experiment was set up in a greenhouse at the IRD Bel-Air Centre in Dakar, Senegal. It consisted of 2 provenances (San Ramón and Flores), 6 mycorrhizal inoculation (5 cultures and one uninoculated control) and 3 soils/substrates (Bel Air, Sangalkam and peat : vermiculite mixture). Each treatment was replicated 10 times, except 6 for peat : vermiculite-grown plants.

Two-day- old seedlings were planted in 15 × 5 cm plastic bags containing sterilised soil that had received 20 g of mycorrhizal culture. All mycorrhizal inoculated plants also received 5 ml of the mixed rhizobial culture (strains CCC22, CCCR1, CCCR15, CCNC26, KCC6, KWN35 and CCK13) containing 10^9 cells ml^{-1} around the root collar area 4 d later. Seedlings were harvested after 4 months. The soil was gently washed from the roots, nodules were collected and aerial parts were separated from the rest of the plant. A sub-sample of roots was taken for clearing and staining as described by Ingleby and Mason (1999), and the mycorrhizal infection was assessed by the gridline intersect method. Nodules, shoot and roots were dried at 60°C and weighed. Shoot N content was measured by the Kjeldahl method (Bremner and Mulvaney 1982). Shoot K and P were determined by atomic absorption spectrophotometry after digesting plant material in a mixture of concentrated hydrochloric and fluoric acids. Data were subjected to a three-way analysis of variance using the Super Anova Computer program, and means were compared with the Fisher's multiple range test.

B.5.2. Comparison between the simple or the mixte inoculation with *Rhizobium* x AM on the growth and the nodulation of *C. calothyrsus* under nursery and field conditions.

A first experiment was set up in September 2001 in Bel Air Centre (Dakar, Senegal) with plants inoculated according to 5 several treatments and cultivated during 105 days in nursery. The treatments are : T1 : no-inoculated plants ; T2 : Plants inoculated with the full cocktail of *Rhizobium* strains ; T3 : plants inoculated with all the strains of the cocktail except CCNC26 ; T4 : Plants inoculated with *Rhizobium* strains KWN35 ; T5 : Plants inoculated with the full cocktail of AM ; T6 : Plants inoculated with the AM isolats GA2. Ten plants from each treatments were harvested in order to measure the shoot, root and nodule dry weight. Results obtained show that the shoot dry weight of plants inoculated with the Rhizobium strains KWN35 is higher compare with values obtained with plants from the others treatments. A measure of height and root collar diameter will be done around 15th Janvier 2002.

A second experiment will be set up after the end of the project (Avril or May 2002) in Bel Air Centre with plants inoculated according to 4 treatments. T1 : no-inoculated plants ; T2 : Plants inoculated with the *Rhizobium* strain KWN35 ; T3 : Plants inoculated with the AM isolats GA2. T4 : Plants inoculated with the both AM isolats GA2 and the *Rhizobium* strains KWN35. Actually, these plants are cultivated in greenhouse.

C. Results.

C.1. Establishment of a collection of rhizobia from nodules of *Calliandra calothyrsus.*

By the method used, 210 rhizobia strains were isolated from the several countries : 20 from Mexico, 23 from Honduras, 41 from New Caledonia, 46 from Guatemala, 29 from Nicaragua, 27 from Costa Rica and 24 from Cameroon. A large majority of them belong to the *Rhizobium* genus (fast growing rhizobia). Usually, *C. calothyrsus* is described as being a species of woody legumes capable to nodulate essentially with *Rhizobium* rather than with *Bradyrhizobium* genus (Turk and Keyser, 1992; Lesueur et al., 1996). However, Peoples et al. (1989) showed that some *Bradyrhizobium* strain can nodulate and form an effective nitrogen-fixing symbiosis with *C. calothyrsus*. Our results show that *C. calothyrsus* can nodulate naturally with *Bradyrhizobium* in Central America, Mexico and New Caledonia. In return, no *Bradyrhizobium* strains were isolated in Cameroon. Now we will have to verify if nodules formed are able to fix nitrogen in symbiosis with *C. calothyrsus*.

C.2. Screening of rhizobia isolated from Central America, New Caledonia and Cameroon.

Our results show important differences for all the parameters between the countries from where rhizobia strains were isolated. It appeared that rhizobia from Costa Rica induced the formation of a high number of nodules, which enhanced an important production of nodules and shoot dry weight (Table 1). In return, plants inoculated with rhizobia strains from Mexico are poorly nodulated and their growth is significantly lower compare to plants of the others inoculation treatments. Symbiotic performances of strains from Guatemala and Nicaragua are similar (not very high) compare to those of strains from New Caledonia and

Costa Rica (Table 1). For the Cameroon, the averages of number of nodules, nodule and shoot dry weight are not really high. It seems indicate that these strains are globally not really interesting for the inoculation of *C. calothyrsus* in field. However, it is important to be careful because all these data are the result of a combination of results obtained with several strains isolated from the same country and one of two strains from Cameroon could be very efficient as in the case of the strain CCCR2.

It is interesting to compare results obtained with rhizobia strains isolated during the first year of the project, and results obtained with our reference strain CCK13. It appeared that plants inoculated with the reference strain are well nodulated (number and biomass) and produce high amount of shoot. In return, the shoot total nitrogen content is low. It seems to indicate that the utilisation of the *Rhizobium* strain CCK13 for the inoculation of *Calliandra calothyrsus* plants is justified. We will discuss of this aspect in the third part of our report.

It was possible to identify, among all the rhizobia strain tested during the screening, the more efficient in terms of nodulation, growth and shoot nitrogen content. According to values of parameters measured, the following strains were chosen : CCCR2 from Cameroon, CCCR1 and CCCR15 from Costa Rica and CCNC26 from New Caledonia. Their results are presented in Table 2. We observe that these four strains are globally more efficient than reference strain CCK13 because for each parameter. values obtained with CCK13 strains are lower than those obtained with the selected strains (Table 2). It confirms that the inoculation of *C.calothyrsus* could be performed in field conditions with one of these strains. However, a second screening experiment will be carry out in greenhouse conditions with them + two rhizobia strains selected by David Odee from Kefri (Kenya) in order to identify in two types of soils, the very best strain able to improve significantly the both nodulation and growth of *C. calothyrsus*. The same experiment was done in Kenya in order to compare the final results and to determine if it is the same rhizobia strain which will be identified as being the more effective with both Flores and San *Ramon C. calothyrsus* provenances.

Table 1 : Effect of the inoculation with rhizobia strains isolated from several countries on nodulation and growth of *Calliandra calothyrsus* (Flores OFI seedlot n°16/96).

Origin and number of rhizobia strains	Number of nodules plant^{-1}	Nodules dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Shoot total N content (%)
Cameroon / 24 strains	50.6b	0.057b	1.76c	1.07f	2.16b
Honduras / 23 strains	61.7d	0.067d	1.98g	1.17f	2.21b
Mexico / 20 strains	65.1e	0.058b	1.78c	1.05d	2.30c
Guatemala / 46 strains	63.7de	0.054a	1.31a	0.67b	2.77f
Nicaragua / 29 strains	65.2f	0.041a	1.33b	0.67a	2.61e

Costa Rica / 27 strains	90.7h	0.064c	1.94e	0.99c	2.55d
New Caledonia / 41 strains	55.0c	0.045a	1.94e	1.07e	2.31c
CCK13 (strain of reference)	83.7g	0.094d	1.97f	1.06d	2.21b
Control (no-inoculated plants)	49.0a	0.057b	1,87d	1.05d	2.11a

For each factor, values (means from 5 replicates) in the same column followed by the same letter are not significantly different according to the Newman & Keuls test at $P < 0.05$.

Table 2 : Effect of the inoculation with the four more efficient strains on nodulation and growth of *Calliandra calothyrsus* (Flores OFI seedlot n°16/96) by comparison with reference strain CCK13 and control.

N° strain	Number of nodules plant^{-1}	Nodules dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Shoot total N content (%)
CCC22	70.5a	0.095c	3.40c	2.08d	2.26c
CCCR1	94.5c	0.079b	3.43c	1.87c	2.27c
CCNC26	109.0cd	0.099d	3.45c	1.71b	2.43d
CCCR15	111.5d	0.100d	3.51d	2.22e	2.38d
CCK13	83.7b	0.094c	1.97ab	1.06a	2.21b
Control	49.0a	0.057a	1.87a	1.05a	2.11a

For each factor, values (means from 5 replicates) in the same column followed by the same letter are not significantly different according to the Newman & Keuls test at $P < 0.05$.

C.3. Optimisation of the inoculation of *C. calothyrsus* with rhizobia under greenhouse conditions.

C.3.1. Characterisation of several carriers available in Cameroon and Central America.

Results obtained with the organic carrier tested for the INCO project are presented in the Table3.

Table 3. Characteristics of the organic carrier tested for the INCO project.

Materials	Origin	pH (H_20)	Amout of lime (g) necessary for the neutralization of 20g of carrier	Water holding capacity (%)
Senna spectabilis	IRAD cameroon	4.82	1g of lime for a pH 5.39	78
Tithonia diversifolia	IRAD Cameroon	6.20	Not necessary	100
Chromoleana odorata	IRAD Cameroon	5.20	No effect of addition of lime	88
Vegetable powder	Conseforth Honduras	7.15	Not necessary	48
Peat	Commercial	3.50	1.5g of lime for a pH 6.7	100

Through these results, it appears that powder of *Senna spectabilis* and *Chromoleana odoratum* cannot been used as carrier, pH is too acid and the addition of lime did not increased significantly the pH. Regarding the others results, *Tithonia diversifolia* and vegetable powder from Honduras can be used without neutralization. For the peat it is necessary to neutralize it with lime (1.5g for 20g of peat).

Survival of rhizobia in organic matter used

In order to be able to choose local supports for the production of inoculum, we tested the survival of some selected rhizobiums strains.

Table 4. Survival of the Rhizobium strain CCK13 of *Calliandra calothyrsus* inoculates in several carrier stored to 28°C (Log of the number of cells.mg^{-1} of carrier).

	T0	T1 : one hour	T2 : one week	T3 : 15 days	T4 : one month
Peat	10	10	11	11.2	11.6
Tithonia diversifolia	10	0	0	-	-
Powder from Honduras	10	9.5	10	-	-

Rhizobium strain CCK13 was inoculated at a concentration of 10^{10} bacteria per mg of carrier in each of the selected carriers and stored to 28°C. For each harvest (1 hour, 1 week, 15 days and 1 month) the number of rhizobia was determinated by dilutions techniques on YEMA media.

The results of Table 4 show that in *Thitonia diversifolia*, the number of rhizobia decreased strongly for disappearing totally one hour after the inoculation of the strain in the carrier. For the two others carriers tested, the rhizobia population seem stable. It suggests that they could be used as carriers for the inoculum of *C. calothyrsus*.

C.3.2. Effect of the size of the inoculum on the nodulation and the growth of *C. calothyrsus*.

The standard inoculum size of 10^9 rhizobia ml^{-1} gave the best overall growth response as it is showed in the Table 5.

C.3.3. Identification of the more performant methodology for the inoculation of *C. calothyrsus* in nursery.

Generally, all methods of inoculation improved shoot, root and total dry weight of plants grown in Sangalkam soil (Table 6). Inoculation with liquid cultures applied either immediately or 1 week after transplanting was superior to other methods. Inoculation with 1 ml of liquid culture 1 week after transplanting gave shoot growth increases of 45 % over non-inoculated control; nearly double the increase with alginate bead inoculation. Nodule dry weight, on the other hand, was greatest in uninoculated control plants thus indicating presence of either a low or less effective indigenous rhizobial population. The gum arabic incorporated treatments, SM1 and SM2, were not significantly different ($P < 0.05$), although the former gave better nodulation and growth response. Inoculation with alginate beads containing rhizobia was comparable to the liquid cultures, except for root dry weight, which was significantly lower.

Table 6 Effects of inoculation method, using rhizobial strain CCK 13, on nodulation and growth of *C. calothyrsus* after 5 months of growth in unsterile Sangalkam soil from Senegal

Inoculation methods[a]	Nodule dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Total dry weight (g plant^{-1})
SM1	0.070**ab**[b]	2.308**bc**	2.305**b**	4.613**bc**
SM2	0.068**ab**	1.928**ab**	2.045**ab**	3.973**ab**
SM3	0.080**bc**	2.527**c**	2.743**c**	5.270**d**
SM4	0.052**a**	2.545**c**	2.378**bc**	4.923**cd**
SM5	0.076**bc**	2.156**abc**	2.210**a**	4.366**bc**
SM6	0.097**c**	1.752**a**	1.722**a**	3.473**a**

[a]SM1, inoculation with 1 ml of liquid culture to pre-treated seed coated with gum arabic; SM2, inoculation with 1 ml of liquid culture and gum arabic mixture (1:1) to pre-treated seed; SM3, inoculation with 1 ml of liquid culture around root collar of seedlings immediately after transplanting; SM4, inoculation with 1 ml of liquid culture around root collar of seedlings 1 week after transplanting; SM5, inoculation with 20 mg of non-dissolved alginate beads containing rhizobia around root collar of seedlings; SM6- control, uninoculated seedlings.
[b]Values in a column followed by the same letter are not significantly different according to the Newman and Keuls test at $P < 0.05$

C.3.4. Effect of the size of the inoculum on the nodulation and the growth of *C. calothyrsus*.

The standard inoculum size of 10^9 rhizobia ml^{-1} gave the best overall growth response (Table 7).

Table 7. Effect of rhizobial inoculum size on nodulation and growth of *C. calothyrsus* seedlings after 5 months of growth in a mixture of potted sterilized peat and vermiculite

Inoculum size (viable cells ml^{-1})	Number of nodules plant^{-1}	Nodule dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Total dry weight (g plant^{-1})
10^{10}	**90a**[a]	0.041**a**	0.213**a**	0.141**a**	0.354**a**
10^9	217**b**	0.082**b**	0.707**b**	0.322**b**	1.029**b**
10^8	93**a**	0.053**ab**	0.367**a**	0.213**ab**	0.580**a**

10^6	156**ab**	0.077**b**	0.383**a**	0.183**ab**	0.566**a**
10^4	133**ab**	0.037**a**	0.296**a**	0.172**ab**	0.468**a**
10^2	147**ab**	0.054**ab**	0.309**a**	0.163**a**	0.472**a**

[a]Values in a column followed by the same letter are not significantly different according to the Newman and Keuls test at $P < 0.05$

C.4. Evaluation of the effect of the inoculation with the both *Rhizobium*-AM on the growth of *C. calothyrsus* cultivated in nursery and under field conditions.

C.4.1. Effect of individual inoculations with AM on growth of plants of *C. calothyrsus* inoculated with a cocktail of rhizobia.

Mycorrhizal root infections were highly variable and appeared to be influenced differently by substrate, provenance and mycorrhizal type (Table 8). Plants grown in Sangalkam soil had higher root infections than those in Bel Air soil. The highest infection (100 %) was scored on Flores plants inoculated with *G. etunicatum* in Bel Air soil. Data for shoot, root and nodule dry wt were combined for both provenances San Ramón and Flores. Generally, mycorrhizal inoculation increased dry matter accumulation. However, we also noted some important differences due to substrate. For example, Sangalkam grown plants inoculated with *Gi. albida* (I) had significantly ($P < 0.05$) higher shoot dry matter than those inoculated with other isolates. In Bel Air soil, the most effective isolates were *G. etunicatum* and *S. verrucosa*. In peat : vermiculite mixture, which was the least productive of the 3 substrates, inoculation with *S. verrucosa* produced better shoot dry matter than the other inoculation treatments.

Results of foliar nutrient content in Table 9 also show major differences between the soils. For example, K foliar content of Sangalkam grown plants were very low compared to those grown in Bel Air soil or peat : vermiculite mixture. Foliar N were greatest in Sangalkam and in peat : vermiculite inoculated with *S. verrucosa* and *G. etunicatum*, respectively. In Bel Air soil, foliar N was highest in uninoculated control plants. This is attributed to concentration effect of N in the leaves due to poor dry matter status. Similar results have also been reported for other woody legumes (Lesueur and Diem, 1997).

Table 8. Mycorrhizal root infection, and shoot, root and nodule dry matter of *C. calothyrsus* seedlings inoculated with selected AM cultures in 3 types of substrates after 4 months of growth under greenhouse conditions at Bel Air, Dakar, Senegal. Shoot, root and nodule dry matter values represent combined data of San Ramón and Flores provenances

Soil/substrate	Mycorrhizal isolate	[a]Mycorrhizal infection (%)	Shoot dry wt (g plant^{-1})	Root dry wt (g plant^{-1})	Nodule dry wt (g plant^{-1})
Sangalkam	*Gigaspora albida* (I)	40, 20	3.389c[b]	1.141b	0.107bc
	Gi. albida (II)	70, 0	2.690b	1.417c	0.127c
	Glomus etunicatum	60, 50	2.825b	1.180bc	0.069a
	Scutellospora calospora	80, 60	2.423b	1.406c	0.111bc
	S. verrucosa	0, 80	2.424b	1.355bc	0.095b
	Control	0, 0	1.783a	0.822a	0.058a
Bel Air	*Gigaspora albida* (I)	10, 0	3.571bc	1.514b	0.142ab

	Gi. albida (II)	0, 50	3.706c	1.629bc	0.102a
	Glomus etunicatum	30, 100	4.115d	1.566bc	0.140ab
	Scutellospora calospora	0, 40	3.306ab	1.632bc	0.100a
	S. verrucosa	40, 10	4.122d	1.729c	0.108a
	Control	0, 0	2.989a	1.186a	0.172b
Peat : vermiculite	*Gigaspora albida* (I)	40, 20	1.118a	0.267a	0.051a
	Gi. albida (II)	0, 0	1.090a	0.277a	0.074ab
	Glomus etunicatum	30, 0	1.736ab	0.421ab	0.101bcd
	Scutellospora calospora	40, 20	1.653ab	0.517b	0.124cd
	S. verrucosa	10, 0	2.061b	0.573b	0.141d
	Control	0, 0	1.590ab	0.530b	0.091abc

[a]Mycorrhizal infection: first and second values represent root infection for San Ramón and Flores, respectively

[b]For each soil/substrate parameter (shoot, root and nodule dry wt), values followed by the same letter within a column are not significantly different according to Fisher's multiple range test at $P < 0.05$

Table 9. Foliar nutrient (N, P and K) concentration of *C. calothyrsus* seedlings inoculated with selected AM cultures in 3 types of substrates after 4 months of growth under greenhouse conditions at Bel Air, Dakar, Senegal. Values represent combined data of San Ramón and Flores provenances

Soil/substrate	Mycorrhizal isolate	N (%)	P (%)	K (%)
Sangalkam	*Gigaspora albida* (I)	2.26c[a]	0.095b	0.450a
	Gi. albida (II)	2.16b	0.101d	0.470b
	Glomus etunicatum	1.94a	0.094b	0.470b
	Scutellospora calospora	2.15b	0.097c	0.461ab
	S. verrucosa	2.32d	0.100cd	0.460ab
	Control	1.96a	0.075a	0.510c
Bel Air	*Gigaspora albida* (I)	2.00b	0.194c	0.990c
	Gi. albida (II)	1.81a	0.138b	1.038d
	Glomus etunicatum	1.95b	0.141b	1.005cd
	Scutellospora calospora	2.17d	0.110a	0.850a
	S. verrucosa	2.11c	0.111a	0.907b
	Control	2.29e	0.133b	1.024cd
Peat : vermiculite	*Gigaspora albida* (I)	2.18cd	0.184c	1.011b
	Gi. albida (II)	1.98a	0.166c	1.091c
	Glomus etunicatum	2.27d	0.120b	1.084c
	Scutellospora	2.17c	0.097ab	1.034bc

	calospora			
	S. verrucosa	2.04ab	0.088a	0.834a
	Control	2.14bc	0.095ab	0.845a

[a]For each soil/substrate parameter (N, P and K concentration), values followed by the same letter within a column are not significantly different according to Fisher's multiple range test at $P < 0.05$

D. Problems.

As for CEH, the withdrawal of CONSEFORH from the project at the half of the project meant that the training course providedduring two months in Dakar to Mr Gaspar Alvarado was not utilised. It was possible to allow to Ms Rosemary Bradley from SCA to incorporate the project. She is a specialist of rhizobia and she knows very well how producethe inoculum. Unfortunetaly, the lack of funds for Ms Bradley put a lot of problems for starting the activities in Costa Rica, especially during the third year of the project. Hopefully, situation was better during the last year, and Ms Bradley produced interesting results on the effect of inoculation with the both rhizobia and AM. No resuts were obtained in Cameroon regarding the effect of the inoculation with rhizobia whereas the UPIL system was set up in Nkolbisson station and the technician, Mr Badjel Badjel was trained during 2 months in Senegal and two others weeks in Cameroon with Mr Pierre Beunard. However, we could hope that the material will be used by others people from IRAD who are working on annual legumes and who was very interested when the UPIL system was installed.

E. Technology implementation plan.

There are no plan to commercially develop any of the rhizobia methodology used and the strains isolated .

F. Publications and papers.

F.1. Papers in refereed journals.

Forestier S, Alvarado G, Dadjel Badjel S, Lesueur D (2001) Effect of inoculation methodologies with *Rhizobium* on nodulation and growth of *Leucaena leucocephala.* World J. Microbiol. Biotechnol. 17: 359-362.

Lesueur D, Ingleby K, Odee, D, Chamberlain J, Wilson J, Tiki Manga T, Sarrailh JM, Pottinger A (2001) Improvement of forage production in *Calliandra calothyrsus* : Methodology for the identification of an effective inoculum containing *Rhizobium* strains and arbuscular mycorrhizal isolates. J. Biotechnol. 91:269-282.

Odee DW, Indieka SA and Lesueur D Inoculation of *Calliandra calothyrsus* in sterile and unsterile (soil) conditions: effect of rhizobial inoculum size and method of inoculation. Article submitted to Biology and Fertility of Soils on 26th November 2001.

F.2. Papers in no-refereed journals.

Lesueur D (2000) Improving forage production of *Calliandra calothyrsus* through symbiotic association in Senegal (INCO/DXII Project). NFT News, 3: 5-6.

Lesueur D (1999) Evaluation and utilization of the biodiversity of the microsybionts of *Calliandra calothyrsus*. Eur. Tropical For. Res. Network News. 29 : 50-51.

F3. Production of books.

A proceedings will be produced with several articlesdescribing the more important results obtained through the four year of the project. Alan Pottinger from the CNRD and Didier Lesueur will assume the scientific edition of the book. Didier Lesueur is also associated to four articles presented in the Proceedings.

F.4. Poster presentations.

Lesueur D, Diouf D (2001) Combined effects of the inoculation with rhizobia and origin of the host plant on the growth and nodulation in *Calliandra calothyrsus*. 13th International Congress on Nitrogen Fixation, Hamilton, 2-7 juillet 2001.

Diouf D, Forestier S, Neyra M, Lesueur D (2001) Optimisation of inoculation of *Calliandra calothyrsus*, *Leucaena leucocephala* and *Acacia mangium* with rhizobium. 13th International Congress on Nitrogen Fixation, Hamilton, 2-7 juillet 2001.

F.5. Production of pamphlets and posters.

Diouf D, Lesueur D (1999). Poster presentation of the forage tree legumes, *Calliandra calothyrsus*. CIRAD-Forêt, Programme Trees and Plantations.

Diouf D, Lesueur D (1999) Pamphlet on "*Calliandra calothyrsus* : Production, Inoculation and Plantation". CIRAD-Forêt, Programme Trees and Plantations.

G. Conclusions

The main objectives of the project were achieved. We have established a large collection of rhizobia, and selected effective strains, which is now available for inoculation of *C. calothyrsus*

A copy of these selected strains were provided to partners involved in the inoculation experiments. An UPIL system was set up in Cameroon and there, they are able to produce a good liquid inoculum of rhizobia. Training in the production of rhizobia inoculum was provided to partners and the inoculation of plants of *C. calothrysus* can be optimised in nursery. Some field trials were setup in Dakar station. It is possible to carry out the analysis of nodules by using ELISAtechnic.

All these results obtained during the four years of the project are available for the farmers who are interested for planting *C. calothyrsus* trees.

Partner 2 : Servicios Científicos Agropecuarios

Rosemary Bradley

Objectives

Context: Natural distribution of *Calliandra calothyrsus* in Costa Rica is mainly in areas of forest regeneration on steep slopes, along roadsides, and along rocky river banks. The soil characteristics in these areas are very variable, from highly acidic Ultisols, to fertile black organic soils. The use of shrub legumes in Central America, for forage, tinder or soil conservation is currently very limited, although several species, including *Calliandra calothyrsus* are native. However, it is expected that incentives for utilization of such sustainable technologies will be introduced, and this increases the possibility that farmers may adopt them. *Calliandra calothyrsus* is considered by CIAT to have limited value as a forage plant, due to its high tannin content, but for the same reason could be useful as a green manure, due to low decomposition rates (CIAT, 2000).

Objectives:
1) Replace CONSEFORH, Honduras as collaborator on the project in Central America, as from February 2000.
2) Undergo training for evaluation and infection by arbuscular mycorrhizae, and production of inoculum, in Edinburgh, Scotland.
3) Equip a laboratory for carrying out the evaluations.
4) Determine the amount of native nodulation and infection by arbuscular mycorrhizae of *Calliandra calothyrsus* in representative soils of Costa Rica.
5) Produce inoculum of different mycorrhizae.
6) Determine the effectiveness of the native strains, in comparison with the inoculum produced from strains selected by the project.

Activities

1) In April 2000, a 3-day training was received on the evaluation of inocula and infection by arbuscular mycorrhizae in Edinburgh, Scotland, as well as participating in the project meeting for two days.
2) Lists of equipment and reagents needed for the laboratory were prepared, and quotes obtained from different sources and shipping companies for sending the equipment to Costa Rica and obtaining the necessary documentation for importation.
3) Soil was collected and sterilized for reproduction of the mycorrhizal inocula, and planted with sorghum and maintained for six months, for subsequent collection and evaluation of the inoculum produced.
   Rhizobial strains were maintained and multiplied in sterile media.
   Five representative soil types were collected and analysed, and an experiment to determine native infection and response to rhizobium inoculation was set up, harvested and analysed.

Results achieved

1) The training course in Edinburgh was a very useful introduction to the methodology necessary for reproducing and evaluating mycorrhizal fungi, and the information will be a useful basis for setting up commercial production of mycorrhizal inoculants, when and if some effective lines can be identified for use under local conditions.
2) The quotes which were obtained for equipping the laboratory are still available for use, when/if the funding arrives.

3) The reproduction of the mycorrhizal inocula is still in progress. The growth of the sorghum plants has been very slow, possibly due to the fertility being too low. It has therefore been decided to replant the same pots after fertilizing with rock phosphate.
4) The greenhouse experiment designed to evaluate effectiveness of native rhizobia and infection by native arbuscular mycorrhizae was terminated in November 2001. The results (see attached paper) showed natural rhizobial infection in three of the five soils tested, and negligible mycorrhizal infection. Rhizobial inoculation had no significant effect in any soil. Plant growth was significantly greater in soil from General Viejo and San Carlos, which respectively showed the lowest and highest nodulation. These results are difficult to interpret without further experimentation designed to separate effects of nitrogen mineralization and soil fertility.

Problems encountered

The major limitation to being able to carry out the project is the way the funding has been disembursed. The estimated total amount needed to support our activities was ECU 40362. This amount when calculated in January 2000 was equivalent to US$ 39281, but at the current exchange rate is equivalent to US$ 45865, meaning that unless the EC is prepared to cover this difference of US$ 6584, SCA will have to do so.
Also, taking into account that the work had to be done in two years rather than four, and the fact that the EC applied the rules which agree to disemburse only 40% of the funding needed in the first year, combined with the fact that we only received this money in August 2000, made it impossible for us to carry out the work as planned. It should be clear that if the funding is for a four year period, a disembursement of 40% covers more than the costs of the first year, but in our case, since the funding was only for two years, obviously 40% did not cover our costs for the first year. Although a considerable effort was made to explain the problem to the EC, they did not accept that there was any reason to disemburse the small amount of additional money which would have allowed us to buy the laboratory equipment we needed.
In addition, even the amount of money which was approved by the EC has not been sent to us. We have been informed by the EC that an amount of ¢3,989,596 at an exchange rate of US$ 1 = ¢315 was approved and sent to CIRAD in February 2001, but we were only sent ¢2,847,759.33 in April when the exchange rate was already US$ 1 = ¢323 (i.e. US$ 8816). We have received no explanation regarding the difference (US$3849), if or when we can expect to receive it.

SCA is a small company which does not have a special fund for supporting research activities. This year we have suffered extreme financial difficulties due to the refusal of the Costa Rican government to allow us to import seeds, due to the foot and mouth epidemic. We have suffered a loss of approximately US$60,000.

We are extremely disappointed in the way the EC has responded to our difficulties.
We have set up the experiments using expensive borrowed money in order to demonstrate that our intentions are honourable, but we have had to use every possible artifice in order to obtain the results, persuading friends at the University to lend us equipment etc.
We request that this complaint be sent in its entirety to the EC.

Thank you for your attention.

Partner 3 : IRAD

Théophile TIKI MANGA

Introduction

Three major components are hereby reported as parts of the present final project report. These are:

1. The assessment of native rhizobia and arbuscular myccorhizae fungi in three soil types of southern Cameroon.
2. The effects of phosphorus amendments on *Calliandra calothyrsus* early growth in acid soil.
3. Assessment of local organic material as carrier for rhizobia culture.

The overall objective of the project was to improve the forage production potentials of *Calliandra calothyrsus* through rhizobium and myccorhizae inoculation for smallholder' farmers of the humid tropics.

I. Activities and results achieved

Various activities were undertaken during the project lifetime notably:

a)- The evaluation of the biodiversity within collection of rhizobia and myccorhizae isolated

b)- The production of inoculum.

c) - The effect of the inoculation with rhizobia on forage production in *C. calothyrsus* cultivated in both research station and field conditions

I.1. Assessment of native rhizobia and arbuscular myccorhizae fungi in three soil types of southern Cameroon

In order to assess indigenous rhizobia and AM fungi diversity, a massive collection survey was then carried out in 1998 in three provinces (Centre, south and north west) of the southern Cameroon where Calliandra calothyrsus species has been introduced since 1985 for agroforestry research activities. A subsequent objective of the study was to evaluate the effects of the sites (soil, climate, vegetation...etc.) on the quantity and quality of rhizobium and myccorhizae types.

Material and methods

Sampling sites

Three sites located in the centre and western highland zones of Cameroon were surveyed. The sites are known to be areas where *Calliandra calothyrsus* has been introduced for about 15 years (since 1985). The three sites (Yaoundé, Ebolowa and Bamenda) are different in terms of climatic characteristics (rainfall pattern and quantity, temperature), altitude and vegetation land cover types (Table 1).

Table 1: Sampling site characteristics

Parameters	Site location

	Yaoundé (Minkoameyos)	Ebolowa (Nkoemvone)	Bamenda (Fonta)
Mean annual rainfall (mm)	1692	1720	2568
Rainfall pattern	Bimodal	Bimodal	Monomodal
Mean temperature (0°)	23.5	24	24.6
Altitude (m)	783	615	1680
Latitude	3° 57' N	25°4' N	6°00' N
Longitude	11°32' E	11°11' E	10°15' N
Vegetation cover	Bush fallow dominated with *Chromoleana odorata*	Primary as well as mature secondary forest	Typical savannah grasses land with few isolated shrubs

Soil sampling and nodule collection

For myccorhizae, several trowels of soil were collected from the top 20 cm at about 1 meter from *Calliandra calothyrsus* tree stands. Coarse surface litter was removed and retaining haft decomposed organic material and plant roots were retained in the sample. For each site, the trowels of soil were pooled, mixed thoroughly and a sub-sample of about 500 g packed into sealable poly bags.

While for chemical analysis, trowels of soil samples were collected from the top 0-20 and 20-40 cm at each site as above. But for rhizobium assessment, the samples were kept in a cool box during the sampling period until the time they were brought to the laboratory. Where available, Calliandra root nodules were also collected and stored in vials containing silica gel until isolation done.

Soil analysis

Composite soil sample for each site was used for analysis according to the methods described by Anderson and Ingram (1989). Soil pH was measured in H_2O, total nitrogen was assessed by Kjeldahl digestion technique and available phosphorus was determined by the Olsen method.

Results

Soil characteristics of the study sites

Table 2 shows how different are the three pilot soil types with Ebolowa being the most acid site (pH: 3.5). While Fonta (Bamenda) site contains four times more organic matter (33.44 %) in the top 40 cm depth as compared to Minkoameyos (8.18 %) and Nkoemvone (8.86 %). Soil from Fonta has the highest CEC (42.42 meq/100g) in the top 20 cm depth as compared to Minkoameyos and Nkoemvone (14.10 and 13.65 meq/100g respectively).

According to USDA soil taxonomy, the sampled soil sites are classified as Ultisols, Oxisols and Humic ferralsols for Yaoundé, Ebolowa and Bamenda respectively.

Table 2: Soil characteristics of the three pilot sites in Cameroon.

Soil characteristics	Sites /Depth

	Minkoameyos (Yaoundé)		Nkoemvone (Ebolowa)		Fonta (Bamenda)	
	0 – 20 cm	20 – 40 cm	0 – 20 cm	20 – 40 cm	0 – 20 cm	20 – 40 cm
Physical						
- clay (%)	46.4	48.9	42.5	46.5	24.5	54.2
- loam (%)	11.4	15.1	8.3	9.2	44.8	17.4
- sand (%)	36.5	33.3	41.4	40.2	20.2	17.5
Organic matter						
- o.m (%)	5.4	2.78	6.14	2.72	15.34	18.10
- o.c (%)	3.13	1.61	3.56	1.58	8.9	10.50
- N (%)	0.25	0.16	0.31	0.13	0.62	0.70
- C/N	12.5	10.1	11.5	12.2	14.4	15.0
pH (H_20)	5.0	6.1	3.8	3.5	5.4	5.7
pH (KCL)	4.3	4.9	3.1	3.5	4.4	4.4
Chemical properties						
-Al^++ Acidity (meq %)	0.19	0.10	3.70	3.08	0.67	0.82
-Ca (meq/100 g)	17.43	17.43	17.43	17.43	19.26	19.26
-Mg (meq/100 g)	2.07	2.07	1.90	1.90	2.29	2.10
-K (meq/100 g)	0.11	0.05	0.05	0.05	0.23	0.17
-Na (meq/100 g)	0.11	0.11	0.11	0.10	0.12	0.12
-CEC (meq/100 g)	14.10	12.00	13.65	7.80	42.42	47.06
Soil class — USDA taxonomy	Ultisols		Oxisols		Humic Ferralsols	
FAO/UNESCO	Ferric Acrisols		Xanthic Ferralsols		Typic Haplumox	

Native arbuscular myccorhizae (AM)

The results show (Table 3) humic ferralsols produced more (43 spores/50 g) arbuscular mycchorhizae spores than the oxisols and ultisols (27 and 18 spores/50 g respectively). It also shows that soil with less spores in the original samples (ultisols with 18 spores /50 g of soil) produces more spores after culturing (600 spores /25 g of soil) in the fiber/sand substrate. While soil with more spores (humic ferralsols with 43 spores per 50 g of soil) produces less spores after culturing (325 spores/25 g of soil). This indicates that the number of spores in the 'trap' culture is inversely proportional to the quantity of spores in the original soil. *Acaulospora*, *Glomus* and *Scutellospora* are the most frequent myccorhizae species found both in the original soil as well as in the 'trap' cultures. This implies that 'culturing' does not induce a significant change in the quality of arbuscular myccorhizae species. The results also indicate that *Calliandra calothyrsus* is very effective when used as host plant in the "trap" cultures. In future, crop plants may only need to be used in the final phase of inoculum production, when large quantities of infected (and non-nodulated) roots are required.

Table 3: Spores number and species of AM fungi identified in three sites of southern Cameroon

Site	Soil type		Mean spore per 50g	Spore species[1]	Host plant specie	Substrate	Mean spore per 25g	Spore species[2]
	USDA/Soil taxonomy	FAO/ UNESCO						
Yaoundé	Ultisols	Ferric Acrisols	18 (20.5 %)	S. scu., S. pel, & A. scr.	Calliandra	Fiber/sand	600	A. sp, G.sp
Ebolowa	Oxisols	Xanthic Ferralsols	27 (30.7 %)	A.dil, S.pel, G. occ.	Calliandra	Fiber/sand	500	A.dil, S.pel., G.sp
Bamenda	Humic Ferralsols	Typic Haplumox	43 (48.8 %)	A.scr, Gi. sp, S.pel.,G.sp	Calliandra	Fiber/sand	325	A.scr, S.ver., G.sp

1 Species identified in the original soil and 2 represents spore species identified after culturing in the fiber/sand substrate

A.dil: Acaulospora dilatata
A.sp.: Acaulospora species
A.scr: Acaulospora scrobiculata
G.occ: Glomus occultum
G.sp: Glomus species
Gi.sp: Gigaspora species
S.pel: Scutellospora pellucida
S.scu: Scutellospora scutata
S.ver: Scutellospora verrucosa

Rhizobia strains

Table 4: Rhizobium strains isolated from nodules of Calliandra calothyrsus harvested in three sites of southern Cameroon

Sites	Soil type	Calliandra provenance	Code number	Rhizobium genus Strain
Ebolowa	Oxisols	San ramon	CCC 6	Rhizobium
			CCC 7	Rhizobium
			CCC 8	Rhizobium
			CCC 14	Rhizobium
			CCC 36	Rhizobium
			CCC 40	Rhizobium
		Flores	CCC 16	Rhizobium
			CCC 18	Rhizobium
			CCC 19	Rhizobium
			CCC 20	Rhizobium
			CCC 26	Rhizobium
			CCC 27	Rhizobium
			CCC 28	Rhizobium
			CCC 29	Rhizobium
			CCC 30	Rhizobium
Bamenda	Humic ferralsols	San ramon	CCC 41	Bradyrhizobium
		Flores	CCC 9	Bradyrhizobium
			CCC 24	Bradyrhizobium

			CCC 25	Bradyrhizobium
Yaoundé	Ultisols	San Ramon	CCC 1	Rhizobium
			CCC 4	Rhizobium
			CCC 31	Rhizobium
			CCC 32	Rhizobium
			CCC 33	Rhizobium
			CCC 34	Rhizobium
			CCC 35	Rhizobium
		Flores	CCC 15	Rhizobium
			CCC 21	Rhizobium
			CCC 22	Rhizobium
			CCC 23	Rhizobium

Thirty rhizobia strains in total were isolated from the collection (Table 4) with the large majority (26 rhizobia representing 85 %) belonging to the *Rhizobium* genus (fast-growing rhizobia) and only 15 % belongs to the *Bradyrhizobium* genus. Usually, *Calliandra calothyrsus* is described as a woody legume capable to nodulate only with Rhizobium and not with *Bradyrhizobium* genus (Turk and Keyser, 1992; Lesueur et al., 1996). However, as shown in the results, Peoples et al. (1989) reported also that *Bradyrhizobium* strain can nodulate and form an effective N-fixing symbiosis with *Calliandra*. The results also show that all the *Bradyrhizobium* genus strains were isolated from the Bamenda site (Humic ferralsols) indicating that the soil type affects the nature of native *Rhizobium* strains in a given zone.

Conclusion

Calliandra calothyrsus can effectively nodulate with *Bradyrhizobium* strains. Soil types affect both the quality rhizobium strain and the quantity of arbuscular myccorhizae spores. The quantity of AM spores found in the original soil is inversely proportional to the quantity of AM fungi after 'bait' culturing. *Glomus*, *Acaulospora* and *Scutellospora* species are the most frequent AM fungi found in Oxisol, Ultisol and humic ferralsol of the southern Cameroon.

I. 2. Effects of phosphorus amendments on *Calliandra calothyrsus* early growth in acid soil

The most limiting factors for *Calliandra* growth in the humid rainforest are aluminium toxicity and low P that ranges between 2 to 8 meq/100g. Although *Calliandra* strikes well on relatively high acid soils, results obtained so far indicate that lack of available P impedes crops as well as leguminous trees early growth. A nursery experiment was then set so as to assess the effect of phosphorus fertilizer and chicken manure on *Calliandra calothyrsus* growth performances and nodulation.

Methods:

Arable soil was collected from an old fallow land (see chemical composition in Annex, on table 1), sieved and filled in perforated polythene bags at a rate of 2 kg of soil per pot. Pre-treated (by manual scarification) seeds of San Ramon provenance were then planted (1 seed/pot) and phosphorus fertilizer (triple super phosphate 48%) and chicken manure (63% P) were respectively applied at the rates of 1g (100ppm) and 2 g. A no-fertilizer treatment was also considered as the control. The trial was set in a completely randomised block design replicated 4 times with treatment unit made up of 10 seedlings per replicate. Growth

parameters such as number of leaves, plant height, biomass dry matter and root length were recorded as well as the number of nodules at 9 and 12 months after planting.

***Results*:**

The results show that the amendment treatments have a significant ($P<0.05$) effect on *Calliandra* growth parameters (notably the leaves number, the root length and biomass dry matter production) mostly at 12 weeks after planting.

Meanwhile the triple super phosphate had a more pronounced effect as compared to the chicken manure treatment. These results indicate the relatively importance of the phosphorus nutrient for the initial growth of *C. calothyrsus*.

Figure 4 below also shows that at 12 weeks after planting, there is a significant effect of triple super phosphate fertilizer and chicken manure on *Calliandra* nodulation (number of nodules). Unlike for the growth parameters, the chemical fertilizer had a more pronounced effect as compared to the chicken manure. This is probably due to the rapid liberalization and mobilization of P by the fertilizer.

I. 3. Assessment of local organic material as carrier for rhizobia culture

The purpose of the study was to identify the best carrier among existing local material that should be used to stabilize rhizobia culture as long as possible until seed inoculation have been performed. The powered inoculants are the most used ones and peat that generally gives the best results does not exist in Cameroon. Meanwhile other carriers could be used such as crop waste (straw, compost), sugar waste, mineral (talc, vermiculite, clay...), charcoal, lignite whenever possible. Hence existing low-cost local material can also perform as carrier.

Methodology

Fresh leaves of *Tithonia diversifolia*, *Cajanus cajan* and straws of *Arachis hypogea* (peanut) were oven-dried at 70°C for 48 hours then crushed and sieved in a 200 mesh. The powder obtained was then placed into autoclavable bag for sterilization at 120°C for 30 minutes, cooled down and re-sterilized 24 hours later after their pH has been cross-checked and then neutralized i.e. pH=6.5.

The Cameroon rhizobia strain CCC_{22} was then used after been controlled disease-free with YEM (Vincent, 1970) for 4 days until a regular growth of 10^9 bacteria/ml. It was then inoculated into ten (10) poly pots for each "carrier" at a ratio of 20 ml for *Cajanus cajan* and peanut and 30 ml for *Tithonia diversifolia.* The inoculated poly pots were then placed in 4°C cool atmosphere and a survival testing was to be undertaken on a monthly interval basis with colony counting to be done at the same time through successive dilution in petric dishes with the YEM-Agar.

Results

Table 1 below presents results obtained after four months.

Table 1: Bacteria population colony (average) obtained in three organic material two months after inoculation

Organic matter type	pH	WHC[1]	Mean bacterial colony/month	
			First month	Second month

Cajanus cajan	6.1	240 %	51.16×10^8	67.27×10^8
Arachis hypogea	6.0	250 %	46.38×10^8	58.40×10^8
Tithonia diversifolia	6.5	600 %	51.6×10^8	68.67×10^8

[1] WHC: Water Holding Capacity

Table 2: Survival of rhizobium in *Tithonia* carrier

Tube	Dilution ration	Concentration	Dilution factor (y)	Number of colonies in dishes			Average colonies per dishes (x)	(x).(y)
				1	2	3		
1	1/10	10^8	10	-	-	-	-	-
2	$1/10^2$	10^7	10^2	-	-	-	-	-
3	$1/10^3$	10^6	10^3	-	-	-	-	-
4	$1/10^4$	10^5	10^4	-	-	-	-	-
5	$1/10^5$	10^4	10^5	432	588	484	501	501×10^5
6	$1/10^6$	10^3	10^6	86	81	82	83	83×10^6
7	$1/10^7$	10^2	10^7	21	23	25	23	23×10^7
8	$1/10^8$	10	10^8	3	5	2	3	3×10^8

(x) . (y)= Population of rhizobium
N.B.: 10g of *Tithonia* inoculum in 90 ml of sterilized water and 1ml of inoculum solution as initial sample.

Tithonia diversifolia seems to be the best among the material tested but its high water holding capacity can be very detrimental to the bacteria survival.

The monitoring of the this study was interrupted after a certain time due to late funds release as the test needed a minimum of chemicals to be used.

II. Problems encountered

One major problem encountered in the whole project lifetime was the late release and transfer of project funds. This created a lot of distortion for the activities planned in such a way that one of the major activities to be undertaken in Cameroon couldn't take place.

Another problem was the complete refusal of purchasing a project car Cameroon while working on-farm. Because our national institution doesn't have vehicle allocated to researchers.

III. Publications and papers

Lesueur D, Ingleby K, Odee, D, Chamberlain J, Wilson J, Tiki Manga T, Sarrailh JM, Pottinger A (2001) Improvement of forage production in *Calliandra calothyrsus* : Methodology for the identification of an effective inoculum containing *Rhizobium* strains and arbuscular mycorrhizal isolates. J. Biotechnol. 91:269-282.

IV. Conclusion and recommendation

Quite a number of benefits can be spelled out as positive project output such as:

1. The acquisition a complete laboratory inoculum unit for IRAD.
2. The training of a full flesh technician for both rhizobium and myccorhizae production and inoculation.
3. Computer equipment and the connection into Internet have been well taken by the institution.

Based on the benefits mentioned above and considering the pre-planned activities to be undertaken by IRAD for the project, we strongly recommend that the project should ***be extended for a 2-3 years new phase in order to transfer the acquired technologies to the farmers' level***. This was supposedly to be the ultimate project goal and as such, the later is considered as not achieving its purpose for Cameroon.

Partner 4 : CEH/ITE

K. INGLEBY, J. WILSON and P.A. MASON

1. Objectives

The specific objectives of CEH Edinburgh (Partner 4) in the project were to:

1. Evaluate the biodiversity of AM fungi present in soils where *Calliandra calothyrsus* is growing in its native and introduced range.
2. Obtain arbuscular mycorrhizal (AM) isolates from *C. calothyrsus* growing in its native and introduced range.
3. Conduct screening tests in glasshouses at CEH Edinburgh to identify effective AM isolates.
4. Produce starter cultures of the most effective isolates for use by project partners in their own country.
5. Provide training and assist nurseries in Kenya, Honduras and Cameroon in the production and evaluation of AM inoculants.
6. Assist with the establishment of nursery and field trials in these countries to monitor long-term growth of inoculated trees.

2. Activities

2.1. Evaluation of biodiversity

Soil samples were collected by partners from under *C. calothyrsus* trees in Central America, Cameroon, Kenya and New Caledonia, and sent to CEH Edinburgh. Each sample was thoroughly mixed, a sub-sample removed for assessment of biodiversity and the remaining soil used to establish 'trap' cultures from which AM cultures would be isolated. AM diversity was examined by assessing populations of spores in the soils. AM spores were extracted from the field soil samples and 'trap' cultures by sucrose centrifugation. Abundance and diversity of spores were assessed using traditional microscopic methods, making identifications on the basis of morphological characteristics of crushed spores. Reference specimens of the dominant spore types were preserved on microscope slides.

2.2. Establishment of AM collection

'Trap' cultures were used to 'bait out' the AM fungi present in the soils and produce fresh, viable spores from which single species pot cultures could be initiated. 'Trap' cultures were grown in the glasshouse in a sterilised mixture of loam, sand and Terragreen (Oil-Dri UK Ltd), using *C. calothyrsus*, Sorghum or millet as host plants. After 4 months growth,

spore populations in the soils were examined and repeat cycling of cultures was done by re-sowing the pots, in order to 'bait out' AM fungi that were dormant during the first culture cycle. Single species isolations were made using freshly extracted spores from the 'trap' cultures, inoculating these spores onto the roots of pre-germinated Sorghum seedlings and growing in a controlled environment cabinet to ensure strict hygiene and prevent ingress by contaminant AM fungi. After 6 months, root samples were stained in Trypan Blue and examined for AM infection. If infection had established, cultures were transferred to the glasshouse where they were maintained on isolated, free draining benches and watered with filtered (50 _) water.

2.3. Selection of the most effective isolates

AM isolates were screened for effectiveness on *C. calothyrsus* in a single, large glasshouse pot experiment. Seedlings of San Ramon provenance (11/91) were used to test 19 inoculants (12 single species and 7 mixed species cultures) and compared with uninoculated control plants which received
autoclaved inoculum. All plants were inoculated with the same rhizobium suspension. Pots were laid out in randomised blocks with each treatment represented once in each block. Plants were harvested after 12 weeks growth.

2.3.1. Student project to examine AM dependency of *Calliandra calothyrsus*

Because little was known about the response of *C. calothyrsus* to AM infection, an experiment was conducted by an honours year student, which examined the dependency and response of *C. calothyrsus* to AM inoculation. *C. calothyrsus* seedlings (Seedlot 12/91, Honduras) were inoculated with *Glomus intraradices* (INVAM isolate, UT 143-2): mycorrhizal and non-mycorrhizal seedlings were grown in a sterilised mixture of coconut fibre and sand, supplied with varying levels of phosphorus and harvested after 8 weeks growth.

2.4. Inoculum production

Pot cultures were grown in the CEH glasshouse using Sorghum as the host plant. Plants were grown in a sterilised substrate consisting of a 1:1:1 mixture of loam, sand and Terragreen. A slow-release insecticide containing 10% chlorpyrifos was incorporated in the substrate to eliminate mites and nematodes. Inoculum quality of the cultures was monitored by removing soil cores to determine the level of AM infection in the roots, the number of spores and to ensure that no contaminant AM fungi were present. Pot cultures were grown for 3-4 months, then watering was gradually reduced and the cultures were slowly dried, before finally removing the shoots and storing in sealed poly bags or containers at cool temperatures.

For nursery production of AM inoculum in partner countries, preliminary tests were carried out on a range of local soils to identify which soils would produce optimal levels of root infection and sporulation.

2.4.1. Training workshop

In order to facilitate the transfer of the selected AM cultures to partner countries and the implementation of AM inoculum production and evaluation, a training workshop for technicians from KEFRI, IRAD and CONSEFORH was held at CEH Edinburgh from 11-20 October 1999. The first part of the workshop aimed to familiarise participants with the general features of AM and the methods used to recognise and examine AM spores and infected roots. The second part dealt specifically with the nursery facilities and methods required by each partner in order to establish AM cultures and begin production of AM inoculum when they

returned to their own country. An illustrated manual was produced for the workshop that was made available to all partners.

2.5. Inoculation techniques

Standard AM inoculation techniques were used. Inoculum consisted of a root/soil/spore mixture obtained from the pot cultures. For glasshouse or nursery inoculation, about 20 g of this mixture was added as a layer to the pots/bags about 1 cm below the surface, and prior to sowing or planting of the seedlings.

2.6. Effects of dual inoculation on growth of *Calliandra calothyrsus* under nursery and field conditions

Dual inoculation experiments were conducted in Kenya in collaboration with Partner 5 (KEFRI). Two contrasting sites were chosen; one at Embu in Central Kenya, the other at Maseno in NW Kenya. For both sites, nursery and field experiments were set up and the same four inoculation treatments were applied: Seedlings of *C. calothyrsus* were inoculated with rhizobium and AM inoculants, either singly or in combination, with appropriate uninoculated control treatments. Both rhizobium and AM inoculants consisted of a cocktail of isolates. The experiments also compared the response of different provenances to inoculation, and the effect of soil solarisation on the performance of the inoculants. All experiments were set up in unsterilised local soils and harvested after 16 weeks.

In the final year of the project (2001), large on-station and smaller on-farm field experiments were designed and set up. Treatments were laid out in randomised blocks to account for within-site variation. Each treatment plot consisted of 9 trees planted at 3 m spacing. Split-plot designs were adopted for experiments that included more than one provenance.

3. Results

3.1. Evaluation of biodiversity

Over 30 different species of AM fungi were identified in the field soils, although the poor condition of spores meant that it was sometimes only possible to assign spores to the level of genus. Abundance, diversity and species composition of AM fungi varied widely between the field soils, and soils that were rich and deficient in AM fungi could be distinguished. These differences were not related to country of origin i.e. countries where *C. calothyrsus* was native or had been introduced.

3.2. Establishment of AM collection

Spore production in the 'trap' cultures tended to reflect the abundance and diversity of spores in the original field soils. As a result, several isolates of the fungi that occurred most often in the field soils were successfully cultured e.g. *Gigaspora albida*, *Glomus etunicatum* and *Scutellospora* spp. In all, 47 single species isolations were attempted in the growth cabinet, of which 23 were successfully potted on and cultured in the glasshouse.

Figure 1. Single species isolations in growth cabinet.

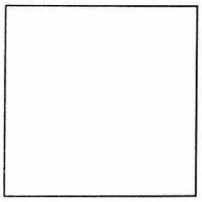


Figure 2. Pot cultures in glasshouse

3.3. Selection of the most effective isolates

Results from the screening experiment showed that mycorrizal inoculation increased the shoot growth, nutrient allocation and nodulation of *C. calothyrsus* seedlings. Fourteen of the 19 inoculants significantly increased stem dry mass compared with the uninoculated control treatment (Figure 1). Mycorrhizal infection formed by the inoculants ranged from 19-62% and was associated with increased shoot growth, nodulation and the proportion of nutrients (N,P,K) allocated to the shoot. On the basis of these results, 5 of the most effective isolates were selected for testing under nursery and field conditions. The experiment also showed that single species inoculants performed as well as mixed species inoculants, and that isolates originating from countries in Central America and Africa could be equally effective on *C. calothyrsus,* which is indigenous to Central America.

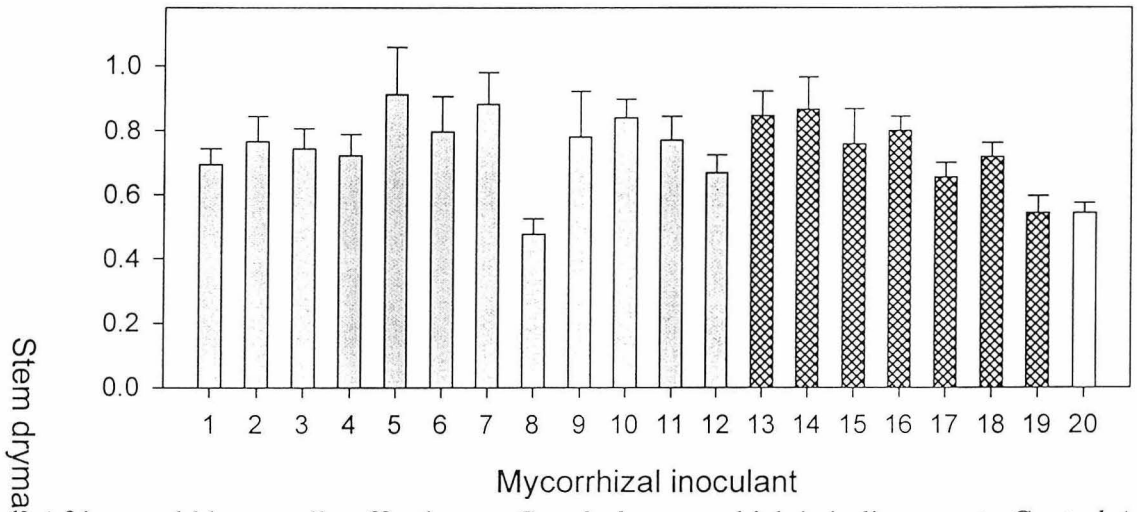
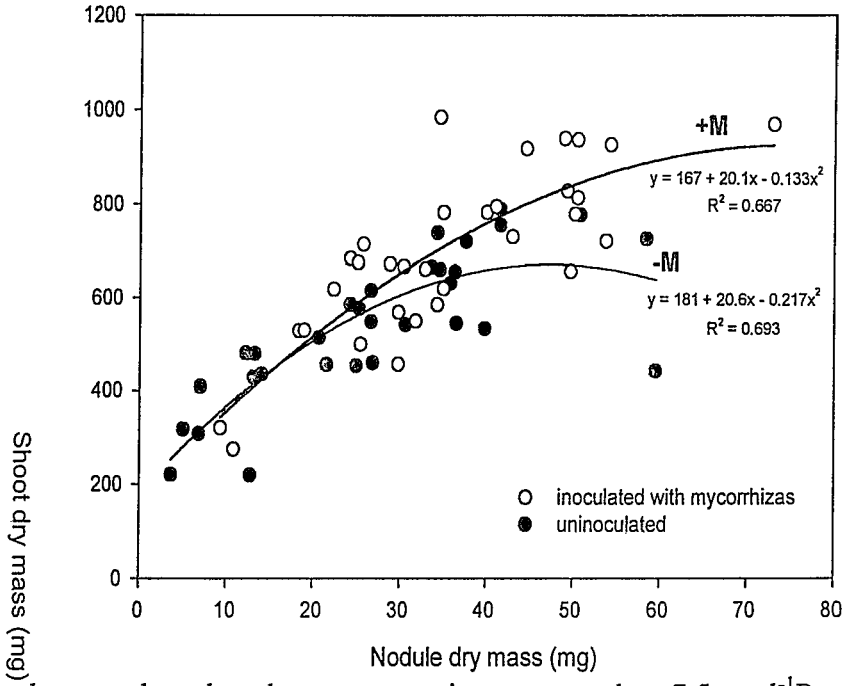


Figure 1. Effect of inoculation with 12 single species inoculants (1-12) and 7 mixed species inoculants (13-19) on the stem dry mass of *Calliandra calothyrsus* seedlings compared with uninoculated control plants (20). Error bars indicate +SE.

3.3.1. Student project to examine AM dependency of *Calliandra calothyrsus*

This experiment showed that AM inoculation of *C. calothyrsus* increased seedling growth and nodulation most at lower concentrations of supplied phosphorus (0-7.5 mg.l^{-1}P). Mycorrhizal



infection was also greatly reduced at concentrations greater than 7.5 mg.l^{-1}P, and growth response of seedlings to increasing nodulation was maintained in mycorrhizal plants, but not in non-mycorrhizal plants (Figure 2). This study suggests that *C. calothyrsus* will be largely dependent on AM fungi in soils of low fertility and will only respond to moderate applications of phosphorus fertilizers.

Figure 2. Shoot growth (shoot dry mass) response curves of mycorrhizal (+M) and non-mycorrhizal (-M) *Calliandra calothyrsus* seedlings to nodulation (nodule dry mass).

3.4. Inoculum production

During the course of the project, monitoring of pot culture quality through the examination of AM spore numbers and root infection levels, indicated that spore production in the loam/sand/Terragreen substrate was usually very high, whereas infection levels on the Sorghum roots were sometimes rather low. These quality checks also revealed that, with one exception, ingress by contaminant AM fungi had not occurred, and that use of a slow-release insecticide had eliminated mites and nematodes.

3.4.1. Training workshop

During the workshop, AM spore populations in field soils, 'trap' cultures and pure cultures were compared. This exercise not only familiarised the participants with the characteristics of the different inoculant fungi, but also clearly demonstrated the need for culture and isolation of mycorrhizal fungi from field soils. Participants saw that field soils contained relatively few spores, most of which were dead, and also contained fungal parasites, mites and nematodes. 'Trap' cultures made using the same field soils, and the resulting single species cultures, markedly increased numbers of 'live' spores and eliminated harmful organisms (Table 1).

Field soils			Trap cultures			Single species cultures		
Origin	Live spores 50 g^{-1}	Dead spores 50 g^{-1}	Test soil	Live spores 50 g^{-1}	Dead spores 50 g^{-1}	Isolate	Live spores 50 g^{-1}	Dead spores 50 g^{-1}

Kenya - Embu	111	63	Embu K10	1543	10	*S. verrucosa* 2c	830	0
Cameroon - Ebolowa	20	137	Ebolowa C2	783	10	*S. calospora* 2	485	0
Honduras - La Ceiba	12	63	La Ceiba H2	1852	10	*Gl. etunicatum* 1	4430	0
Cameroon - Bamenda	24	168	Maguga K2	435	12	*Gi. albida* 1b	365	0
			Ocoman H3	1433	50	*Gi. albida* 2	875	0

Table 1. Comparison of AM spore numbers in field soils, trap cultures and single species cultures

3.4.2. Inoculum production in-country

After despatch of the 5 selected inoculants to partners, subsequent nursery tests in Senegal have indicated that storage at 6°C has little or no detrimental effect on viability of the inoculum. This suggests that spores, rather than infected root fragments, are the most important component of the AM inoculants. An experiment conducted in Kenya, growing the 5 AM inoculants in contrasting local soils, has shown a differential response to the soils by the AM fungi in terms of infection and sporulation (Figure 3). This work shows that, in pot culturing, factors such as soil type, pH, nutrient regimes etc. need to be tailored to suit specific fungi.

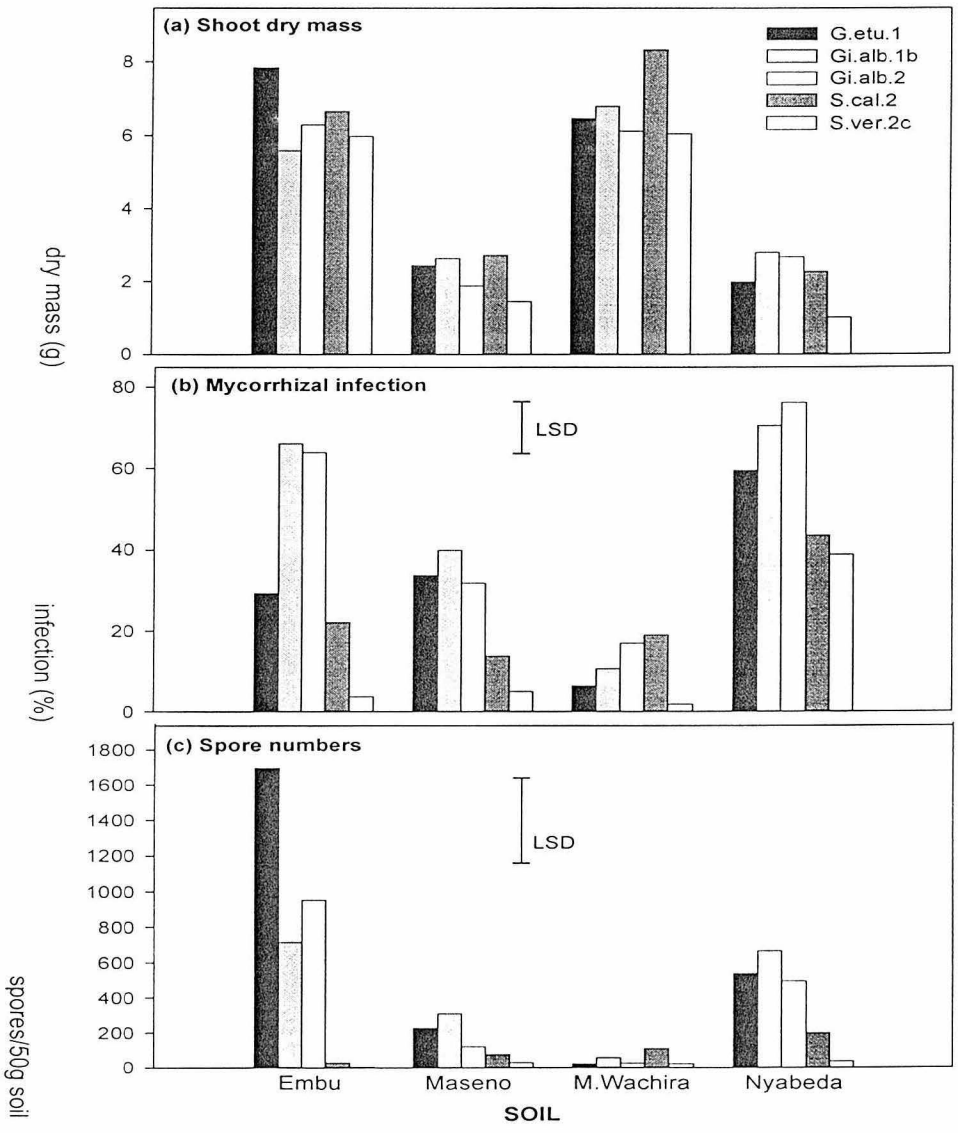


Figure 3. Shoot growth, mycorrhizal infection and spore production of five AM inoculants growing in Sorghum pot cultures using four different Kenyan soils.

3.5. Inoculation techniques

Standard nursery AM inoculation techniques were used successfully during the course of the project.

3.6. Effects of dual inoculation on growth of *Calliandra calothyrsus* under nursery and field conditions

Both nursery experiments in Kenya showed that all inoculation treatments increased seedling growth when compared with the uninoculated control seedlings, and that dual inoculation with rhizobium and AM inoculants increased plant growth more than single inoculation with either microsymbiont. The results at the Muguga nursery in the Embu soil (Table 2), also showed a differential growth response of the seedlings to the inoculants: those receiving rhizobium alone had more leaves, greater leaf dry mass and leaf area than seedlings receiving AM alone. In contrast, seedlings receiving AM alone had greater root collar diameter, stem and root dry mass than seedlings receiving rhizobium alone, and had a significantly greater root/shoot ratio than all other inoculation treatments. This indicates that dual inoculation benefits many more aspects of plant growth and vitality than single inoculation with either microsymbiont.

	Inoculation treatment				
	+R+M	+R-M	-R+M	-R-M	*P* value
RCD (mm)	4.50 a[1]	3.94 c	4.19 b	3.89 c	<0.001
Stem DW (mg)	471 a	355 bc	401 b	311 c	<0.001
Leaf area (cm^2)	188 a	172 a	168 a	135 b	0.003
Leaf DW (g)	2.05 a	1.74 b	1.67 b	1.44 c	<0.001
Leaf number	9.73 a	10.18 a	8.60 b	8.75 b	<0.001
Root DW (g)	1.45 a	1.14 b	1.40 a	1.00 b	<0.001
RS ratio	0.61 b	0.56 b	0.76 a	0.62 b	0.001
Leaf N (%)	1.80	1.85	1.74	1.88	0.068
Leaf C (%)	44.2	43.9	44.2	44.0	0.949
Leaf P (ppm)	1571 a	1185 bc	1336 b	1053 c	<0.001
Nodule number	401 a	324 a	136 b	117 b	<0.001
Nodule DW (mg)	0.182	0.169	0.163	0.140	0.163
Myc. infection (%)	51.8	53.9	55.2	52.8	0.396

Table 2. Overall effects of inoculation treatments on growth of *Calliandra calothyrsus* seedlings growing in Embu soil.
[1] letters indicate significant differences within each row at $P<0.05$ as determined by ANOVA and Fisher's LSD test.

Levels of AM infection and nodulation in inoculated seedlings and uninoculated seedlings were the same, which was not surprising given that the plants had been growing 16 weeks in soils known to contain active populations of both microsymbionts. These growth benefits therefore indicate that the inoculants were able to successfully colonise *C. calothyrsus* roots in the presence of naturally occurring microbes, and form more effective symbiotic associations than the naturally occurring microsymbionts.

Comparisons can be made between the nursery experiments at Muguga and Maseno, because the inoculants, provenances and solarisation treatments were identical at both nurseries. These showed that San Ramon was the faster-growing provenance and tended to be more responsive to the inoculation treatments in both soils. It could also be seen that the response to inoculation was better in the Embu soil than the Maseno soil which was the less fertile soil but, in contrast to the Embu soil, was rich in populations of naturally occurring AM fungi. This contrast might also partly explain the opposite effect of solarisation on these soils, as the plants grew better in the untreated Maseno soil, where solarisation may have significantly reduced the activity of naturally occurring AM fungi compared the Embu soil which was deficient in AM fungi anyway. This would not explain the positive effect of solarisation on plant growth in the Embu soil, which may be attributable to changes in soil chemistry resulting in a release of nutrients, or a reduction in the level of soil pathogens.

The results of the nursery experiments in Kenya suggest that dual inoculation with rhizobium and AM will increase the growth of *C. calothyrsus* in the majority of cases, but that before inoculation is undertaken, the soil chemistry and microbial activity of the planting site should be characterised in order to gauge the potential benefits of inoculation.

4. Problems

The withdrawal of CONSEFORH from the project in 2000, meant that the training provided to the technician at the workshop in October 1999 was not utilised, and the scheduled programme of production and testing of AM inoculants under nursery and field conditions in Honduras was not completed. The substitution of SCA into the project to continue the Central American work in Costa Rica, was hampered by the time needed to provide training and establish the laboratory and nursery facilities needed to undertake the work. In Cameroon, collaboration with IRAD was hindered by staffing problems, usually the availability of the technician assigned to the project, who had also received training at the workshop in Edinburgh. This also limited progress with nursery inoculation work in Cameroon. As a result of these problems, a more extensive programme of collaboration was developed with KEFRI (Partner 5), and this report outlines some of the results achieved in developing nursery inoculation systems and testing the performance of inoculants under nursery and field conditions.

5. Technology implementation plan

There are no plans to commercially develop any of the AM methodology used.

6. Publications and papers

6.1. Papers in refereed journals

Ingleby, K., Fahmer, A., Wilson, J., Mason, P. A., & Smith, R. I. (2001). Interactions between mycorrhizal colonisation, nodulation and growth of *Calliandra calothyrsus* seedlings supplied with different concentrations of phosphorus solution. *Symbiosis*, 30: 15-28.

Lesueur, D., Ingleby, K., Odee, D., Chamberlain, J., Wilson, J., Manga, T. T., Sarrailh, J-M. & Pottinger, A. (2001). Improvement of forage production in *Calliandra calothyrsus*: methodology for the identification of an effective inoculum containing *Rhizobium* strains and arbuscular mycorrhizal isolates. *Journal of Biotechnology*, 91: 269-282.

Odee, Ingleby, et al., Growth of arbuscular mycorrhizal fungi in pot cultures using different Kenyan soils and the implications for inoculum production. (in preparation)

6.2. Training manual

Ingleby, K. & Mason, P.A. (1999). Production of arbuscular mycorrhizal inoculum in the glasshouse and nursery. Workshop at CEH Edinburgh, 11-20 October 1999. 20 pp.

7. Conclusions

Overall conclusion:

The main objectives of the project were achieved. The biodiversity of AM fungi was evaluated in soils where *C. calothyrsus* was growing in its native and introduced range, and isolates of frequently occurring fungi were obtained and cultured. From these cultures, effective isolates were identified and starter cultures were produced for project partners. Training in the production and evaluation of AM inoculants was provided to partners. Limited progress was made in the evaluation of inoculants under nursery and field conditions in Central America and Cameroon, but this meant that a more extensive and productive programme of work was developed with Partner 5 (KEFRI).

Specific conclusions of the work were that:

1. Populations of AM fungi vary greatly between sites, these differences are not related to country of origin but to factors such as soil fertility and land use.
2. Field soils should not be used as AM inoculum: pot culturing will greatly increase inoculum quality, but selection of soils and culture methods often needs to be tailored to suit specific fungi.
3. Single species AM inoculants performed as well as mixed species inoculants: differences in effectiveness were also not related to country of origin.
4. *C. calothyrsus* is largely dependent on AM fungi in soils of low fertility and will only respond to moderate applications of phosphorus fertilizers.
5. AM inoculants formed more effective symbiotic associations, and were competitive with, naturally occurring fungi.
6. Dual inoculation with selected AM and rhizobium inoculants benefits many more aspects of plant growth and vitality than single inoculation with either microsymbiont.
7. Before inoculation is undertaken, the soil chemistry and microbial activity of the planting site should be characterised in order to gauge the potential benefits of inoculation.

Partner 5 : KEFRI

David Odee

Objectives

The overall objective of the project was to evaluate and utilize the microsymbiont (rhizobia and arbuscular mycorrhiza [AM]) biodiversity of *Calliandra calothyrsus* in order to optimize fodder production in the smallholdings of the humid zone.

The specific objectives of KEFRI (Partner 5) in the project were to:

1. Establish a culture collection of *Calliandra*-rhizobia found in Kenyan conditions
2. Evaluate biodiversity of *Calliandra*-rhizobia in Kenya
3. Select effective strains of rhizobia originating from a wide range of environments
4. Produce rhizobial inoculants for *C. calothyrsus*

5. Develop nursery inoculation techniques for *C. calothyrsus*
6. Study the effects of dual inoculation with selected rhizobia and AM in the nursery and field conditions

Activities

Culture collection establishment

In order to establish a culture collection of *C. calothyrsus*, soils or root nodules were collected from ecologically and agriculturally diverse sites located mainly in the western and central highlands of Kenya.

Special soil collection was done for sending out to CEH (formerly ITE) to evaluate biodiversity and establish pure AM cultures taking care not to cross-contaminate between soils from different sites. Each soil was labeled appropriately and shipped to CEH laboratory, Edinburgh for spore isolation and pure culture development.

Soils for rhizobia trapping rhizobia were collected and kept in a cool box during isolation and transportation back to the laboratory. These soils were used to inoculate *C. calothyrsus* seedlings grown in Leonard jars (Vincent, 1970) under glasshouse conditions in order to enumerate and trap indigenous rhizobia.

Assessment of indigenous rhizobia and genetic compatibility with provenances of C. calothyrsus

Soils collected for rhizobial trapping under glasshouse conditions were used to enumerate indigenous rhizobia compatible with provenances of *C. calothyrsus*. The soils for enumeration had been specially sampled to represent (a) collections made under calliandra and (b) away from calliandra from various agro-ecological conditions of Kenya in order to establish whether history of calliandra cultivation and genotype influenced the population sizes of indigenous rhizobia. Standard most probable number (MPN) plant infection assays were used.

Selection of effective strains of rhizobia

(i) Selection in Leonard jars (sterile conditions)

A subset of 17 indigenous *Rhizobium* strains was selected from the Kenyan rhizobial collection for this study. The selection was based on visual observation of shoot colour and biomass production during trapping experiments and/or authentication experiments with provenances of *C. calothyrsus*. Two reference strains, TAL 1145 from NifTAL and MUN 24 from Moi University were included for comparisons TAL1145. Symbiotic N_2-fixation potential of these strains was evaluated with provenances San Ramón and Flores. They were tested on 2 provenances: San Ramón and Flores. *Rhizobium* cultures were grown to late exponential phase in yeast mannitol broth prior to inoculation of seedlings in Leonard jars (Vincent, 1970). The experimental design was a randomized complete block design with 8 replications. Plants were grown in glasshouse conditions (temperature 30/18 °C [day/night]); natural light of 12 h photoperiod) at the Kenya Forestry Research Institute Headquarters, Muguga, Kenya. They were harvested after 12 weeks of growth. Data on shoot height, shoot dry weight, root dry weight, nodule number, nodule dry weight and nodule specific weight were analyzed by ANOVA and means separated by Least Significant Difference (LSD) at $P < 0.05$. Symbiotic nitrogen-fixation potential, referred to as **relative effectiveness index**, was calculated as the difference between mean shoot dry weight of inoculated plants and non-inoculated (-N) treatment divided by -N uninoculated treatment.

(ii) Selection in potted field soils (unsterile conditions)

The strains used were KCC6, KCC17, CCK13, KCC39 and KWN35 from Kenya, CCCR1 and CCCR15 from Costa Rica, CCNC26 from New Caledonia, and, CCC22 from Cameroon. Two types of soils were used: Muguga nursery and Maseno field soils. Black polyethylene bags (size 6 × 9 cm flat dimensions) with drainage holes at the bottom were filled with 1 kg of respective soil per bag. Two seedlings were planted per bag. Within 2 days after planting, each plant received a 1 ml of late log phase culture (about 10^9) culture grown in yeast mannitol broth. The experiment was a complete randomized block design consisting of 2 soil types, 2 provenances, 9 *Rhizobium* strains inoculation treatments plus non-inoculated control treatment. All treatments were replicated 20 times. The experiment was carried out over a period of 24 weeks (6 months) under glasshouse conditions with watering of pots maintained near field capacity. Shoot, root and nodule dry wt were determined after oven drying.

(iii) Dual rhizobial and mycorrhizal inoculation

Five AM isolates namely *Glomus etunicatum*, *Gigaspora albida* (type I), *Gi. albida* (type II), *Scutellospora verrucosa* and *S. calospora* had previously been selected by Partner 4, and bulked up on *Sorghum bicolor* at KEFRI.

Two soils collected from farms in Nyabeda (near Maseno Agroforestry Centre) and Mama Wachira (near Embu Agroforestry Centre) were used. Soils were sieved (<5 mm) and thoroughly mixed with sterile river sand at the ratio of 3:1 and 1:1 (soil : sand) for Mama Wachira and Nyabeda, respectively. White PVC pots of 3.5 l capacity and 15 cm diameter were lined with polythene bags and filled with approximately 2 kg of one soil type (mixture) per pot. Each pot received a mycorrhizal inoculum of 20 g consisting of root, spores and soil from 6 month old *Sorghum* cultures. Pre-germinated seedlings were transplanted in pairs per pot. Every pot was inoculated with 1 ml of a mixture of fully-grown rhizobial cultures (10^9 ml^{-1}) 3 d after seedling transplanting. The rhizobial mixture consisted of 9 effective strains namely KCC6, KCC17, KCC39, CCCR1, CCCR15, CCNC26, CCK13, KWN35 and CCC22. Number of replicates per treatment, experimental conditions and duration were as for rhizobial strain evaluation in potted soils described above.

Inoculant production

In Kenya, filtermud is the principal carrier for rhizobial inoculants. Filtermud, a waste product of the sugarcane mills, is a fine deposit obtained during the filtration and clarification processes of crushed cane juice. Muhoroni filtermud, the type used routinely for inoculant production at KEFRI, is neutral (around pH 6.4), and therefore requires no additional treatment. The methods of Anyango *et al.* (1985) were used in the preparation of filtermud inoculants with the selected strains containing 10^9 rhizobia g^{-1}.

Inoculation techniques for Calliandra

Inoculation methods used in Kenya for *C. calothyrsus* seedlings raised in (i) Leonard jars and (ii) potted Muguga nursery soil

Pre-treated seeds were germinated in trays of sterile sand. Inoculation was carried out either in the germination trays or after transplanting to Leonard jars and potted Muguga nursery soil. A filtermud inoculant was used containing effective strain KCC 17 originally isolated from *C. calothyrsus* seedling grown in the nursery test soil.

Pre-sowing inoculation (treatment KM1) was carried out by applying the filtermud inoculant to pre-treated seed coated with sucrose (10 % sucrose sticker solution) to a final inoculum concentration of *c.* 10^8 rhizobia $seed^{-1}$. Post-sowing inoculation treatments, i.e., KM2, KM3 and KM4 were carried out with liquid inoculant prepared as a suspension of filtermud culture to give a final inoculation rate of $>10^7$ rhizobia $seed^{-1}$ or $seedling^{-1}$. Inoculation of treatment

KM3 was carried out 5 d after sowing. Inoculation of treatment KM4 was done on 11-day old seedlings transplanted to Leonard jars or potted Muguga nursery soil, one seedling per jar or pot (see Table 4 for detailed treatment descriptions).
Seedlings grown in Leonard jars were replicated 5 times. They were replenished with sterile N-free nutrient solution, watered as required and harvested 3 months after transplanting. Seedlings grown in Muguga nursery soil were replicated 10 times. They were watered regularly to field capacity and harvested at 2, 4 and 6 months after transplanting. Both experiments were laid out in randomized complete block designs.
Analysis of variance (ANOVA) and the Newman and Keuls test were performed on all data to detect significant differences between treatment means.

Evaluation of selected rhizobia and AM for in nursery and field conditions

Dual inoculation experiments were conducted in Kenya in collaboration with Partner 4 (CEH) using 2 contrasting soil: Embu and Maseno. For both sites, nursery and field experiments were set up and the same four inoculation treatments were applied: Seedlings of *C. calothyrsus* were inoculated with the multi-strain *Rhizobium* culture and AM inoculants, either singly or in combination, with appropriate uninoculated control treatments. The experiments also compared the response of 2 provenances (San Ramón and Flores) to inoculation, and the effect of soil solarisation on the performance of the inoculants. These experiments were set up such that one part was harvested after 16 weeks of growth in the respective nurseries and another transplanted to field trials located in Maseno and Embu area under on-station and on-farm conditions.

Results achieved

Culture collection

A total of 221 rhizobia were isolated, either directly from nodules collected in field conditions or trap host plants under glasshouse conditions. Isolates originated from nodules of various *C. calothyrsus* provenances, which also included naturalized Kenyan populations. The isolates were all fast growing on yeast extract mannitol agar (YEMA) media. Results of mycorrhizal isolation work carried out by CEH indicated that the various soils contained several AM species with the poorest site, Nyabeda in western highlands, having the highest AM species diversity among the Kenyan soils (Lesueur *et al.*, 2001).

Assessment of indigenous rhizobia and genetic compatibility with provenances of C. calothyrsus

Results of MPN showed that the Kenyan soils had varying indigenous population densities. These densities were, in most cases, higher in soils proximal to *C. calothyrsus* plants, indicating localized build up around the roots (Fig. 1). The host genotype also appeared to influence the size of indigenous rhizobia (Fig. 2). This result demonstrated the genetic variation in nodulation and N_2-fixation, but also emphasized the need to exercise caution in interpreting MPN results by plant infection methods. The practical implication of these results, which estimated indigenous populations of up to 10^5 g^{-1} of soil in some sites, is that inoculation response would only be achieved by an effective and highly effective inoculant strain in cases of ineffective indigenous rhizobia.

Table 1. Effect of *Rhizobium* strains on shoot dry wt, nodule numbers $plant^{-1}$, nodule dry wt, nodule specific wt and relative effectiveness index of *C. calothyrsus* provenances Flores and San Ramón after 12 weeks of growth in Leonard jars. Each value is the mean of 8 replicates. This is an abridged Table showing only 4 Kenyan strains representing effective and ineffective types.

Provenance	Strain[a]	Shoot dry wt (mg)	No. of nodules plant^{-1}	Nodule dry wt (mg)	Nodule specific wt (mg)	Effectiveness index
Flores	Uninoculated (-N)	110	0	0	-	-
	Uninoculated (+N)	573	0	0	-	-
	KCC1	461	158	48	0.17	3.2
	KCC6	571	56	47	0.61	4.2
	KCC11	141	76	13	0.21	0.3
	MUN 24	447	49	30	0.55	3.1
	TAL 1145	382	40	31	0.38	2.5
	KCC39	547	73	45	0.65	4.0
San Ramón	Uninoculated (-N)	128	0	0	-	-
	Uninoculated (+N)	400	0	0	-	-
	KCC1	91	22	10	0.72	-0.3
	KCC6	650	60	62	1.29	4.1
	MUN 24	524	45	27	0.54	3.1
	TAL 1145	492	30	21	0.76	2.8
	KCC39	511	29	29	0.75	3.0
	LSD[b]	304	51	28	0.63	-

[a]KCC, Kenya calliandra collection; MUN, Moi University collection, TAL, NifTAL Project collection, University of Hawaii
[b]LSD, Least significant difference at $P< 0.05$

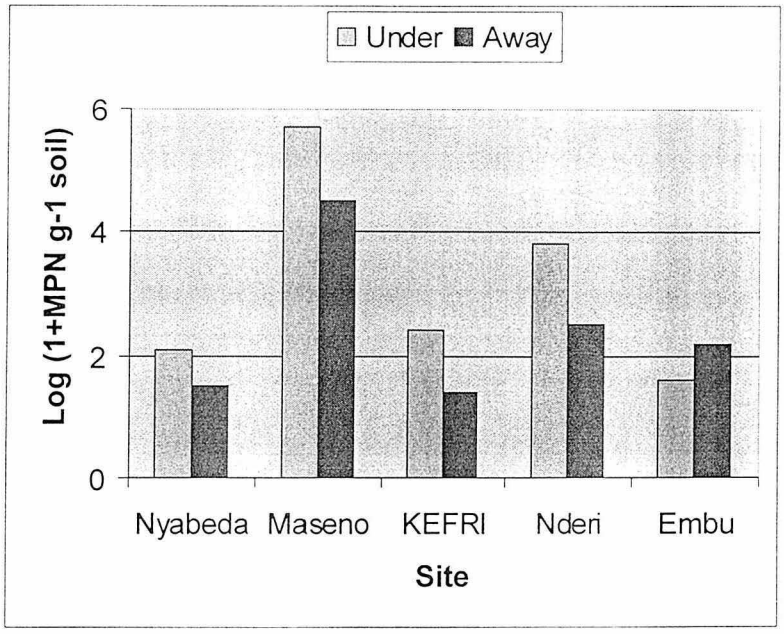


Fig. 1. Effect of *C. calothyrsus* (San Ramón) proximity on the size of indigenous rhizobia in various Kenyan sites

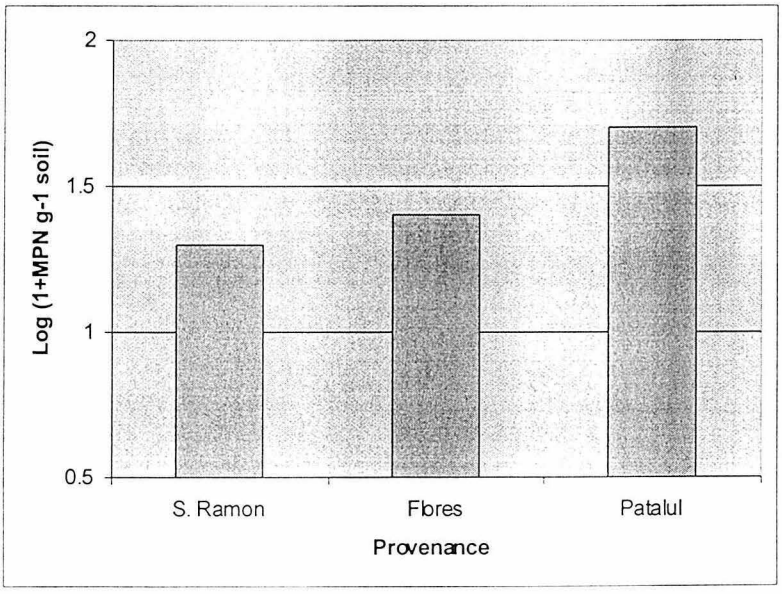


Fig. 2. Variation in genetic compatibility between indigenous rhizobia and *C. calliandra* genotypes in Muguga soil, Kenya

Strain selection
(i) Selection in sterile conditions (Leonard jars)

Most of the strains were effective either on one or both provenances, with only 2 (KCC1 and KCC11) showing effectiveness indices ≤ 0 (Table 1).

(ii) Strain selection in potted field soils

Generally, there was lack of rhizobial inoculation response in Muguga soil (data not shown). This was attributed to the relatively high fertility and presence of effective indigenous rhizobia (see Fig. 2). One of the strains, KCC17, isolated from this soil was also selected as an inoculant strain. On the other hand, there was significant ($P<0.0001$) rhizobial strain inoculation response with a number of strains in number of nodules plant^{-1} in Maseno soil (Table 2). Significant ($P<0.01$) strain effect was also observed in root dry wt. Significant ($P<0.05$) provenance effects were observed in nodule and total plant dry wt. There was also significant ($P<0.01$) interaction effect between strain and provenance in nodule numbers plant^{-1}. However, there was no single strain that consistently showed inoculation response in all parameters. It was therefore decided to use a mixture of the most effective strains as multi-strain inoculant for unsterile conditions (nursery and field environments).

(iii) Dual rhizobial and mycorrhizal inoculation

ANOVA results indicated significant differences ($P=0.0067$) due to mycorrhizal inoculation only for nodule numbers plant^{-1} in Mama Wachira soil (Tables 3). Plant height, number of nodules plant^{-1}, and nodule dry wt parameters were also significantly ($P<0.01$) different in both soils due to provenance effect. Significant provenance ($P<0.01$) effect was also observed for shoot and total plant dry wt in Mama Wachira soil and root dry wt in Nyabeda soil. Mycorrhizal inoculation response was highly variable, and depended on soil, provenance and parameter. In some parameters, uninoculated controls performed better than inoculated treatments. Greater dry matter was accumulated in Mama Wachira soil than Nyabeda soil (data for Nyabeda soil not shown), thus reflecting differences in soil fertility. Flores appeared to produce more shoot and total plant dry wt than San Ramón in the more fertile Mama Wachira soil, whereas the reverse was true for root dry wt. Nodulation (number of nodules plant^{-1} and nodule dry wt) was also more prolific in Flores than San Ramón, and in both soils.

Inoculation techniques for Calliandra

There were significant growth responses due to inoculation in seedlings grown in Leonard jars (Table 4). Among inoculated treatments, significant differences were observed only in nodule number plant^{-1} and height. Growth responses due to seed inoculation (treatments KM1 and KM2) were comparable to seedling inoculation (treatments KM3 and KM4) except for seedling height parameter. There was more than a 2-fold increase in both seedling height and total dry weight over non-inoculated control due to inoculation at 50 % seedling emergence. In Muguga nursery soil, seedling nodulation and growth were optimal even without inoculation (data not shown). This result was not surprising because the effective inoculant strain (KCC17) was originally isolated from this soil with an indigenous population density of up to 30 rhizobia g^{-1}. On the basis of this study, we recommend that *C. calothyrsus* seedlings raised in unsterile soil conditions should be inoculated soon (≤ 1 week) after sowing.

Table 2. Effect of rhizobial inoculation on shoot, root, nodule and total plant wt, and number of nodules plant^{-1} of *C. calothyrsus* provenances San Ramón and Flores after 24 weeks of growth in potted Maseno soil, Kenya

Provenance	Strain	Shoot dry wt (g plant^{-1})	Root dry wt (g plant^{-1}).	No. of nodules plant^{-1}	Nodule dry wt (g plant^{-1}).	Total dry wt (g plant^{-1})
San Ramón	KCC6	1.39	1.06	64	0.092	2.53
	CCCR15	1.46	1.53	19	0.062	2.94
	CCNC26	1.05	1.11	13	0.061	2.20
	CCK13	1.05	0.86	14	0.037	1.89
	CCCR1	1.39	1.07	20	0.053	2.49
	CCC22	1.30	1.41	9	0.050	2.58
	KCC39	2.19	1.23	30	0.040	2.39
	KCC17	1.64	1.30	26	0.056	3.00
	KWN35	1.15	1.03	14	0.032	2.12
	Control	1.15	0.86	11	0.032	1.80
Flores	KCC6	1.66	1.43	43	0.081	3.16
	CCCR15	1.51	1.23	24	0.081	2.76
	CCNC26	1.69	1.16	25	0.085	2.91
	CCK13	1.74	1.27	27	0.093	3.05
	CCCR1	1.65	0.95	24	0.062	2.66
	CCC22	1.71	1.39	20	0.071	3.15
	KCC39	1.68	1.08	42	0.127	2.86
	KCC17	1.28	0.91	26	0.095	2.30
	KWN35	2.68	1.04	27	0.080	2.65
	Control	1.22	0.84	9	0.044	2.08
	LSD[a]	NS[b]	0.38	13	0.048	0.81

[a]LSD, Least significant difference at $P < 0.05$
[b]NS, not significant

Table 4. *Effect of mycorrhizal cultures on plant height, number of nodules plant^{-1}, and shoot, root, nodule and total plant dry wt of* C. calothyrsus *provenances San Ramón and Flores after 24 weeks of growth in potted Mama Wachira field soil, Kenya*

Provenance	Strain	Plant height (cm)	No. of nodules plant^{-1}	Nodule dry wt (g).	Shoot dry wt (g)	Root dry wt (g).	Total dry wt (g)
San Ramón		41.8	325	0.231	5.86	2.32	8.41
	Gi. albida II	49.8	267	0.127	5.61	1.73	7.46
	G.etunicatum	40.4	263	0.164	5.73	2.16	8.06
	S. calospora	37.9	363	0.207	4.29	2.16	6.66
	S.verrucosa	36.5	183	0.162	4.36	2.37	6.20
	Control	31.3	228	0.175	4.33	2.03	6.52

Flores		44.7	628	0.264	6.09	2.17	8.49
	Gi. albida II	45.0	642	0.257	6.39	2.14	8.78
	G.etunicatum	52.8	446	0.362	7.49	2.31	10.05
	S. calospora	48:6	701	0.310	5.86	2.14	8.40
	S.verrucosa	44.4	417	0.291	6.03	1.71	8.01
	Control	50.3	470	0.243	7.11	2.47	9.82
	*LSD[a]	13.1	194	0.136	2.06	NS[b]	2.64

[a]LSD, Least significant difference at $P < 0.05$
[b]NS, not significant

Inoculant production
Filtermud is widely used for legume inoculant production in Kenya. The quality of *Calliandra* inoculants was to inoculants produced for other species. There has a growing interest in *Calliandra* inoculants since KEFRI started production for this species 2 years ago. Production of *C. calothyrsus* rhizobial inoculants presently accounts for 50 % of the total production by KEFRI (Fig. 3). Other species for which rhizobial inoculants are produced include *Leuceana leucocephala, L. trichandra, Acacia tortilis, A. polyacantha, A. senegal* and *A.* (syn. *Faidherbia*) *albida.*

Evaluation of selected rhizobia and AM in nursery and field conditions
Both nursery experiments in Kenya showed that all inoculation treatments increased seedling growth when compared with the uninoculated control seedlings, and that dual inoculation with the multi-strain *Rhizobium* and AM inoculants increased plant growth more than single

inoculation with either microsymbiont, underlining the synergistic benefits of dual inoculation. We obtained better inoculation responses in this experiment than the dual inoculation experiment using Mama Wachira (Embu) and Nyabeda

Table 4. Effect of inoculation method, using strain KCC 17, on nodulation and growth of *C. calothyrsus* seedlings after 3 months of growth in Leonard jars

Method of inoculation[a]	Number of nodules plant^{-1}	Nodule dry weight (g plant^{-1})	Seedling height (cm)	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Total dry weight (g plant^{-1})
KM1	89b[b]	0.037a	13.9bc	0.312b	0.101a	0.444b
KM2	59a	0.031a	14.9cd	0.290b	0.088a	0.393b
KM3	67a	0.034a	17.1de	0.343b	0.097a	0.482b
KM4	54a	0.028a	11.5b	0.263b	0.089a	0.381b
KM5	NA[c]	NA	7.8a	0.136a	0.085a	0.227a

[a]KM1, inoculation of pre-treated seeds prior to sowing in germination trays; KM2, inoculation of pre-treated seed immediately after sowing in germination trays; KM3, inoculation after 50 % seedling emergence in germination trays; KM4, inoculation of seedling immediately after transplanting; KM5, control - uninoculated seedlings

[b]Values in a column followed by the same letter are not significantly different according to the Newman and Keuls test at $P < 0.05$

[c]Not applicable

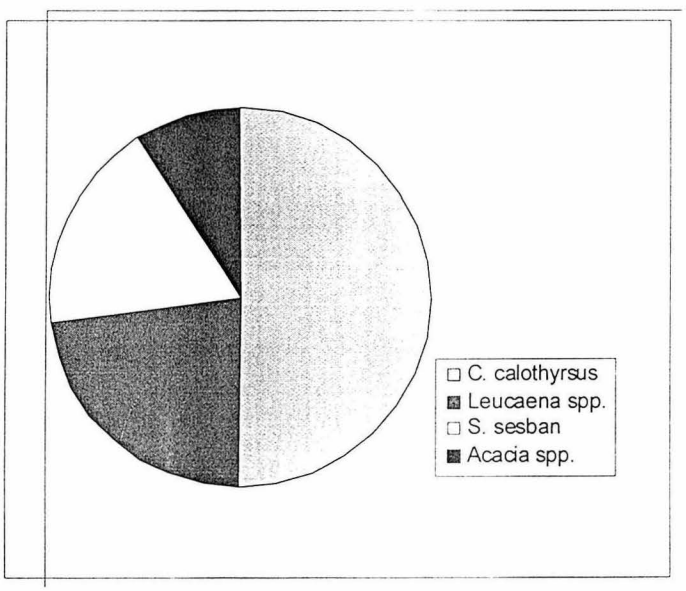


Fig. 3. Inoculant production at KEFRI

(Maseno) soils. This may be attributed to differences in site conditions and natural microsymbionts fluctuations due to temporal or seasonal variations. Partner 4 has presented detailed results.
Part of the experimental seedlings was also transplanted and will be assessed regularly to evaluate the long-term impact of dual inoculation under field conditions.

Problems
There was no problem encountered.

Technology implementation plan
There are no plans to commercially exploit the inoculant techniques developed.

Publications and papers
Papers
Lesueur, D., Ingleby, K., Odee, D., Chamberlain, J., Wilson, J., Manga, T. T., Sarrailh, J-M., Pottinger, A. (2001). Improvement of forage production in *Calliandra calothyrsus*: methodology for the identification of an effective inoculum containing *Rhizobium* strains and arbuscular mycorrhizal isolates. *Journal of Biotechnology* **91**: 269-282.
Muok, B.O., Gudu, S.O., Odee, D.W. 1998. A broad-range inoculant for legume trees in acid soils: fixing nitrogen in sub-Saharan Africa. *Agroforestry Today* **10(3):** 12-13.
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. Submitted to *Biology and Fertility of Soils.*
Odee, D., Ingleby, K. *et al.*, Growth of arbuscular mycorrhizal fungi in pot cultures using different Kenyan soils and the implications for inoculum production. (in preparation)

Posters
Odee, D.W. 1998. Biodiversity and ecology of African tree rhizobia. *In* Dakora, F.D. (ed.), pp 193-194, Proceedings of the Eighth Congress of African Association for Biological Nitrogen Fixation, November 23-27, Cape Town, South Africa.
Odee, D.W., Njoroge, J., Esitubi, M., Oyoo, E., Ochieng, J., Mugwe, J., Niang, A. 1998. Microsymbiont status of *Calliandra calothyrsus* grown in the highlands of Kenya for soil fertility and fodder production. *In* Dakora, F.D. (ed.), pp 196, Proceedings of the Eighth Congress of African Association for Biological Nitrogen Fixation, November 23-27, Cape Town, South Africa.

Conclusions
Through this project, we have established an ecologically diverse collection of microsymbionts, and selected effective rhizobia and AM, which is now available for inoculation of *C. calothyrsus* for fodder and soil fertility improvement in Kenya and the East and central African region. Our capacities in studying and utilizing these microsymbionts have been improved through training, research equipment and active interactions within the project and several scientific fora. The technical information obtained in this project is applicable to other future legume-microsymbiont research and development, and should be repackaged for wider adoption by the smallholder farmers. It was a successful project.

Partner 6 : CNRD

Alan Pottinger

1. Objectives

The objectives of the contribution to the project by OFI and CNRD were as follows:

- ***Advise on matters relating to seed selection***
  - Using results from 10 years of research on provenances selection of *Calliandra calothyrsus* coordinated by the researchers to select provenances suitable for use in the project.
- ***Arrange despatch of seed of C. calothyrsus to project partners***
  - Using the seed store of the Oxford Forestry Institute, UK, to send quantities of seed to project partners for experimentation.
- ***Arrange collection and despatch of microsymbionts from Central America and Mexico***
  - Organise and carry out a field visit to Central America and Mexico to collect and despatch to project partners a range of Rhizobia and michorrizae from their native range.
- ***Design, construct and maintain a project website***
  - Using relevant software, create a website that displays project objectives, activities and publications in order to provide an up-to-date source of information on project progress and outputs.

2. Activities

Advise on matters relating to seed selection

From 1990 onwards the Oxford Forestry Institute (OFI) carried out research into the characteristics of *C. calothtyrsus* that make it suitable as an agroforestry tree for poor farmers in the tropics. This research included exploration of the tree in its native range of Central America and Mexico, identification of potential uses in local agroforestry systems, reproductive biology, collection of seed from throughout the native range, and management of an international provenance trial network of the species.

The introduction of a tree species into a country is rarely carried out in a planned and systematic manner. Ideally, the full range of genetic variability should be evaluated for productivity and assessed for environmental impact prior to release of seed or vegetative material for planting programmes. In practice, however, most tree introductions take place in an *ad hoc* manner, sometimes through the interests of plant collectors, often by travellers or colonists bringing with them trees which they know to be useful in their homelands, and sometimes by foresters wishing to experiment with new species. Whatever the means of introduction, the eagerness to investigate new species coupled with a lack of appreciation of the genetic diversity contained within most species generally means that introductions are made without any prior formal evaluation and are usually of narrow genetic base (Hughes, 1994). Although introduced species frequently grow well in comparison with native species, it is the experience of foresters in both tropical and temperate countries that the initial source of seed used for introductions is rarely the most productive (Zobel and Talbert, 1984; Barnes and Simons, 1994).

Provenance trials enable a range of seed sources of a single species to be evaluated on a given site and provide evidence upon which to base future decisions surrounding seed procurement. However, they are frequently overlooked in introduction programmes partly due to the expense and practical difficulty in obtaining a wide range of seed sources, but also often due to a lack of understanding of their fundamental importance to future planting decisions. Large gains in productivity can be made through the selection of the best provenance of a species for a given site and purpose.

For *C. calothyrsus*, along with many other agroforestry tree species, the initial interest in the performance of the species developed from a limited selection of the genetic diversity contained within the species (Pottinger, 1996). *C. calothyrsus* is planted widely in Indonesia, and until recently almost all seed available to researchers and those interested in planting programmes, came from this source. This 'land race' was itself derived from seed originating from a small area of Guatemala. In view of the growing international interest in *C. calothyrsus* for use on farms, combined with the lack of knowledge surrounding its genetic resources, a programme was initiated in 1990 at the Oxford Forestry Institute (OFI) to carry out a comprehensive exploration, collection and evaluation of *C. calothyrsus* and its close relatives (Macqueen 1991; Pottinger 1996).

The results from provenance trials carried out in over 30 countries indicated that seed from Flores in Guatemala and from San Ramon in Nicaragua performed best overall, although there was significant variation in performance amongst sites (Chamberlain, 2002). Project partners in **IC18-CT97-0194** were encouraged to use seed from these sources for experiments. In addition, however, it was agreed that the criteria by which the provenances were evaluated in the international provenance trials (i.e. production of fuelwood and fodder) may be influenced by the interaction of microsymbiont inoculation and, as a result, additional provenances and closely related species were also included in the project.

Arrange despatch of seed of C. calothyrsus to project partners

Seed was sent from the seed store of the OFI to project partners as outlined in Table 1.

TABLE 1 Seed despatched to project partners

Date of order	Content of order			Recipient
	Species	**Provenance**	**Weight (gms)**	
April 1998	*C. calothyrsus*	Flores	100	Theophile Tiki Manga (IRAD, Cameroon)
Sept. 1998	*C. calothyrsus*	Flores	400	David Odee (KEFRI, Kenya)
January 1999	*C. calothyrsus*	Colotilda	20	Didier Lesueur, (CIRAD, Senegal)
	C. acapulsensis	El Mesquite.	15	"
	" "	Playa Azul	30	"
	C. grandiflora	Yahuitlan	7	"
	C. houstoniana	Rio Hondo	30	"
	" "	La Esmeraldo	50	"
	" "	Minatitlan	50	"
	" "	Tuxtepec	100	"
	C, juzepczukii	Cintalapa	30	"
	C. longepedicellata	Las Joyas	30	"
	C. physocalyx	Putla de Guerrero	30	"
March 1999	*C. calothyrsus*	Patulul	40	"
	" "	Santa Maria de Jesus	40	"
	" "	Patulul	40	"
	" "	Santa Maria de Jesus	40	"
	" "	Patulul	40	"
	" "	Santa Maria de Jesus	20	"
March 1999	*C. calothyrsus*	Flores	30	Kevin Ingleby (CEH, UK)
April 1999	*C. calothyrsus*	San Ramon	100	"
May 1999	*C. calothyrsus*	Flores	14	Jean Michel Sarrailh (CIRAD, New Caledonia)

Arrange collection and despatch of microsymbionts from Central America and Mexico

The collection of soil and root nodule samples for culturing and subsequent evaluation with *Calliandra calothyrsus* was undertaken in Honduras, Nicaragua, Costa Rica, Guatemala and Mexico. The collections were made in collaboration with CONSEFORH (Conservation and Silviculture of Honduran Dry Zone Forest Species Project) in Honduras, ICTA (Instituto de Ciencas Tecnicas Agricolas) in Guatemala, and UNAM (Universidad Nacional Autonoma de Mexico), the Instituto de Ecología, Jalapa, and the British Council in Mexico (collecting permit No. DOO750-1803/98).

In general, the success in finding fresh, white to pink root nodules that contained live *Rhizobia* was varied, but in three sites no nodules were found (Ocoman, Honduras; Zihuatenjo, Mexico; Playa Azul, Mexico). Old nodules (mid- to dark brown in colour) were found in the majority of the sites, and seemed to be characteristic of the drier sites that receive less rainfall. A small sample of soil (~100 g) was collected from each site and was sent with the nodules to Didier Lesueur at CIRAD-For_t, ORSTOM-Bel Air, Dakar, Senegal.

Soil samples were collected from within the first 20 cm depth of soil, and within a radius of 50 cm from the main stem of the tree. Soil samples were taken from 8-12 trees scattered across the population and the samples bulked. At least one kilo of soil was sampled, usually more, and placed in a sealable plastic bag. Both the soil and nodule samples were kept in a cool box during transit. The nodules were kept dry and maintained at a temperature of 15-20 °C, and the soil was kept moist and maintained at the same temperature. The soil samples were sent, along with a kilo of sorghum seed from Honduras, for mycorrhiza culturing to ITE, Scotland, UK.

Details of the site locations, mean annual rainfalls, soil types, vegetation cover, etc., were copied from the OFI seed collection manual and were included with each soil and nodule sample. Table 2 is a summary of the collections made.

TABLE 2 Summary of the populations of *Calliandra calothyrsus* sampled for soil and root nodules.

Date	Population	Nodules	Soil	Vegetation
30/1/98	La Ceiba, Honduras	120 new white nodules from 12 trees	Soil samples from below 12 trees	Collection made on riverbanks - not much vegetation below trees.
31/1/98	Ocoman, Honduras	No nodules - one or two dead ones observed	Soil samples from below 8 trees	Collection made in a roadside population with a sparse covering of grass.
2/2/98	Santa Maria, Honduras	60 old nodules from 10 trees (3 trees had only 2 or 3 nodules)	Soil samples from below 10 trees	Collection made in a roadside with a sparse covering of grass and trees, and from a maize field.
5/2/98	San Ramon, Nicaragua	80 old nodules from 12 trees	Soil samples from below 12 trees	Grass and other leaf litter present. Collection made in a roadside population.
6/2/98	Santo Tomos, Nicaragua	70 old nodules from 8 trees	Soil samples from below 10 trees	Grass and other leaf litter present over hard, dry soil. Collection made in the roadside.
7/2/98	La Puerta, Nicaragua	100 old nodules from 12 trees (3 trees had the majority of the nodules)	Soil samples from below 10 trees	Vegetation cover generally grass and other small bushes/ trees. Collection made in field margins. Soil hard and compacted.

12/2/98	Fortuna, Costa Rica	50 new and 50 old nodules from 10 trees	Soil samples from below 10 trees	Vegetation cover generally grass, sandy soils in some places.
13/2/98	Turrialba, Costa Rica	70 new and 20 old nodules from 10 trees	Soil samples from below 10 trees	Lots of vegetation cover hence other tree, grass and vine roots. Collection from river site only.
14/2/98	San Isidro, Costa Rica	40 new and 60 old nodules from 7 trees	Soil samples from below 10 trees	Lots of leaf litter, other plant roots and large stones. Collections made in the Rivas to Caanan site.
24/2/98	Patulul, Guatemala	70 new and 20 old nodules from 8 trees (more trees with no or 2-3 nodules)	Soil samples from below 12 trees	Loose soil, low density of vegetation. Soil sampled from a second area - drier, more vegetation (king grass & thorny legumes).
25/2/98	Santa Maria de Jesus, Guatemala	More than 150 nodules from 10 trees	Soil samples from below 10 trees	Loose, fine soil sampled from trees on a slope. Associated vegetation mostly woody.
25/2/98	Santa Maria de Jesus, Guatemala	None collected	Soil samples from below 8 trees in a coffee plantation.	Similar soil type as above but with more organic matter.
26/2/98	Cuyuta, Guatemala	100 nodules from 8 trees. Large amounts of old nodules attached to fine roots in leaf litter. Trees had not been inoculated.	Soil samples from below 8 trees	Loose, dry soil. Very little other vegetation due to closed canopy. Mostly *Calliandra* leaf litter.
27/2/98	Flores, Guatemala	80 nodules from 7 trees (a few more trees with 2-3 nodules per tree)	Soil samples from below 10 trees	Generally dry and hard soil with many stems and roots. Lot of associated woody vegetation and vines, and leaf litter.
6/3/98	Zihuatenjo, Mexico	No nodules, either old or new	Soil samples from below 10 trees	Dry and rocky soil. Lot of associated woody legume vegetation and vines. Little surface vegetation due to a canopy of vines covering trees and bushes.
7/3/98	Playa Azul, Mexico	Generally, no nodules, either old or new; two nodules collected from one tree.	Soil samples from below 8 trees	Dry and rocky soil with many stems and roots. Lots of associated woody vegetation and vines, and surface litter composed of leaves and dead stems.
10/3/98	Plan del Rio, Mexico	110 nodules collected from 10 trees. Lots of large, fresh nodules observed. Duplicate left in Mexico.	Soil samples from below 10 trees	Generally moist, loose soil. Lots of associated woody legume vegetation. Soil surface litter mainly dead stems and leaves.

A small proportion of the nodules collected from Plan del Rio, Mexico were left with Dra. Valeria Souza at the Instituto de Ecología, UNAM, AP 70-275 Coyoacan 04510, Mexico D.F. tel: (5) 622-9006, fax: (5) 622-8995, email: souza@servidor.unam.mx. It was planned that Dra. Souza would culture the *Rhizobium* from this sample and store should it be required at a later date by Didier Lesueur. Dra. Souza also expressed the willingness to multiply inoculum for CONSEFORH for a fee

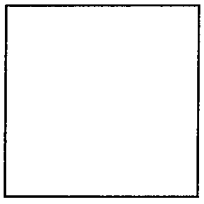
and as required for their field experiments. Dra. Souza's laboratory is very well-equipped, and the multiplication of inoculum is routine for them. This seems to be a sensible arrangement given the unsuitability of the UPIL equipment for CONSEFORH. (Subsequent discussions did not lead to a significant contribution to the project by Dra Souza).

Dra. Souza also suggested an alternative way of storing root nodules, i.e., on a small piece of root in Eppendorf tubes containing a small piece of slightly damp cotton wool. The nodules from Plan del Rio were therefore split into two lots and sent to Didier Lesueur, one lot as described above, and the second as Dr Lesueur had suggested, wrapped in dry paper.

Acknowledgements: The collection trip was completed successfully with the help and goodwill of many people. Thanks go to Kevin Crockford, Ernesto Ponce and Gaspar Alvarado at CONSEFORH; Carlos Rodriguez and Juan Alberto Qui_onez at ICTA; Hector Hern_ndez and Valeria Souza at UNAM; Raul Benet at the British Council, Mexico for his abundant help, advice and contacts; Michael Keyes at the Instituto de Ecología, Jalapa for his goodwill, help and enthusiasm; Felipe Ramirez Ruiz de Velasco at SEMARNAP, Mexico for issuing the necessary collecting permit.

Design, construct and maintain a project website

A project website was developed and stored by the University of Oxford, UK. The website can be accessed at http://users.ox.ac.uk/~dops0024/EUmicrosymbionts/index.htm



The website was constructed in Microsoft FrontPage and is divided into the following sections:

- Introcudtion to the project
- Introduction to *Calliandra calothyrsus*
- Project activities (each partner had a separate page on which to report their on-going activites)
- Project publications
- Annual reports (downloadable directly from the website)

The website is updated frequently through suggestions and inputs from partners.

3. Publications

Lesueur D, Ingleby K, Odee, D, Chamberlain J, Wilson J, Tiki Manga T, Sarrailh JM, Pottinger A (2001) Improvement of forage production in *Calliandra calothyrsus* : Methodology for the identification of an effective inoculum containing *Rhizobium* strains and arbuscular mycorrhizal isolates. J. Biotechnol. 91:269-282.

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Chamberlain, J.R. (2002) (ed.) Calliandra calothyrsus: an agroforestry tree for the humid tropics. OFI Tropical Forestry Paper 35. 280 pp

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Partner 7 : University of Helsinki

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OBJECTIVES

Calliandra calothyrsus can form nodules and fix nitrogen in symbiosis with root-nodule bacteria (rhizobia). Rhizobia are in general a diverse group of gram-negative soil bacteria including the genera *Rhizobium*, *Agrobacterium*, *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium*. Previous studies suggest that most of the tropical woody legumes are nodulated by a wide range of different rhizobia (de Lajudie et al. 1994, Bala and Giller 2001). However, not all of them are capable of inducing effective, nitrogen fixing nodules (Räsänen et al. 2001). One aim of the whole project was to find tested and effective rhizobial strains to use for inoculation *C. calothyrsus* tree seedlings in the field. Therefore, a large collection of bacteria isolated from root nodules of *C. calothyrsus* was established, and the ability of each strain to nodulate and fix nitrogen was investigated by other partners.

The objective of our work was to evaluate the genetic diversity of 293 rhizobial isolates collected from the root nodules of *C. calothyrsus* growing in its native region in Central America (Mexico, Honduras, Guatemala, Nicaragua and Costa Rica) and in areas, to which the tree has been introduced (New Caledonia, Cameroon and Kenya). The diversity of rhizobial strains was evaluated by PCR-RFLP analysis of the 16S rDNA gene (PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism). Ribosomal 16S and 23S rDNA genes are present in all bacteria and are regarded as highly conserved. Because they evolve slowly, they are useful for studying distantly related organisms at the level of genus or above (Gürtler et al. 1991, Laguerre et al. 1994, Nick 1998). However, in the case a large collection of bacteria, the traditionally used methods, including a distinct DNA isolation phase, was too laborious, time consuming, and more susceptible to mistakes. Use of integrated workstations (biorobots) is still only rarely possible in the laboratories. Therefore, in order to be able to screen 293 isolates, we developed an optimised and rapid semi-automated system for PCR-RFLP analysis of 16S rDNA gene.

ACTIVITIES

Methodology used

Bacterial strains and growth conditions. Nodule samples were collected year 1998 from several sites in Central America by ITE and CONSEFORH, in Cameroon by IRAD, in Kenya by KEFRI and in New Caledonia by CIRAD-Forêt. Later, rhizobial strains were isolated, inoculated with *C. calothyrsus* seedlings and reisolated from nodules by CIRAD-Forêt and KEFRI. We obtained most of the isolates (238) in the beginning of year 1999 and Kenyan isolates (19) in spring 2000. In order to check the purity and uniformity of the obtained strains, additional recultivation tests were carried out at University of Helsinki in spring 1999. To separate possible different bacteria from each other, the strains, which were cultivated in yeast-mannitol (YEM) broth, were diluted with Tween buffer so that the first dilution was shaken at least for half an hour. After dilution rhizobia were plated on YEM agar containing Congo red.

Genetic analysis. The preliminary 16S rDNA PCR-RFLP analysis of 24 strains in summer 1999 suggested that the bacterial collection contained several different taxa. Therefore,

the isolates were in future screened by 16S rDNA PCR-RFLP analysis, which separated bacteria at the genus level.

In RFLP, PCR products (amplified DNA) are digested with different frequently cutting endonucleases and restriction site differences of PCR-amplified 16S rDNA can be detected. The resulting fragments are separated by electrophoresis and compared pairwise. According to the information obtained from digestion with different enzymes, a DNA genotype is generated and compared to other samples. Variations in RFLP genotypes are often displayed as dendrograms based on genetic similarities.

During the work it became evident that the traditional method was too laborious and time consuming for analysis of around 300 bacteria. Therefore, in collaboration with Lars Paulin, Institute of Biotechnology, Helsinki, we started to develope a semi-automated system for screening bacteria in the winter of 2001. To facilitate the identification of the genotypes obtained, representatives of each genotype were run together with the 30 reference strains representing different, defined *Rhizobium*, *Agrobacterium*, *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium* species (Fig. 1).

Procedure for the semi-automated 16S rDNA PCR-RFLP analysis

Preparation of template DNA for PCR. For PCR reactions, all strains were grown in 250 µl yeast-mannitol medium for 2 days at 28°C in 2.2 ml 96 Deep Well Plates (ABgene®House, UK). After 2 days of cultivation, all 250 µl of culture was carefully washed two times with 250 µl of distilled, autoclaved water. Finally, washed cells were suspended in 10 µl water and stored at -70°C until use.

PCR reaction. PCR reactions were carried out in 0.2 ml Skirted Termo-Fast®96 Tube Plates (ABgene®House, UK) in 50 µl volume containing 15 pmol of rD1 and fD1 primers (Weisburg et al., 1991), 200 µM each of dATP, dCTP, dGTP, dTTP, 2U of Dynazyme polymerase, 1_ buffer supplied with polymerase and 1 µl of previously prepared intact cells as template. The parameters of the cycles included an initial denaturation at 95°C for 5 min (to destroy the cells), followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min with slow ramp to 72°C, extension at 72°C for 3 min and finally 72°C for 10 min. Aliquots of 5 µl of each amplified DNA (96 samples at a time) were examined by horizontal electrophoresis in 1% agarose using the Maxicell® Primo Submarine Gel System (TermoQuest, U.S.A).

Restriction fragment analysis. For RFLP analysis aliquots (8 to 15 µl) of PCR products were digested with three four-base recognizing restriction endonucleases (*Hae I*, *Mbo I*, *Msp I*) as described by Laguerre et al. (1994). Restricted samples were loaded into three different gels, analysed by horizontal electrophoresis in 3% agarose gel (96 samples at a time) and stained with ethidium bromide.

In order to facilitate comparisons, the molecular size standard pGEM (Promega, U.S.A.) was loaded every 8 samples. Electrophoresis was carried out at 100 V for 1 h, followed by 200V for 1 h. The gels were photographed by using KODAK DC290 Zoom Digital Camera and images were stored in TIFF files.

Stored restriction genotypes were analysed with GelCompar 4.1 (Applied Maths, Belgium). To create one file, combined-gels option was used when analysing RFLP genotypes

obtained by using three restriction endonucleases. Following conversion and normalisation, the levels of similarity between all RFLP genotypes were calculated by using the Dice similarity coefficient, S_D, equal to the ratio of twice the number of common bands in two compared patterns, to the sum of all bands in both patterns. Finally, the unweighted pair group method with arithmetic averages (UPGMA) was used to cluster genotypes obtained from representative and reference strains. To verify if the dendrogram faithfully represented the calculated similarity matrix, a cophenetic correlation value (CC) was calculated. The CC value is the product-moment correlation between all original matrix similarities and all corresponding similarity values derived from the dendrogram. If the CC value is high (more than 90%), the similarity matrix can faithfully be represented by an UPGMA dendrogram. The CC value for created dendrogram was 95%, indicating a good quality of the cluster analysis (Fig. 1).

Other activities

Leena Räsänen represented University of Helsinki at the first meeting held in Dakar 1998 and organised by CIRAD-Forêt, and at the third meeting held in Edinburgh 2000 and organised by ITE. Kristina Lindström participated in the fourth, last meeting hold in Montpellier 2001 and organised by CIRAD-Forêt. The poster was presented July 2001 by Kristina Lindström at 13th International Congress on Nitrogen Fixation in Hamilton, Canada.

RESULTS ACHIEVED

Isolates with different colony morphologies. During the testing of purity and uniformity of the obtained isolates, we observed that about 40 strains had at least two different colony types. Thus, the number of isolates increased from 257 to 293. All duplicates were treated as separate isolates during whole analysis.

RFLP analysis showed that most of the duplicate isolates had identical 16S rDNA genotype. In the case of five strains (CCC40/1, 2; CCN13/1, 2; CCH32/1, 2; CCM55/1, 2; CCCR16/1, 2), the duplicate strains belonged to different genotypes. *S. arboris* strain was able to change the colony morphology from slimy to dry after incubation at high temperatures or after resuscitation of lyophilised or frozen cultures (Räsänen et al. 2001). In our work it is, however, still open whether the duplicate strains actually represent two different colony morphologies of the same strain, or if it is an indication that some nodules were occupied by two different rhizobia (strains/species).

Semi-automated PCR-RFLP analysis of the 16S rDNA gene proved to be a relatively rapid and good method for screening a large number of isolates. To analyse 96 samples at a time, a high-speed pipetting system, microtiter plates with 96 tubes for growing bacteria and for PCR reactions, and a gel tray with suitable well combs were used. Hence, the order of the samples should be conserved during analysis. When universal primers fD1 and rD1 were used to amplify the 16S rDNA gene from intact cells without isolating DNA, all strains produced a single band of about 1500 bp, corresponding to the expected size the 16S rDNA gene (Laguerre et al. 1994).

Biodiversity of *Calliandra* rhizobia. The biodiversity of rhizobia nodulating *C. calothyrsus* was large. Fifteen different genotypes were distinguished with the three restriction

enzymes (*Hae I, Mbo I, Msp I*; Fig. 1, Table 1). Six major genotypes had 12-116 strains and eight minor genotypes contained eight strains or less. Major genotypes were represented by genotypes as follows: **A** (116 strains), **B** (63), **C** (40), **D** (24), **E** (14) and **F** (12). Minor groups with low numbers of strains were represented by the following genotypes: **G** (8 strains), **H** (6), **I** (4) and **J, K, L, M, N, O** (1 strain; Fig. 1, Table 1).

Genotypes **A, C, E, G, H** and **I** were clustered with established rhizobial species from the genera *Agrobacterium, Mesorhizobium, Sinorhizobium* and *Rhizobium*, whereas genotypes **B, D,** and **F** remained unclustered (Fig. 1). The biggest group, genotype **A,** included 40% of the 293 bacterial isolates analysed (Fig. 2). *A. rhizogenes, R. tropici* and *R. hainanense*, which clustered together with the representative of genotype **A,** were not distinguished in our work (Fig. 1). This was in agreement with the work of Young and Haukka (1996) and Nick (1998). Nevertheless, strains resembling *R. tropici/R. leguminosarum* were recently found to induce nitrogen-fixing nodules on *C. calothyrsus* (Bala and Giller 2001). This result together with our results suggest that most of the 116 strains in genotype **A** belong to the *R. tropici* species. The representative of genotype **E** had exactly the same 16S rDNA-RFLP pattern as *A. radiobacter, A. tumefaciens* and *M. tianshanense*. The representative of genotype **C** showed 100% similarity with *S. medicae* and *S. meliloti* and the representative of genotype **G** showed 100% similarity with *S. saheli* and genotype was identical with *R. etli* (Fig. 1).

Surprisingly, representatives of the big groups **B, D** and **F** stayed unclustered (Fig. 1). They may represent new groups of rhizobia or be a result of intraspecies of variability. The type strains of *M. amorphae* , *S. fredii* and *S. arboris* remained unclustered. Genotypes **J, K, L, M, N** and **O,** containing only one isolate, did not cluster with defined rhizobial species (Fig. 1). Nevertheless, these strains were reported by CIRAD-Forêt being able to nodulate *C. calothyrsus* tree seedlings.

The grouping of 293 strains presented here is still an initial measure of diversity of large group of bacteria. Partial or full-length sequencing of the 16S rDNA gene will explain, do they belong to described species or do they represent new taxa. Especially the big groups **B, D,** and **F,** which stayed unclustered (no clear similarity to any defined rhizobia) are especially interesting.

Effective and ineffective strains. Most of the isolated strains have formed nitrogen-fixing nodules with *C. calothyrsus*. Only nine strains were reported by CIRAD-Forêt to be unable to form nodules. In our study, these nine strains belonged to several, different genotypes, namely **A** (CCG44), **B** (CCG9, CCNC97, CCC40), **D** (CCN10), **E** (CCNC73), **G** (CCH31) and **H** (CCG1, CCCR33), respectively.

Six strains were found to be very effective in forming nitrogen fixing nodules on *C. calothyrsus* seedlings, and were subsequently used in field tests by other partners. According to the 16S rDNA analysis these strains represented different rhizobial groups: **A** (CCCR15, KWN35, KCC6), **C** (CCCR1, CCC22) and **D** (CCNC26) (Fig. 1). In conclusion, it seems that bacteria belonging to different genera can induce effective nodules on *C. calothyrsus* roots. However, the same groups can contain strains incapable of symbiotic performance.

***Bradyrhizobium*-like strains.** Generally, rhizobia have been divided into fast-growing rhizobia and slow-growing bradyrhizobia (*Bradyrhizobium* sp). Traditionally, *Bradyrhizobium* species have been differentiated from fast growers, because they grow slowly and alkalise YEM agar plates supplemented with pH indicator, bromothymol blue. The majority of

Calliandra strains were fast growers, only 23 isolates were tentatively identified by CIRAD-Forêt according to the pH reaction test as *Bradyrhizobium*-like rhizobia. Except three strains representing genotypes **A, L** and **M**, most of the examined *Bradyrhizobium*-like strains (20/23), belonged to genotype **B**. Surprisingly, genotype **B** contained both alkali producing strains (23) and acid producing strains (40). The genus *Mesorhizobium* harbors strains, which often have a growth rate intermediate between fast-growing and slow-growing. Perhaps these strains are *Mesorhizobium* sp.

Geographical distribution of 16S rDNA genotypes. Generally, the biodiversity of *C. calothyrsus* nodulating strains was greater in Central America and New Caledonia than in Kenya and Cameroon (Fig. 3). It is, however, still open whether the nodulation of *C. calothyrsus* trees in Kenya, Cameroon and New Caledonia was induced by local indigenous rhizobia or rhizobia originating from the native area of *C. calothyrsus*, Central America. Restriction patterns and sequence analysis of the symbiotic genes *nodA* and *nifH* would give more information about their origin (Haukka et al. 1998). Genotype A dominated in almost every country except Costa Rica and Cameroon. (Table 2, Fig. 3).

Problems encountered

The project was scientifically very interesting and challenging to our laboratory. Unfortunately, we were not able to obtain matching funds from national sources, and therefore the amount of funding we had for the work was inadequate. Nevertheless, we were in the end able to analyse a threefold amount of strains than originally promised, by stretching the funds and relying partly on other projects.

Publications and papers

Lindström Kristina, Aneta Dresler-Nurmi, Leena A. Räsänen and Zewdu Terefework. Use of automated systems to study the biodiversity of rhizobia. 13th International Congress on Nitrogen Fixation, 2-9.7.2001, Hamilton, Canada. Poster and abstract.

Conclusion

Semi-automated PCR analysis of 16S rDNA showed that the biodiversity of rhizobia nodulating *C. calothyrsus* was large; the tree can be effectively nodulated by taxonomically diverse rhizobia. Fifteen different genotypes were distinguished with three restriction enzymes (*Hae I, Mbo I, Msp I*). Six major genotypes had 12-116 strains and eight minor genotypes contained eight strains or less. Genotypes **A, C, E, G, H** and **I** were clustered with established rhizobial species, whereas genotypes **B, D, F** remained unclustered. The largest genotype **A** possibly represent the species *R. tropici*. Inoculant strains used in field test by other partners represented genotypes **A** (CCCR15, KWN35, KCC6), **C** (CCCR1, CCC22) and **D** (CCNC26). The biodiversity of isolates was greater in Central America and New Caledonia than in Kenya

and Cameroon. The semi-automated PCR-RFLP method, used in this study, appeared to be a rapid and reliable tool for the initial evaluation of the biodiversity of large number of isolates.

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Haukka, K., Lindström, K., and Young, P. 1998. **Three phylogenetic groups of** ***nodA*** **and** ***nifH*** **genes in** ***Sinorhizobium*** **and** ***Mesorhizobium*** **isolates from leguminous trees growing in Africa and Latin America.** ***Applied and Environmental Microbiology*** **64:419-426.**

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Table 1. Geographical origins of the *Calliandra* isolates and results obtained with the 16S rDNA PCR-RFLP isolates.

PROFILE / ORIGIN	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	No of isolates
MEXICO																total 25
Plan de Rio	2	2	-	1	1	-	-	-	1	-	-	-	-	-		7
Playa Azul	1	2	-	-	-	-	-	-	-	-	-	-	-	-		3
Zihuatanejo	7	3		1		2								1	1	15
HONDURAS																total 28
Santa Maria	6	1	-	1	1	2	-	-	-	-	-	-	-	-		11
La Ceiba	4	3	-	3	3	2	2	-	-	-	-	-	-	-		17
NEW CALEDONIA																total 58
La Couléc	2	-	1	3	1	-	5	-	-	-	-	-	-	-		12
Col Pirogue	4	1	-	1	-	-	-	-	-	-	-	1	-	-		7
Ile de Mar_	8	3	1	-	-	-	-	-	-	-	-	-	1	-		13
Champs de Bataille	2	5	-	1	2	-	-	2	-	-	-	-	-	-		12
Port Laguerre	5	7	-	-	2	-	-	-	-	-	-	-	-	-		14
GUATEMALA																total 57
Cuyata	9	4	1	2	-	-	-	1	-	-	-	-	-	-		17
Patulul	15	2	-	-	-	1	-	-	-	-	-	-	-	-		18
Flores	2	2	1	1	-	-	-	-	3	-	-	-	-	-		9
Santa Maria de Jesus	6	7	-	-	-	-	-	-	-	-	-	-	-	-		13
NICARAGUA																total 36
La Puerta	5	-	1	1	2	-	-	-	-	1	-	-	-	-		10
Nicaragua	1	-	-	2	-	-	-	-	-	-	-	-	-	-		3
San Ramon	6	5	-	-	-	2	-	-	-	-	-	-	-	-		13
Santo Tornes	6	4	-	-	-	-	-	-	-	-	-	-	-	-		10
COSTA RICA																total 34
Turrialba	1	2	5	2	-	-	-	-	-	-	-	-	-	-		10
Fortuna	3	5	2	1	1	1	1	-	-	-	-	-	-	-		14
San Isidro	3	3	-	1	-	-	-	3	-	-	-	-	-	-		10
CAMEROON																total 35
Yaoundé	-	-	14	-	-	-	-	-	-	-	1	-	-	-		15
Ebolowa	2	3	14	-	1	-	-	-	-	-	-	-	-	-		20
KENYA																total 20
Owila Farm, Siaya	1	-	-	-	-	-	-	-	-	-	-	-	-	-		1
Maseno	3	-	-	1	-	-	-	-	-	-	-	-	-	-		4
Nderi, agro-farm	2	-	-	-	-	-	-	-	-	-	-	-	-	-		2
Nyabeda	2	-	-	-	-	2	-	-	-	-	-	-	-	-		4

KEFRI Office	2	-	-	-	-	-	-	-	-	-	-	-	-	-		2
Mama Elizabeth Farm	1	-	-	-	-	-	-	-	-	-	-	-	-	-		1
Embu	-	-	-	2	-	-	-	-	-	-	-	-	-	-		2
C.C. Kari	1	-	-	-	-	-	-	-	-	-	-	-	-	-		1
Muguga nursery	1	-	-	-	-	-	-	-	-	-	-	-	-	-		1
Narobi	1	-	-	-	-	-	-	-	-	-	-	-	-	-		1
Kenya	1	-	-	-	-	-	-	-	-	-	-	-	-	-		1
TOTAL	116	63	40	24	14	12	8	6	4	1	1	1	1	1	1	293

Fig. 2. Proportion of different 16S rDNA genotypes within 293 rhizobial strains isolated from *Calliandra calothyrsus* nodules in Central America, New Caledonia, Cameroon and Kenya

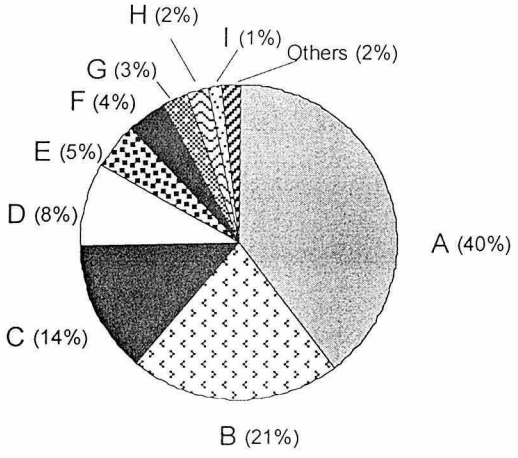
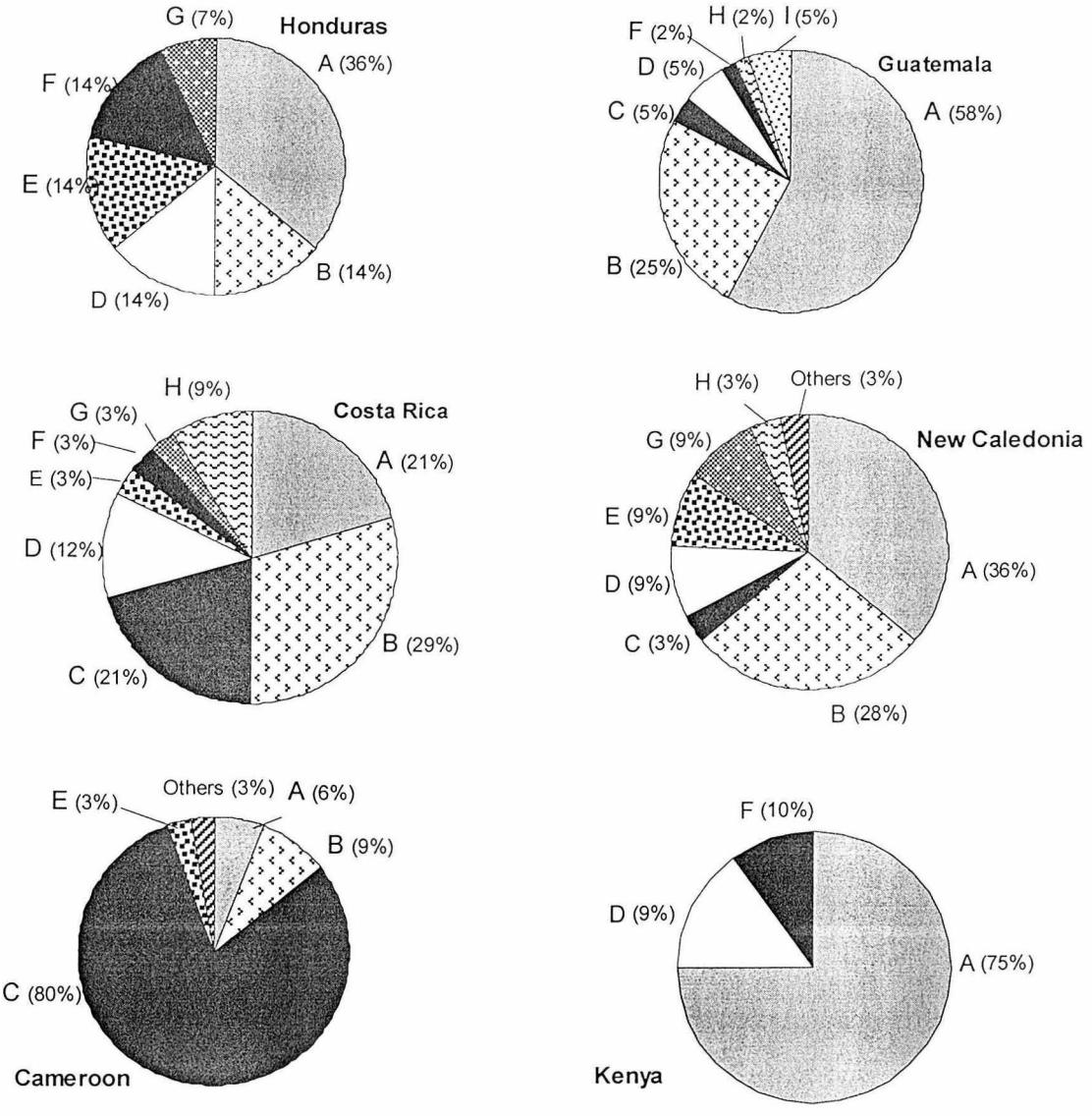


Fig. 3. Distribution of rhizobial strains isolated from the nodules of *Calliandra calothyrsus*.
Others = genotypes J, K, L, M, N, O).



ANNEXES

IUFRO

NFT News

Improvement and Culture of Nitrogen Fixing Trees

February 2000 Volume 3 No. 1

Working Party 2.08.02

Web page at : *http://iufro.boku.ac.at/iufro/iufronet/d2/wu20802/nl20802.htm*

Message from the Coordinator WP 2.08.02

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I take this opportunity to welcome all NFT members and supporters to the new millennium. I am looking forward to rewarding achievement in our activities especially for the promotion of NFT species for the social benefits of our communities. Once more it is with great pleasure to acknowledge many members who kindly sent their comments on previous NFT News and those who contributed to this issue.

Although great effort has been put into conservation of ecosystems, major areas of the world's forests have been destroyed last century and tropical forests continue to be lost at alarming rates. Associated with substantial decline of forest cover is a concomitant loss in species, including NFT species, and reduction of genetic diversity within surviving species. Consequently the loss of forest cover has led to alarming soil erosion in tropical ecosystems and to the depletion of fertility of arable land especially for the farmers in developing countries. During the past years, research on NFT species covered mainly the screening of species in various countries. The genetic resources for most experiments and protocols for the trials were coordinated by international institutions (e.g. NFT Association, ICRAF, CSIRO, CIRAD, FAO) and national research agencies. Researchers involved are encouraged to publish data from such experiments.

The promotion of trees in the farming systems for the contribution to the improvement of soil fertility will boost productivity of lands on most farms, especially for poor farmers with less resources for alternative inputs. Thus more funds should be made available for research and publication on farm trees.

As usual I welcome any comments or suggestions regarding the NFT News, and contributions to the next issue. I look forward to meeting you next August in Kuala Lumpur for the IUFRO Congress 2000.

The Role of Acacia Hybrids in the Reforestation Program in Vietnam

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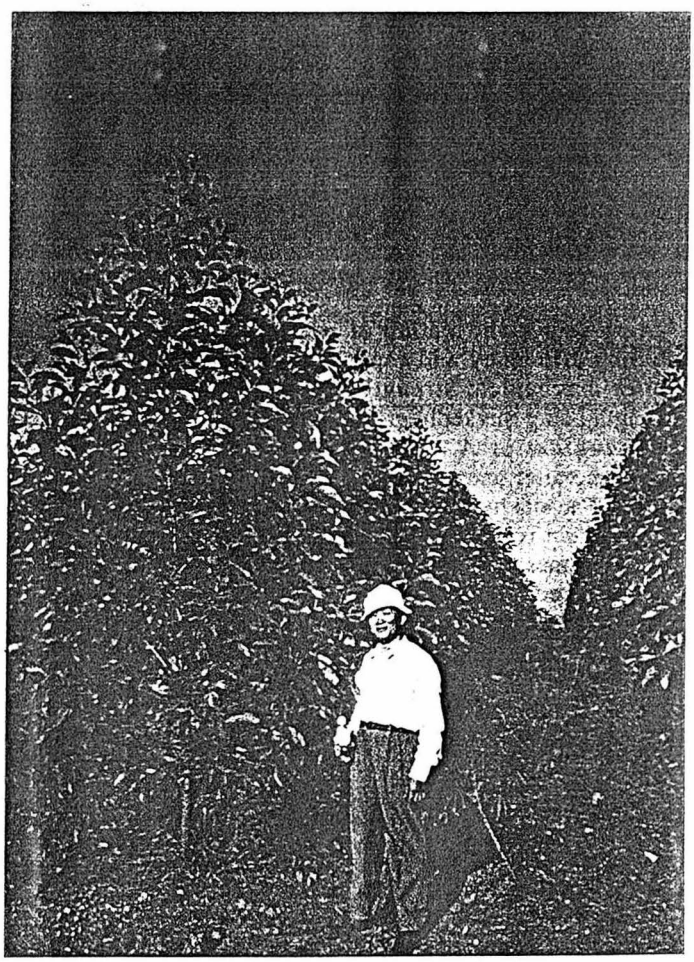
The forest cover in Vietnam has decreased from 43% in 1945 to 29% at present. Wars and over exploitation of natural forests have both contributed to the reduction. In 1998 the Vietnamese government embarked on a national reforestation program. Under this program 2 million ha of production forest and 1 million ha of protection forest will be established over the period 1999 – 2010.



A 5-month old acacia hybrid plantation in Ha Tay province, Vietnam

examined. One of these is *Haplormosia*, common in tropical West Africa. Trees may be up to 20m tall and produce a very high quality timber, which is resistant to decay, and termite attack (Allen and Allen 1981). It is currently classified in the tribe Sophoreae, which is almost certain to be split soon and which currently contains some genera, for example *Cladastris* which appears not to nodulate (Allen and Allen 1981; Faria *et al.* 1989). Another important genus is *Pterocarpus*. This is consistently reported to nodulate in Africa, with positive reports also from Asia and Venezuela, but never in Brazil in spite of extensive searching and inoculation experiments (Faria *et al.* 1989). African species grown in Brazil may nodulate freely with indigenous rhizobia (Faria and Lima 1998).

Dalbergia is a genus, which appears to nodulate freely, with nodules of the aeschynomenoid type, normally associated with peanut (*Arachis hypogaea*). The genus contains many fine timber trees, such as rosewoods, a number of which are threatened with extinction, according to the latest IUCN list. If it is to be replanted sustainably, its ability to fix nitrogen needs to be taken into account more than it appears to be at present (see recommendations in Westley and Roshetko 1994).

General comments and conclusions

Even when a genus can nodulate, it may not do so under a particular set of conditions. It is always wise to check when working in a location for which there is no information. Further, there is much work to be done in checking all the remaining genera for their ability to nodulate and to try to assess the amount of nitrogen fixed in the field (Sprent and Parsons 1999).

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Improving Forage Production of *Calliandra calothyrsus* through Symbiotic Association in Senegal (INCO/DGXII Project)

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Introduction

The global interest in tree legumes for production of forag green manure, mulch, shade, fuelwood, firebreaks and so erosion control has resulted in increased research interest i their multiple uses. They can offer advantages ove herbaceous species in terms of perennial nature, higher dr matter yields, and better resistance to mismanagement an capacity to retain high-quality foliage under environmentall stressful conditions. *Calliandra calothyrsus* is a wood legume with a high agroforestry potential. It is a thornles fodder tree legume native to Mexico and Central America which although not used in its native range, has bee introduced with success to many tropical regions mainly fo soil erosion control and livestock forage. Like mos leguminous trees, *C. calothyrsus* can form nodules and fi nitrogen in symbiosis with rhizobia. In many tropical soils the presence of inadequate or non-effective rhizobia population may limit nitrogen fixation. In such soils inoculation with appropriate rhizobia but also mycorrhiza ma improve yield, but there is currently a lack of information o the symbiotic relationship of the microbial partner and th host plant.

Objectives and research activities

The main objective of the project is to optimise the forag production of *C. calothyrsus* in field conditions by inoculatio with highly efficient strains of rhizobia and/or mycorrhiz strains selected in laboratory, greenhouse and in field con- ditions. However, in order to achieve this objective, it is nec- essary to investigate the main symbiotic characteristics of *C. calothyrsus*, evaluate a range of potential inocula, and to be able to produce a suitable microbial inoculum for inoculating plants in field conditions. Consequently, research activities are concentrated in two main areas:

(i) Collection of microsymbionts of *C. calothyrsus*. The project will establish a large collection of rhizobia and mycorrhiza isolated respectively from nodules and roots of this species harvested in its native range (Honduras, Costa Rica, Nicaragua, Guatemala and Mexico), and in humid countries where it has been introduced with success (Cameroon, Kenya and New Caledonia). After evaluation of the biodiversity within the collection of microsymbionts, a symbiotic screening will be carried out in laboratory and in greenhouse in order to select the most efficient strains for inoculation in field conditions.

(ii) Field inoculation of *C. calothyrsus*. Existing methodologies for producing rhizobial inoculum and inoculating plants in field conditions will be developed further for inoculation in field stations, and finally, under farm conditions.

Results obtained during the first year of the project

The aim of the first year of the project was to establish a collection of microsymbionts (rhizobium and Arbuscular

Mycorrhizae or AM) of *Calliandra calothyrsus*. For this reason, all the tropical partners harvested samples of soils and nodules in the place where future field experiments with *C. calothyrsus* will be set up by the project. Oxford Forestry Institute (OFI) harvested soil and nodule samples from Mexico (3 sites), Honduras (2 sites), Guatemala (4 sites), Nicaragua (3 sites) and Costa Rica (3 sites). OFI were assisted by the Project for Conservation and Silviculture of Forest Tree Species for Honduras (CONSEFORH) during this trip. In Cameroon, the Agricultural Research Institute for Development (IRAD) harvested soil samples at 3 sites but nodules at only 2 sites. Regarding New Caledonia, samples were harvested at 5 sites. In Kenya, soil samples were harvested at 8 sites located in the Western and Central Highlands. One part of all the soil samples were sent to the Institute of Terrestrial Ecology (ITE) for the mycorrhizal studies, and the remainder of soil and nodule samples were sent to CIRAD-Forêt in Senegal for rhizobia work. The isolation of rhizobia and their preliminary characterisation (*Rhizobium* genus) was carried out by the Kenya Forestry Research Institute (KEFRI).

From soil samples, ITE has established "trap" cultures using *C. calothyrsus*, sorghum, millet and cowpea as host plants to "bait out" the AM fungi present in the soils. Thirty-five singles of species isolations were set up in a controlled environment cabinet using AM spores extracted from the "trap" culture soils. The soils from Honduras (H2), Guatemala (G2 and G4), Cameroon (C2) and Kenya (K7 and K9) contained most spores. AM fungal species that occurred most frequently included *Gigaspora albida, Glomus etunicatum, Scutellospora heterogama, Scutellospora verrucosa* and *Acaulospora scrobiculata*. Regarding the rhizobia, a "trapping" experiment was carried out in nursery from nodules samples by using four seedlots of *C. calothyrsus* (2 seedlots of Flores and 2 seedlots of San Ramon). After two months of culture, young fresh nodules were observed and harvested on the root system of *C. calothyrsus* plants. It was possible to isolate rhizobia contained in these nodules and to obtain a pure culture of each of them. By this methodology, 242 rhizobium strains were isolated. A large majority (219) of these rhizobia belong to the *Rhizobium* genus or fast-growing rhizobia but only 23 strains are of *Bradyrhizobium* or slow-growing rhizobia. All these rhizobia strains were tested in an inoculation experiment with *C. calothyrsus* in a greenhouse. By this means, we could confirm that all these strains are able to nodulate with *C. calothyrsus*, and we could identify those, which are the more efficient with the host plant. This experiment started in November 1998 and results should be obtained in March 1999. A copy of the 242 rhizobia strains has been sent to Leena Rasanen at the Department of Applied Chemistry and Microbiology of the University of Helsinki for molecular characterisation.

Concurrently, CIRAD-Forêt assessed the effect of important factors on nodulation and nitrogen fixation in *C. calothyrsus*. It was demonstrated that the factors "provenances of the host plant", "soils characteristics" and "defoliation of plant" could modify significantly the success of the inoculation and the efficiency of the symbiosis formed. It suggests that for further experiments in field conditions, it will be important to take in consideration these factors, especially for the choice of the rhizobia strains, which will be inoculated, to the plants. KEFRI enumerated indigenous rhizobial populations in soil of the 8 sites where samples were harvested. The results showed some differences between the sites according to whether soil was harvested under or away from a plant of *C. calothyrsus*. The difference is very important in soil of the Maseno Centre. For comparison, this work should be duplicated in the harvested soils of Central America, New Caledonia and Cameroon. This information will be very useful for the interpretation of the results of future inoculation experiments with *C. calothyrsus* at these sites. Regarding the mycorrhizal aspect of the *C. calothyrsus* symbiosis, KEFRI has assessed the amount of root length infected by AM fungi. Results showed those AM fungi root infections ranged from 4 to 64 %. It was also observed that the highest mean root infection was scored on root samples from Maseno Centre (Kenya). ITE has examined, through an experiment carried out in greenhouse, the effect of the mycorrhizal inoculation with *Glomus intraradices* on the growth of *C. calothyrsus* seedlings. The results showed that leaf, stem, root and nodule dry mass, stem diameter and leaf area were increased by mycorrhizal inoculation at the lower P concentrations (0 and 7.5 ppm P). However, increasing P application reduced mycorrhizal infection and diminished or eliminated these beneficial effects of inoculation.

Lastly, for limiting the problem of providing seed of *C. calothyrsus* during the period of the project, seed orchards of Flores (OFI seedlot 6/97) were set up by IRAD and KEFRI in Cameroon and Kenya. In Senegal, CIRAD-Forêt also set up several plots for seed production (Flores 16/96 and San Ramon 11/91) in the Bel Air Research Centre. Further activities are planned in the implementation of the project including training, laboratory and field experiments based on the above results.

A New Record on Casuarina Blister Bark Disease in Southern China

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Casuarinas, especially *Casuarina equisetifolia*, have been planted extensively in the coastal areas of southern China for wood production and for stabilisation of moving sands. There are currently about 300 000 ha of casuarina plantations in the coastal areas of Guangdong, Hainan, Fujian, Zhejiang and Guangxi Autonomous Region bordering the South China Sea.

Some diseases have been found to attack casuarina trees in China but only a bacterial wilt, *Pseudomonas solanacearum*, poses a serious threat (Bai and Zhong 1996). This disease has caused widespread death of trees, up to 90% in some plantations. The pathogen attacks root, branch and stem. It is believed that wounding caused by excessive branch lopping for firewood is the primary cause but trees damaged by typhoons have also been reported to be infested by *P. solanacearum*. The symptoms of infested trees are characterised by yellowing of bottom needles which gradually proceeds upwards resulting in wilt and death.

There is evidence of a new disease associated with the death of casuarina trees in an 8-year-old provenance trial of *C. equisetifolia* planted at Yangxi in Guangdong province. Many trees in the trial were damaged by a typhoon in September 1996. In a recent visit to the trial as many as ten

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Balaban, Philadelphia/Rehovot

Interactions Between Mycorrhizal Colonisation, Nodulation and Growth of *Calliandra calothyrsus* Seedlings Supplied with Different Concentrations of Phosphorus Solution

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Abstract

Interactions between arbuscular mycorrhizal colonisation, nodulation and growth of *Calliandra calothyrsus* were investigated in an experiment in which mycorrhizal and non-mycorrhizal seedlings were grown in a glasshouse environment, in pots containing a sterilised substrate and supplied with four different concentrations of phosphorus solution (0, 7.5, 15 and 30 mg l^{-1} P). All seedlings were inoculated with *Rhizobium*. After eight weeks growth, assessments were made of stem, leaf and root growth, nodule dry mass and percentage mycorrhizal colonisation. Mycorrhizal colonisation was highest at 0 mg l^{-1} P and was reduced with increasing phosphorus application. Mycorrhizal inoculation increased seedling growth and nodulation most at lower concentrations (0 and 7.5 mg l^{-1} P) of phosphorus application. Phosphorus application improved growth and

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nodulation at 7.5 mg l^{-1} P compared with 0 mg l^{-1} P, but further increase in phosphorus application did not result in further growth benefits. Seedling growth was positively correlated with mycorrhizal colonisation and nodule dry mass with strongest relationships occurring for nodule dry mass. Growth response of seedlings to greater nodulation was maintained in mycorrhizal plants but not in non-mycorrhizal plants, even at high levels of P application when available P was not limiting. It is concluded that *C. calothyrsus* is dependent on mycorrhizal association in P-deficient soils, and that mycorrhizal inoculation has the potential to enhance its growth and nodulation under these conditions.

Keywords: Arbuscular mycorrhizas, nitrogen-fixing tree, growth responses

1. Introduction

Calliandra calothyrsus Meissner is a small, thornless, leguminous tree native to humid and sub-humid regions of Central America. In recent years its value as a multi-purpose tree for use in agroforestry has been recognised, both as a source of firewood and high quality fodder and as a hedgerow plant in alley cropping systems. In addition, the species grows well in acidic, infertile soils typical of many parts of the humid tropics. As a result, *C. calothyrsus* has been increasingly planted as an exotic in many tropical countries and evaluation of its potential benefits and optimisation of its growth has been undertaken (Lesueur et al., 1996; Mugendi et al., 1999a; 1999b; Ndufa et al., 1999).

Like many tree legumes, *C. calothyrsus* forms symbiotic relationships with both nitrogen-fixing bacteria and arbuscular mycorrhizal (AM) fungi. The main benefit of AM association to the plant is through enhanced phosphorus uptake and, because *C. calothyrsus* forms associations with AM fungi and nitrogen-fixing *Rhizobia*, it is able to sustain growth in both phosphorus (P) and nitrogen (N) deficient soils. Positive interactions between AM fungi and *Rhizobia* have also been demonstrated for many legumes: mycorrhizal colonisation has been shown to stimulate nodule formation and nitrogen-fixing activity in the host plant and, as effective nodulation depends on an adequate supply of P, it is thought that these benefits are largely P-mediated (Barea and Azcón-Aguilar, 1983).

To a large extent, plant growth response to AM colonisation is determined by soil fertility and in particular the availability of P. However, plant species which form AM differ in their susceptibility to root colonisation and in their dependence on AM formation to stimulate plant growth. Janos (1980) has shown that tropical trees exhibit a wide range of dependency on AM colonisation and more recent studies (Habte and Turk, 1991; Manjunath and Habte, 1992; Habte,

1995) on tree legumes used in agroforestry systems have also shown that some species show high dependency (e.g. *Leucaena leucocephala, Cassia siamea, Glyricidia sepium*) whereas others are less dependent (e.g. *Cassia reticulata, Sesbania pachycarpa*). As a result, dependent species are more likely to grow poorly when indigenous AM propagules present in field soils are deficient or ineffective.

Although extensive research programmes are in progress which evaluate the use of *C. calothyrsus* in agroforestry, comparatively few studies have examined its symbiotic relationships, which could be crucial in low-input farms and infertile soils. Reena and Bagyaraj (1990) and Ibrahim et al. (1996) have shown the potential for improving establishment and growth of introduced *C. calothyrsus* by mycorrhizal inoculation and P application, but do not relate mycorrhizal colonisation and plant growth to different concentrations of available P in the soil. Desmond (1995) found that increasing concentrations of available P improved plant growth and mycorrhizal colonisation of *C. calothyrsus,* but levels of colonisation observed were very low and may well have precluded any growth response due to mycorrhizal colonisation alone.

In this study, we aimed to examine the interactions between mycorrhizal formation, nodulation and plant growth and to evaluate the mycorrhizal dependency of *C. calothyrsus*. To do this, we compared the growth of mycorrhizal and non-mycorrhizal seedlings (both inoculated with *Rhizobium*) in soil containing increasing concentrations of available P. The research formed part of a wider study aimed at optimisation of growth and forage production by *C. calothyrsus* through evaluation and application of microsymbiont diversity (Lesueur, 2000).

2. Materials and Methods

Experimental design

A factorial experiment was used to test 2 mycorrhizal and 4 nutrient treatments. Treatments were laid out in 8 randomised blocks with each treatment represented once within each block.

Plant material

Seeds of *Calliandra calothyrsus* seedlot 12/91 ex. Honduras, supplied by the Centre for Natural Resources and Development, Oxford, UK (CNRD), were scarified by chipping off a small piece of the seedcoat and pre-germinated in petri-dishes on moistened, sterilised filter paper for 5 days.

Mycorrhizal inoculum

Pot cultures of *Glomus intraradices* Schenck & Smith (isolate UT 143-2, supplied by the International Culture Collection of Arbuscular and VA Mycorrhizal Fungi, West Virginia University, USA) were grown for 4 months in a sterilised loam/sand/grit mixture using cowpea (*Vigna unguiculata* L.) and millet (*Pennisetum typhoides* L.) as host plants.

Inoculation and set up of the experiment

The experiment was conducted in a glasshouse set to provide a day/night temperature regime of 28/20°C and relative humidity of 30–50%. Natural sunlight was supplemented with high-pressure mercury vapour lamps when necessary, to produce a day length of 14 h.

Free-draining, 1 litre pots were filled with a sterilised (autoclaved at 121°C and 1.03. 10^5 Nm^2 for 1 hour) mixture of horticultural coconut fibre and sand (1:1 by volume). Mycorrhizal pot cultures were thoroughly mixed and 14 g of inoculum (soil, spores and root fragments) was added to each pot at about 1 cm depth prior to planting. For control pots (i.e. non-mycorrhizal plants) the same amount of autoclaved inoculum was added. Three pre-germinated seedlings were then transplanted to each pot. At this point, the seedlings were inoculated with a *Rhizobium* suspension, consisting of isolate TAL 1455 (also supplied by CNRD, Oxford, UK) and crushed *C. calothyrsus* nodules, to ensure that all seedlings became nodulated. One week after planting, the seedlings were thinned to one per pot, retaining the seedling nearest to average height.

Phosphorus treatments and nutrient supply

Four phosphorus treatments were applied in the form of H_3PO_4 solution at three different concentrations (7.5, 15 and 30 mg l^{-1} P), and in the form of de-ionised water for 0 mg l^{-1} P. In addition to daily watering, all plants were supplied with Ingestad's solution (Ingestad, 1971) which had been modified to remove the phosphorus by replacing KH_2PO4 with KOH. Ingestad's and phosphorus solutions were applied to seedlings twice weekly, increasing the amount added from 10 cm^3 of each solution per pot per application in weeks one and two, to 15 cm^3 per pot per application in weeks three and four, and 20 cm^3 per pot per application in weeks five, six and seven.

Chemical analysis of the sterilised coconut fibre/sand mixture showed that it contained 345 mg K kg^{-1}, 9.4 mg NH_4^-N kg^{-1}, <0.5 mg NO_3^-N kg^{-1} and <0.2 mg P kg^{-1}. The mixture contained 4.05% organic matter and had a pH of 5.3.

Assessments

During the growth period, weekly measurements of plant height were made. Plants were harvested eight weeks after the experiment was set up. Shoots were removed and measurements were made of stem diameter, stem dry mass, leaf area, and leaf dry mass. After root washing, nodules were counted and removed for determination of dry mass. Mycorrhizal colonisation was assessed on a sub-sample of the roots so that root dry mass could be determined using the remaining roots. For sub-sampling, the root system was cut into root fragments about 1 cm long, thoroughly mixed and then 100 root fragments were removed at random. Fresh mass of the sub-sample and remaining roots was obtained and then the dry mass of the remaining roots was determined, so that the total root dry mass could be estimated by proportion. For assessment of mycorrhizal colonisation, root sub-samples were stained with trypan blue (Koske and Gemma, 1989) using a modified syringe method (Claasen and Zasoski, 1992) and percentage mycorrhizal colonisation was estimated using the gridline intersect method (Tennant, 1975).

Statistical analysis

Data were examined for normality and homogeneity of variances (Bartlett's test; Sokal and Rohlf, 1995), and transformed where necessary. Nodule dry mass data was normally distributed but showed heterogeneity of variance, so analysis was carried out on square root transformed data. One plant, which failed to nodulate and produced outliers in several data sets, was excluded from the analysis. Data were examined by two-way analysis of variance (ANOVA) using mycorrhizal inoculation and phosphorus application as treatment factors. Means were compared using Fisher's LSD test when the F-test from ANOVA was significant at $P<0.05$. Correlation coefficients and regression analysis were used to examine relationships between variates and P response curves were fitted when variates were significantly affected by P application.

3. Results

Weekly height measurements

After thinning to one seedling per pot, the initial height measurement showed that inoculated plants were significantly ($P=0.011$) taller than non-inoculated plants. For this reason ANOVA of weekly plant heights was adjusted using the initial height as a covariate. During the first 6 weeks of growth, plant height was not significantly affected by mycorrhizal inoculation

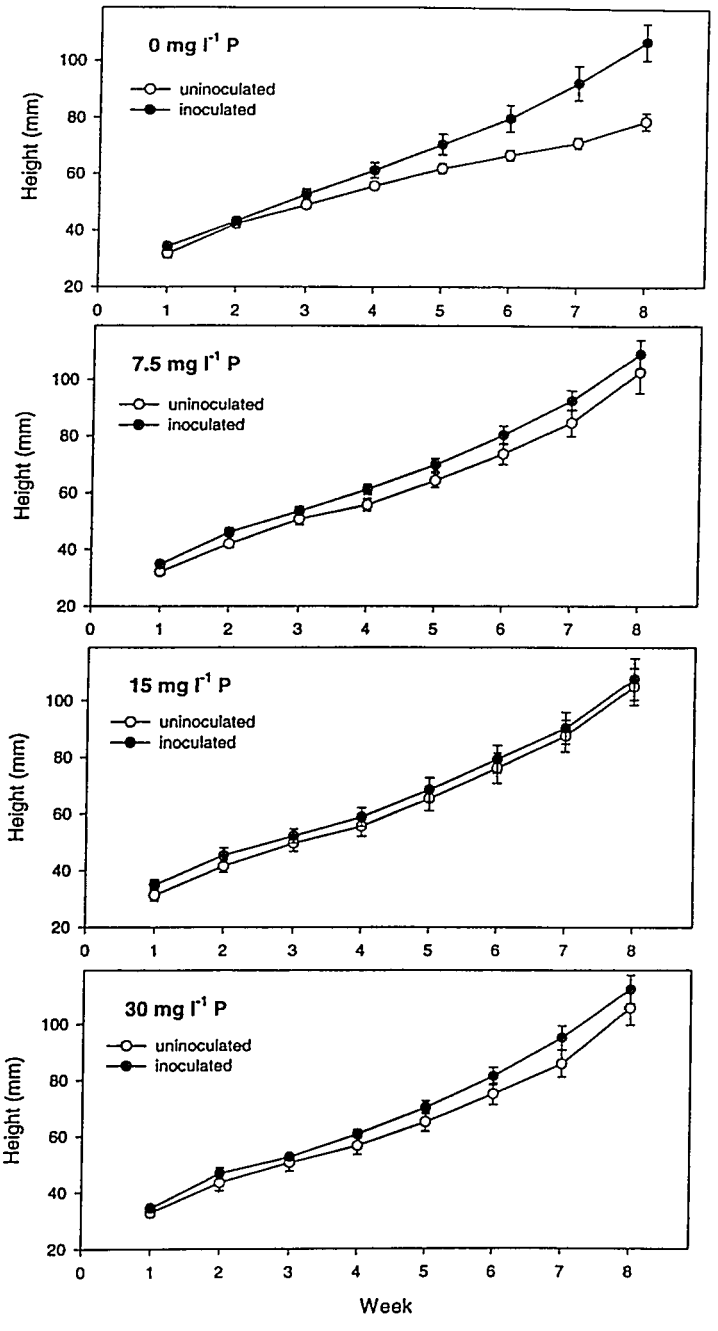


Figure 1. Weekly height growth of *Calliandra calothyrsus* seedlings in response to mycorrhizal inoculation and application of four different concentrations of phosphorus solutions. Error bars = ±SE.

Table 1. Effect of mycorrhizal inoculation on the growth of *Calliandra calothyrsus* seedlings after eight weeks

	Uninoculated	Inoculated	P value
Stem diameter (mm)	2.17 b[a]	2.37 a	0.004
Stem dry mass (mg)	99 b	127 a	<0.001
Leaf dry mass (mg)	430 b	567 a	<0.001
Leaf area (cm^2)	103 b	135 a	<0.001
Root dry mass (mg)	166 b	207 a	0.008
Nodule dry mass (mg)[b]	26.4 b	36.0 a	<0.001
Mycorrhizal colonisation (%)	0 b	29.1 a	<0.001

[a]Letters indicate significant differences within each row at $P<0.05$ as determined by ANOVA and Fisher's LSD test. [b]Square root transformations were performed on nodule dry mass data for analysis, significance is given against untransformed data.

Table 2. Effect of application of four different phosphorus solutions on the growth of *Calliandra calothyrsus* seedlings after eight weeks

	0 mg l^{-1} P	7.5 mg l^{-1} P	15 mg l^{-1} P	30 mg l^{-1} P	P value
Stem diameter (mm)	2.16	2.31	2.29	2.32	0.269
Stem dry mass (mg)	101	116	115	120	0.325
Leaf dry mass (mg)	416 b[a]	540 a	515 a	521 a	0.026
Leaf area (cm^2)	100 b	130 a	123 a	121 a	0.034
Root dry mass (mg)	179	199	178	189	0.732
Nodule dry mass (mg)[b]	17.2 b	34.3 a	34.8 a	38.6 a	<0.001
Mycorrhizal colonisation (%)[c]	44.7 a	31.5 b	23.9 c	16.1 d	<0.001

[a]Letters indicate significant differences within each row at $P<0.05$ as determined by ANOVA and Fisher's LSD test. [b]Square root transformations were performed on nodule dry mass data for analysis, significance is given against untransformed data. [c]Means given are for inoculated plants only.

or P application. After week seven however, inoculated plants were significantly ($P=0.030$) taller than uninoculated plants. Although no significant interactions were found, Fig. 1 shows that this effect was almost entirely due to uninoculated plants being smaller when no phosphorus was applied. At week eight, there was a significant ($P=0.037$) effect of P application on plant height, with seedlings given no phosphorus being smaller than those given 7.5, 15 and 30 mg l^{-1} P. As with week seven, no interactions between mycorrhizal inoculation and P application were found.

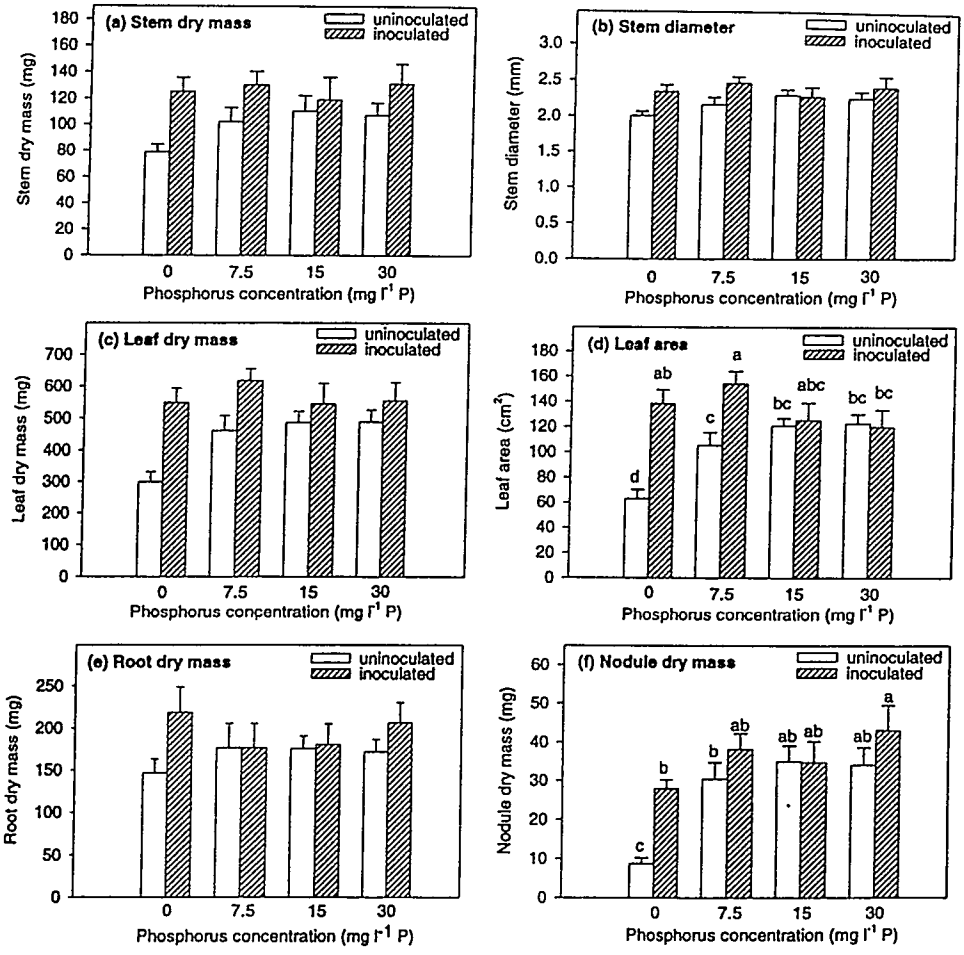
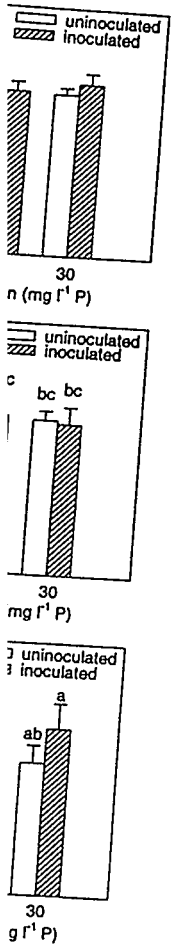


Figure 2. Effect of mycorrhizal inoculation and application of four different concentrations of phosphorus solutions on growth of *Calliandra calothyrsus* seedlings after eight weeks. Error bars = ±SE. Columns with different letters are significantly different at $P<0.05$ as determined by ANOVA and Fisher's LSD test.

Plant harvest

Main effects of mycorrhizal inoculation on seedling growth are summarised in Table 1. Mycorrhizal inoculation significantly increased stem dry mass, stem diameter, leaf dry mass, leaf area, root dry mass and nodule dry mass. All plants that received mycorrhizal inoculum became colonised by AM, whereas no colonisation was found in uninoculated control plants.



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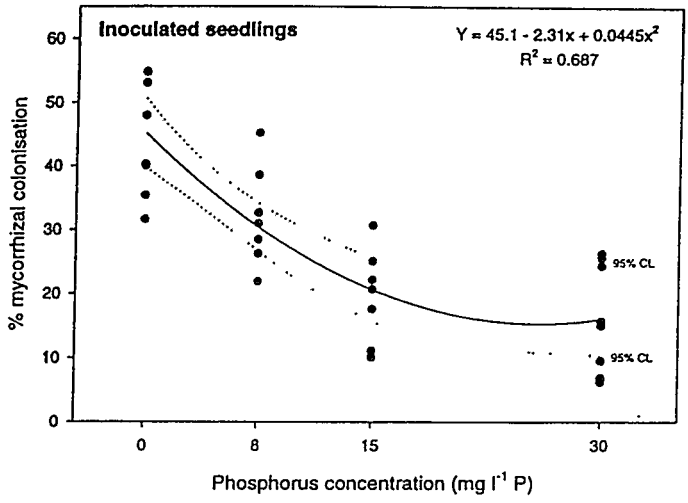

Figure 3. Fitted response curve of mycorrhizal colonisation of inoculated *Calliandra calothyrsus* seedlings to application of four different concentrations of phosphorus solution.

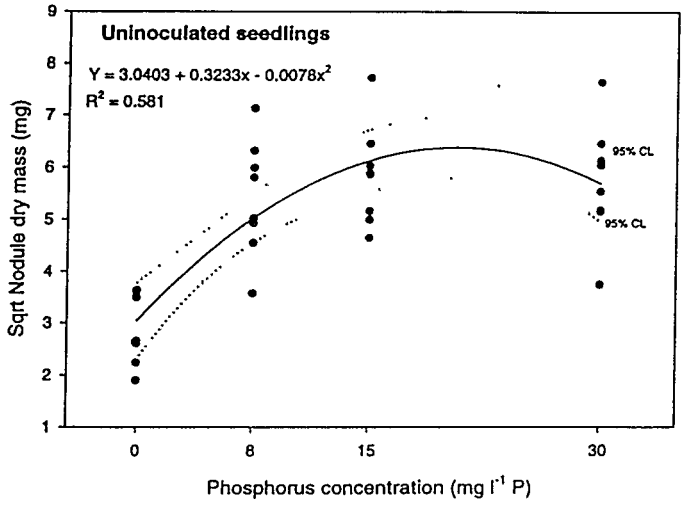

Figure 4. Fitted response curve of nodule dry mass of uninoculated *Calliandra calothyrsus* seedlings to application of four different concentrations of phosphorus solution.

Main effects of P application on seedling growth are summarised in Table 2. P application reduced percent mycorrhizal colonisation from 44.7% when 0 mg l^{-1} P was applied to 16.1% when 30 mg l^{-1} P was applied. Nodule dry mass was

also strongly affected by P application, with seedlings receiving 7.5, 15 and 30 mg l^{-1} P having twice the nodule dry mass of those receiving no phosphorus. Generally, P application had less impact on seedling growth than mycorrhizal inoculation: only significant differences in leaf dry mass and leaf area were noted, with seedlings receiving no phosphorus having smaller leaf area and leaf dry mass.

Significant interactions between mycorrhizal inoculation and P application were found for nodule dry mass ($P=0.001$) and leaf area ($P<0.001$). Nodule dry mass of non-mycorrhizal plants grown at 0 mg l^{-1} P was less than that of all other treatment combinations (Fig. 2f). Leaf area of non-mycorrhizal plants grown at 0 mg l^{-1} P was smaller than all other treatment combinations, while leaf area of non-mycorrhizal plants grown at 7.5 mg l^{-1} P was less than that of mycorrhizal plants grown at 0 mg l^{-1} P and 7.5 mg l^{-1} P (Fig. 2d). Similar differences were found for other growth parameters measured, with mycorrhizal inoculation increasing stem dry mass (Fig. 2a), stem diameter (Fig. 2b), leaf dry mass (Fig. 2c) and root dry mass (Fig. 2e) most at lower levels of P application.

Relationships between seedling growth, mycorrhizal colonisation, nodulation and phosphorus application

All parameters of seedling growth were positively correlated with both percent mycorrhizal colonisation and nodule dry mass, although correlation coefficients were much greater for nodule dry mass than for mycorrhizal colonisation (Table 3).

For mycorrhizal (inoculated) seedlings, percent mycorrhizal colonisation was negatively correlated with increasing P application. The P response curve (Fig. 3) showed that mycorrhizal colonisation decreased markedly from 0 mg l^{-1} P to 15 mg l^{-1} P, but that the rate of decrease slowed between 15 mg l^{-1} P and 30 mg l^{-1} P.

For non-mycorrhizal (uninoculated) seedlings, nodule dry mass was positively correlated to P application, whereas no relationship was found with mycorrhizal seedlings. The P response curve (Fig. 4) showed that nodulation of non-mycorrhizal seedlings increased up to P applications of 15 mg l^{-1} P, but did not increase at 30 mg l^{-1} P.

The strong positive relationship between seedling growth and nodulation was examined further by fitting separate nodule dry mass/shoot dry mass response curves for mycorrhizal and non-mycorrhizal seedlings (Fig. 5). ANOVA showed a significant difference ($P=0.004$) between these curves, which suggested that seedlings were more responsive to high levels of nodulation when they were mycorrhizal.

Table 3. Correlation coefficients (r) between growth, mycorrhizal colonisation and nodulation of *Calliandra calothyrsus* seedlings (n = 63)

	Mycorrhizal colonisation (%)	Nodule dry mass (mg)[a]
Stem diameter (mm)	0.323 *	0.745 ***
Stem dry mass (mg)	0.372 **	0.750 ***
Leaf dry mass (mg)	0.430 ***	0.811 ***
Leaf area (cm^2)	0.432 ***	0.739 ***
Shoot dry mass (mg)	0.424 ***	0.809 ***
Root dry mass (mg)	0.402 **	0.639 ***
Mycorrhizal colonisation vs. nodule dry mass 0.256*		

*, **, ***Significant at P<0.05, P<0.01 and P<0.001 respectively. [a]Square root transformations were performed on nodule dry mass data for analysis.

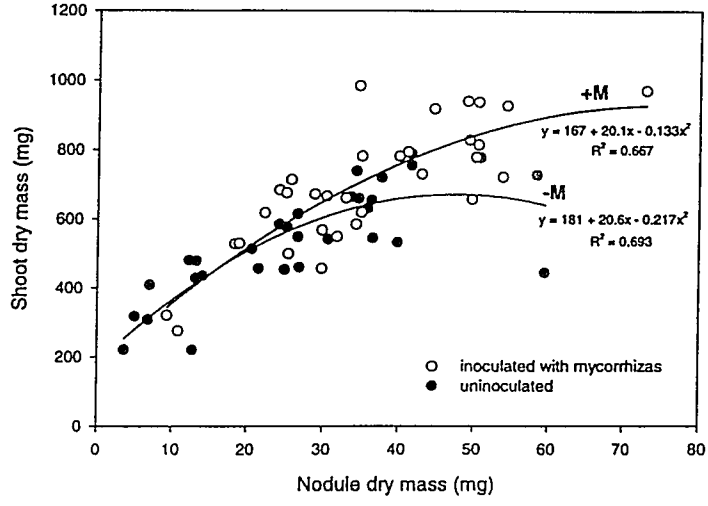

Figure 5. Fitted response curve of shoot dry mass of mycorrhizal (+M) and non-mycorrhizal (–M) *Calliandra calothyrsus* seedlings to nodulation (nodule dry mass).

4. Discussion

This study has shown that mycorrhizal inoculation benefits the growth of *Calliandra calothyrsus*, and supports the results of previous studies by Reena and Bagyaraj (1990), Desmond (1995) and Ibrahim et al. (1996). In our study

however, application of increasing levels of P reduced mycorrhizal colonisation and eliminated growth benefits attributable to mycorrhizal inoculation, whereas studies by Desmond (1995) and Ibrahim et al. (1996) showed that mycorrhizal inoculation only improved plant growth in P-deficient soils when P was applied. It is difficult to compare our study with that of Ibrahim et al. (1996) as mycorrhizal colonisation levels were not reported, while Desmond (1995) found much lower levels of mycorrhizal colonisation (1.5–18.4% after 13 weeks growth), which may have precluded any growth response to mycorrhizal inoculation alone. Our data would support this view and, had the 8-week growth period of our study been extended, colonisation levels and resulting growth benefits to plants growing at low P applications may have been much greater.

The results also show that the *C. calothyrsus* seedlings had a poor growth response to added P, which supports results obtained in field plots by Ndufa et al. (1999). This lack of response to added P and the concomitant low levels of AM colonisation suggests that, without adequate mycorrhizal colonisation, the *C. calothyrsus* seedlings were unable to take up sufficient P to maintain growth and therefore exhibited a high degree of mycorrhizal dependency.

The growth of mycorrhizal and non-mycorrhizal plants at 0 and 7.5 mg l^{-1} P indicates that, when available P was limiting plant growth, mycorrhizal plants were able to compensate for this. It is widely accepted that the main benefit plants receive from AM association is increased P uptake, and this suggests that the mycorrhizal *C. calothyrsus* seedlings were able to either prevent leaching of P from the pots or access normally unavailable P sources in the coconut fibre/sand substrate. At low P applications, mycorrhizal plants also increased leaf area and leaf dry mass more than stem or root growth. The enhanced leaf growth of mycorrhizal plants may have resulted in increased photosynthesis, carbon acquisition and greater allocation of carbon to the roots, which in turn may have stimulated AM colonisation and nodulation.

Even at high levels of P application (when available P was not limiting), mycorrhizal plants were able to maintain a positive growth response of seedlings to increasing nodulation whereas non-mycorrhizal plants were not. Kucey and Paul (1982) and Barea et al. (1987) found that N-fixation was greater in mycorrhizal than in non-mycorrhizal plants and it is possible that nodulation of the *C. calothyrsus* seedlings may have been generally ineffective to some degree and that N-fixation processes were stimulated by mycorrhizal plants. AM fungi are also known to significantly increase uptake of ammonium and trace elements such as copper and zinc (Smith and Read, 1997). Therefore at high P applications, mycorrhizal plants may have sustained plant growth by stimulating N-fixation and/or by directly supplementing N uptake.

This study has shown that mycorrhizal inoculation of *C. calothyrsus* increases plant growth in conditions of low P availability. The study also

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Improvement of forage production in *Calliandra calothyrsus*: methodology for the identification of an effective inoculum containing *Rhizobium* strains and arbuscular mycorrhizal isolates

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Abstract

The overall aim of this paper is to describe the selection of effective rhizobia and arbuscular mycorrhizas (AM), which after inoculation, will significantly improve the forage production of *Calliandra calothyrsus* under field conditions. To achieve this objective, the following activities were carried out: (i) establishment from both nodules and soil samples of a collection of microsymbionts (rhizobium and AM) of *C. calothyrsus* from Central America (Mexico, Honduras, Guatemala, Nicaragua and Costa Rica), also from outside its native range in Cameroon, Kenya and New Caledonia; (ii) identification under glasshouse conditions of the most effective rhizobia and AM isolates; (iii) production of a solid selected inoculum for field trials; (iv) examination of the impact of the inoculation on the growth of *C. calothyrsus* monitored under nursery conditions. We have screened 446 rhizobia strains in the nursery and identified six as being very effective at nodulating the host plant. They originated from Costa Rica (CCCR15 and CCCR1), from New Caledonia (CCNC26), from Cameroon (CCC22) and from Kenya (KWN35 and KCC6). In relation to AM, five isolates have been selected for the ability to infect and promote growth of the host plant—two isolates of *Gigaspora albida* isolated from Kenya (GA1b and GA2); one isolate of *Scutellospora verrucosa* isolated from Kenya (SV2c); one isolate of *Scutellospora calospora* isolated from Guatemala (SC2) and one isolate of *Glomus etunicatum* isolated from Honduras (GE1). Further experiments will test these selected inocula, singly and in mixtures, in order to obtain an inoculant which significantly improves the growth of *C. calothyrsus* and to enable its distribution

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to farmers who use this woody legume for forage production on their farms.

Keywords: Arbuscular mycorrhiza; *Calliandra calothyrsus*; Inoculum; Nitrogen fixation; Rhizobia; Symbiosis

1. Introduction

Trees are a very important component of many tropical agricultural systems, especially the woody legumes. They provide local populations with animal forage and firewood, and they contribute to the reduction of soil erosion and the improvement of soil fertility (Nair, 1993). *Calliandra calothyrsus* is one of the most important woody legumes and is widely used in agroforestry systems in countries in Asia, Africa, the Indian Ocean and South Pacific. In comparison with another widely used woody legume, *Leucaena leucocephala*, *C. calothyrsus* is able to grow on a wide range of soils types, including acid soils (Palmer et al., 1994). *C. calothyrsus* produces a high protein forage, and is associated with successful growth of trees, field crops and grasses (Kanmegne et al., 1999).

Like most leguminous trees, *C. calothyrsus* can form nodules and fix N_2 in symbiosis with rhizobia, a root nodule bacteria. It can also form a symbiotic relationship with arbuscular mycorrhiza (AM) and improve the phosphorus nutrition of plants. According to Dommergues (1995), *C. calothyrsus* is a woody legume with a high symbiotic nitrogen-fixing potential. In many tropical soils, the presence of inadequate or non-efficient rhizobial and AM populations may limit N_2 fixation and phosphorus nutrition (Singleton et al., 1992; Dommergues et al., 1999). In such soils, inoculation with the appropriate rhizobia and AM can improve yields provided no other constraints limit growth. In the nursery, Lesueur et al. (1996) and Purwantari et al. (1996) showed that growth and nitrogen fixation in *C. calothyrsus* could be significantly improved by inoculation with selected rhizobia strains. Reena and Bagyaraj (1990) and Ibrahim et al. (1996) obtained the same positive effect on the growth of *C. calothyrsus* after inoculation with AM fungi.

Currently, a comprehensive collection of rhizobia and AM isolates able to form an efficient symbiosis with *C. calothyrsus* is not available. Halliday and Somasegaran (1984) stated that some strains of rhizobia in the NifTAL collection were isolated from nodules of *C. calothyrsus* but they are not effective (Lesueur et al., 1996). Macqueen (1993) explained that it is possible to inoculate seedlings of *C. calothyrsus* with a cocktail of strains isolated from *Leucaena leucocephala* (TAL 1145) and *Gliricidia sepium* (TAL 1806) in order to increase the plant's growth. Lesueur et al. (1996) showed that from among 37 strains of rhizobia isolated from nodules of *C. calothyrsus* harvested in Kenya and Reunion Island, CCK13 strain significantly improved the growth of plants cultivated in nursery in comparison with strains TAL 1145 and TAL 1806. According to these authors, this strain could be used as inoculum for increasing forage production in field conditions. However, under irrigated conditions in Senegal, beneficial effects of inoculation with strain CCK 13 were not observed on the growth of plants from three provenances of *C. calothyrsus* until 2 years after planting (unpublished data). These results suggested that an optimum inoculum for *C. calothyrsus* was not available and further sampling and research will be needed in order to be able to improve significantly the growth of *C. calothyrsus* cultivated in various ecological conditions. An example of what could be achieved is the use of the *Bradyrhizobium* strain Aust 13ct for the inoculation of *Acacia mangium* plants in nursery (Galiana et al., 1998).

For this reason, a large collection of microsymbionts (rhizobia and AM) was established from both soils and nodules harvested in several countries from Central America, Cameroon, Kenya and New Caledonia. Using this collection, several experiments were conducted in order to identify an inoculum composed of effective rhizobial strains and AM isolates.

The objective of this paper is to describe the experiments that have been carried out so far in identifying the selected inocula which will be tested in field conditions for the improvement of forage production by *C. calothyrsus* in Kenya, Cameroon, and New Caledonia. There were three main stages to this work: (i) establishment of a large collection of rhizobia and AM; (ii) first phase screening of rhizobia and AM in the nursery; (iii) second phase screening and the assessment of interactions between rhizobia and AM. In this paper, only the first two stages are described and discussed.

2. Materials and methods

2.1. Establishment of a large collection of rhizobia and AM

2.1.1. Sampling of rhizobia and AM

Soil and root nodule samples were collected from 22 sites in Honduras, Nicaragua, Costa Rica, Guatemala, Mexico, New Caledonia, and Cameroon (Table 1). Soil samples were collected from within the first 20 cm depth of soil, and within a radius of 50 cm from the main stem of the tree. Soil samples were taken from 8 to 12 trees scattered across a population and the samples bulked. Upto 120 nodules were sampled from the same number of trees and bulked. The soils were placed in re-sealable plastic bags, kept moist and maintained at a temperature of 15–20 °C. The nodules were stored in paper, kept dry and maintained at the same temperature.

In Kenya, a similar type of harvest was done in eight sites (Table 2).

2.1.2. Establishment of a collection of rhizobia

For the establishment of this collection, it was necessary to carry out trapping experiments in the glasshouse in order to isolate rhizobia. It could be possible to isolate directly from dried nodules harvested in field. But usually, you have a lot of

Table 1
List of rhizobia isolated from nodules harvested from the root system of trees of *C. calothyrsus* in Central America, Cameroon and New Caledonia

Country	Sites	Number of fast-growing rhizobia	Number of slow-growing rhizobia
Mexico	Plan del Rio	5	1
	Playa Azul	1	1
	Zihuatanejo	14	
Honduras	Santa María	11	-
	La Ceiba	13	1
Guatemala	Cuyata	13	2
	Patulul	12	2
	Flores	10	
	Santa María de Jesus	10	1
Nicaragua	La Puerta	10	
	San Ramón	10	3
	Santo Tomes	9	1
Costa Rica	Turrialba	8	1
	Fortuna	11	1
	San Isidro	7	1
New Caledonia	La Coulée	9	-
	Col Pirogue	5	1
	Ile de Maré	10	2
	Champ de Bataille	8	2
	Port Laguerre	8	2
Cameroon	Yaounde	10	-
	Ebolowa	15	-

Table 2
List of rhizobia isolated from nodules harvested on the root system of trees of *C. calothyrsus* in Kenya

Sites of collection	Provenances/variety of *C. calothyrsus*	Number of strains
Maseno veterinary	Local variety	49
Maseno Centre	San Ramon	31
Maseno Centre	Flores	53
Nyabeda, Siaya	Local variety	10
Mama Elizabeth Farm, Embu	Local variety	1
Mama Elizabeth Farm, Embu	San Ramon	42
Mama Elizabeth Farm, Embu	Flores	26
Agro-farm, Nderi	Local variety	4
KEFRI Office	Local variety	2
Nyabeda farm	Local variety	2
Owila farm, Siaya	Local variety	1

problems with contaminations and the risk to isolate other strains than rhizobia is high. For this reason, it is better to carry out a trapping experiment. These experiments were carried out in Senegal and Kenya. For the trapping experiment in Senegal, two provenances of *C. calothyrsus* were used, Flores (OFI seedlots references 16/96 and 10/91) from Guatemala and San Ramón (OFI seedlots references 110/94 and 11/91) from Nicaragua. The Oxford Forestry Institute (OFI), UK, supplied the seeds which were scarified mechanically. A small nick was made in the outer coat as described by Macqueen (1993). The scarified seeds were germinated on sand under glasshouse conditions.

Eight-day-old seedlings were transplanted into 12 × 8 cm plastic bags filled with sand. A watering regime of N-free nutrient solution (Broughton and Dilworth, 1971) and distilled water were alternately added. Nodules of *C. calothyrsus* sampled from sites in eight countries (Mexico, Honduras, Guatemala, Nicaragua, Costa Rica, New Caledonia, Cameroon) were crushed in distilled water and the solution obtained was applied to the root collar of the plants. There were five replicates per site of harvest and per *C. calothyrsus* provenance.

In the Kenyan trapping experiments, three accessions of *C. calothyrsus* were used: the 'local variety' designated as *C. calothyrsus* ex-Guatemala K-16, ex-San Ramón (Embu) ICRAF seedlot and Flores (OFI seedlot reference 6/97). Nodules were collected either from field-grown plants or trapping experiments under glasshouse conditions as described above.

After 2 months of culture, all fresh young nodules were harvested from the plants for the isolation of rhizobia in the laboratory. The same number of nodules (five per treatment) harvested on each *C. calothyrsus* provenance were sterilised superficially in 95% ethanol for 30 s and rinsed with sterile distilled water. After that, nodules were placed in solution of $HgCl_2$ (0.1%) for 3 min as a second sterilisation. After several rinses in sterile distilled water, each nodule was crushed quickly on a sterile glass slide and streaked onto YEM agar plates. In this way, risk of osmotic shock is much reduced. When the cultures obtained were pure, all the strains were maintained in YEM medium containing 20% (v/v) glycerol at −80 °C. In our article, we will speak about rhizobia rather than bacterial isolate even if all results of the molecular characterisation demonstrating that each bacterial isolates originated from the same soil is different from the others are not already available.

In order to distinguish fast-growing rhizobia from slow-growing rhizobia, strains of the collection were streaked onto YEM agar plates supplemented with bromothymol blue, which is a pH indicator. After 5 days of culture at 28 °C, we observed whether the rhizobia had acidified the medium (yellow colour around the colonies for the fast-growing) or alkalised it (blue colour for the slow-growing).

2.1.3. Establishment of a collection of AM isolates

The collection of AM isolates was established using 'trap' cultures to produce fresh, viable spores which were then used to initiate single species pot cultures. Thirty three different field soils were collected for trap culturing (Table 3). After thorough mixing of each sample, spores were extracted from 50 g portions of each soil (Walker et al., 1982), so that the original numbers

Table 3
Numbers of arbuscular mycorrhizal fungal spores present in the original soils collected under *C. calothyrsus* in Central America, Cameroon, Kenya and New Caledonia

Country	Site	Soil sample	Number of spores per 50 g of soil	Species present
Kenya	Muguga (KEFRI)	K1-5	41	Gi. alb., A. scr., S. pel., S. het.
	Muguga (KEFRI)	K6	29	S. pel., A. scr., S. sp., G. sp.
	Maseno (Agrocentre)	K7	43	S. dip., S. nig., S. sp., A. scr., A. sp.
	Maseno (Nyabeda)	K8	182	S. ver., S. nig., S. pel., A. spi., A. lon., A. scr., A. sp.
	Maseno (Owila)	K9	60	A. lon., S. ver., G. sp.
	Embu (Agrocentre)	K10	13	A. spi., S. ver.
	Embu (M. Eliz. farm)	K11	9	G. sp.
	Embu (M. Wach. farm)	K12	0	
Cameroon	Yaounde	C1	18	S. scu., S. pel., A. scr.
	Ebolowa	C2	27	A. dil., S. pel., G. occ.
	Bamenda	C3	43	A. scr., Gi. sp., S. pel., G. sp.
New Caledonia	Port Laguerre	NC1	3	G. sp.
Mexico	Zihuatanejo	M1	8	S. ver., G. geo.
	Plan del Rio	M2	9	G. cal., A. spi.
	Playa Azul	M3	13	A. scr.
Nicaragua	San Ramón	N1	95	A. scr.
	La Puerta	N2	29	G. cal., A. scr., A. spi.
	Santo Tomes	N3	79	G. sp., G. mos., A. scr., A. sp., S. pel.
Honduras	Santa Maria	H1	251	A. scr., Gi. sp., S. pel., S. ery., S. dip., S. scu., G. mos., G. geo., Scl. sp.
	La Ceiba	H2	19	Gi. sp., A. scr.
	Ocoman	H3	179	A. sp., A. spi., A. exc., A. mel., S. pel., S. sp., G. geo., G. cal., Scl. sp.
Costa Rica	San Isidro	CR1	23	A. spi., A. sp.
	Turrialba	CR2	37	G. mos., A. mor., A. scr.
	Fortuna	CR3	22	A. scr., A. spi., A. mor., A. fov., Scl. sp., G. geo.
Guatemala	Flores	G1	45	S. scu., A. scr., A. spi., A. sp., G. geo., G. mos.
	Santa Maria de Jesus	G2	19	Gi. gig., A. scr., G. mos., Scl. sp.
	Patulul	G3	53	A. scr., A. fov., A. exc., Gi. gig., G. sp.
	Santa Maria de Jesus + Calliandra	G4	8	Gi. sp., A. scr.
	Cuyata	G5	31	Gi. sp., A. scr.

Fungal species abbreviations. G. sp., *Glomus* sp.; A. lae., *Acaulospora laevis*; G. etu., *Glomus etunicatum*; A. dil., *Acaulospora dilatata*; G. cal., *Glomus callosum*; S. sp., *Scutellospora* sp.; G. mos., *Glomus mosseae*; S. pel., *Scutellospora pellucida*; G. geo., *Glomus geosporum*; S. het., *Scutellospora heterogama*; G. occ., *Glomus occultum*; S. ery., *Scutellospora erythropa*; Scl. sp., *Sclerocystis* sp.; S. dip., *Scutellospora dipapillosa*; A. sp., *Acaulospora* sp.; S. scu., *Scutellospora scutata*; A. scr., *Acaulospora scrobiculata*; S. ver., *Scutellospora verrucosa*; A. spi., *Acaulospora spinosa*; S. nig., *Scutellospora nigra*; A. exc., *Acaulospora excavata*; S. cor., *Scutellospora corralloidea*; A. mel., *Acaulospora mellea*; S. cal., *Scutellospora calospora*; A. mor., *Acaulospora morrowiae*; Gi. sp., *Gigaspora* sp.; A. fov., *Acaulospora foveata*; Gi. aib., *Gigaspora albida*; Gi. gig., *Gigaspora gigantea*.

and species of AM spores present in the soils could be determined.

Trap cultures were set up in the glasshouse. One litre pots were half-filled with sterilised substrate (a 1:1:1 mixture of loam/sand/Terragreen (granules of compressed clay obtained from Oil-Dri UK Ltd)), a layer of test soil added and covered with more substrate. Pots were then sown with seed of *C. calothyrsus* (OFI seedlot 6/97 ex. Flores, Guatemala), *Pennisetum typhoides* or *Sorghum* sp.

After 4 months, soil cores were removed from each culture, spores were extracted from the soil and populations of spores assessed. Cultures were potted-on using more sterilised substrate or re-sown, as repeat cycling of 'trap' cultures has been shown to 'bait out' AM fungal species that were dormant during the first cycle.

To establish single species cultures, freshly extracted spores of different species were separated into petri dishes, cleaned and rinsed thoroughly to remove any contaminant hyphae, then pipetted onto the roots of 1-week-old Sorghum seedlings. These seedlings were then planted into 'cones' of filter paper placed in small 80 ml pots and filled with sterilised substrate. The filter paper ensured that the spores were not washed from the roots by subsequent watering of the seedlings. The seedlings were grown in a controlled environment cabinet to ensure strict hygiene and prevent ingress by other AM fungi.

Sub-samples of roots were examined after 6 months to see if AM infection had been established. Roots were placed in modified syringes (Claassen and Zasoski, 1992) and stained using the method of Koske and Gemma (1989). If infection had been established, cultures were transferred to the glasshouse and potted on in 1-l pots filled with fresh sterilised substrate. Single species isolates were maintained in the glasshouse under stricter conditions of hygiene than the trap cultures, pots were placed on an isolated, free-draining bench, and were separated by at least 20 cm to prevent cross contamination; all watering was done using water which had been passed through a 5 micron polypropylene filter unit (Pall Filtration Ltd, UK).

2.2. Large-scale screening of rhizobia and AM in the glasshouse and nursery

2.2.1. Screening of rhizobia

Under glasshouse conditions, all rhizobial strains were used to inoculate seedlings of *C. calothyrsus* in order to identify among them the efficient ones. *Rhizobium* strain CCK13 was included as a reference strain because Lesueur et al. (1996) had demonstrated that it was very effective with *C. calothyrsus* under glasshouse conditions. Seedlings from Flores (OFI seedlot number 16/96) were scarified and set up as described above. Seedlings were cultivated in sand for 3 months. After this period, plants were transferred to sterilised soil from Sangalkam (50 km from Dakar, Senegal). Its physiochemical characteristics were as follows—pH H_2O 6.5; fine silt 7.4%; coarse silt 24.5%; fine sand 36.6%; coarse sand 21.5%; total carbon 0.54%; total nitrogen 0.06% and Olsen phosphorus 8.8 mg kg^{-1}. Plants were harvested after 6 months of growth and several parameters were measured—number of nodules, dry weight of nodules, and shoot and root dry weights. The shoot total nitrogen content was determined by the Kjeldahl method (Bremner and Mulvaney, 1982).

2.2.2. Screening of AM

Mycorrhizal inocula were screened in a single, large experiment, which tested all inocula under identical growing conditions. The quality of each inoculant used in the experiment was assessed by removing a soil core from each pot and determining the level of AM infection and spore concentration. Results are given in Table 5.

The experiment was set up in the glass house. Three pre-germinated seeds (San Ramón 11/91) were transferred to 1-l pots filled with a sterilised sand/Terragreen mixture (1:1 by volume). Approximately, 20 g of chopped roots/soil/spores inoculum was added to the pots at about 1 cm below the soil surface. Control treatment plants were inoculated with 20 g of a sterilised mixture of all 19 inoculants. All seedlings were inoculated with 1 ml of *Rhizobium* suspension containing 16.7×10^4 cells ml^{-1}. After 1 week, seedlings were thinned to one per pot. Treatments were laid out

on benches in eight randomised blocks with each treatment represented once within each block.

Chemical analysis of the substrate showed that unexpectedly high levels of phosphorus were available (NPK: 24, 10, 305 mg kg^{-1}) and no additional nutrients were supplied during the course of the experiment. Plants were harvested after 12 weeks and measurements made of shoot and root growth, AM infection and nodulation, and nutrient allocation to the shoots.

3. Results

3.1. Establishment of a large collection of rhizobia and AM

3.1.1. Collection of rhizobia

Tables 1 and 2 show that 446 rhizobia were isolated, 22 from Mexico, 25 from Honduras, 47 from New Caledonia, 50 from Guatemala, 33 from Nicaragua, 29 from Costa Rica, 25 from Cameroon and 215 from Kenya. A large majority of these rhizobia are fast growing rhizobia. No slow growing rhizobia were isolated from Cameroon or Kenya. It was confirmed under glasshouse conditions that all these bacterial isolates were able to nodulate and fix nitrogen in symbiosis with *C. calothyrsus*.

3.1.2. Collection, isolation and growth of AM

Many species of AM spores occurred naturally in soils collected from Central America, Kenya, Cameroon and New Caledonia (Table 3). However, it is possible to distinguish soils that were rich in both numbers and diversity of AM spores like K8 from Maseno in Kenya and H1 and H3 from Santa María and Ocoman in Honduras. In contrast, only a few AM spores were found in soils collected from Embu, Kenya (K10-12), New Caledonia (NC1), Mexico (M1-3) and Guatemala (G4). Due to the poor condition of spores in field soils, it was sometimes only possible to assign spores to the level of genus, however, permanent reference slides of the dominant spore types were preserved.

Spore production in the trap cultures tended to reflect the abundance and diversity of spores in the original field soil. As single species isolations were made according to the availability of fresh spores from the trap cultures, they are also similarly related.

Table 4 shows single species isolations that were attempted and whether or not AM infection had established on the Sorghum roots after 6 months. Several isolates of the fungi that occurred most frequently in the field soils and trap cultures were obtained e.g. *Gigaspora albida*, *Glomus etunicatum* and *Scutellospora verrucosa*. The results also indicate that *C. calothyrsus* seedlings were as effective as the crop plants (sorghum and millet) for use in the trap cultures.

Table 5 shows the level of AM infection and spore concentration found in the inoculants tested in the screening experiment. Generally, sporulation in the loam/sand/Terragreen substrate was very high, whereas infection levels on the Sorghum roots varied (11–62%) and were sometimes rather low.

3.2. Screening of rhizobia and AM in glasshouse and nursery

3.2.1. Rhizobia

Results presented in Table 6 are grouped by country in order to facilitate the presentation even if this type of global presentation is not usual. In this way, it is possible to show a general putative effect of the origin of the strains on the positive improvement of the *C. calothyrsus*'s plant growth. Our results show important differences, for all the parameters measured between the countries from where these strains were isolated. In terms of number of nodules per plant, strains from Costa Rica were most effective and those from New Caledonia and Cameroon were least effective. In terms of shoot dry weight, strains from Costa Rica, New Caledonia and Honduras were best and those from Guatemala and Nicaragua were worst. However, interpretation of this data requires caution as one of the 22 strains from the Cameroon (i.e. strain CCC22) was highly effective while the others were ineffective.

It was interesting to compare results obtained using strains isolated during the first year of the project, with results obtained using our reference

Table 4
List of AM single species isolations attempted from trapping experiments

Fungal species	Isolate number	Soil number	Trap host plant	Infection established after 6 months
Gi. albida	1a	K1	*C. calothyrsus*	✓
Gi. albida	1b	K1	*C. calothyrsus*	✓
Gi. albida	3	K1	*C. calothyrsus*	✓
S. heterogama	2	K1	*C. calothyrsus*	
S. heterogama	5	K1	*C. calothyrsus*	
S. heterogama	4	K2	*C. calothyrsus*	
Gi. albida	2	K3	Millet	✓
S. heterogama	1	K3	Millet	
S. scutata	1	K7	*C. calothyrsus*	
S. heterogama	3	K7	*C. calothyrsus*	
S. heterogama	6	K7	*C. calothyrsus*	✓
S. nigra	1	K7	*C. calothyrsus*	
S. verrucosa	3	K7	*C. calothyrsus*	✓
S. verrucosa	2a	K9	*C. calothyrsus*	
S. verrucosa	2b	K9	*C. calothyrsus*	
S. verrucosa	2c	K9	*C. calothyrsus*	✓
S. verrucosa	4	K9	*C. calothyrsus*	✓
G. mosseae	4	K10	*C. calothyrsus*	
S. scutata	2	K10	*C. calothyrsus*	
Gi. albida	6	K10	*C. calothyrsus*	✓
G. etunicatum	3	K12	*C. calothyrsus*	
S. pelucida	1	C2	*C. calothyrsus*	
A. dilatata	1a	C2	*C. calothyrsus*	
A. dilatata	1b	C2	*C. calothyrsus*	
A. dilatata	1c	C2	*C. calothyrsus*	
A. scrobiculata	1	C3	*C. calothyrsus*	
S. verrucosa	1	C3	*C. calothyrsus*	
G. etunicatum	1	H2	Sorghum	✓
G. etunicatum	2	H2	Millet	✓
G. mosseae	1	H2	Sorghum	
G. mosseae	2	H2	Millet	
G. sp. *nov.*	1	H2	Millet	
G. mosseae	3a	H2	*C. calothyrsus*	✓
G. mosseae	3b	H2	*C. calothyrsus*	✓
G. etunicatum	3a	H2	*C. calothyrsus*	✓
G. etunicatum	3b	H2	*C. calothyrsus*	
G. etunicatum	3c	H2	*C. calothyrsus*	
Gi. albida	4a	G2	Sorghum	
Gi. albida	4b	G2	Sorghum	✓
A. sp. *nov.*	1	G2	*C. calothyrsus*	
S. calospora	2	G2	*C. calothyrsus*	
A. spinosa	1a	G4	Sorghum	✓
A. spinosa	1b	G4	Sorghum	✓
S. calospora	1	G4	Sorghum	✓
Gi. Albida	5a	G4	*C. calothyrsus*	✓
Gi. Albida	5b	G4	*C. calothyrsus*	✓
Gi. Albida	5c	G4	*C. calothyrsus*	✓

strain CCK13. It appeared that plants inoculated with the reference strain were well nodulated (number and biomass) and enhanced shoot dry weight. In contrast, the shoot total nitrogen content was low where shoot dry weight was enhanced. These results confirmed the effectiveness of this strain as demonstrated by Lesueur et al. (1996).

The most efficient bacterial isolates or strains in terms of nodulation, growth and shoot nitrogen content were: CCC22 from Cameroon, CCCR1 and CCCR15 from Costa Rica and CCNC26 from New Caledonia (Table 7). It can be seen that these four strains were globally more effective than the reference strain under glasshouse conditions. However, these results will have to be confirmed through inoculation field trials with *C. calothyrsus*. In a parallel screening experiment carried out in Kenya, two effective rhizobial strains (KCC6 and KWN35) were identified that significantly improved the growth of two provenances (Flores and San Ramón) of *C. calothyrsus* after inoculation.

3.2.2. *Arbuscular mycorrhizas*

Mean mycorrhizal infection for each inoculant ranged from 22 to 65% and no infection was found on uninoculated control plants. Significant effects of inoculation treatment were found on stem diameter ($P < 0.006$), stem dry mass ($P < 0.003$), nodule dry mass ($P < 0.017$), total amount of N ($P < 0.037$) and P ($P < 0.001$) allocated to the shoot and the proportion (%) of P ($P < 0.021$) allocated to the shoot. Increases in shoot growth due to mycorrhizal inoculation were largest for stem dry mass, with 14 of the inoculants having significantly

Table 5
Spore concentrations and mycorrhizal infection found in each inoculant tested in the screening experiment

Treatment code		Isolate	Spores per 25 ml soil	Species present	Mycorrhizal infection (%)
Single species cultures					
1	*Gigaspora albida*	1a	254	*Gi. alb*	21
2	*Gigaspora albida*	1b	732	*Gi. alb*	28
3	*Gigaspora albida*	2	497	*Gi. alb*	30
4	*Gigaspora albida*	3	304	*Gi. alb*	35
5	*Glomus etunicatum*	1	3201	*G. etu*	30
6	*Glomus etunicatum*	2	1566	*G. etu*	24
7	*Acaulospora spinosa*	1a	1223	*A. spi*	23
8	*Gigaspora albida*	5b	166	*Gi. alb*	23
9	*Scutellospora calospora*	2	996	*S. cal*	38
10	*Glomus etunicatum*	3	1596	*G. etu*	11
11	*Scutellospora verrucosa*	2b	7	*S. ver*	41
12	*Scutellospora verrucosa*	2c	149	*S. ver*	19
Mixed cultures					
13	K2 Calliandra	b	163	*Gi. alb, S. het, G. etu*	45
14	K7 Calliandra	b	88	*S. het, S. nig, S. ver, A. ver, G.* sp.	47
15	K10 Calliandra	b	151	*S. pel, Gi. alb, G.* sp., *A.* sp.	40
16	H12 Calliandra	a	940	99% *G. etu*	44
17	H2 Sorghum	b	2156	90% *G. etu*, A. spp.	46
18	H3 Sorghum	b	173	99% G. sp.	34
19	C2 Calliandra	b	652	99% *G. occ*	62

Table 6
Effect of the inoculation with rhizobia strains isolated from several countries on nodulation and growth of *C. calothyrsus* (Flores OFI seedlot number 16/96)

Origin and number of rhizobia strains	Number of nodules per plant	Nodules dry weight (g per plant)	Shoot dry weight (g per plant)	Root dry weight (g per plant)	Shoot total N content (%)
Cameroon/24 strains	50.6b	0.057b	1.76c	1.07f	2.16b
Honduras/23 strains	61.7d	0.067d	1.98g	1.17f	2.21b
Mexico/20 strains	65.1e	0.058b	1.78c	1.05d	2.30c
Guatemala/46 strains	63.7de	0.054a	1.31a	0.67b	2.77f
Nicaragua/29 strains	65.2f	0.041a	1.33b	0.67a	2.61e
Costa Rica/27 strains	90.7h	0.064c	1.94e	0.99c	2.55d
New Caledonia/41 strains	55.0c	0.045a	1.94e	1.07e	2.31c
CCK13 (strain of reference)	83.7g	0.094d	1.97f	1.06d	2.21b
Control (uninoculated plants)	49.0a	0.057b	1.87d	1.05d	2.11a

For each factor, values (means from five replicates) in the same column followed by the same letter are not significantly different according to the Newman and Keuls test at $P<0.05$.

greater stem dry mass than the uninoculated control (Fig. 1). Mycorrhizal inoculation also stimulated nodulation and the proportion (%) of P allocated to the shoots, nine of the inoculants produced significantly greater nodule dry mass than the uninoculated control and 11 of the inoculants produced significantly greater percentage of P in the shoots than the uninoculated control.

Relationships between seedling growth, mycorrhizal infection and nodulation are shown in Table 8. Mycorrhizal infection and nodule dry mass were positively correlated with each other, and both increased shoot growth and allocation of nutrients to the shoot, although relationships were generally stronger for nodule dry mass. Root dry mass was not affected by mycorrhizal infection or nodulation, and the significant decrease in root/shoot ratio can be attributed to the increases in shoot growth. As mycorrhizal infection and nodulation both increased shoot growth, it was not surprising that they also increased total amounts of NPK allocated to the shoot. However, only mycorrhizal infection increased the proportion (%) of P and K allocated to the shoot.

The experiment indicated that differences between isolate effectiveness did not appear to be related to the country or site of origin. In addition, mixed cultures did not perform any better than single species cultures. As a result, five single species isolates have been selected to initiate the production and testing of inoculum under nursery and field conditions. Single species cultures are clearly identifiable and can be combined, if necessary to produce equally well-defined mixed cultures. The five isolates comprise a selection of species from three of the main genera of AM fungi, and all formed high levels of infection in the screening experiment. Although benefits to shoot growth, nodulation and nutrient allocation varied between isolates, the selected isolates include those that benefited plant growth in some, if not all, of these respects.

4. Discussion

The principal objective of our work was to identify symbionts (rhizobia and arbuscular mycorrhiza) able to improve the growth of *C. calothyrsus* in the nursery and in the field. To do this, it was necessary to establish a collection of strains of rhizobia from nodules harvested on the root system of trees of *C. calothyrsus* located in their area of origin (Central America and Mexico), and in countries where the species has been introduced successfully (Cameroon, Kenya and

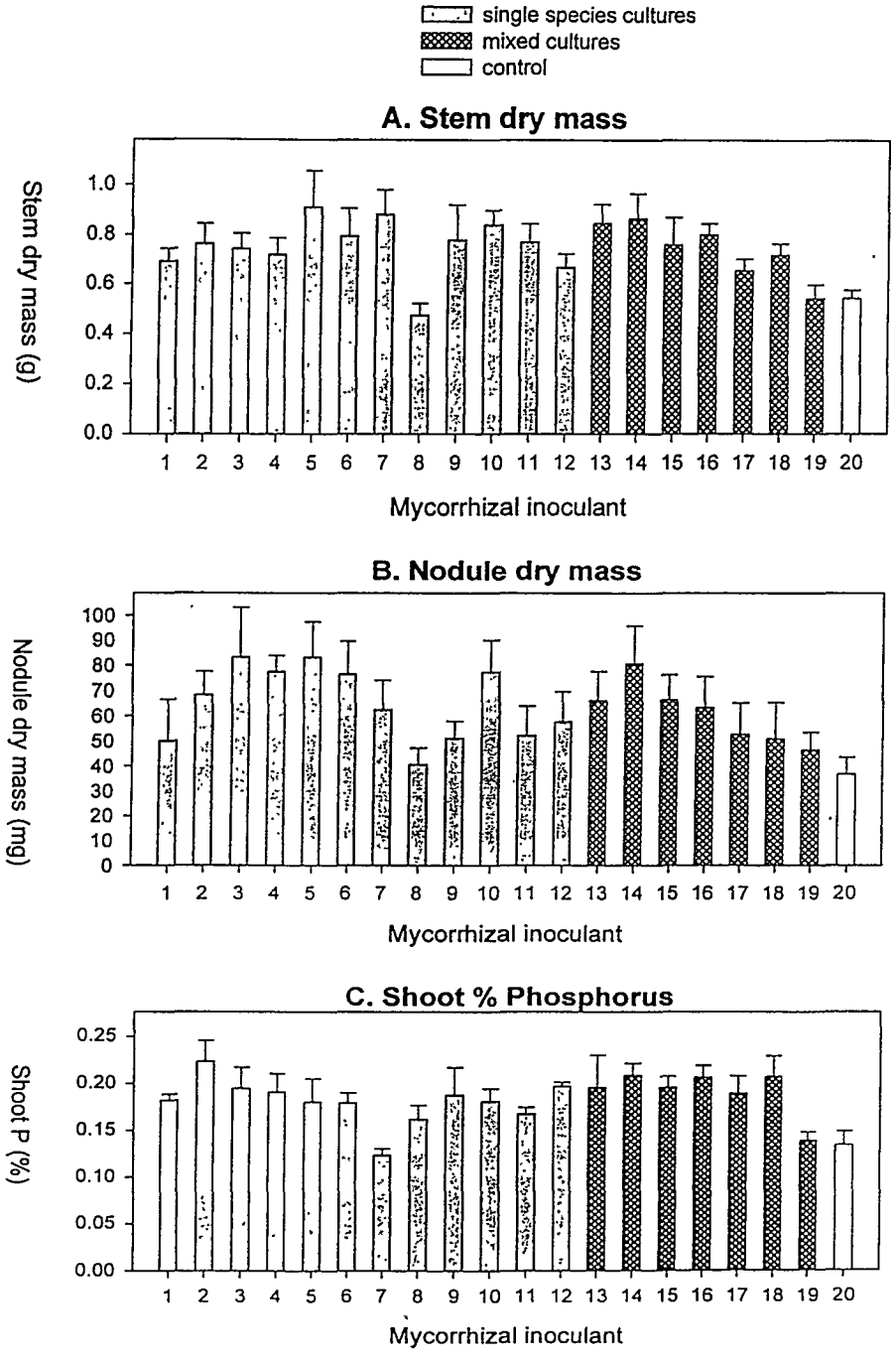

Fig. 1. Effects of inoculation with AM fungi on stem dry mass (Fig. 1a), nodule dry mass (Fig. 1b) and ^{33}P allocated to the shoot (Fig. 1c) of *C. calothyrsus* seedlings in comparison with uninoculated control plants. Bars indicate ± S.E. (see Table 5 for details of inoculants).

Table 7
Effect of inoculation with the four most efficient strains on nodulation and growth of *Calliandra calothyrsus* (Flores OFI seedlot number 16/96) by comparison with reference strain CCK13 and control

Number of strain	Number of nodules per plant	Nodules dry weight (g per plant)	Shoot dry weight (g per plant)	Root dry weight (g per plant)	Shoot total N content (%)
CCC22	70.5a	0.095c	3.40c	2.08d	2.26c
CCCR1	94.5c	0.079b	3.43c	1.87c	2.27c
CCNC26	109.0cd	0.099d	3.45c	1.71b	2.43d
Control	49.0a	0.057a	1.87a	1.05a	2.11a

For each factor, values (means from five replicates) in the same column followed by the same letter are not significantly different according to the Newman and Keuls test at $P<0.05$.

New Caledonia) (Duguma and Mollet, 1996; Paterson et al., 1996; Sarrailh et al., 1996). Soil samples were collected from the same sites as the nodules, and AM spores were identified, isolated and cultured with regard to rhizobia, 446 bacterial isolates were isolated, the majority of which have fast growth (Tables 1 and 2). In the majority of countries, a small number of *Bradyrhizobium* strains (slow-growing rhizobia) were isolated which suggests that in nature, *C. calothyrsus* can be nodulated by *Bradyrhizobium*, as well as by *Rhizobium*. In contrast, in Cameroon, only *Rhizobium* strains were isolated. These results are in accordance which those described by Lesueur et al. (1996) in Kenya and in Reunion Island. These new observations add to discussion on the contradictory results already published by Lesueur et al. (1996) and Purwantari et al. (1996) concerning the specificity of the species for nodulating with rhizobium. However, to conclude on this aspect, one can say that *C. calothyrsus* nodulates preferentially with fast growing rhizobia, but that under certain conditions, it can also form N-fixing symbiosis with slow-growing rhizobia. Through observations carried out in the greenhouse, it can be noted that the strains which are most efficient with *C. calothyrsus* are of fast-growing rather than slow-growing rhizobia origin.

Globally, in terms of symbiotic effectiveness, there are significant differences between the rhizobia strains from different countries. The strains which form the highest number of nodules are from Honduras, Mexico and Costa Rica, and those that simulate greater growth of the plant are from Costa Rica, New Caledonia and Honduras. Among the least effective strains are those from Nicaragua which form many small nodules, but are inefficient, and those of Cameroon which have a limited effect on the growth of the trees (Table 6). By way of comparison, Galiana et al. (1990) studied the infectivity and the effectivity of Australian and African strains of *Bradyrhizobium* from *Acacia mangium*, a woody leguminous plant originating in Australia (Queensland) and Papouasia/New Guinea. These authors demonstrated that the most powerful strains were those

Table 8
Correlation coefficients (r) found between mycorrhizal infection, nodulation and growth and nutrient allocation of *C. calothyrsus* seedlings

Seedling growth	% mycorrhizal infection	Nodule dry mass
Shoot height	0.203*	0.250*
Stem diameter	0.144	0.257**
Stem dry mass	0.262**	0.397***
Leaf dry mass	0.147	0.403***
Leaf number	−0.043	−0.122
Root dry mass	−0.100	0.154
Root/shoot ratio	−0.258**	−0.240*
% mycorrhizal infection v nodule dry mass	0.337***	
Nutrient allocation		
%shoot K	0.292**	0.192
%shoot N	0.414***	0.545***
%shoot P	0.415***	0.139
Total shoot K	0.379***	0.554***
Total shoot N	0.346**	0.634***
Total shoot P	0.480***	0.536***

*, **, *** significant at $P<0.05$, 0.01 and 0.001, respectively.

isolated from nodules collected in the native range of the species (Australia). It should be noted, however, that the symbiotic performance of the African strains (from Senegal and Côte d'Ivoire) were not much less effective than those of the Australian strains. Although the number of strains tested by these authors is lower than that of the current study, a supremacy of the strains from Central America in terms of symbiotic effectiveness for growth improvement of *C. calothyrsus* was not observed. Among all of the strains which were tested, superior strains were identified that could be used for the inoculation of *C. calothyrsus*. Two strains from Kenya (KCC6 and KWN35), two strains from Costa Rica (CCCR1 and CCCR15), a strain from New-Caledonia (CCNC26) and a strain from Cameroon (CCC22) allowed a greater development of the *C. calothyrsus* seedlings. These six strains will be used separately to inoculate seedlings of *C. calothyrsus* cultivated in two different soils in order to identify the most powerful strain. The behaviour of these selected strains of rhizobia will also be tested in the presence of the five isolates of arbuscular mycorrhiza selected for their positive effect on the growth of *C. calothyrsus*.

In relation to AM, it can be noted that spore populations found in the field soils collected from established *C. calothyrsus* sites suggest that some soils may be deficient in AM fungi, e.g. K10, K11 and K12 from Kenya, NC1 from New Caledonia, M1, M2 and M3 from Mexico and G4 from Guatemala (Table 3). Degraded sites or sites not already colonised by *C. calothyrsus* (i.e. sites that may be targeted for planting with *C. calothyrsus*) may show more severe mycorrhizal deficiencies. Of the remaining soils, K8 from Kenya and H1 and H3 from Honduras were notably high in spore numbers and diversity, which serves to emphasise that these differences are not related to country of origin i.e. countries where *C. calothyrsus* is native or has been introduced. In terms of diversity, spores of many AM fungal species were identified. However, the species most widely represented in the trap cultures were *Gigaspora albida*, *Glomus etunicatum* and *Scutellospora verrucosa* (Table 4). The screening experiment showed that mycorrhizal inoculation stimulated seedling growth, nodulation and nutrient allocation to the shoot. Although overall levels of mycorrhizal infection were good, available P in the sand/Terragreen substrate was measured at the comparatively high level of 10 mg kg^{-1} P. Mycorrhizal growth responses are known to be reduced when soil available P is high, therefore, growth responses could be greater in unfertile soils. Our results are in accordance with those obtained by Ibrahim et al. (1996) which showed that *C. calothyrsus* grew better in a P-deficient soil, when VAM-infected brachiaria roots were added to the pots. These authors demonstrated that early growth of inoculated *C. calothyrsus* seedlings was between 4 and 31 times greater than uninoculated plants according to the level of P application. From this work, we predict that mycorrhizal inoculation will be effective and necessary ,if soils at planting sites are P-deficient and indigenous AM fungi are ineffective or absent.

In conclusion, we can say that the establishment of a large collection of both rhizobia and AM fungi able to form a symbiosis with *C. calothyrsus*, should enable the selection of effective microsymbionts. Selection of the best rhizobia–AM combinations will be made through single and dual inoculations under greenhouse and field conditions and also using several provenances of *C. calothyrsus* because Lesueur (2001) showed that there is a strong interaction between rhizobia strains and plant provenances. From this work we can also say that differences between effective and ineffective isolates of rhizobia and AM fungi do not appear to be related to country of origin. It will be also interesting to compare the effectiveness of these rhizobia strains with their distribution in the several genomic groups determined by using molecular biology techniques such as PCR-RFLP of the 16S rRNA gene.

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Effect of *Rhizobium* inoculation methodologies on nodulation and growth of *Leucaena leucocephala*

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Summary

The aim of this study was to evaluate the effect of five methods of *Rhizobium* inoculum application on nodulation and nitrogen fixation in *Leucaena leucocephala* seedlings cultivated for 6 months in the greenhouse. Plants inoculated with alginate beads were significantly more developed and more nodulated than plants inoculated with the other methodologies used.

Introduction

The *Leucaena* genus is composed of several species which offer real potentialities for agroforestry (Shelton 1998). Like many other leguminous plants, the *Leucaena* species nodulate with *Rhizobium* and fix atmospheric nitrogen. Various workers have shown that *Leucaena leucocephala* and other *Leucaena* species are nodulated by a specific group of rhizobia (Turk & Keyser 1992; Lesueur *et al.* 1998). Consequently, the introduction of *L. leucocephala* into a new location generally results in the absence of effective nodulation due to the lack of specific rhizobia in the soil (Sanginga *et al.* 1988). In this situation, systematic inoculation of the seedlings with selected strains of *Rhizobium* is essential for symbiotic efficiency. Field studies using single and mixed strain inocula have demonstrated that the early growth of *L. leucocephala* plants is significantly improved by inoculation with specific *Rhizobium* (Norris 1973; Bushby 1982; Homchan *et al.* 1989; Sanginga *et al.* 1989; Wong *et al.* 1989). However, in several instances (Norris 1973; Bushby 1982; Homchan *et al.* 1989) the growth differences between inoculated and uninoculated plants were not evident 1-2 years after sowing. Loss of benefit from inoculation with an effective strain may be due to poor adaptation of the *Rhizobium* strain to soil conditions and to competition from ineffective indigenous rhizobial strains. Moawad & Bohlool (1984), Somasegaran & Martin (1986), Homchan *et al.* (1989), and Wong *et al.* (1989) advocated the application of the *Rhizobium* strain TAL 1145, which is an elite strain for *L. leucocephala* and *L. diversifolia* with high nitrogen fixation ability. However, in greenhouse conditions, Lemkine & Lesueur (1998) obtained excellent results on the growth of several species of *Leucaena* with the strain LdK4, higher than those inoculated with strain TAL 1145. Therefore, the most generalized solution is to use a cocktail of strains that effectively nodulate woody leguminous plant species like *L. leucocephala*, *Calliandra calothyrsus* and *Gliricidia sepium*. This type of inoculum is distributed by the Oxford Forestry Institute and the NIFTAL Project of the University of Hawaii for field inoculation with all seedlots of *Leucaena* (Hughes 1993). From a practical point of view, the inoculation of woody leguminous plants, and more particularly *L. leucocephala*, can be carried out in various ways. Different inoculation methodologies reported in literature are presented in Table 1. We note that the seeds can be coated directly with the inoculum using gum arabic, or even with sweetened water.

Young seedlings can be inoculated classically with a liquid culture of the selected strain of *Rhizobium*, or by the intermediary of an inoculum adsorbed on an inert support like peat (Table 1). As we can see in this table, the inoculation of *L. leucocephala* is usually carried out by coating seeds with peat inoculum containing rhizobia. But several questions arise regarding the success of nodulation when the inoculum is coated around the seed. For this reason, it seemed interesting for us to compare this methodology of inoculation with others which involve the utilization of liquid inoculum.

Another method of *Rhizobium* inoculum application to legumes using alginate beads was developed by Diem *et al.* (1989). This method has not been tested on

Table 1. Methodologies used for the inoculation of *L. leucocephala* with rhizobia

Strain used	Methodology of inoculation	Size of inoculum	Type of experiment	Type of sowing	Reference
TAL 582 TAL 82 TAL 1145 = CB3060	Seed coating with peat inoculum	10^7 10^8 cells per seed	Field conditions	Direct sowing	Thies *et al.* (1990)
Irc 1045 Irc 1050	Seed coating with peat	10^7 cells per seed	Field conditions	Direct sowing	Sanginga *et al* (1989)
TAL 1145 = CB3060	Seed coating with peat inoculum	4×10^7 cells per seed	Field conditions	Direct sowing	Homchan *et al.* (1989)
LdK4	Liquid inoculum	2×10^9 cells per seedling	Greenhouse conditions	Sowing in soil and after putting in soil	Lemkine & Lesueur (1998)
$CB8_1$ and NGR8	Seed coating with peat inoculum	1.6×10^5 cells per seed	Field conditions	Sowing in paper sacks and after putting in soil	Norris (1973)

Leucaena. The production of these beads is carried out in the laboratory. Once dried, these beads are deposited in contact with the root system of plant or hand before being dissolved in a phosphate buffer solution.

To our knowledge, no study has been undertaken to identify the most effective method of inoculation to optimize nodulation and the growth of *L. leucocephala*. We describe in this article our results obtained on this topic.

Materials and Methods

Source of Rhizobium *and* Leucaena *seed*

The *Rhizobium* strain used in our experiments was LDK4 which was isolated in Kenya. It was grown in YEM medium (Vincent 1970). *Leucaena* seeds were harvested from an orchard in the ISRA/IRD Bel Air centre (Dakar, Sénégal).

Leucaena seeds were scarified for 30 min in concentrated sulphuric acid, washed with sterile water and germinated in petri plates containing 8 g of agar per litre. Seeds were placed in an incubator for 2 days at 28 °C.

Methodologies used for the inoculation of plants

We tested five different methodologies for the inoculation of *Leucaena* seedlings:

1. Inoculation with 20 mg of nondissolved alginate beads containing a culture of LDK4 strain.
2. Inoculation with a pure liquid culture of the LDK4 strain 1 week after sowing at the surface of the soil, around the root system of plant (1 ml of inoculum).
3. Coating of *Leucaena* seedlings with gum arabic and then of pure liquid culture of the LDK4.
4. Mixing of gum arabic and pure liquid culture of LDK4 and then putting the mixture in contact with the *Leucaena* seedlings.
5. Inoculation at the level of plant collar (1 ml) of the seedlings with a pure liquid culture of LDK4 at the same time that the seedlings are planted in the plastic bags.

Description of the experiment

Seedlings inoculated with *Rhizobium* were placed into plastic bags containing nonsterile soil from Bel Air Centre (pH 7.35; Carbon 0.36%; Organic matter 0.62%; Nitrogen content 0.44‰). Each inoculation treatment comprised 12 replicates. Plants were grown for 6 months (March–August 1999) 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.

Results and Discussion

Plants inoculated with the alginate beads had more nodules compared with those inoculated by other methods (Table 2). There were no significant differences in nodulation among the other methods. On the other hand, uninoculated control plants also formed some nodules (46 per plant) indicating nodulation by indigenous rhizobia present in the soil. When the biomass of nodules was analysed, it was noted that this superiority of the number of nodules of the control plants did not result in a higher biomass of nodules than the values obtained with the inoculated plants (Table 2). This was also true with regard to the shoot and root dry weight. We can thus conclude that the control plants were nodulated by ineffective native strains which induce the formation of many small, ineffective nodules.

The plants inoculated with the alginate beads produced the highest dry matter of nodules and shoot. The difference is very significant, because it corresponds to an increase of 35% in the biomass of the nodules, and approximately 50% of the shoot dry weight compared to the inoculated plants with the other four classical methods. This type of inoculum has already been tested

Table 2. Effects of the methodologies used for the inoculation practiced with the *Rhizobium* strain LdK4 on nodulation and growth of *L. leucocephala.*

Methodologies used for the inoculation	Number of nodules per plant	Nodule dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Shoot total nitrogen content (%)
Control	46b	0.187bc	4.28ab	5.00c	3.10c
Alginate beads[1]	75c	0.288d	8.80b	4.04ab	2.20a
Classical[2]	21a	0.188bc	4.34ab	4.81bc	3.15c
Method 1[3]	24a	0.115a	3.68a	2.56a	2.42b
Method 2[4]	29a	0.244cd	3.05a	2.63a	2.53b
Immediately[5]	20a	0.144ab	3.92a	4.05b	3.16c

Note: For each factor, the values (average of 12 repetitions) in the same column followed by the same letter are not significantly different according to the Fisher test ($P < 0.05$).

[1] Inoculation with a pinch of nondissolved alginate beads.

[2] Inoculation 1 week after sowing at the level of plant collar (1 ml of inoculum).

[3] Coating of seeds pre-germinated with gum arabic and then of inoculum.

[4] Mix gum arabic and inoculum then put in contact with the pre-germinated seeds.

[5] Inoculation the same day at the level of plant collar (1 ml of inoculum).

Control means non-inoculated plants.

successfully in field conditions (Brunck *et al.* 1990), and on several species of nitrogen-fixing trees like *Acacia mangium* (Galiana *et al.* 1994; Lesueur *et al.* 1994) and *Casuarina equisitifolia* (Sougoufara *et al.* 1989), and it allowed significant increases in production compared to the uninoculated trees. Our results confirm the effectiveness of this type of inoculum in a nonsterile soil. However, it is difficult to explain the low shoot nitrogen content measured in these plants. The increased production of shoot biomass could induce a dilution of the amount of fixed nitrogen in plant tissue. But this has to be confirmed by further investigations.

One of the constraints of the alginate beads is that they should be dissolved in a solution of buffer phosphate (Diem *et al.* 1989). This means that during inoculation, the plant also benefits from phosphate fertilization. One of the goals of our work is to distribute the local farmers with a complete inoculum in the broad sense (*Rhizobium*, endomycorrhizae and ectomycorrhizae). However, there is an inverse relationship between phosphate concentration and mycorrhizal colonization of seedlings. For this reason the alginate beads were used directly, without solubilization in phosphate, by depositing them on the surface of the soil so that they were dissolved gradually during daily watering. The results so obtained were very positive, and it is very encouraging for distribution of inoculum to the local farmers.

Concerning the technique of direct sowing, and coating of the inoculum on the surface of the seeds, our results show very clearly that in terms of nodulation, the seedlings inoculated with methods 1 and 2 are less nodulated than those inoculated with the alginate beads. Moreover, both shoot and root dry weights of these seedlings are 50% lower than those observed in plants inoculated with the alginate beads.

Lastly, it will be noted that the three techniques (methods 1, 2 and 5) which involve application of *Rhizobium* inoculation directly to the roots of the young seedlings did not lead to significant nodulation. The delivery of the inoculum 5–6 days after the establishment of the plant remains in our view the best period of inoculation.

In conclusion, our work shows the importance of the mode of inoculation on nodulation and growth of seedlings of *L. leucocephala.* The use of alginate beads as inoculum gave the best results (in particular compared to the direct sowing of coated seed with peat inoculum), even if they were not dissolved in a phosphate solution. Consequently, it seems that it is this type of inoculum which must be provided to local farmers for inoculation of their trees, because the storage of the alginate beads is easy and the method of application is simple and is more likely to lead to successful nodulation.

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Rapport de mission effectuée au Sénégal du 14 au 22 octobre 2001 dans le cadre du projet INCO Calliandra de l'Union Européenne

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Rapport de mission effectuée au Sénégal du 14 au 22 octobre 2001 dans le cadre du projet INCO Calliandra de l'Union Européenne :

Objet de la mission :

Cette mission avait pour objectif de rendre opérationnel le système ELISA (Enzyme Linked Immunosorbent Assay), équipement financé dans le cadre du projet INCO Calliandra, avant de former différentes personnes impliquées dans le projet à cette technique de caractérisation immunologique.

Intérêt de l'utilisation de la méthode ELISA dans le cadre du projet INCO Calliandra :

Bien que d'autres méthodes d'identification de souches de rhizobium plus performantes que la méthode ELISA soient déjà opérationnelles au laboratoire de Dakar, notamment de biologie moléculaire, les méthodes immunologiques restent particulièrement performantes pour étudier la dynamique des populations bactériennes, tant au niveau qualitatif que quantitatif. En effet, sous condition que cette méthode immunologique soit utilisée dans le contexte particulier d'études sur la compétition et la survie au cours du temps de souches bactériennes connues introduites dans le sol ou tout autre substrat, elle a l'avantage par rapport aux autres méthodes de pouvoir traiter une très grande quantité de nodosités racinaires (siège de la fixation de l'azote contenant les rhizobiums chez les Légumineuses) en un minimum de temps.
Dans le cadre du projet INCO, de nombreuses études de compétition *in situ* ou en conditions contrôlées ont déjà été entreprises ou sont prévues chez *Calliandra calothyrsus* et chez d'autres espèces d'arbres étudiées en comparaison.

Déroulement de la mission et personnes rencontrées :

- Dimanche 14 octobre :

Départ de Montpellier et arrivée à Dakar à 21 h.

- Lundi 15 octobre :

- Matin :

1) Visite du laboratoire de microbiologie de l'IRD (Institut de Recherche pour le Développement) à Bel-Air (Dakar) ;
2) Réunion sur l'objet de la mission et les objectifs du projet avec Didier LESUEUR, chercheur CIRAD-Forêt coordinateur du projet INCO Calliandra travaillant au laboratoire de microbiologie, Marc NEYRA, chercheur IRD Directeur du laboratoire de microbiologie, et Amadou SARR, doctorant de l'Université de Marrakech (Maroc) travaillant au laboratoire de microbiologie sous la direction de D. LESUEUR ;
3) Participation à la réunion de laboratoire hebdomadaire avec présentation des objectifs de la mission et de mes activités de recherche ;

4) Rencontre avec Mamadou GUEYE, chercheur de l'ISRA (Institut Sénégalais de Recherche Agricole), Ibrahima N'DOYE et Samba SYLLA, respectivement Maitre de Conférence et Maitre assistant du Département de Biologie Végétale à l'UCAD et travaillant au laboratoire de microbiologie et discussion sur leurs activités de recherche.

- Après-midi :

Déballage des appareils (spectrophotomètre ELISA de la marque Labsystems Multiskan EX, imprimante en sortie, laveur de plaques ELISA et agitateur de plaques), montage, programmation et mise en route.

- Mardi 16 octobre :

- Matin :

Démarrage d'une expérimentation pour calibrer l'appareillage ELISA et à but démonstratif :
1) Définition d'un protocole expérimental ;
2) Préparation des milieux de culture bactériens et ensemencement de différentes souches de rhizobium pures à tester.

- Après-midi :

Préparation des réactifs, tampons et autres solutions chimiques nécessaires à la réalisation du test ELISA.

- Mercredi 17 octobre :

- Matin :

Démarrage du test ELISA de démonstration : Dépôt des antigènes à différentes dilutions, fixation des antigènes, incubation 1 h, lavage, dépôt des 1ers anticorps (sérums) à différentes dilutions, incubation 1 h, lavage (voir protocole détaillé en Annexe 1).

- Après-midi :

Dépôt des 2^{nds} anticorps (conjugué) à différentes dilutions, incubation 3 h, lavage (voir protocole détaillé en Annexe 1), conservation des plaques ELISA en chambre froide.

- Jeudi 18 octobre :

Sortie sur le terrain à Keur Momar Sarr, à 200 km au Nord de Dakar, en présence de Didier LESUEUR, Marc NEYRA et Jacques BIAGUI (Technicien sur financement CIRAD-Forêt) : Visite d'une ferme pilote de cultures maraîchères en irrigué mise en place dans le cadre d'un financement Sénégalo-Israélien. Visite des essais sylvicoles mis en place au sein des périmètres irrigués : plantations pures ou mixtes d'*A. auriculiformis*, d'*A. mangium* et d'*A. crassicarpa* ; utilisation de *Leucaena leucocephala* en « alley cropping ».

Retour au laboratoire de Microbiologie à Bel Air et rencontre avec Bassirou SOUGOUFARA, Responsable de la Division Suivi et Evaluation de la Direction des Eaux et Forêts, Chasse et Conservation des Sols (DEFCCS).

- Vendredi 19 octobre :

- Matin :

1) Suite et fin de l'expérimentation ELISA initiée et interrompue le mercredi 17 octobre : Dépôt du substrat de l'enzyme, lecture des densités optiques au spectrophotomètre après plusieurs temps d'incubation (voir protocole détaillé en Annexe 1) puis interprétation des résultats.

2) Départ pour la Faculté des Sciences de Dakar et visite du laboratoire de Biotechnologie de l'UCAD en compagnie de Diaga DIOUF, Maître Assitant du Département de Biologie Végétale à l'UCAD.
Rencontre et discussion sur les recherches effectuées à l'UCAD avec Tidiane BA, chef du Département de Biologie Végétale de l'UCAD, Yaye Kene Gassama DIA et Mame Oureye SY, respectivement Maitre de Conférence et Maitre Assistant du Département de Biologie Végétale à l'UCAD.

- Après-midi :

1) Visite de l'URCI, laboratoire de culture in vitro situé à Bel Air et mitoyen du laboratoire de microbiologie ;

2) Visite des essais sylvicoles mis en place par D. LESUEUR sur le centre IRD de Bel Air à proximité du laboratoire de microbiologie :
Essai d'inoculation rhizobium sur *Calliandra calothyrsus* ; essais comportementaux chez *Gliricidia sepium*, *Tamarindus indica*, *Eucalyptus camaldulensis*, *Leucaena leucocephala* et *Casuarina equisetifolia* ; verger à graines multiprovenances de *Calliandra calothyrsus* ; collection multiclonale d'hybrides *Acacia mangium x A. auriculiformis* ; essai compétition souches de rhizobium en fonction de la provenance chez *Acacia senegal* et *Acacia nilotica* ;

3) Bilan de la mission avec D. LESUEUR et discussion sur les suites à y donner (poursuite des expérimentations ELISA dans la cadre du projet INCO Calliandra, notamment par le biais d'un stage de 3 semaines d'A. SARR à Montpellier au Laboratoire des Symbioses Tropicales et Méditerranéennes en Décembre 2001,...).

- Samedi 20 octobre :

Rédaction et récapitulation d'un protocole ELISA standard adapté à des cultures pures de rhizobium et des nodosités racinaires d'arbres fixateurs d'azote.

- Lundi 22 octobre :

Départ pour Paris et arrivée à Montpellier à 21 h.

Conclusion :

Cette mission a permis de rendre l'appareillage ELISA opérationnel sans problème technique particulier.
Les résultats de réactions homologues et hétérologues obtenus à partir de deux souches de rhizobium de référence se sont révélés positifs. Cependant, le développement trop rapide de la coloration du substrat de l'enzyme en fin de test implique l'utilisation ultérieure du 2^{nd} anticorps (conjugué Goat anti rabbit-phosphatase alkaline) à une dilution beaucoup plus élevée (de l'ordre de $1/10.000^{ème}$) pour déterminer avec précision le titre des différents sérums.
Ces mises au point devront être répétées et élargies aux 7 sérums disponibles en utilisant comme antigènes des cultures de rhizobium pures puis des nodosités racinaires contenant les souches correspondantes afin de détecter des réactions croisées souches-anticorps éventuelles. Ces travaux préliminaires permettront dans une seconde étape d'appliquer la technique sur des nodosités contenant des souches à identifier dans le cadre d'études de compétition ou de survie après inoculation.
Il est proposé qu'une partie de ces travaux de mise au point soient réalisés avant la fin de l'année 2001 au LSTM à Montpellier dans le cadre d'un stage de 3 à 4 semaines d'Amadou SARR financé par le projet INCO Calliandra.

Annexe 1 :

Protocole ELISA testé sur les souches de rhizobium LDK4 et CCK13 de *Calliandra Calothyrsus* (réactions homologues et hétérologues) :

- Dépôt des antigènes (cultures bactériennes en fin de croissance exponentielle) à deux dilutions différentes : 1/100ème (10^7 bactéries/ml) et 1/10.000^{ème} (10^5 bactéries/ml) (voir figure du plan de plaque en Annexe 2) ;

  Dilution des suspensions bactériennes dans des tubes Eppendorf en prélevant 0,15 ml de chaque suspension à une concentration de 10^9 bactéries/ml à rajouter dans 1,35 ml de Phosphate Buffer Saline (1,5 ml total) -> dilution 1/10ème puis répéter l'opération pour obtenir une dilution de 1/100ème.
  Faire la dilution au 1/10.000^{ème} de la même façon (vortexer entre chaque dilution avant de prélever).
  Dépôt de 100 µl par puits de A1 à H9 (voir plan de plaque en Annexe 2), ne pas déposer d'antigène dans les puits A10 à H12 (témoins négatifs) ;

- Placer la plaque dans un bain-marie à 100°C et à 5 cm de la surface de l'eau bouillante avec couvercle fermé ;

- Séchage des puits au sèche-cheveux pendant 15 min (intensité maximale à 20 cm au-dessus de la plaque) ;

- Dêpôt du Coating Buffer (carbonate buffer pH 9,8 -> ajustement du pH non nécessaire, voir composition du tampon dans l'Appendix 4 de Somasegaran et Hoben, Handbook for Rhizobia, Springer-Verlag, 1994) à raison de 100 µl par puits ;

- Agitation pendant 1 min sur agitateur de plaques réglé sur puissance 7 ;

- Incubation pendant 1 h à l'étuve à 37°C en posant un couvercle sur la plaque ;

- Lavage avec du PBS-Tween : 3 fois de suite avec 5 s de trempage entre chaque bain de lavage (programme 1 du laveur de microplaques : nombre de cycles : 3 ; soak : 0 ; volume : 0,45 ml ; horizontal : 20 ; vertical : 250) ;

- Dépôt de 100 µl du 1er anticorps (sérum lapin-anti rhizobium) dans chaque puits* à une dilution de 1/2560ème (titre FITC des sérums donné par le fournisseur : 1/1000ème) pour les deux sérums testés : anti-LDK4 et anti-CCK13 -> dilution obtenue par ajout de 10 µl de chacun des sérums dans 26 ml de PBS (mélange dans des tubes Corning à centrifugation avec bouchons à vis) et passage au vortex.
  * de A1 à H9 (lignes A, C, E, G pour anti-CCK13 et B, D, F et H pour anti-LDK4) (voir plan de plaques en Annexe 2) ;

- Agitation pendant 1 min sur agitateur de plaques réglé sur puissance 7 ;

- Incubation pendant 1 h à l'étuve à 37°C en posant un couvercle sur la plaque ;

- Lavage 3 fois avec du PBS-Tween comme précédemment ;

- Dépôt du 2nd anticorps GAR-PA (Goat anti-rabbit conjugué à la phosphatase alkaline) à différentes dilutions : 1/2000ème ; 1/4000ème et 1/8000ème -> prise de 3 µl de conjugué mélangé à 6 ml de PBS (= 1/2000ème), puis dilution de 3 ml de la dilution à 1/2000ème rajouté à 3 ml de PBS (= 1/4000ème) et reprise de 3 ml à 1/4000ème rajoutés à 3 ml de PBS (= 1/8000ème).
  Dépôt de 100 µl de chacune des 3 dilutions dans les puits : de A1 à H3 pour GAR-PA à 1/2000ème, de A4 à H6 pour GAR-PA à 1/4000ème et de A7 à H9 pour GAR-PA à 1/8000ème (voir plan de plaques en Annexe 2).
  Puis dépôt de 100 µl de GAR-PA à 1/2000ème dans le puits A10 et 50 µl dans le puits A11 ; idem pour GAR-PA à 1/4000ème dans les puits C10 et C11 et GAR-PA à 1/8000ème dans les puits E10 et E11 (pas assez pour les autres puits) ;

- Incubation du GAR-PA pendant 3 h (mais 1h30 minimum possible) dans l'étuve à 37°C avec un couvercle posé sur la plaque ;

- Lavage 3 fois avec du PBS-Tween comme précédemment ;

  (à ce stade, conservation de la plaque en chambre froide à 4°C pendant une nuit + 24 h supplémentaires)

- Préparation du PNPP (p-Nitrophenyl Phosphate, substrat de l'enzyme phosphatase alkaline) : à préparer le jour même de l'utilisation dans 100 µl d'un tampon Diéthanolamine à pH 9,8 (voir composition dans l'Appendix 4 de Somasegaran et Hoben), ce dernier devant être conservé dans une bouteille teintée -> Prise de 10 µl de ce dernier tampon et dissolution de 10 mg de PNPP ;

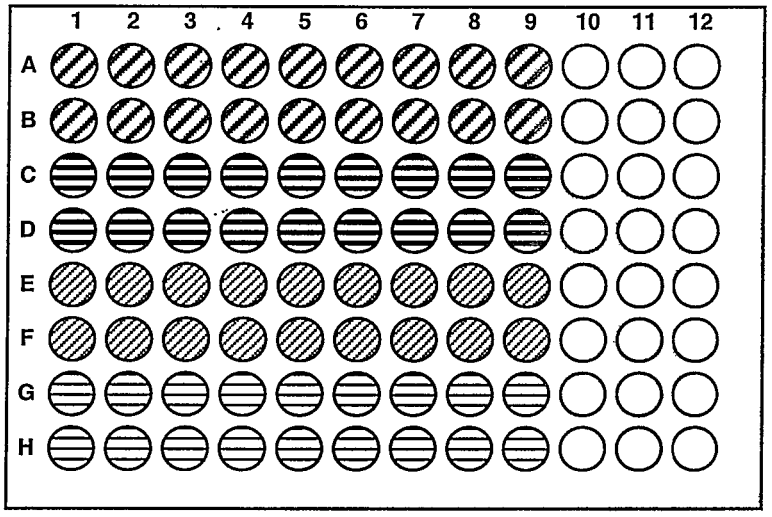
- Dépôt de 100 µl dans chacune des 96 puits de la plaque (distribués avec la pipette multicanaux comme lors des précédentes étapes) et mise en incubation de la plaque à l'étuve à 37°C ;

- Lecture de la plaque pour mesures des absorbances à 405 nm à 16 et 30 min (voir résultats bruts en Annexe 3 -> remarque : développement d'une coloration jaune immédiat mais normalement, les mesures sont effectuées après 30 mn puis 60 mn d'incubation) ;

- Stoppage de la réaction de coloration par ajout de NaOH à 1 M à raison de 50 µl par puits (normalement NaOH à 3 M).

Annexe 2 :

1) Dépôt de l'antigène :



Souche LDK4 à 10^7 bact./ml

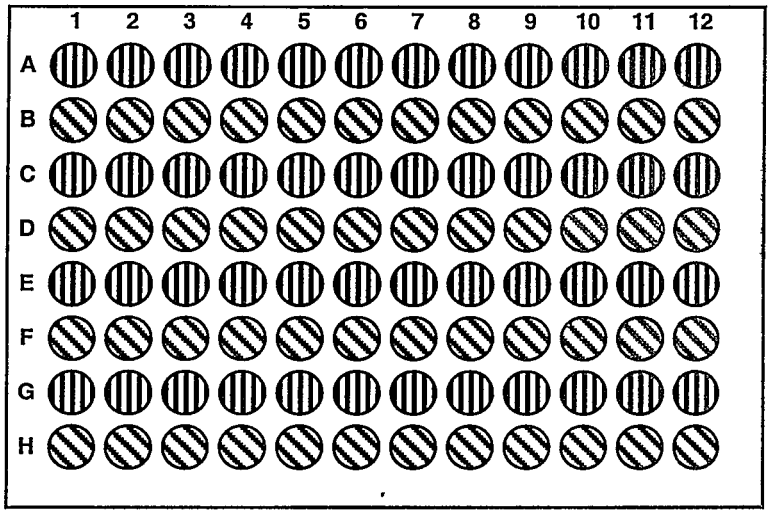
Souche CCK13 à 10^7 bact./ml

Souche LDK4 à 10^5 bact./ml

Souche CCK13 à 10^5 bact./ml

Témoin sans souche

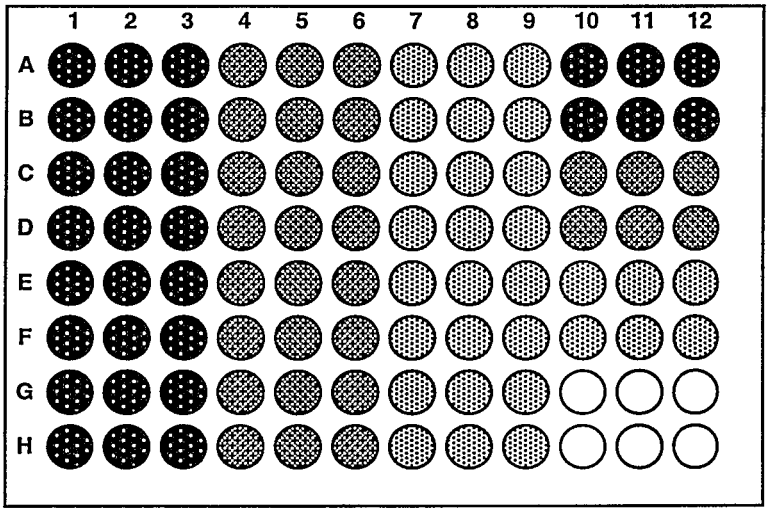
2) Dépôt du 1er anticorps :



Sérum anti-CCK13 dilué au 1/2560ème

Sérum anti-LDK4 dilué au 1/2560ème

3) Dépôt du 2nd anticorps (Conjugué Goat Anti-Rabbit/Phosphatase Alkaline) :



GAR-PA dilué au 1/2000ème

GAR-PA dilué au 1/4000ème

GAR-PA dilué au 1/8000ème

Témoin sans GAR-PA

Rapport de stage effectué à Montpellier du 10 au 30 novembre 2001 dans le cadre du projet INCO Calliandra de l'Union Européenne

Amadou SARR

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BP : 1386 Dakar, Sénégal

Maître de Stage : Antoine GALIANA du Programme Arbre et Plantation, CIRAD-Forêt

Laboratoire des Symbioses Tropicales et Méditerranéennes, UMR 113, CIRAD-Forêt/IRD/INRA/ENSAM,
Campus International de Baillarguet
Montpellier

Initiation à la technique d'analyse des nodules de légumineuses forestières par la technique immunologique ELISA

Principe :

L'ELISA (Enzyme-Linked Immunosorbent assay) est une technique immunologique et colorimétrique, qui consiste tout d'abord à immobiliser des antigènes dans les cupules des microplaques de microtitration. Après une étape de lavage l'antigène est mis en contact avec des anticorps primaires spécifiques. Après une deuxième étape de lavage, les anticorps primaires attachés à l'antigène dans les cupules réagissent avec les anticorps secondaires conjugués à de la phosphatase alcaline qui va se lier spécifiquement aux anticorps primaires. La réaction avec un substrat d'enzyme (p-nitrophenyl phosphate) cause un changement coloré, dont l'intensité correspond à la quantité d'anticorps primaire présent.
À la différence de la plupart des autres méthodes sérologiques, l'ELISA peut être employée pour analyser un grand nombre d'échantillons simultanément, Ce qui fait qu'elle est particulièrement utile pour identifier des souches de *Rhizobium* dans de nombreux nodules.

Objectif : Analyser des nodules et de souches pures de *Rhizobium* par la technique ELISA

1- Nodules : il s'agit de voir si la souche inoculée de *Rhizobium* CCK13 dans un sol de Sangalkam non stérilisé, est présente dans les nodules de *Calliandra calothyrsus*.

2- Souches : nous testons toutes les réactions homologues (réaction de la souche avec son anticorps) et hétérologues (réaction de la souche avec des anticorps qui ne lui sont pas propres) des souches de *Rhizobium* : GSK4, LDK4, CCK13, 11C et 13C.

Analyses de nodules de *Calliandra calothyrsus* récoltés sur des plants cultivés dans un sol non stérile de Sangalkam

Origine des nodules

Essai d'inoculation de *Calliandra calothyrsus* avec la souche CCK13 dans des buses contenant du sol de Sangalkam non stérilisé. Après récolte, un aliquot de nodules est collecté sur chaque plant et puis est mis à sécher.

Préparation des nodules secs : Antigène

Cent nodules secs ont été écrasés sur une plaque en verre, la poudre de nodules a été récupérée dans des tubes Eppendorf, dans lesquels ont été ajoutés 0,5 ml de PBS (phosphate Buffered Saline ; voir annexe). La poudre de nodules forme l'antigène. Les tubes Eppendorf ont été placés dans un bain-Marie à 80°C et à 5 cm de la surface de l'eau couvercle fermé pendant 1 h. Lorsque les extraits de nodules ont sédimentés au fond des tubes Eppendorf, une partie du des culots a été re-suspendue dans 200 µl de coating buffer (pH9,8) et l'autre moitié dans 200 µl de PBS sans coating. Deux microplaques ont été utilisées. L'une avec coating buffer et l'autre sans coating. 100 µl ont été déposés dans les 96 cupules sur les microplaques ELISA à l'aide d'une pipette multicanaux. Les microplaques ont été incubées à l'étuve à 37°C. Après 3 h d'incubation, l'antigène est versé dans l'évier. Les microplaques, qui n'avaient pas reçues le coating buffer, ont été séchées au sèche-cheveux pendant 15 min avec l'intensité maximale et à 20 cm au-dessus de la microplaque. Les autres ont été séchées à l'air ambiant. Toutes les microplaques ont été lavées avec du PBS-Tween environ 3 fois avec 5 secondes de trempage entre chaque lavage (Programme 1 du laveur microplaques : NB cycle 3 ; Soak 0 ; volume 0,450 ml ; horizontal = 280 ; vertical = 250).

Sérum ou Anticorps primaire :

Deux sérums (Rabbit-anti-rhizobium) ou anticorps primaires ont été utilisés (anti-CCK13 et anti-KWN35). Les anticorps I lyophilisés ont été dilués dans 10 ml de PBS et aliquotés à raison de 1 ml par tube Eppendorf. Les aliquotes ont été stockées à –20°C. 100 µl d'anticorps I ont été déposés dans chaque cupule. Quatre dilutions (320^e, 640^e, 1280^e, 2560^e) de chaque anticorps I ont été testées. Les dilutions ont été réalisées dans des tubes corning à centrifugation avec bouchon à vis. Les microplaques ont été incubées à 37°C. Après 1 h d'incubation, l'anticorps est versés et les microplaques lavées comme précédemment au laveur de microplaques avec PBS-Tween.

Anticorps secondaire : GAR-PA

Dépôt de l'anticorps II ou GAR-PA (Coagulant Rabbit Conjugué à la Phosphatase Alkaline) à une dilution : $1/8000^e$ (5µl de GAR-PA dans 40 ml de PBS). 100 µl de l'anticorps II ont été déposés par cupule. Incubation de GAR-PA pendant 2 h dans l'étuve à 37°C couvercle de microplaque déposé.

Lavage au PBS-Tween 3 fois comme précédemment (conservation de la microplaque en chambre froide).

Préparation du substrat de l'enzyme (diéthanolamine à pH 9,8) à conserver dans une bouteille ambrée. Dix mg de PNPP (*p*-nitrophenyl phosphate) 1mg/ml ont été dissouts dans 10 ml de ce dernier tampon (à préparer extemporanément). 100 µl ont été déposés dans chacune des 96 cupules de la microplaque (distribués avec une pipette multicanaux), le tout étant incubé à l'étuve à 37°C.

La lecture de la microplaque pour les mesures des absorbances à 405 nm se fait après 30 min et 60 min.

Remarque : développement d'une coloration jaune immédiate.

Arrêt de la coloration par ajout de NaOH 3M à raison de 50 µl par cupule.

Résultats :

Les souches de *Rhizobium* présentes dans les nodules réagissent avec l'anticorps anti-CCK13 et donnent une DO à 405nm supérieure à 1 après 30 min qui va évoluer vers 1,4 après 60 min d'incubation a 37°C, ceci montre que cette souche inoculée dans le sol de Sangalkam a bien colonisé les nodules de cette plante. L'anticorps anti-KWN35 par contre réagit avec les nodules et donne DO à 405 nm de 0,6 qui reste constant même après 60 min d'incubation. (voir graphiques).

Réactions homologues et Réactions hétérologues pour deux souches : anticorps anti-GSK4, anticorps anti-LdK4, anticorps anti-CCK13, anticorps anti-11C et anticorps anti-13C :

Les souches de *Rhizobium* 13C, CCK13, 11C, LdK4, et GSK4 ont été cultivées sur du milieu YM pendant 48 à 72h. 1 ml de chaque culture a été mis dans un tube Eppendorf, puis centrifugé à 13000 rpm pendant 5 min. Le culot a été re-suspendu dans 1 ml de PBS. Cette opération a été répétée 3 fois. Les culots ont été dilués à 10^{-7}. Cent µl de chaque antigène ont été déposés par cupule. Pour cela, 7 microplaques ont été utilisées et identiquement organisées. Ces microplaques ont ensuite été incubées dans un bain-Marie à 80°C. Après 30 min d'incubation, les microplaques ont reçu du coating buffer à raison de 200 µl par cupules et ont été incubées en chambre froide à 5°C pendant toute la nuit. Après cette étape, le

contenu des microplaques a été versé, ensuite nous avons procédé au lavage des microplaques par le PBS-Tween à l'aide du laveur de microplaques.

Dilutions de l'Anticorps I : Six dilutions ont été utilisées (320^{e}, 640^{e}, 1280^{e}, 2560^{e}, 5120^{e} et 10240^{e}). L'incubation a été faite dans une étuve à 37°C. Après 1 h d'incubation, les microplaques ont été lavées comme précédemment.

Dilution de l'anticorps II (GAR-PA) : 1/14000^{e} (5µl de GAR-PA dans 70 ml de PBS). 100 µl de l'anticorps II ont été déposés dans les cupules. L'incubation à été faite a 37°C. Après 2 h, les microplaques ont été lavées au PBS-Tween.

Dépôt du PNPP : Toutes les microplaques reçoivent 100 µl de PNPP par cupule, l'incubation ayant été faite dans une étuve à 37°C. Les lectures ont été faites à 405 nm après 30 min et 60 min d'incubation.

La réaction a été arrêtée par ajout de NaOH 3M à raison de 50 µl par cupule.

Résultats :

Les souches 11C et 13C ont des affinités immunologiques. Ces deux souches réagissent en effet bien avec les anticorps anti-13C et anti-11C. (voir graphique)

La souche GSK4 réagit avec son anticorps ce qui est normal, mais en revanche elle réagit aussi avec l'anticorps l'anti-CCK13, ceci qui peut être éventuellement dû à une erreur de manipulation. Ceci est à confirmer.

Les anticorps anti-KWN35 et anti-KCC6 n'ont pas donné de réactions hétérologues avec les souches.

La souche LDK4, n'a pas réagi avec son anticorps anti-LdK4, ceci est à vérifier par la réalisation d'un autre test ELISA.

Annexe

PBS :

Dissoudre 5 pastilles de phosphate buffered saline dans 1 litre d'eau. Stocker à 4°C.

PBS-Tween :

Diluer 0,5 ml de tween 20 dans un litre de PBS et stocker à 4°C

Coating buffer :

Na_2CO_3	1,59 g
$NaHCO_3$	2,93 g
NaN_3	0,2 g

Dissoudre dans de l'eau distillée, ajusté à pH 9,6 avec du NaOH et compléter a 1 litre. Stocker à 4°C mais pas plus de deux semaines.

Tampon substrat de l'enzyme : Diethanolamine

Diethanolamine	97 ml
NaN_3	0,2 g
$MgCl_2$ ($6H_2O$)	100 mg

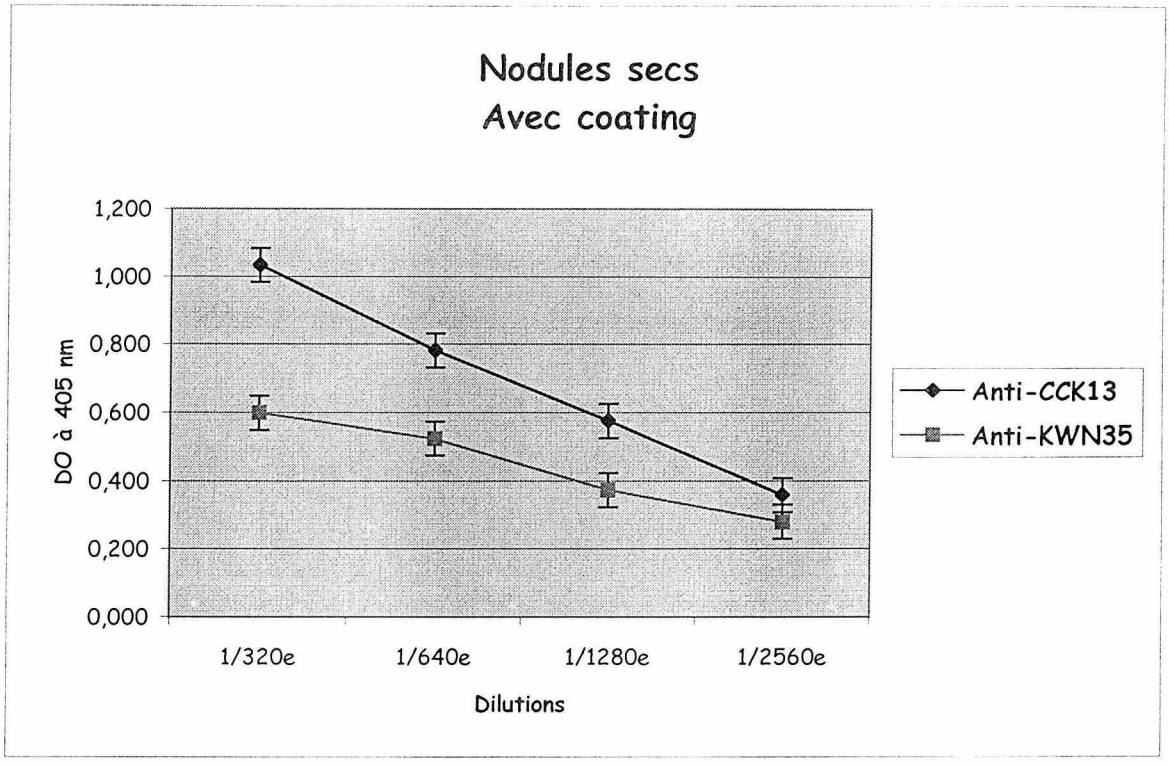
Dissoudre dans 800 ml d'eau distillée et ajuster le pH à 9,8 avec HCl. Ajuster le volume a 1 litre avec l'eau distillée. Stocker à température ambiante dans une bouteille ambrée.

Substrat de l'enzyme : p-nitrophenylphosphate

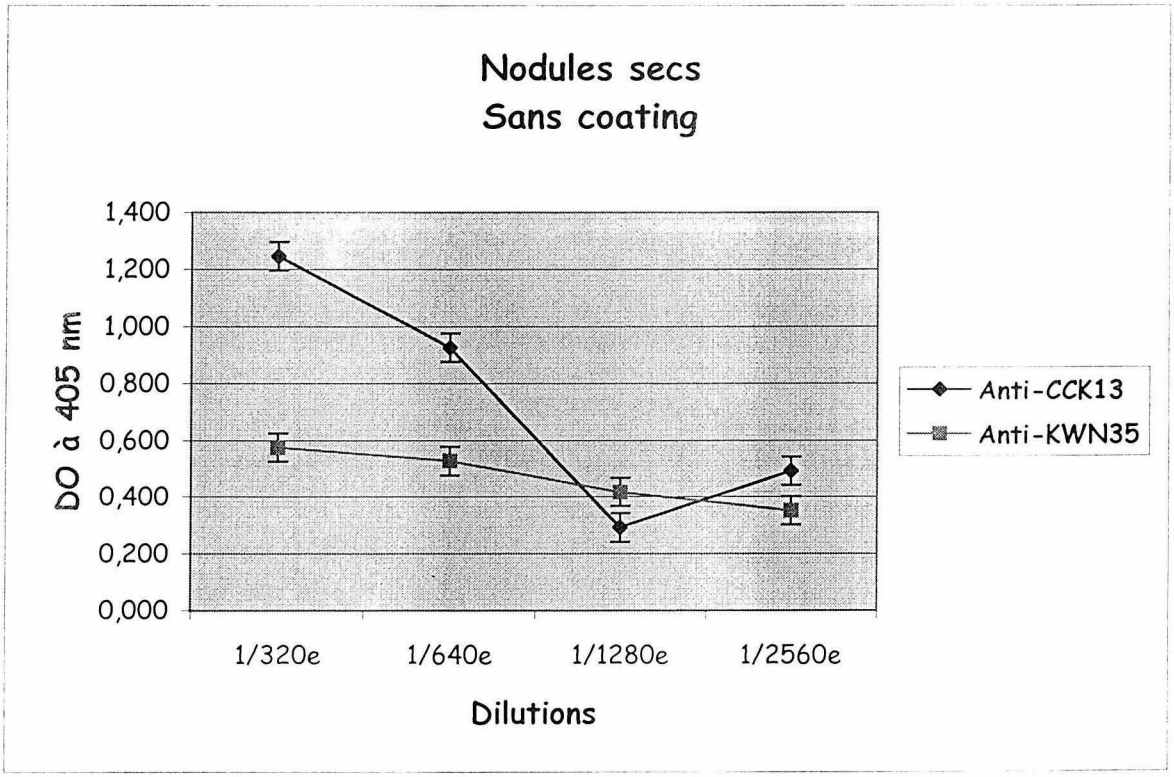
Dissoudre 5 mg de PNPP dans 5 ml du tampon diethanolamine.

A préparer extemporanément. Dissoudre le PNPP dans le tampon substrat de l'enzyme à température ambiante. Le PNPP est stocké a –20°C à l'obscurité avant l'utilisation.

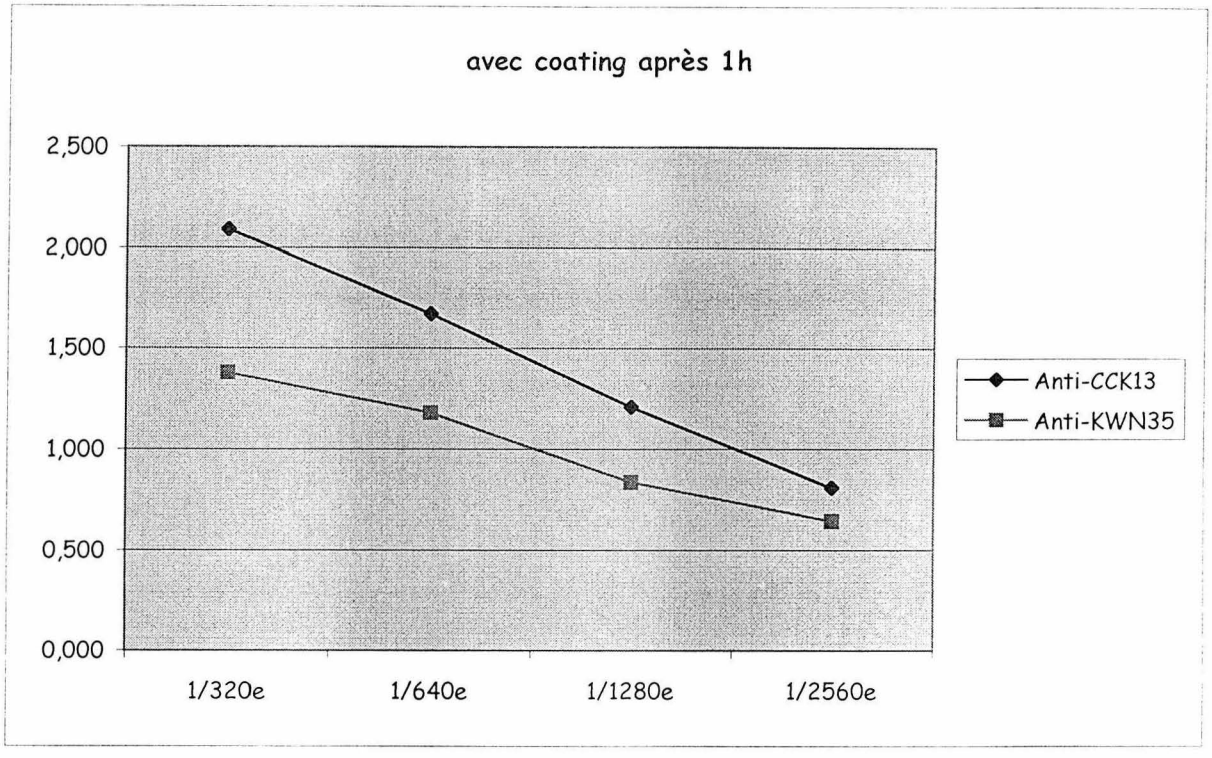
Graphique 1



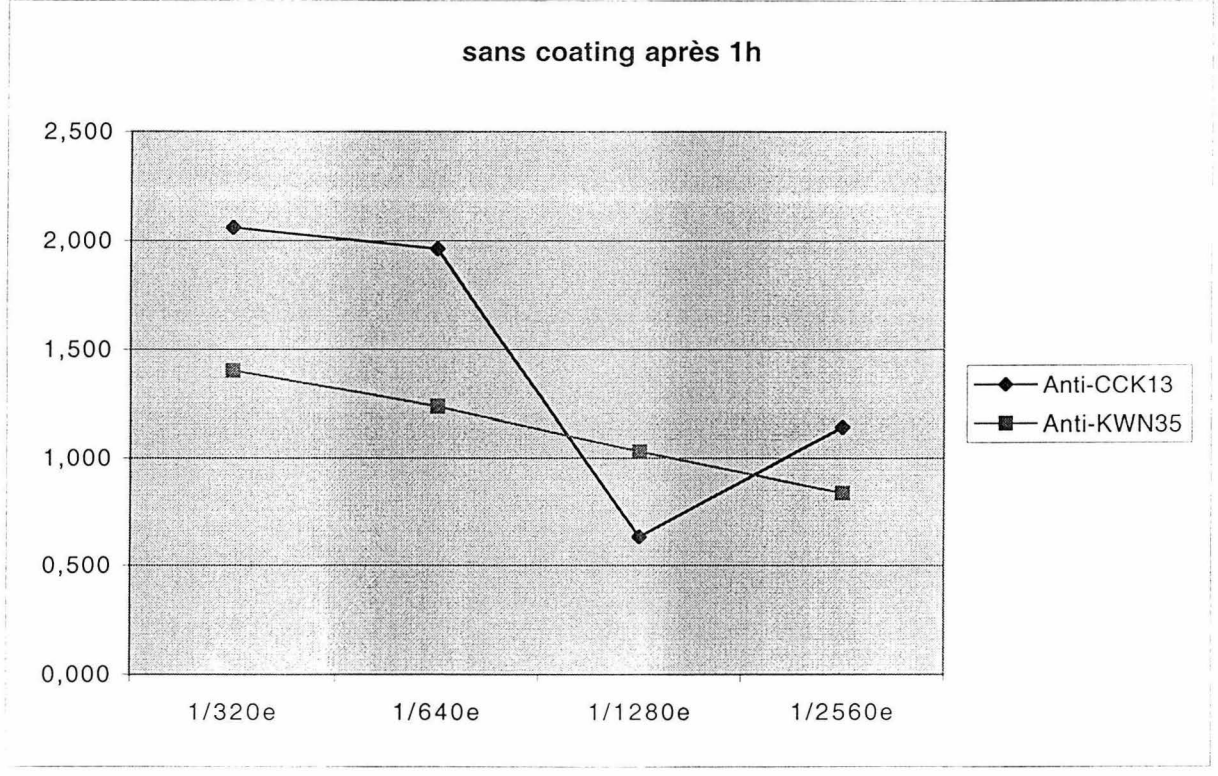
Graphique 2

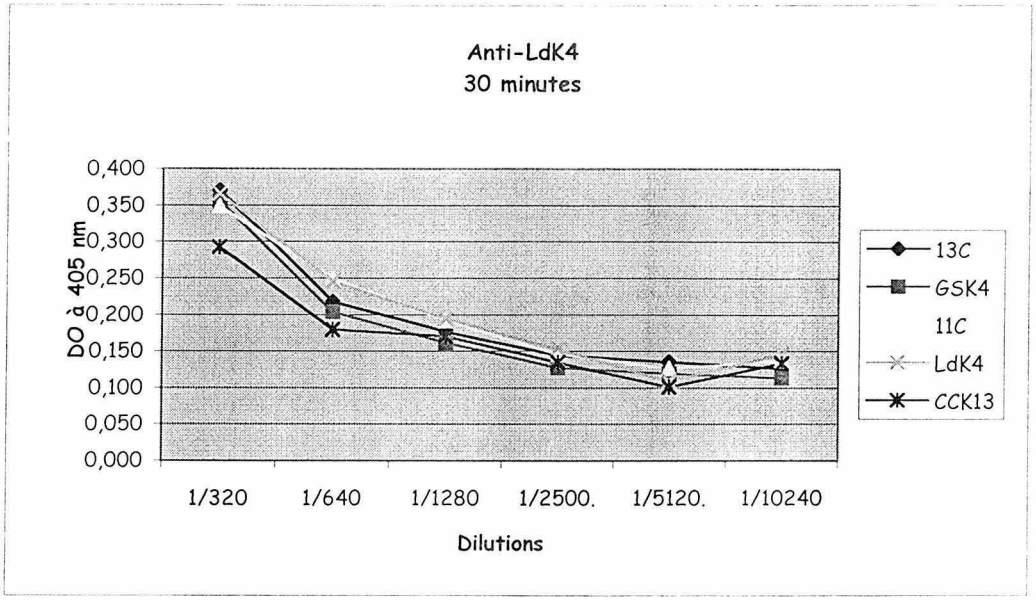


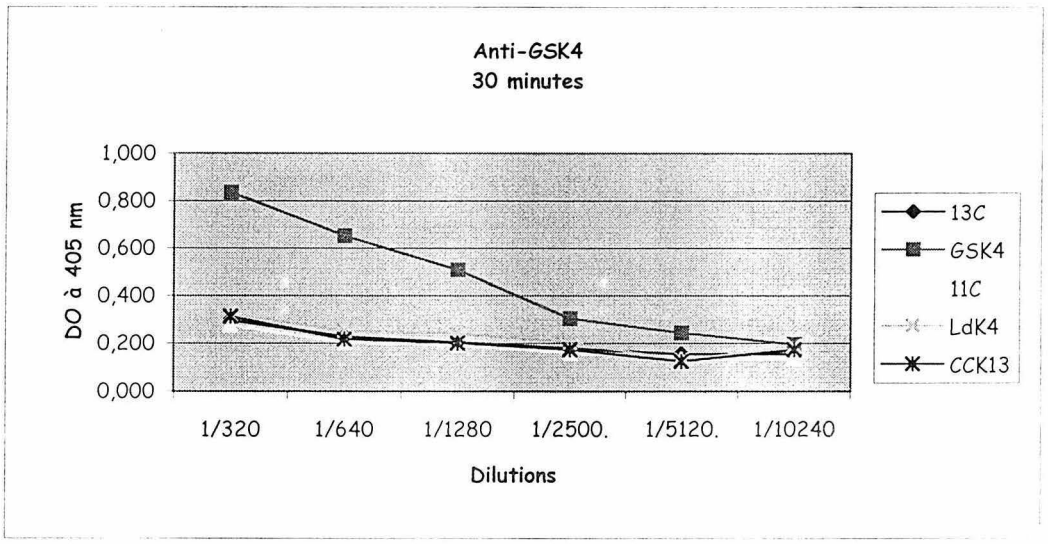
Graphique 3

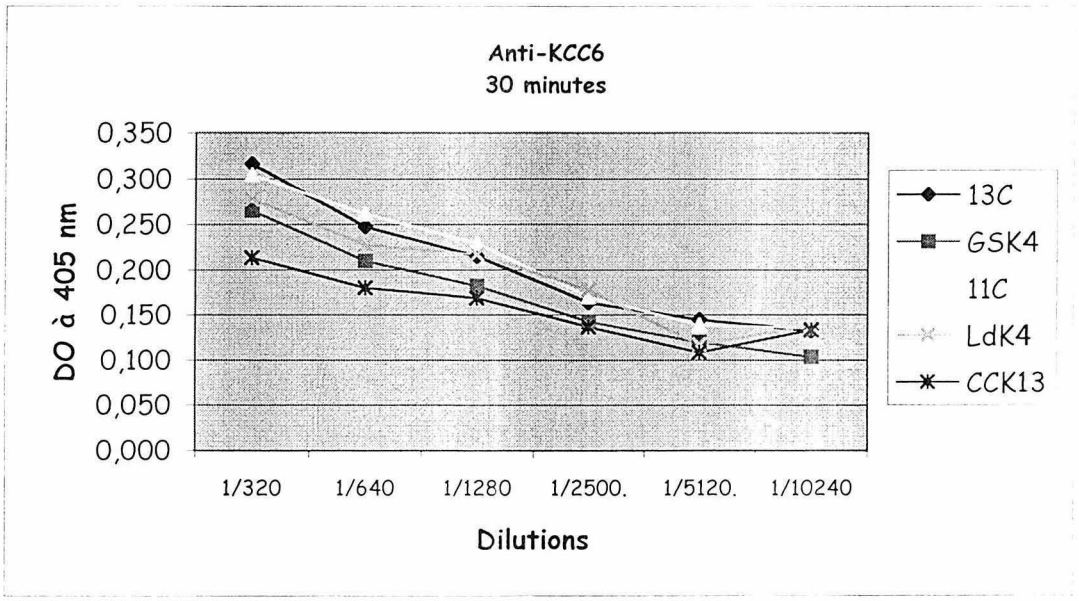


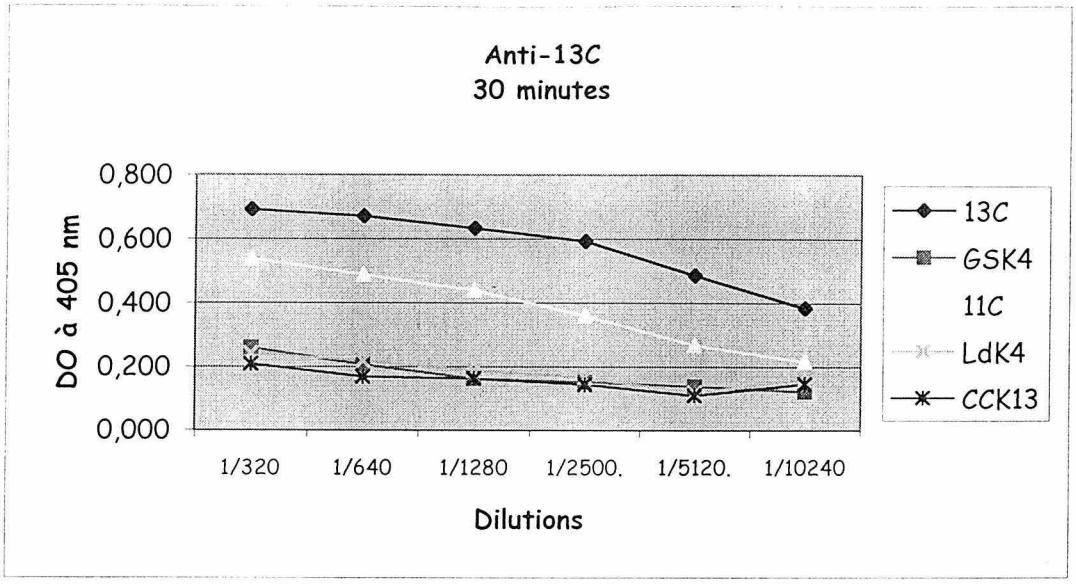
Graphique 4

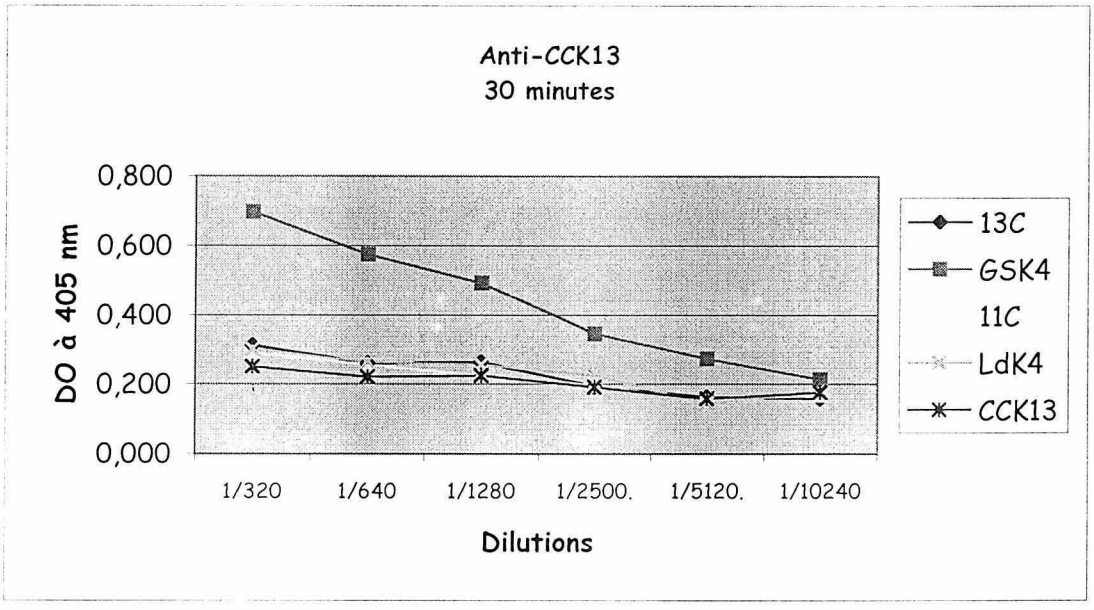


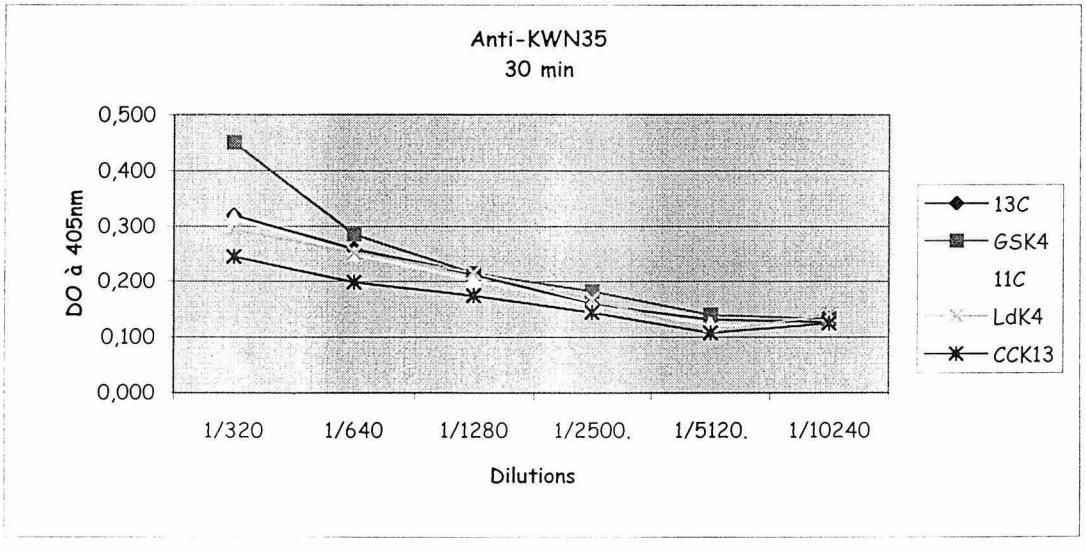

Anti-LdK4
30 minutes
DO à 405 nm
0,400
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
1/320
1/640
1/1280
1/2500.
1/5120.
1/10240
Dilutions
13C
GSK4
11C
LdK4
CCK13

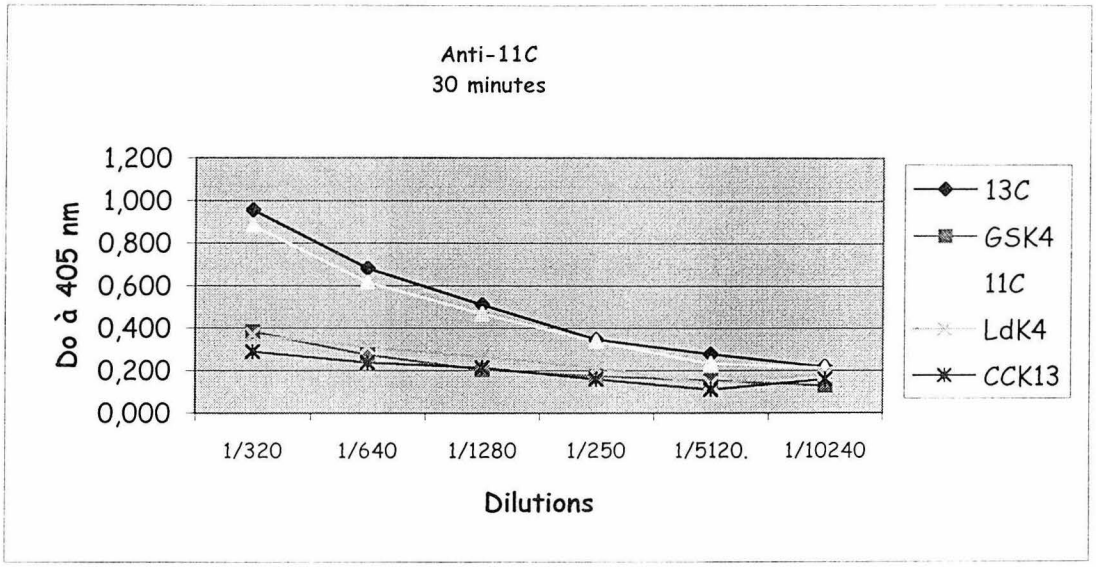

Anti-GSK4
30 minutes
DO à 405 nm
1,000
0,800
0,600
0,400
0,200
0,000
1/320
1/640
1/1280
1/2500.
1/5120.
1/10240
Dilutions
13C
GSK4
11C
LdK4
CCK13


Anti-KCC6
30 minutes
DO à 405 nm
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
1/320
1/640
1/1280
1/2500.
1/5120.
1/10240
Dilutions
13C
GSK4
11C
LdK4
CCK13

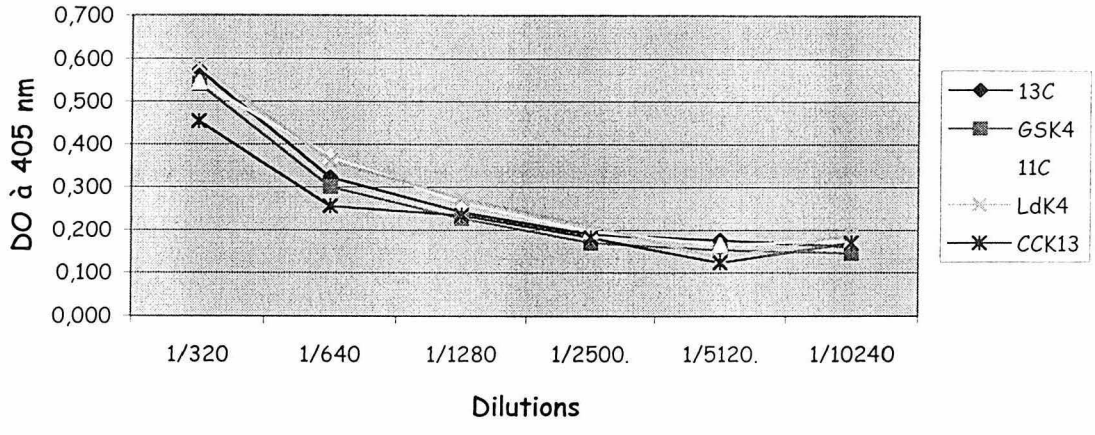

Anti-13C
30 minutes
DO à 405 nm
0,800
0,600
0,400
0,200
0,000
1/320
1/640
1/1280
1/2500.
1/5120.
1/10240
Dilutions
13C
GSK4
11C
LdK4
CCK13


Anti-CCK13
30 minutes
DO à 405 nm
0,800
0,600
0,400
0,200
0,000
1/320
1/640
1/1280
1/2500.
1/5120.
1/10240
Dilutions
13C
GSK4
11C
LdK4
CCK13

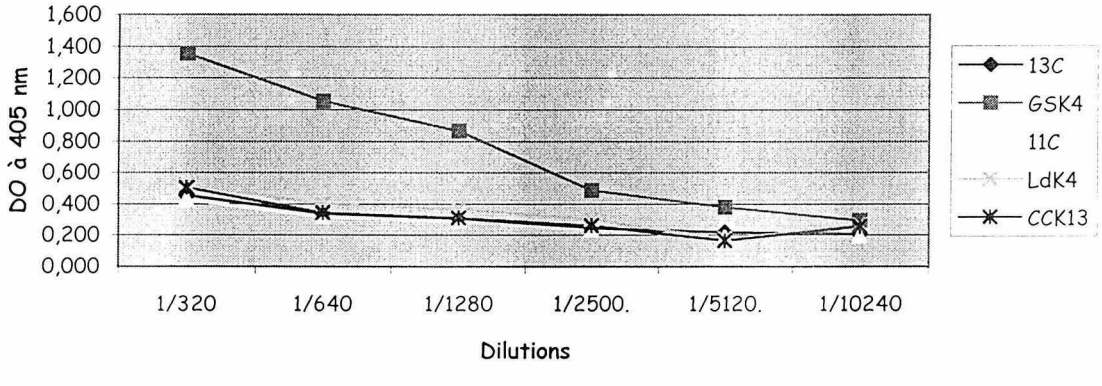

Anti-KWN35
30 min
DO à 405nm
0,500
0,400
0,300
0,200
0,100
0,000
1/320
1/640
1/1280
1/2500.
1/5120.
1/10240
Dilutions
13C
GSK4
11C
LdK4
CCK13


Anti-11C
30 minutes
Do à 405 nm
1,200
1,000
0,800
0,600
0,400
0,200
0,000
1/320
1/640
1/1280
1/250
1/5120.
1/10240
Dilutions
13C
GSK4
11C
LdK4
CCK13

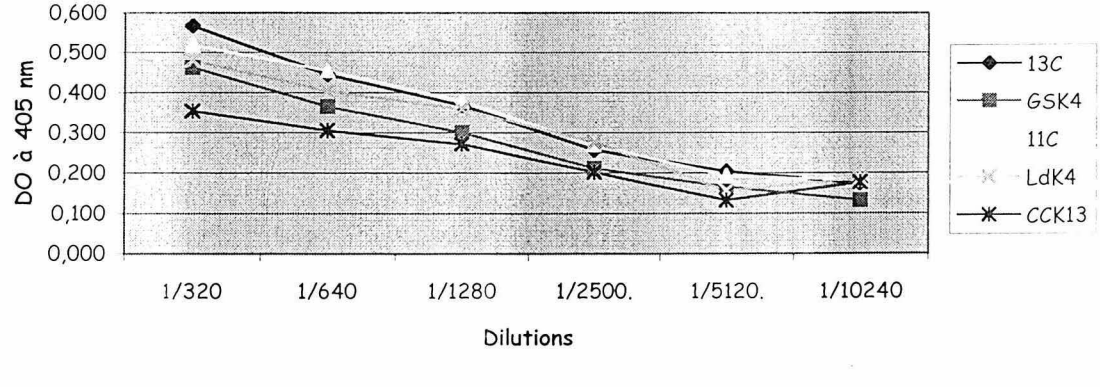
Anti-LdK4
après 1h

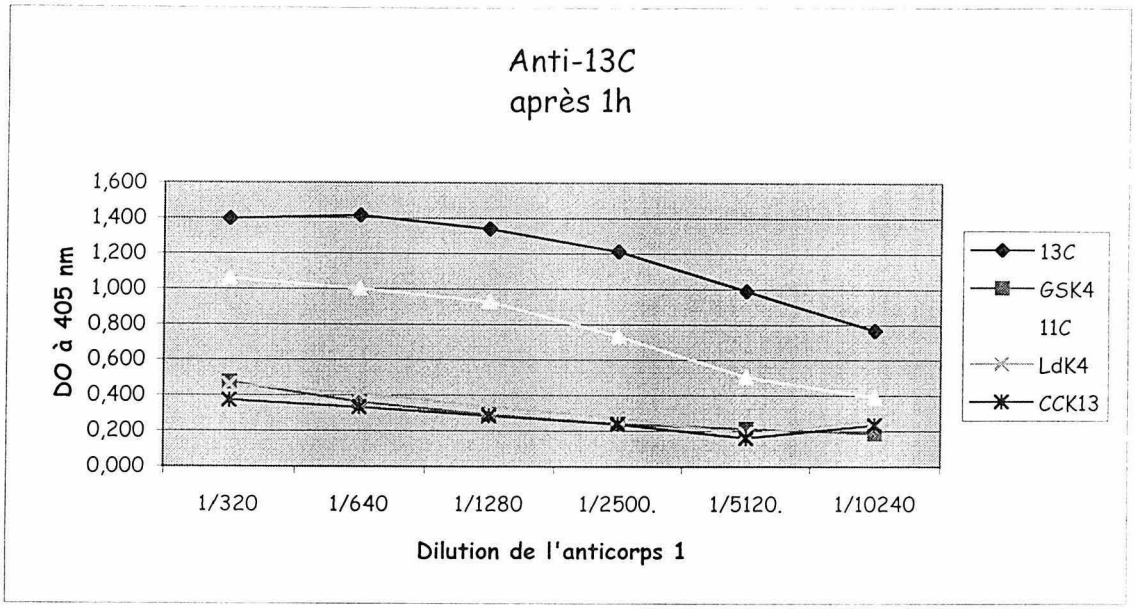


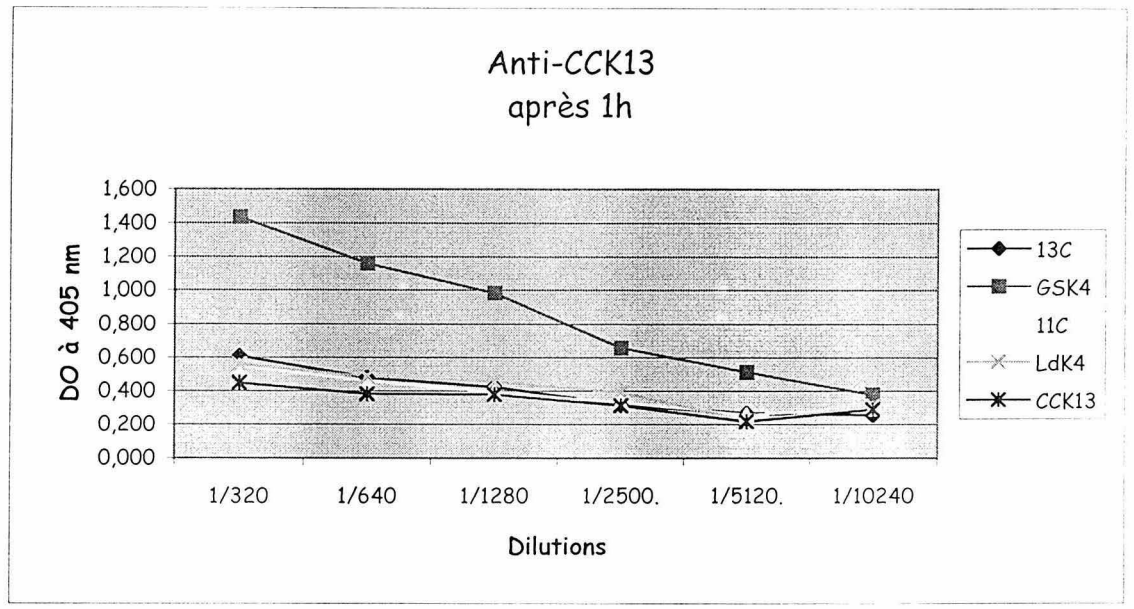
Anti-GSK4
après 1h

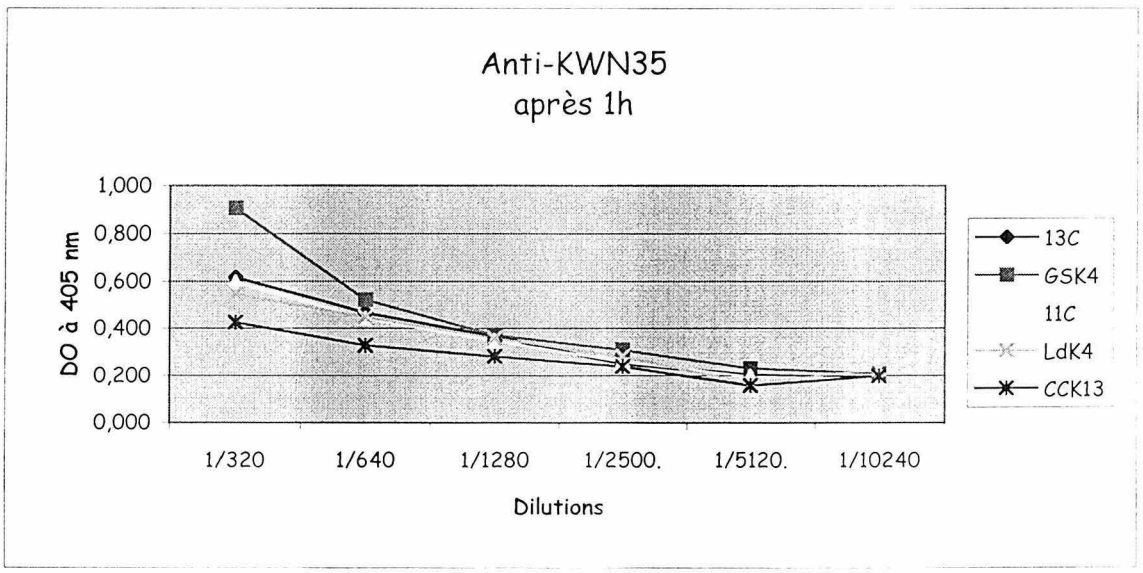


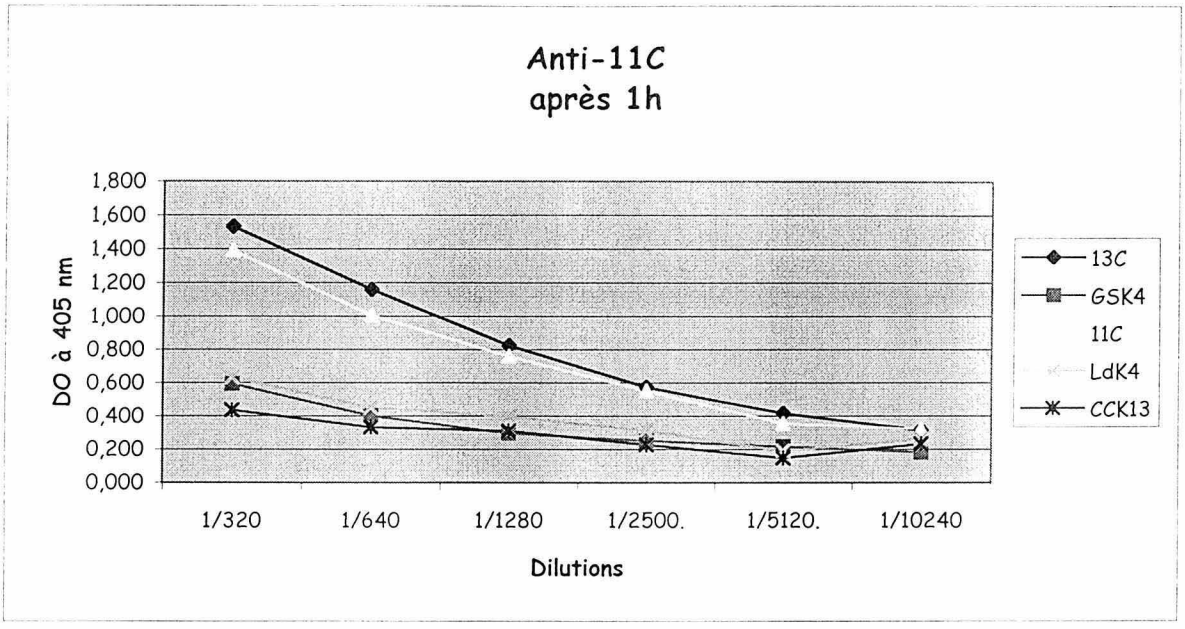
Anti-KCC6
après 1h




Anti-13C
après 1h
1,600
1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
DO à 405 nm
1/320
1/640
1/1280
1/2500.
1/5120.
1/10240
Dilution de l'anticorps 1
13C
GSK4
11C
LdK4
CCK13


Anti-CCK13
après 1h
1,600
1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
DO à 405 nm
1/320
1/640
1/1280
1/2500.
1/5120.
1/10240
Dilutions
13C
GSK4
11C
LdK4
CCK13


Anti-KWN35
après 1h
1,000
0,800
0,600
0,400
0,200
0,000
DO à 405 nm
1/320
1/640
1/1280
1/2500.
1/5120.
1/10240
Dilutions
13C
GSK4
11C
LdK4
CCK13


Anti-11C
après 1h
DO à 405 nm
1,800
1,600
1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
1/320
1/640
1/1280
1/2500.
1/5120.
1/10240
Dilutions
13C
GSK4
11C
LdK4
CCK13