

Original Research Article

Competitiveness among Rhizobia for Nodulation of Introduced Leguminous Tree *Gliricidia sepium* in a Sub-Saharan Sandy Soil

ABSTRACT

Agroforestry systems are progressively integrated by small farmerholder to mitigate agricultural production charge and contribute to sustainable agriculture by restoring and maintaining sandy soil fertility. A greenhouse experiment was carried out to test the nodulation level of introduced *Gliricidia sepium* tree with foreign rhizobial reference strains TAL1769 and TAL1770 against native rhizobial community using PCR/RFLP techniques. Restriction patterns of the two inoculated reference strains obtained for 16S – 23S rDNA - IGS were different to those of indigenous rhizobia detected in all remained nodules collected from root plants regarding the restriction enzymes *Hae*III and *Msp*I. Nodule occupancy rates of the reference strains and native rhizobial strains of profile F ranged from 18.36 to 22.45 and were higher compared to others rhizobial strains detected in *gliricidia* rhizosphere. Therefore, regarding its high competitiveness level, the native rhizobial strain may be considered as a good candidate to inoculate the introduced legume tree *gliricidia*.

Keywords: competitiveness, gliricidia, nodule occupancy, rhizobial strains

1. INTRODUCTION

Agro-ecological zone named Niayes in Senegal is an off-season cultivation area of the country where agriculture is mainly seasonal and rain-fed. Long-term agricultural practices in this zone are detrimental to the sandy soil structure. Thus, the physico-chemical properties of soils are affected. Furthermore declining soil fertility is one of the main causes of the poor performance of agricultural production. On soil nutrients, nitrogen is one of the most influential on crop growth and productivity [1]. Unfortunately, to overcome this problem, the use of chemical fertilizers is increased, which contributes for the pollution of groundwater and constitute a real public health problem. Thereby, improving soil fertility in a sustainable way has been a major joint concern for policy makers and local farmers. From then on, agroforestry systems based on legume plants are increasingly being developed. In addition, successful legume inoculation is known to raise the amount of N fixed and improve agrosystem management. There has been increasing evidence that the rhizobial inoculation biotechnology have an important role in the development of sustainable agriculture [2]. The legume tree *Gliricidia sepium*, native to Central America and Mexico [3], is introduced in Senegal with rhizobial reference strains. This fast-growing perennial tree is integrated into farming multipurpose cropping system for soil nutrient enrichment through N₂-fixation. Use of *G. sepium* mulch are reported to increase crop yield [4].

For playing its expected role, plant seedlings have to be inoculated with effective rhizobia. This biotechnology is widely experimented in the agro-ecological zones for cultivation of different legume crops. However presence of indigenous rhizobia population may limit rhizobial inoculation efficiency. Rhizobial bioinoculants fail to nodulate their host mainly because of highly competitive indigenous rhizobia capable of nodulating the same legume species. Indeed, depending on the environment, a legume could select its preferential rhizobial partner in order to establish symbiosis. Acosta-Duran and Martinez-Romero [5] identified diverse rhizobial strains that induce nodules in *G. sepium*, namely *Sinorhizobium* spp., *Rhizobium tropici* and *Rhizobium etli*, depending on the soil sampled. Different studies described the presence of these rhizobial species/strains in Senegalese soils [6,7]. In fact, Giller and Cadisch [8] reported that if large populations of compatible effective rhizobia are present in the soil, then the responses to inoculation will often not be found using standard available inocula.

According to Toro [9] the success of inoculation depends the competitiveness of new rhizobial strains inoculated. Triplett [10] indicated that a high competitiveness of soil inoculated strains in comparison with native rhizobia strains, is as important as the effectiveness of the symbiotic N₂ fixation itself. The objective of this study is to evaluate the nodulation ability of introduced foreign strains relative to the native rhizobial population, in order to obtain an inoculum for growing legumes gliricidia in Senegal.

2. MATERIAL AND METHODS

2.1. Experimental design

The experiment was carried out at Dakar Research Station located at Bel Air (latitude 14°44'N, longitude 17°30'W). The sandy soil type used in this study was collected from Niayes agro-ecological zone which presented the main following characteristics: 7.0 pH, 0.025% N, 0.04% C and 26 ppm available P. The soil sampled from the 5 - 30 cm topsoil was sieved (1 mm), and homogenized. Unsterilized soil portions (1.6 kg) containing approximately 10² rhizobia/g were weighed into pots for plant cultivation. Seeds of *G. sepium* were surface scarified and sterilized for 15 min immersion in sulphuric acid (95%) and washed vigorously in sterile distilled water. They were germinated at 30°C in Petri dishes containing water-agar medium (0.8%). Plant seedlings were transferred into pots containing unsterilized soil and watered daily at field capacity with tap water. Rhizobial foreign strains TAL1769 and TAL1770 provided by the NifTAL (Nitrogen Fixation for Tropical Agriculture Legumes) collection of American University of Hawaii were used for inoculation of seedlings grown on non-sterile potted soil. The inoculum containing approximately 10⁹ cells/ml is supplied in liquid form at the rate of 1 ml per plant. A non-inoculated treatment was also set up. Each treatment was repeated 4 times.

2.2. Rhizobial molecular identification

PCR amplification of 16S – 23S rDNA *Intergenic Transcribed Spacer* region of the rhizobium which has induced nodulation was performed on nodules. Height nodules per plant were randomly collected from gliricidia root systems, 45 days after planting. In order to detect the rhizobium strain, RFLP techniques was applied on nodules and thereby determine indigenous rhizobial diversity. Fresh nodules collected from roots were surface sterilized with calcium hypochlorite (5%, 5 min) followed by pure ethanol (96%, 5 min) and thoroughly rinsed with sterile water. Sterilized nodules were peeled and individually crushed in an extraction buffer. For each nodule, 150 µl of ground material were extracted in micro-tube and added to 150 µl of CTAB/PVPP2X buffer (0.2 M Tris-HCl, pH 8 ; 0.04 M EDTA pH 8 ; 2.8 M NaCl ; CTAB 4% w/v ; PVPP 2% w/v (Sigma)). The tubes were incubated (65°C, 60 min) for bacterial cell lysis. A series of centrifugations (15 min, 13000 x g) was performed to

recover the supernatant aqueous phase containing bacterial DNA. The DNA was then precipitated (13,000 x g, 4 °C, 30 min) and subsequently washed and dissolved in 20 µl of ultrapure water. The purity and amount of DNA suspension obtained from each nodule were evaluated by nucleic acid spectrum scanning spectrophotometry (Pharmacia Biotech ; 200 - 350 nm) and by agarose gel control 1% (w/v).

Primers FGPS 1490-72 (5'-TGCGGCTGGATCCCCTCCTT-3') defined by Navarro *et al.*[11] and FGPL 132'-38 (5'-CCGGGTTTCCCATTCCGG-3') by Ponsonnet and Nesme[12] were used for PCR amplification. PCR reaction tube contained a *Ready To Go* PCR beads (Pharmacia Biotech) mixed with 2.5 µl of each the primers, 18 µl of bidistilled water and 2 µl of rhizobial DNA suspension. Each Ready-To-Go beads contained for 25 µl volume reaction: 2.5 U Taq ADN polymerase, 10 mM Tris HCl (9.0 pH), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP (dATP, dCTP, dGTP, dTTP).

DNA amplification was done with a Perkin-Elmer thermocycler (GeneAmpSyst 2400). The following temperature cycle was used : initial denaturation at 95°C for 5 min ; 35 amplification cycles (denaturation at 95°C for 30 s, primer annealing at 50 °C for 30 s and extension at 72 °C for 30 s), final extension at 72°C for 5 min. An excess of restriction enzymes (5 U/reaction) was used to digest a 7 or 10 µl aliquot of PCR product. Restricted DNAs with *Hae*III (Amersham) and *Msp*I (Boehringer Mannheim) were analysed by horizontal agarose (metaPhor, 2.5%) gel electrophoresis carried out at 80 V for 3 h. Gels were stained in an aqueous solution of ethidium bromide (1 mg/ml) and photographed under UV illumination with a Gel Doc 1000 Bio Rad.

RFLP analysis for the two restriction enzymes *Hae*III and *Msp*I led to determine of the genetic relationships of the different isolates. The profiles of the inoculated reference strains were thus monitored among the strains that induced nodulation of plants' root system. The DNA digestion profiles of PCR product of native nodule-inducing rhizobia were compared with inoculated reference strains. Analysis of the IGS profiles was made by comparing the calculated band size with the Gel Analyst software. The representativity of the different strain profiles allowed to evaluate their nodule occupancy rate, which expresses the competitiveness of the strain under consideration.

3. RESULTS

Restriction patterns of 16S-23S IGS of the reference strains TAL1769 and TAL1770 were distinct for the two *Hae*III and *Msp*I enzymes used (Figure 1). These profiles were also different to those detected in nodules collected from non-inoculated plants. For inoculated gliricidia plants, the restriction profiles of the two reference strains are obviously found. In addition, new profiles were observed corresponding to indigenous rhizobial strains that could induce nodulation of gliricidia. Thus whatever the enzyme considered, six different profiles were revealed corresponding to diverse rhizobia population able to nodulate gliricidia plants in the soil of Niayes zone.

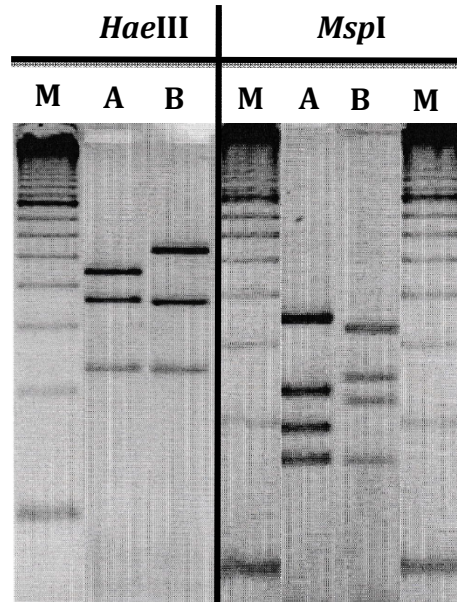


Figure 1 :Restriction patterns of inoculated reference strains TAL 1769 (A) and TAL 1770 (B) revealed by RFLP analysis of IGS PCR products digested with *HaeIII* and *MspI*.

Gliricidia plants grown on non-sterilized soil were inoculated with reference strains TAL 1769 and TAL 1770. PCR products of 16S-23S intergen obtained from all nodules sampled were treated separately with both restriction enzymes. The different profiles detected are presented in Figure 2 for *HaeIII* restriction enzyme. The restriction fragment length polymorphisms of both enzymes are compared between the inoculated and native strains that induced the nodulation of gliricidia.

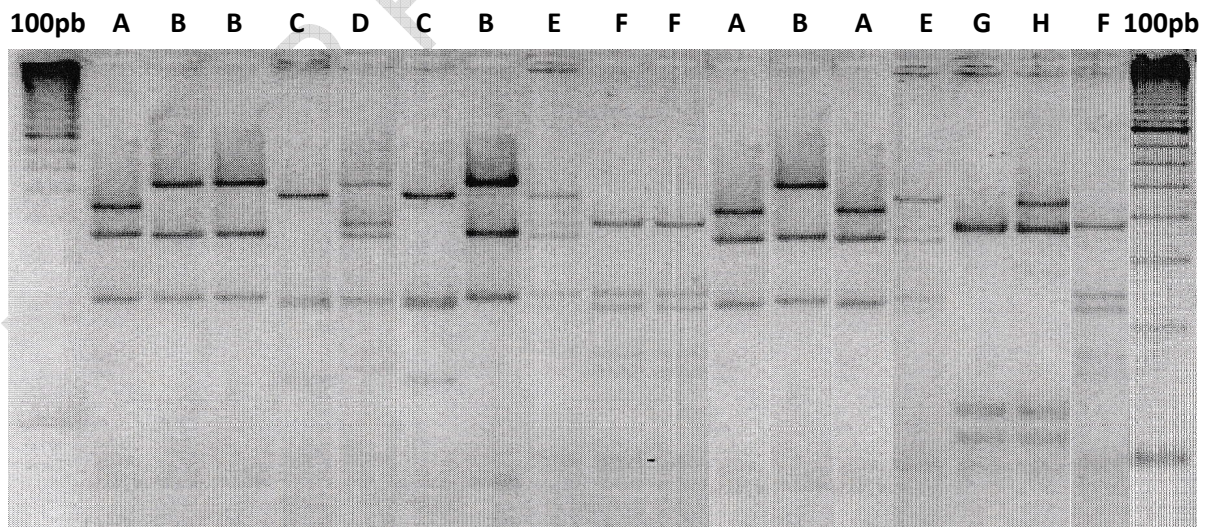


Figure 2 :Composite gel showing restriction patterns of PCR products digested by *HaeIII* from nodules of gliricidia inoculated with reference strains TAL 1769 (A) and TAL1770 (B) in unsterilized soil.

A and B for inoculated strains TAL1769 and TAL1770, respectively.

C, D, E, F, G and H for new profiles from nodules.

The different profiles identified were analysis. Thus, according to their similarity, 8 types of profiles noted from A to H have been listed (Figure 2, Table 1). For the two restriction enzymes applied in this study, the profile groups identified were the same for all analyzed nodules. The occupancy rate of the nodules for each strain were determined when compared the number each profile to the total number of profiles. Thus, nodule occupancy rates are highly variable and ranged from 22.45 to 4.08% (Table 1). The two reference strains did not have the same behavior compared to the native rhizobial population. When inoculated, the strain TAL1769 was more efficient than TAL 1770 for the induction of nodules with respectively an occupation rate of 22.45 against 18.36. However, the inoculated reference strains and the native rhizobium of profile F showed the highest occupancy rates.

Table 1 : Nodule occupancy of the reference and native strains according to the IGS patterns of the analyzed nodules.

Rhizobial strains	Percentages of restriction pattern of IGS genes							
	A	B	C	D	E	F	G	H
TAL 1769	22.45	-						
TAL 1770	-	18.36	-					
Native strains		-	14.29	6.12	10.20	18.36	4.08	6.12

The native rhizobial populations classified according to their restriction patterns are distributed unequally in six groups corresponding to the different profiles. Among these strains, those with the F profile performed better in nodule occupancy with 18.36%. At the same time, the lowest percentage of nodule occupancy was encountered with the G profile (4.08%). The percentages of nodule occupancy are globally important for indigenous strains. In fact, these strains distributed in the 6 profiles dominated the foreign strains with 59.19% against 40.81% respectively.

4. DISCUSSION AND CONCLUSION

Domestication of foreign legume species into a new geographical area is generally made with effective nodulating rhizobia strains. This practice has been observed for several species of agronomic or forest interests.

Thus, two rhizobial strains provided by the NifTAL were used for inoculation of *G. sepium* plants.

Field cultivation of gliricidia in Senegal soils showed root nodulation without inoculation [13]. This attests to the presence of a rhizobial native population able to induce gliricidia nodulation. The rhizobial population assessment of Niayes soil showed a low number of infective rhizobia of gliricidia. Because of the presence of indigenous rhizobia in this soil, efficiency of introduced strains requires verification against native strains. For the competitiveness study of rhizobia, the 16S-23S inter-gene space which constitutes a highly variable zone of the bacterial genome was used. The PCR-RFLP technique of this inter-gene has been often applied in taxonomic studies of bacterial strains [11,14,7]. The fragment sizes obtained with the two restriction enzymes are different for the PCR products of the strains that induced nodules. According to Bala et al. [15] isolates with the same RFLP profiles of IGS are considered as clone of the same strain. However, when two strains have different profiles, they are considered to be genotypically different. The use of this technique allowed to differentiate profiles between the reference strains themselves and the native strains. In this study, difference in profiles was also mentioned between the foreign strains and indigenous strains. Analysis of the RFLP profiles of reference strains also showed differences between the two strains regardless of the enzyme considered. Infectivity rates of the different competing strains were determined. The two inoculated rhizobial strains

induced nodulation of gliricidia in the presence of native rhizobia. The six rhizobia RFLP profiles obtained from native rhizobial population revealed to the high level of diversity. Profiles of foreign and indigenous strains found in nodules are different. The presence of the profile of strain TAL1769 was more frequent than that of strain TAL1770. Moreover, it is often noted the absence of restriction profiles of the two strains inoculated into some nodules in favor of the native strains. This means a limited degree of infectivity of the inoculated foreign strains compared to the native strains. According to Olivares [16], native rhizobial populations, often poorly effective for symbiotic nitrogen fixation, are well adapted to soil conditions and are able to occupy most of the nodules formed in the presence of bacterial strains applied to seeds. So overall, the two reference strains were not dominant in the formation of nodules of gliricidia compared to the native rhizobial population. Given the relative weakness of the native rhizobia population compared to the high density of the inoculum, 10^2 bacteria per gram of soil against 10^9 bacterial cells per liter respectively, the reference strains are not more competitive than the native strains. In the same agro-ecological area, Diouf et al. [17] reported in *Phaseolus vulgaris* very high nodule occupancy ratios for a strain inoculated compared to native strains with nodule occupancy rates for the inoculated strain often ranged from 90 to 98%. Thies et al. [18] described as highly competitive a strain that forms more nodules despite a smaller proportion of the population compared to other competing strains. In addition, many authors have reported a lack of competitiveness of introduced strains compared to native strains [19, 20]. Many works have reported that native rhizobiums can be considered competitive because the size of their population is a priori lower than that of the inoculum and they have succeeded in inducing the formation of nodules [18,19]. The results of this work are in contrast to those of Moawad and Bohlool [21] who showed that inoculated strain alone or in a mixture was able to completely dominate the native rhizobia of two types of tropical soils for the nodulation of the plant, in a competitiveness study of the infective rhizobial strains of *Leucaena leucocephala*. In short, even though the native rhizobial population of Niayes zone is in low proportion compared to the inoculated reference strains, their ability to nodulate gliricidia may be considered as very important. In fact, under natural conditions, inoculated rhizobial strains are at a survival disadvantage as compared to indigenous strains. Naturally occurring rhizobia are known to be more persistent in soils than introduced strains [22,23]. Nodule occupancy rates of the most favorable rhizobial strain depended on the competitiveness of other rhizobial strains in the rhizosphere and the environmental adaptability of the favorable rhizobial strain [24]. In sandy Sub-Saharan soil of Senegalese Niayes agro-ecological zone, native rhizobia population shown a high level of competitiveness able to ensure nodulation of *G. sepium* tree. In addition these rhizobia are reported to be highly N_2 -fixation efficient [25]. Thereby, these rhizobia, well adapted to environmental conditions can represent a useful germplasm for inoculum production due to their better competitiveness.

REFERENCES

1. Leghari SJ, Wahocho AN, Laghari GM, Laghari AH, Bhabhan GM, Tulpar KN, et al. Role of nitrogen for plant growth and development : A review. Adv. Environ. Biol. 2016;10:209-218.
2. Buresh RJ, Tian G. Soil improvement by trees in sub-Saharan Africa. Agro. Systems. 1998; 38: 51-76.
- Moawad H, Bohlool B.B. 1984. Competition among *Rhizobium* spp. for nodulation of *Leucaena leucocephala* in two tropical soils. Appl. Envir. Microbiol. 48:5-9
3. Simons AJ, Steward JL. *Gliricidia sepium* – a multipurpose forage tree legume. In Gutteridge R.C., Shelton H.M. (eds): Forage tree legume in tropical agriculture CAB International, Wallingford, UK, pp 30-48. 1994.

4. Budelman, A. Nutrient composition of the leaf biomass of three selected woody leguminous species. *Agro. Systems*.1989; 8: 39-51.
5. Acosta-Duran C, Martinez-Romero E. Diversity of rhizobia from nodules of leguminous tree *Gliricidia sepium*, a natural host of *Rhizobium tropici*. *Arc Microbiol.* 2002; 178: 161–164.
6. de Lajudie P, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyra M, et al. Polyphasic taxonomy of rhizobia : Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium saheli* sp. nov. and *Sinorhizobium teranga* sp. nov. *Int. J. Syst. Bacteriol.* 1994;44:715-733.
7. Diouf A, de Lajudie P, Neyra M, Kersters K, Gillis M, Martfnez-Romero E, et al. Polyphasic characterization of rhizobia that nodulate *Phaseolus vulgaris* in West Africa (Senegal and Gambia). *Int. J. Syst. Evol. Microbiol.* 2000;50: 159-170.
8. Giller KE, Cadisch G. Future benefits from biological nitrogen fixation: An ecological approach to agriculture. *Plant Soil.* 1995;174:255–277.
9. Toro N. Nodulation competitiveness in the Rhizobium-legume symbiosis. *World J. Microb and Biotech.* 1996;12: 157-162.
10. Triplett EW. Construction of symbiotically effective strain of *Rhizobium leguminosarum* bv. trifolii with increased nodulation competitiveness. *Appl. Environ. Microbiol.*, 1990 ; 56: 574–576.
11. Navarro E, Simonet P, Normand P, Bardin R. Characterization of natural population of *Nitrobacterspp.* using PCR/RFLP analysis of ribosomal intergenic spacer. *Arch. Microbiol.* 1992;157:107-115.
12. Ponsonnet C, Nesme X. Identification of *Agrobacterium* strains by PC/RRFLP analysis of pTi and chromosomal regions. *Arch. Microbiol.* 1994;161:300-309.
13. Ndiaye M, Ganry F. Variation in the biological N₂ fixation by tree legumes in three ecological zones from the North to the South of Senegal. *Arid Soil Res. Rehab.* 1997;11:245-254.
14. Laguerre G, Mavingui P, Allard MR, Charnay MP, Louvrier P, Mazurier SI, et al. Typing of rhizobia by PCR DNA fingerprinting and PCR-Restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *App. Environ. Microbiol.* 1996;62: 2029-2036.
15. Bala A, Murphy P, Giller K. Occurrence and genetic diversity of rhizobia nodulating *Sesbania sesban* in African soils. *Soil Biol. Biochem.* 2002;34: 1759-1768.
16. Olivares J. Competitiveness of rhizobium strains for nodule occupancy. In: Palacios R, Mora J, Newton WE. (eds) *New Horizons in Nitrogen Fixation. Current Plant Science and Biotechnology in Agriculture*, vol 17. Springer, Dordrecht. 1993.
17. Diouf A, Spencer MM, Gueye M. Use of *gusA* gene marker in competition study of common bean (*Phaseolus vulgaris*) nodulating *Rhizobium* in Senegalese soils. *World J. Microbiol. Biotechnol.* 2000; 16: 337 - 340.
18. Thies JE, Bohlóol BB, Singleton PW. Environmental effects on nodulation for nodule occupancy between introduced and indigenous rhizobia and among introduced strains. *Can. J. Microbiol.* 1992;38: 493-500.
19. Brockwell J, Bottomley PJ, Thies JE. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. *Plant Soil.* 1995; 174: 143-180.
20. Aguilar OM, Lapez MY, Ricillio PM. The diversity of rhizobia nodulating beans in Northwest Argentina as a source of more efficient inoculant strains. *J. Biotech.* 2001; 91: 181-188.
21. Moawad H, Bohlóol BB. Competition among *Rhizobium* spp. for nodulation of *Leucaena leucocephala* in two tropical soils. *Applied and Env. Microbiol.* 1984; 48: 5–9.
22. Okogun JA, Sanginga N. Can introduced and indigenous rhizobial strains compete for nodule formation by promiscuous soybean in the moist savanna agroecological zone of Nigeria? *Biol. Fertil. Soils* 2003; 38:26–31.

23. Allito BB, Ewusi-Mensah N, Alemneh AA. Rhizobia strain and host-legume interaction effects on nitrogen fixation and yield of grain legume: A Review. *Molecular Soil Biology*. 2015; 6: 1-6.
24. Ji ZJ, Yan H, Cui QG, Wang ET, Chen WF, Chen WX. Competition between rhizobia under different environmental conditions affects the nodulation of a legume. *Syst. App. Microbiol.* 2017;40:114-119.
25. Diouf A, Ndiaye M, Fall-Ndiaye MA, Diop TA. Maize crop N uptake from organic material of *Gliricidia sepium* coinoculated with rhizobium and arbuscular mycorrhizal fungus in Sub-Saharan Africa sandy soil. *Am. J. Plant Sciences*. 2017, 8:428-440.

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