

Full Length Research Paper

Response of three peanut cultivars toward inoculation with two *Bradyrhizobium* strains and an *Arbuscular mycorrhizal* fungus in Senegal

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The aim of this investigation was to isolate the most specific, effective and competitive strains for peanut, and to determine the level of variability in peanut cultivars response toward single/dual inoculation. Peanut seeds of three cultivars namely 55–437, Fleur 11 and 69–101 were inoculated with two bradyrhizobial strains (LMG9283 and USDA3187) and an AM fungus *Glomus intraradices*, individually or in combination, and were grown in the open–air conditions using a non–sterile sandy soil from Sangalkam. Plant controls were supplied with NPK chemical fertilizer at the rate of 150 and 300 kg ha⁻¹. Results obtained in term of nodule occupancy revealed a high competitiveness of LMG9283 strain with cultivars 55–437 and 69–101. However Fleur 11 cultivar was nodulated by indigenous strains rather than the introduced strains. Assessment of the data on nodulation, shoot biomass and pods yield revealed that, among the single inoculation of the three cultivars, 55–437 and 69–101 produced the largest increase in the parameters studied, however, Fleur 11 showed a higher growth and pods yield with the chemical fertilization. The dual inoculation with bradyrhizobial strains associated with *G. intraradices* further improved the parameters studied for 55–437 and 69–101, demonstrating a synergy between LMG9283 and *G. intraradices*. This better response allowed us to suggest that 55–437 and 69–101 cultivars should be tested in the arable fields with *Bradyrhizobium* strain LMG9283 and *G. intraradices*.

Key words: *Arachis hypogaea* L., *Bradyrhizobium* strains, *Glomus intraradices*, yield, inoculation.

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is a cash crop cultivated in Senegal over a century. It generated 60% of the Gross Domestic Product (GDP) from agriculture and approximately 80% of the export earnings of the country. It is the major oil–yielding crop, and the four oil–factories established in the country constituted the backbone of national industrial fabric. After a long period of decrease,

the yields of peanut have been increased these five last years. However, there is a continuous degradation of the determining factors of such increases, that is, soil fertility with reduction of fallow and low–level use of fertilizers.

Rhizobial inoculants are known to be an alternative to the use of industrial N fertilizers and a means to maintain or improve soil fertility (Peoples, 1995; Alves et al., 2003; Chalk et al., 2006). Rhizobium legume symbiosis is one of the most important nitrogen–fixing systems (Kahindi et al., 1997). The isolation and selection of elite strains is very important because the effective rhizobial strains can be used as inoculants for effective nodulation (Dudeja and Khurana, 1988; Dhery and Dreyfus, 1991; Lanier et

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al., 2005; Bogino et al., 2006). The competitive ability of an inoculant strain is an important factor determining the success of rhizobial inoculation (Triplett, 1990). Selected rhizobial strains often fail to compete with indigenous soil rhizobia (Thies et al., 1991; Castro et al., 1999; Bogino et al., 2006; 2008) and do not increase nodulation.

Many approaches, the use of fluorescent antibodies (Schmidt et al., 1968), antibiotic resistance (Castro et al., 1999; Bogino et al., 2008), and gus marker (Diouf et al., 2000), have been already used as tools for targeting strains responsible for nodules formation. The development of Polymerase Chain Reaction (PCR) primers that specifically target strains of rhizobia (Navarro et al., 1992; Pansonnet and Nesme, 1994), has allowed the direct characterization of rhizobial communities within root nodules of plants (Laguerre et al., 1994). Most of the molecular-based studies have involved the intergenic spacer (IGS), corresponding to the region between 16S and 23S rDNA. This region exhibits a large degree of variability in length and thus offers a suitable target for rhizobial strains differentiation (Laguerre et al., 1994, 1996; Doignon-Bourcier et al., 2000; Bala et al., 2001; Thiao et al., 2004; Krasova-Wade et al., 2006). Restriction Fragment Length Polymorphism (RFLP) analysis of PCR-amplified DNA is now commonly used for competitiveness assay between rhizobial inoculants and indigenous bacteria and has the additional advantage to highlight genetic diversity of rhizobia nodulating a legume plant into soil.

Successful establishment of peanut involves effective symbiotic association between elite strains and compatible host plants. The present study is therefore, designed to isolate the most specific, effective and competitive strains for peanut, and to use elite ones as inoculants.

MATERIALS AND METHODS

Experimental design

An experiment was carried out at Dakar (Bel Air experimental station, 14°44'N, 17°30'W) using a non-sterile soil from Sangalkam, 30 km east of Dakar. This soil has a pH of 6.5 with 58.15, 32.8 and 3.6% of sand, loam and clay respectively and contains 0.06% total N, 0.54% total C, 39 mg P kg⁻¹ total P, 4.8 mg P kg⁻¹ available P. It was sieved (< 1 mm), homogenized and used to fill up with the three quarters buried concrete containers. Seeds of selected cultivars (Fleur 11, 55-437 and 69-101) of peanut (Clavel and Ndoye, 1997) were hand sown in a randomized complete block design with four replicates. The size of the containers was 1 x 1 x 1 m with 10 and 40 cm within and between rows respectively for Fleur 11 and 55-437 cultivars. The plants spacing was 10 and 60 cm for 69-101 cultivar. Plants were grown for 90 days in the open air conditions (temperature of 27-35°C, relative humidity of 70-80% and 12 h light) and were watered daily using tap water without addition of nutrients.

There were six treatments: (i) plants inoculated with rhizobial strains (R); (ii) plants inoculated with arbuscular mycorrhizal strain *Glomus intraradices* (M); (iii) plants inoculated with R and M (RM); (iv) plants supplied with NPK chemical fertilizer at the rate of 150 kg

ha⁻¹ (E1) and (v) NPK 300 kg ha⁻¹ (E2); (vi) control (C) plants not inoculated with rhizobial strains or mycorrhizal strain and not supplied with chemical fertilizer.

Inoculants preparation and inoculation

Each strain of *Bradyrhizobium* was grown in YEM medium (Vincent, 1970) for 5-6 days at 28°C with rotary shaking at 150 rpm. *Rhizobial inoculum* was applied as 10 ml suspension of mixed culture (v/v) of *Bradyrhizobium* strains USDA3187 and LMG9283 containing 10⁸ cells mL⁻¹. The molecular patterns of each rhizobial strain in the mixture were determined before inoculation in order to identify and distinguish them.

The AM fungus *G. intraradices* was multiplied on *Zea mays* for 12 weeks under greenhouse conditions on sterilized substrate (soil and sandy 1:1 v/v). For AM fungal inoculation, 10 g of the substrate containing an average of 40 spores g⁻¹ soil and root fragments with 85% of colonized roots length, were placed below the seedlings. Treatments without bradyrhizobial or AM fungal inoculums received 10 ml and/or 10 g of autoclaved inoculums in order to avoid differences in soil nutrient content linked to rhizobial and/or AM fungal inoculums additions.

Sampling

For each cultivar, plants were harvested at flowering [30 days after sowing (DAS)], pod filling (45 DAS), and pod maturity periods (60 DAS). Plant shoots were dried (80°C for 72 h) and weighed. The soil adhering to roots was removed under running tap water and nodules were picked and counted. For rhizobial diversity, 12 nodules were randomly chosen for each treatment (4 plants with 3 nodules per plant). For competitiveness assessment, a number of 48 nodules were used for each of the inoculated treatments (4 plants with 12 nodules per plant). Nodules were surface sterilized by immersion in 0.1% (w/v) HgCl₂ for 30 s, rinsing in sterile water and then in 96% ethanol for 2-3 min following by rinsing in sterile water and maintained at -80°C in sterilized water containing glycerol (20%). For the third harvest, pods were collected and plant shoots were dried and weighed. Analysis of Variance (ANOVA) and critical difference at 0.05 of the Fischer test were applied to determine the significance of treatments.

Rhizobial diversity and identification of nodule occupants

Nodule occupancy and diversity of peanut rhizobial populations were determined using PCR/RFLP of the IGS 16S-23S rDNA. DNA was extracted from each nodule using Dneasy Plant Mini kit (QIAGEN) and used as template for molecular characterization. The IGS between the 16S and 23S rDNA was amplified as described by Thiao et al. (2004) using primers FGPS1490-72 (5'-TGCGGCTGGATCCCCCTCTT-3') Navarro et al. (1992) and FGPL132-38 (5'-CCGGGTTTCCCCATTCGG-3') Pansonnet and Nesme (1994). The well amplified samples were digested individually in 20 ml final volume with 4-base-cutting restriction enzymes *Msp* I and *Hae* III (Laguerre et al., 1994) as specified by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with an excess of enzyme (10 U per reaction) for 3 h. Restricted DNA was analyzed by horizontal gel electrophoresis in 2.5% (w/v) Metaphor[®] agarose (FMC BioProducts, Rockland, Maine USA) gels in TBE buffer. Electrophoresis was run at 2.3 V cm⁻¹ for 3 h. Gels were stained in an aqueous solution of ethidium bromide (1 mgL⁻¹) and photographed under UV illumination with Gel Doc software (Bio-Rad, Hercules, CA, USA). Restriction profiles for both enzymes were resolved, and the patterns were compared in pairs for all combinations of sampled nodules and for inoculated

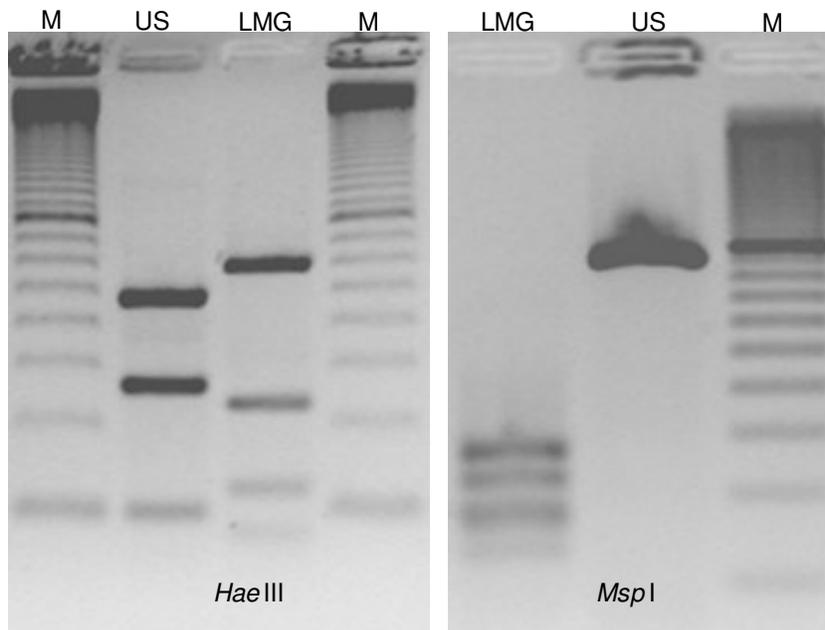


Figure 1. Restriction patterns of the inoculated strains using PCR-RFLP of the 16S–23S rDNA intergenic spacer. PCR products were digested with two restriction enzymes (*Hae* III and *Msp* I). Line M is 100 bp DNA ladder. US: USDA3187; LMG: LMG9283.

Table 1. Nodule occupancy (%) of rhizobial strains in three cultivars of peanut (*Arachis hypogaea* L) cultivated for 30 days and inoculated with a mixed rhizobial inoculum USDA3187 and LMG9283.

Rhizobial strains	Cultivar 55-437						Cultivar 69-101						Cultivar Fleur 11					
	R	RM	M	E1	E2	C	R	RM	M	E1	E2	C	R	RM	M	E1	E2	C
USDA 3187	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LMG 9283	67	54	0	0	0	0	42	52	0	0	0	0	25	29	0	0	0	0
S1	33	42	100	100	91	87	46	48	86	86	70	87	71	67	100	100	81	87
S2	0	0	0	0	0	0	0	0	5	0	0	0	4	0	0	0	0	0
S3	0	0	0	0	0	0	0	0	0	7	10	0	0	0	0	0	0	0
S4	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0
S5	0	4	0	0	9	13	12	0	9	7	10	13	0	4	0	0	19	13

strains.

RESULTS

Nodulation

The PCR-RFLP patterns of each of the inoculated *Bradyrhizobium* strains were specific (Figure 1), and thus this allowed us to identify and distinguish them in the nodules using this particular molecular technique. Results indicated that the patterns corresponding to those of the inoculated *Bradyrhizobium* strains (USDA3187 and LMG9283) were not found in nodules sampled from the uninoculated plants (Table 1). Based on this result, we

may assume that the inoculated strains were naturally absent in soil of Sangalkam. This facilitated the assessment of nodule occupancy. Thus, the nodules showing the patterns of one of the inoculated strains were counted as occupied by this corresponding strain, while the nodules showing the patterns different to those of the inoculated strains were counted as occupied by the natural population.

Because we were unable to well amplify the DNA extracted at 45 and 60 DAS, results on nodule occupancy were only presented at 30 DAS. It has been previously reported for peanut that consistent isolation of quality DNA from mature plants is particularly problematic due to the presence of polyphenols, tannins and polysaccharides that can contaminate DNA during

Table 2. Shoot dry weight (g plant⁻¹) and pods yield (g plant⁻¹) of three cultivars of peanut (*A. hypogaea*) cultivated for 30 days and inoculated with a mixed rhizobial inoculum USDA3187 and LMG9283.

Cultivars	Treatments	30DAS/Mid flowering		45DAS/Filling pod		60DAS/Maturity		
		NNumb	SDW	NNumb	SDW	NNumb	SDW	Pods yield
55-437	R	7.5 b	1.43 b	62.75 c	4.23 a	87.75 b	7.86 a	2.02 a
	M	58.75 a	2.59 b	128.5 abc	7.81 a	148.75 a	8.31 a	3.6 a
	RM	74.75 a	2.83 b	215 a	7.25 a	100.75 ab	7.82 a	2.1 a
	E1	65.75 a	4.05 a	206.5 ab	7.47 a	58.75 bc	8.56 a	2.78 a
	E2	15 b	2.32 b	95.75 abc	6.2 a	84.75 b	8.2 a	2.14 a
69-101	C	20.75 b	1.55 b	66 bc	4.16 a	25.5 c	4.71 b	0.25 b
	R	139 b	3.53 bc	193.5 abc	7.35 bc	297.5 ab	17.55 ab	1.45 ab
	M	153.25 ab	5.05 ab	299 a	10.64 ab	231 bc	14.8 abc	1.58 ab
	RM	201.5 a	6.4 a	277.75 a	15.18 a	350 a	21.22 a	2.4 a
	E1	15.75 c	1.96 cd	113.75 bc	8.72 b	28.75 cd	8.8 cd	0 c
Fleur 11	E2	4.5 c	0.92 d	61.5 c	3.51 c	13.5 d	6.56 d	0 c
	C	50 c	2.35 cd	213.5 ab	7.1 bc	187.5 bc	11.98 bcd	0.7 bc
	R	130.75 a	6.59 a	168.5 abc	12.39 ab	141 b	12.5 b	3.75 bc
	M	97.5 ab	3.94 bc	71.75 c	6.67 ab	156.75 b	8.38 b	3.66 bc
	RM	64.5 bc	2.65 c	140.5 bc	4.91 b	95 b	8.17 b	1.74 c
Fleur 11	E1	72 bc	6.31 ab	266.5 a	10.23 ab	351 a	17.53 a	12.85 a
	E2	30.75 c	3.79 bc	203.75 ab	11.03 ab	150.5 b	13.9 ab	6.35 b
	C	71.75 bc	3.04 c	92.75 bc	9.39 ab	194 b	9.5 b	2.57 c

In the same column and for each cultivar, values followed by the same letters do not significantly differ ($p \leq 0.05$) according to Fisher's LSD (least significant difference) test. R: USDA3187 + LMG9283; M: *G. intraradices*; RM: R + M; E1:15g NPK; E2: 30g NPK; C: control without fertilization, NPK: chemical fertilization NPK (20-10-10); DAS: days after sowing; SDW: shoots dry weight.

isolation, and thus inhibit amplification (Sharma et al., 2000).

Whatever the peanut cultivar was, the profile of *Bradyrhizobium* strain USDA3187 was not found in any of the 432 nodules harvested from all treatments at 30 DAS, meaning that this strain was not competitive. However, in addition to the profiles of *Bradyrhizobium* strain LMG9283, five different profiles corresponding to five indigenous strains S1 to S5 were found (Table 2 and Figure 2).

With cultivar 55-437, *Bradyrhizobium* strain LMG9283 and indigenous strains S1 were found in 67 and 33% of nodules from R treatment respectively. In RM treatment, the respective corresponding nodule occupancy was 54 and 42%. In the other treatments, indigenous strains S1 and S5 prevailed in nodules with an average nodule occupancy of 95 and 5%, whereas strains S2, S3 and S4 were not found in the nodules. These data indicated that *Bradyrhizobium* strain LMG9283 was more competitive than the indigenous strains in cultivar 55-437. However, with cultivar 69-101, *Bradyrhizobium* strain LMG9283 was more competitive than strain S1 in RM treatment only: 52 vs 48%. In cultivar Fleur 11 *Bradyrhizobium* strain LMG9283 was less competitive in all treatments. In

addition, with a very low nodule occupancy, indigenous strains S2, S3, S4 and S5 were also found in cultivar 69-101, S5 being more present than the three other strains. Thus these strains were less competitive than strains LMG9283 and S1.

In comparison with the control plants, inoculation with the mixed bradyrhizobial (R) inoculum did not increase the nodule number of plants except at early stage (30 DAS) of plant growth at which significant increase was recorded on cultivars 69-101 and Fleur11: +178 and +82% respectively. Similarly, no difference was observed in nodulation of cultivars 55-437 and Fleur 11 between the applications of NPK fertilized plants and the control ones, even if the application rate was doubled. In addition, application of the higher NPK rate (30 g plant⁻¹) significantly decreased the nodule number of variety 69-101 at 45 and 60 DAS: less than four and thirteen times respectively.

By contrast, the use of mycorrhizal (M) strain *G. intraradices* alone or associated with the mixed bradyrhizobial inoculum had increased on average three and two times the nodule number recorded on the cultivars 55-437 and 69-101, respectively over the plant growth period.

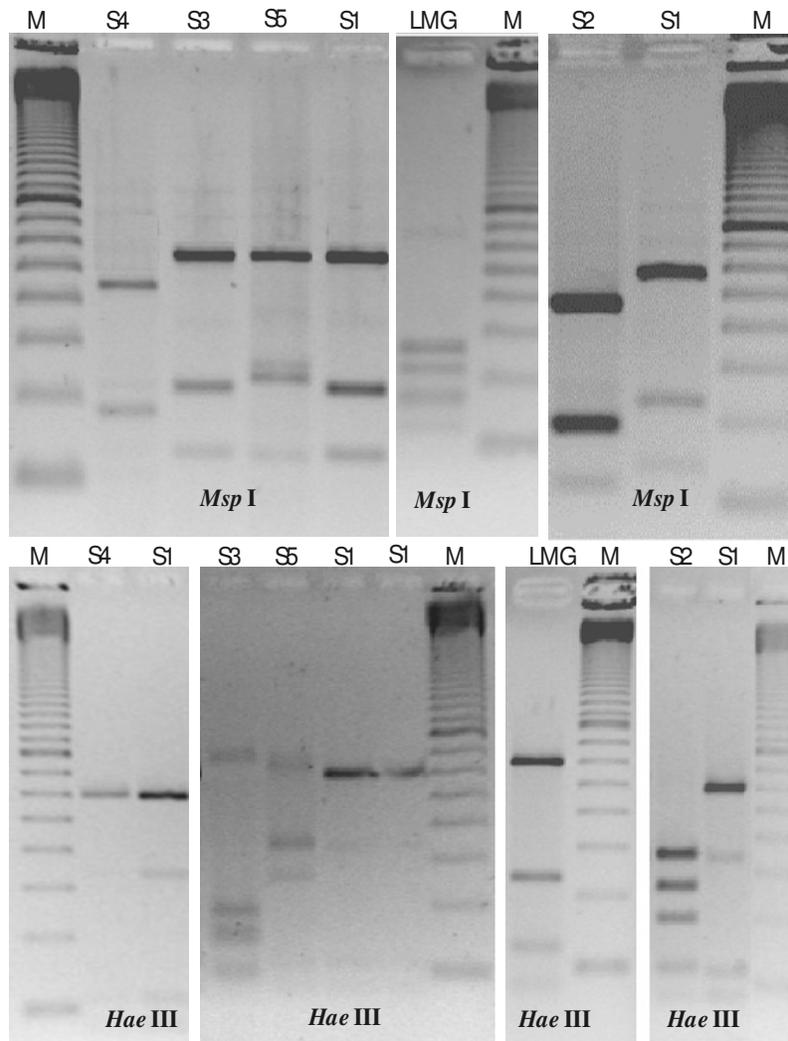


Figure 2. Restriction patterns of the six strains found inside nodules using PCR-RFLP of the 16S–23S rDNA. Line M is 100 bp DNA ladder. Profiles shown were generated by using the restriction enzyme *Hae* III and *Msp* I.

Biomass production

As for the nodulation and up to 45 DAS, there was no significant difference in the Shoot Dry Weight (SDW) of cultivars 55–437 between Control (C) and inoculated plants with the rhizobial (R) or the mycorrhizal (M) strains used alone or with the dual inoculum (RM). However, at 60 DAS, similar to any application rate of NPK fertilizer, inoculation of cultivar 55–437 with R, M or RM had increased the SDW: + 73% over the control plants. Significant increase of SDW of 69–101 cultivars was observed with RM inoculation during all the growth period at 30, 45 and 60 DAS: 172, + 113 and 77%, respectively. In addition, application of 15 g NPK fertilizer had increased (+ 84%) the SDW of variety Fleur 11 at 60 DAS whereas 30 g had no effect.

Pod yields

For cultivar 55–437, pod yields of inoculated (RH, M or RM) and fertilized plants were similar and higher (+11%) than that of control plants. Surprisingly, no pods were recorded on the fertilized plants of cultivar 69–101 for which no difference was observed between inoculated (RH, M or RM) plants and the control plants. Cultivar Fleur 11 had yielded more than the two other varieties with a maximum of 12.8 g plant⁻¹.

DISCUSSION

From this research work, two main points deserve discussion: (i) the success of introduced strains into the

soil in establishing nodules with peanut in competitive situation is highly depending on cultivar genotypes and (ii) dual inoculation with rhizobia and AM fungi could be an option for peanut cultivation when considering the intimacy of compatibility occurring between peanut cultivars and symbiotic partners (rhizobial strains, *Arbuscular mycorrhizal* fungi).

The presence of rhizobial strains inside the nodules were performed using PCR/RFLP of the IGS 16S–23S rDNA (Laguerre et al., 1994, 1996; Doignon–Bourcier et al., 2000; Bala et al., 2001). This technique offers an accurate method, provided that the diversity of rhizobial strains can be highlighted: five types of indigenous strains were detected, which is not possible with other techniques such as the use of fluorescent antibodies (Schmidt et al., 1968), antibiotics (Castro et al., 1999), gus marker (Diouf et al., 2000).

Leguminous can form nodules when they find compatible soil rhizobia. Generally, nodule formation depends on number of available infective rhizobia on roots infection sites. The more there are infective rhizobia, the larger the number of nodules is, even if the nodulation is also governed by both bacteria and intrinsic plant factors. In our study, all peanut cultivars were inoculated with 10 ml of a rhizobia suspension (strain USDA3187 mixed to strain LMG9283) containing 10^8 cells mL^{-1} . Since the soil of Sangalkam contains a very low rhizobial population (10^3 cells g^{-1} , Guene et al., 2004), it should be expected to have a positive response of peanut to inoculation with strain USDA3187 mixed to strain LMG9283 in terms of nodulation. However, strain USDA3187 failed nodulating plants and only strain LMG9283 has been found in the nodules, with nodule occupancies considerably variable, influenced by peanut cultivars. Since competitiveness of a given rhizobium strain is known to be on its ability to induce nodulation of a legume in the presence of other rhizobia (Castro et al., 1999; Bogino et al., 2006, 2008), both introduced (strain LMG9283) and indigenous strains could be considered as competitive on the three peanut cultivars. However, their competitiveness degree is different, the indigenous strains being more competitive than the introduced ones for cultivar Fleur 11 contrary to cultivars 55–437 and 69–101 for which the former strain is more competitive than the latter. This confirmed the role of plant genotype on competitiveness of rhizobial strains already reported in soybean (*Glycine max*) (Keyser and Cregan, 1984; Cregan and Keyser, 1988; Meghvansi et al., 2008).

Data reported by Castro et al. (1999) showing toward inoculation that peanut roots were nodulated by indigenous rhizobia rather than the introduced strain USDA3187, strengthen our results. We were unable to detect strain USDA3187 from any of the nodules obtained; indicating that this introduced strain was not as competitive as naturalized peanut rhizobia. Several factors have been reported as influencing the competition between the introduced strains and the indigenous

rhizobia, for instance, biological factors, such as bacteriophages (Evans et al., 1979) or epiphytic bacteria (Handelsmann et al., 1988), as well as environmental factors, such as temperature, pH, nitrate content, etc. (Beattie et al., 1989). However, in the case of our study, we are tempted to hypothesize that it might be related to its intrinsic factors since USDA3187 was inoculated at the same amount as LMG9283 which was detected from nodules of inoculated plants. Because we have not investigated on the free living persistence of USDA3187 in the soil after inoculation, it could be difficult to conclude on the reason of its lack of nodulation.

Plant response to inoculation is also determined by a variety of factors such as the presence and quality of indigenous rhizobial populations. The positional advantage of those indigenous strains for nodule formation was reported on peanut grown around the world (Diatloff and Langford, 1975; Castro et al., 1999; Bogino et al., 2006; 2008). Thus response of peanut to rhizobial inoculation has always been questionable in the world: India (Gaur et al., 1974; Nambiar, 1985; Wange, 1989; Joshi et al., 2008), Israel (Schiffmann and Alper, 1968), Brazil (Cardoso et al., 2009), Argentina (Castro et al., 1999; Bogino et al., 2006; 2008). In Senegal, such a response has not been observed so far, even in the selected site, that is Sangalkam (Gueye M, pers. com.), where the population size of rhizobial indigenous population is low (Guene et al., 2004). Indeed, most of the inoculated plants nodules of cultivars 55–437 and 69–101 were occupied by the strain LMG 9283 at 30 DAS and positive responses were observed at maturity in terms of shoot dry weight and pod yields. But this cannot be regarded as a success of peanut inoculation with rhizobial strains. Field inoculation over several years is necessary.

The three cultivars were dually inoculated with rhizobial strains and mycorrhizal strain *G. intraradices*. Compared to the effect of simple inoculation with rhizobial strain, the expected synergy already described between rhizobial strains and *G. intraradices* reaching an increase of growth and yield of legume species (Lekberg and Koide, 2005; Duponnois et al., 2005; Meghvansi et al., 2008) has not been recorded in our study although, nodulation of cultivars 55–437 and that of 69–101 were improved. This can be attributed to the fact that native mycorrhizal fungi may provide the potential benefit of this mutualistic association (Schenck and Kinlock, 1980) since no difference in shoot dry weight was recorded between mycorrhizal (M treatment) and non mycorrhizal (C treatment) except for cultivar 55–437 at maturity stage. Because the native mycorrhizal population has not been estimated in our study, it would be difficult to conclude on this.

Conclusion

In Senegal, peanut has not been so far successfully inoculated by rhizobial and/or mycorrhizal strains even in

sites where rhizobial population is low. The competing indigenous rhizobial strains are the main limiting factor to the success of inoculation. In these circumstances, the yield increase of peanut cultivars 55–437 and 69–101 should be considered as new promising data towards the adoption of rhizobium technology for peanut improvement in Senegal.

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