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Genetic diversity of rhizobia nodulating *Arachis hypogaea* L. in diverse land use systems of humid forest zone in Cameroon

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ABSTRACT

Peanut (*Arachis hypogaea*) is one of the most important legume cultivated in the humid forest zone of Cameroon. However, in this country the diversity of rhizobial strains that can nodulate peanut was poorly understood. Forty-two strains from *Arachis hypogaea* were examined by restriction fragment length polymorphism (RFLP) analysis of 16S–23S rDNA genes amplified by polymerase chain reaction (PCR). A considerable level of genetic diversity was determined among those peanut *isolates*. Eight composite genotypes were obtained from the combined data of the RFLP analysis with four endonucleases. A correlation between land use system and the diversity of peanut isolates was observed in the current study. The highest diversity was found in the cocoa farm and fallow and the lowest one in peanut farm. Our results have shown that the genotype richness diversity of peanut isolates depends on the land use system.

1. Introduction

Peanut (*Arachis hypogaea* L.) is an important crop that provides food for direct human subsistence and other several food products. It takes a significant part in the economy of many countries in the world. In the humid forest zone of Cameroon, *Arachis hypogaea* is the major legume crop. The soil in this region is ferruginous and ferralitic, characterized by acidity and low fertility.

Legumes are usually nodulated by indigenous root-nodule bacteria. The symbiosis formed through interaction between rhizobia and legume plants, such as peanut, can transform atmospheric N_2 to ammonia thus supplying nitrogen to the plant and enhancing its ability to withstand stress, even in arid environments (Rodelas et al., 1999). The contribution of biological nitrogen fixation (BNF) was 40.9 kg/ha for *Arachis hypogaea* (Okito et al., 2004). However, in spite of the contribution of this legume to the soil, at the farm level, grain yields are often low and inconsistent. Inoculation of legume seed is an efficient and convenient way of introducing viable rhizobia to the soil and subsequently to the rhizosphere of legumes (Deaker et al., 2004).

Abundant types of indigenous rhizobia are widely distributed in various geographical and ecological areas of the world (Xu et al., 1995; Chen et al., 1997; Tan et al., 1999; Peng et al., 2002). Peanut has been reported to form effective nodules with slow-growing rhizobia (Van Rossum et al., 1995; Urtz and Elkan, 1996; Zhang et al., 1999; Saleena et al., 2001).

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Currently, Taurian et al. (2006) demonstrated that *Arachis hypogaea* L. is nodulated by *Bradyrhizobium* species and also by fast growing rhizobia closely related to *Rhizobium giardini* and *Rhizobium tropici* species.

Nodulation of peanut by indigenous bacteria is usually assumed to be adequate, and inoculation is seldom practiced. However, survival and effective functioning of *Rhizobium* populations are reduced by high soil temperatures, salt and osmotic stress, soil acidity and alkalinity, pesticide and fungicide applications as well as nutrients deficiencies stress (Zahran, 1999). Through inoculation of strains selected from indigenous populations, improved symbiotic efficiency can be achieved, which can lead to an increase in peanut yield and quality. Variations in the acidity tolerance of some Cameroonian strains of rhizobia were evaluated by Nwaga and Ngo Nkot (1998), but little is known about the genetic variation in peanut rhizobial populations indigenous to Cameroon. Knowledge about the indigenous population is necessary before the selection and application of the strains.

In Cameroon, four land use systems are mostly used among which mixed farming, fallow, cocoa farms and forest. The object of this study was to assess the genetic diversity of rhizobia isolated from *Arachis hypogaea* in different land use systems of Cameroon. This was done using RFLP analyse of the PCR amplified 16S–23S DNAr Inter Genic Spacer (IGS) region and host specificity.

2. Materials and methods

2.1. Bacterial strains

Rhizobia were isolated from soil samples in the laboratory using peanut as trapping host. Four different sites located in one agroecological zone of Cameroon, which have no history of *Arachis hypogaea* L. inoculation, were sampled. The rhizobia isolated and reference strains used in this study are listed in Table 1.

A total of 42 peanut-nodulation rhizobia isolates were obtained from the nodules of *Arachis hypogaea* in different land use systems of Cameroon using standard procedures (Vincent, 1970). Two strains described by Nwaga and Ngo Nkot (1998) are included. The purity of cultures was assured by repeated isolation from single selection. All strains were grown and maintained on yeast extract-mannitol (YM) with or without agar (Vincent, 1970).

Table 1 – Original host legume and the land use systems of rhizobia isolates used in this study

Land use systems	No.	Isolates	Host legume
Mixed farming[a]	1	AhBeC11	*Arachis hypogaea* L
Mixed farming[a]	2	AhBeC21	*Arachis hypogaea* L.
Mixed farming[a]	3	AhBeC31	*Arachis hypogaea* L.
Mixed farming[a]	4	AhBeC41	*Arachis hypogaea* L.
Mixed farming[a]	5	AhBoC11	*Arachis hypogaea* L.
Mixed farming[a]	6	AhBoC41	*Arachis hypogaea* L.
Mixed farming[a]	7	AhEC11	*Arachis hypogaea* L.
Mixed farming[a]	8	AhEC12	*Arachis hypogaea* L.
Mixed farming[a]	9	AhEC31	*Arachis hypogaea* L.
Mixed farming[a]	10	AhYC11	*Arachis hypogaea* L.
Mixed farming[a]	11	AhYC21	*Arachis hypogaea* L.
Mixed farming[a]	12	AhYC31	*Arachis hypogaea* L.
Mixed farming[a]	13	AhYC41	*Arachis hypogaea* L.
Mixed farming[a]	14	VUID1	*Vigna unguiculata* L.
Mixed farming[a]	15	VUXY1	*Vigna unguiculata* L.
Fallow	16	AhBeJ11	*Arachis hypogaea* L.
Fallow	17	AhBoJ21	*Arachis hypogaea* L.
Fallow	18	AhBoJ31	*Arachis hypogaea* L.
Fallow	19	AhBoJ41	*Arachis hypogaea* L.
Fallow	20	AhEJ21	*Arachis hypogaea* L.
Fallow	21	AhEJ31	*Arachis hypogaea* L.
Fallow	22	AhEJ41	*Arachis hypogaea* L.
Fallow	23	AhEJ42	*Arachis hypogaea* L.
Fallow	24	AhYJ11	*Arachis hypogaea* L.
Fallow	25	AhYJ41	*Arachis hypogaea* L.
Cocoa	26	AhBoP31	*Arachis hypogaea* L.
Cocoa	27	AhBoP41	*Arachis hypogaea* L.
Cocoa	28	AhEP11	*Arachis hypogaea* L.
Cocoa	29	AhEP21	*Arachis hypogaea* L.
Cocoa	30	AhEP31	*Arachis hypogaea* L.
Cocoa	31	AhYP21	*Arachis hypogaea* L.
Cocoa	32	AhYP31	*Arachis hypogaea* L.
Cocoa	33	AhYP41	*Arachis hypogaea* L.
Forest	34	AhBeF11	*Arachis hypogaea* L.
Forest	35	AhBeF21	*Arachis hypogaea* L.
Forest	36	AhBeF41	*Arachis hypogaea* L.
Forest	37	AhBoF32	*Arachis hypogaea* L.
Forest	38	AhBoF41	*Arachis hypogaea* L.
Forest	39	AhEF11	*Arachis hypogaea* L.
Forest	40	AhEF21	*Arachis hypogaea* L.
Forest	41	AhEF41	*Arachis hypogaea* L.
Forest	42	AhYF31	*Arachis hypogaea* L.

[a] Soil samples were collected during the peanut crop cycle.

2.2. 16S–23S DNAr PCR-RFLP

A couple of primer, 16S–23S primers designed from conserved regions of *Frankia* sp. rrn, FGPS1490-72 (located at positions 1490–1510 of the 16 S rDNA, 5′-TGCGGCTGGATCCCCTCCTT-3′) (Normand et al., 1996), and FGPL132-38 (located at positions 132-114 of the 23 S rDNA, 5′-CCGGGTTTCCCCATTCGG-3′) (Norman et al., 1992), were used for PCR amplification of 16S–23S rDNA IGS region. After having been washed two times, bacterial suspensions were used as template for the PCR amplification.

Polymerase Chain Reaction (PCR) was performed in 25 μl mixture containing 1.25 μl of each primer, ultra pure water 20.5 μl; 1.5 U of Taq polymerase (Ready-to-Go PCR beads, Pharmacia Biotech) and 2 μl of bacterial cells suspension. The temperature profile was as follows: initial denaturation at 94 °C fot 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; and a final extension step at 72 °C for 7 min.

Amplification was conducted in a thermocycler GeneAmp PCR System 2400 (Perkin Elmer), using the procedure described by Van Berkum et al. (1996). Restriction endonucleases, HinfI, HhaI, HaeIII and MspI were used to digest PCR products as specified by the manufacturer using and aliquot of 6 or 8 μl. The DNA fragments were separated electrophoretically on a 2.5% agarose Metaphor gel at 3.3 V cm^{-1} for 1 h. The gels were stained in aqueous solution of 1 mg ml^{-1} ethidium bromide

and photographed under UV illumination with Gel Doc 1000 (BIO-RAD).

2.3. Host specificity

Seeds of *Macroptilium atropurpureum* were scarified and sterilized by immersing in concentrated H_2SO_4 for 5 min, thoroughly rinsed with sterile water. Seeds were distributed on the surface of 0.9% agar plates, then incubated for 1–2 days at 28 °C for germination. After germination, seedlings were transferred aseptically to Gibson tubes under sterile conditions. Suspension of 42 peanut rhizobia strains grown in YM medium were inoculated onto the roots of plants. Seeds were inoculated by adding 1 ml of rhizobial cultures at the middle of the exponential growth phase with about 10^8 cells ml^{-1} for each tube. Plants were cultivated in controlled growth chambers at 28 °C with 16 h in the light and 8 h in the dark. Light intensity was 120 μmole m^{-2} s^{-2}. Plants were harvested 21days after inoculation.

3. Results

3.1. PCR-RFLP analysis of 16S–23S rDNA IGS regions

Eighteen isolates produced a single band ranging from 750 and 850 bp which correspond to the expected size of the 16S–23S rDNA IGS fragment (Normand et al., 1996). Most strains (24 over 42), of which the purity was carefully checked, reproducibly exhibited an additional band in the same range of size.

Tree 4-base-cutting restriction endonucleases and one 5-base cutting restriction endonuclease were used to digest the 16S–23S rDNA IGS fragments amplified from the 42 peanut isolates. Results for the RFLP patterns obtained after digesting the amplified DNA with endonucleases HaeIII, HhaI, Hinf and MspI were presented in Table 2. Great variation was found by PCR-RFLP analysis of the 16S–23S rDNA inter genic spacer among the tested strains. The isolates were placed into groups on the basis of restriction fragment generated by digestion of product PCR with the enzymes. From 4 to 8 distinct restriction patterns were detected with each of the four endonucleases and the isolates used. The numbers of restriction patterns obtained with each of the four restriction enzymes were as follows: HinfI (6 patterns), HhaI (8 patterns), MspI (5 patterns), HaeIII (4 patterns). The result indicated that there was no digestion of amplified product from some strains by MspI and HaeIII (Table 2).

The types A, D, F, G and H were obtained by fragments with double band. The types B, C and E had isolates with a single band.

Endonuclease HhaI and HinfI were found to be the most discriminative with 8 and 6 genotypes (A–H) respectively detected among the Cameroonian rhizobia compared to HaeIII and MspI.

8 composite restriction pattern types were obtained by combining data from the digestions. The digestion of rrn fragment with enzyme HhaI generated some different DNA fragment types as produced with HinfI enzyme (Fig. 1).

Isolates originated from different land use systems in humid forest could be assigned to the same genotype for example (genotype A: AhBeC11, AhBeJ11, AhBoP31, AhBeF11; genotype B: AhYC21, AhBoJ41, AhYP21, AhYF31). Other genotypes were found only with one isolate (AhBoF32, AhEJ21, AhYP31 and VUID1).

Types D and F were regrouped with three endonucleases. These types showed the same restriction patterns with HaeIII, HinfI and MspI digestions, but they showed different patterns when digested with HhaI. This could be due to species differences on intraspecies level.

In the soils use in this study, no inoculant strains have been used. Our results showed that the highest diversity was found in cocoa farm where no peanut had ever been planted and in fallow. On the other hand, the presence of the leguminous crop tended to decrease the diversity of the *Rhizobium* population.

Table 2 – 16S–23S genotypes and restriction patterns revealed by PCR-RFLP of the tested rhizobia

Isolates[a]	Restriction patterns[b]				16S–23S genotype[c]
	HaeIII	HhaI	Hinf	MspI	
AhBeC11, AhBeC21, AhBeC31, AhBeC41, AhBeF11, AhBeF21, AhBoC11, AhBoC41, AhBoJ21, AhBoP31, AhEC11, AhEC12, AhEC31, AhEJ42, AhEP31, AhEF11, AhEF21, AhEF41, AhYP41, VUXY1	a	a	a	a	A
AhBoJ41, AhEP11, AhYC11, AhYC21, AhYC31, AhYC41, AhYJ11, AhYJ41, AhYP21, AhYF31	nd	b	b	nd	B
AhBeJ11, AhBeF41, AhBoF41, AhEP21	nd	c	c	c	C
AhBoJ31, AhBoP41, AhEJ31, AhEJ41	a	d	a	a	D
AhBoF32	nd	e	e	e	E
AhEJ21	a	f	a	a	F
AhYP31	g	g	g	g	G
VUID1	h	h	nd	a	H

[a] Isolates of peanut from the four land use system. nd: not determined.
[b] Different restriction patterns obtained with each restriction enzyme among the 42 isolates analyzed (Fig. 1 are shown); isolates with the same letter has the same restriction patterns obtained for one restriction enzyme, vice versa.
[c] The 16S–23S rDNA genotypes, which are numbered from A to H, represent combination of restriction patterns obtained with the four endonucleases.

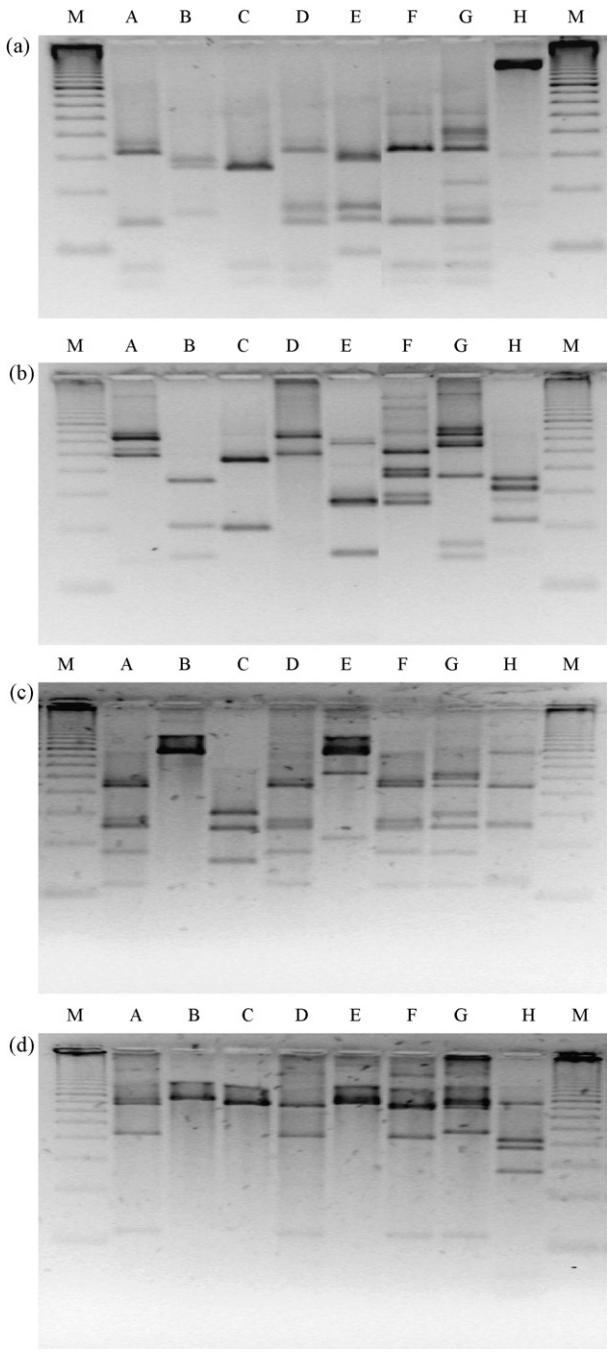

Fig. 1 – Types of restriction patterns of PCR-amplified 16S–23S rDNA intergenic spacer digested with HinfI (a); HhaI (b); MspI (c) and HaeIII (d) of isolates used in this study. The lane assignments (lanes A–H) are given in Table 2. Lane M, molecular weight marker 100 pb ladder (Pharmacia Biotech). From left to right: representatives of the IGS types, AhBeC21, AhBoJ41, AhBeJ11, AhBoJ31, AhBoF32, AhEJ21, AhYP31 and VUID1.

The highest species richness was found in cocoa and fallow. Eight or ten respective isolates were grouped to five different IGS types, whereas nine isolates originated from forest, in four IGS types follow-up of 15 isolates of peanut mixed farming grouped in three IGS types (Table 3).

Table 3 – Number of peanut isolates per land use system and IGS types

Land use system	Number of isolates	IGS types
Peanut mixed farming	15	A, B, H
Fallow	10	A, B, C, D, F
Cocoa	8	A, B, C, D, G
Forest	9	A, B, C, E

These results indicate the existence of different levels of heterogeneity in these land systems. Cocoa monoculture and fallow seem to be the most heterogeneous systems, while low level of diversity is observed in the peanut farm.

In the other hand, genotypes A (the most abundant genotype in these soils) and B were the largest types in this study, these two types are found in all the land use systems. IGS type C was common to all land use systems except mixed farming system. Type H is found in mixed farming system; type F in fallow; type G in cocoa monoculture and type E in the forest. In this study we demonstrated that genotype richness diversity of peanut isolates depends on the land use system.

Representative isolates of different IGS types obtained are: AhBeC21, AhBoJ41, AhBeJ11, AhBoJ31, AhBoF32, AhEJ21, AhYP31 and VUID1. The dendrogram derived from RFLP patterns clearly showed the existence of two divergent groups among peanut rhizobia (Fig. 2). Group I consisted of isolates of genotypes A, D, C and F represented by AhBeC21, AhBoJ31, AhBeJ11 and AhEJ21 respectively. Group II comprised genotypes B, G, E and H and were represented by AhBoJ41, AhYP31, AhBoF32 and VUID1 respectively. Group I and group II included the isolates of all the land use systems. Endonuclease HhaI was found to be the most discriminative. MspI is intermediary. Isolates 2, AhBeC21; 5, AhBoJ31; 4, AhBeJ11; 7, AhEJ21 are very close to each other with HaeIII, HinfI and MspI (data not shown). From the dendrogram, isolates from the same land use system were separated into different groups.

3.2. *Host specificity*

All the 42 peanut rhizobia strains isolates used in this test were able to nodulate *Macroptilium atropurpureum*.

4. Discussion

The data of this study indicated that from 4 to 8 distinct restriction patterns were detected with each of the four endonucleases and the isolates used. The numbers of restriction patterns obtained with each of the four restriction enzymes were as follows: HinfI (6 patterns), HhaI (8 patterns), MspI (5 patterns), HaeIII (4 patterns). The reason why there was no digestion of amplified product from some strains by MspI and HaeIII could be due to absence of the restriction sites. To examine the diversity of the isolates of peanut using PCR–RFLP analysis, determination of the minimum number set of the restriction enzymes will be needed. Moyer et al. (1996) reported that combination of the three or four tetrameric restriction enzymes gave good resolution results. By PCR–RFLP analysis of 16S–23S rDNA IGS regions, the discriminating power was sufficient to group chromosomally closely related

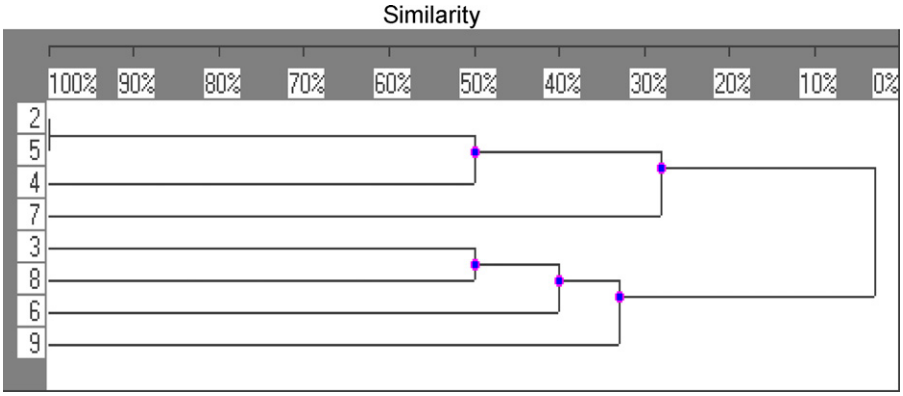

Fig. 2 – Dendrogram of genetic relationships of indigenous peanut rhizobia and representative isolates derived from HhaI restriction patterns of amplified 16S–23S rDNA (2, AhBeC21; 5, AhBoJ31; 4, AhBeJ11; 7, AhEJ21; 3, AhBoJ41; 8, AhYP31; 6, AhBoF32; 9, VUID1).

strains on the basis of the simple, reproducible and hence easy to analyse patterns of restriction fragments.

The highest diversity was found in cocoa farm where no peanut had ever been planted and in fallow. On the other hand, the presence of the leguminous crop tended to decrease the diversity of the *Rhizobium* population. These results are in agreement with data from Coutinho et al. (1999) and Zilli et al. (2004) that found decrease in rhizobia diversity associated with the presence of the host. The authors suggest that the introduction of a leguminous plant is capable of promoting the selection of particular *Rhizobium* taxa. Tamimi and Young (2004) reported a restricted genetic diversity of the bean rhizobial population in the cultivated soils of various locations of Jordan.

Such a high genetic homogeneity was observed among rhizobia strains associated with *Arachis pintoï* in Cerrados soils (Pereira Pinto et al., 2004). These authors discussed that the presence of *A. pintoï* in the original site may favor the selection of similar genetic strains. Nine isolates originated from forest. Results described by Chen and Huang (2002) according to the 16S–23S RFLP patterns, also revealed different genotypes of soybean rhizobia in an unexploited forest region in central China with no soybean planting history.

The fact that highest level of diversity was recorded in soils under cocoa monoculture as compared to soils under mixed peanut farming corroborates the results of Depret et al. (2004) who have reported highest level of diversity in soils under wheat monoculture. However, the diversity of *R. leguminosarum bv. viciae* was said to be strongly dependent on the crop species and/or the agricultural practices involved.

The restricted genetic diversity of the peanut rhizobial population in the mixed farming could be related to environmental factors. This is in agreement with the results obtained by Venkateswarlu et al. (1997) who reported that crop related factors have more critical influence on the abundance of native rhizobial population than soil or climatic factors. Zilli et al. (2004) also reported that the presence of the legumes provides ecological conditions to specific *Rhizobium* groups, which acquire competitiveness determinant traits and become successfully established. In this study we demonstrated that genotype richness diversity of peanut isolates depends on the land use system.

5. Conclusions

In this work, we studied the diversity of peanut rhizobia in four land use systems. A total of 8 IGS types were recorded among the 42 isolates. Further studies to characterize these isolates need to be carried out. The highest level of diversity was recorded in soils under cocoa monoculture as compared to soils under mixed peanut farming, suggesting that the presence of the host legume may reduce the native rhizobia population. The understanding of the native rhizobia population diversity will contribute to the selection of inoculants for peanut in the humid forest zone of Cameroon.

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