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Effects of a rock phosphate on indigenous rhizobia associated with *Sesbania sesban*

Ousmane Sacko [a,*], Inamoud Ibny Yattara [a], Messaoud Lahbib [b], Marc Neyra [c,1]

[a] Laboratoire de Microbiologie des Sols, Département de Biologie, Faculté des Sciences et Techniques, Université de Bamako, BP. E 3206, Mali, France
[b] Chaire UNESCO pour l'Environment, Campus Universitaire, Badalabougou, Villa N°9/MEN, BP. E 1023, Bamako, Mali, France
[c] IRD. UR O40, Laboratoire Commun de Microbiologie IRD/ISRA/UCAD, Centre de Recherche de Bel-Air, Dakar, Senegal

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ABSTRACT

Tilemsi rock phosphate (TRP) of Mali is one of the most promising rock phosphate in West Africa for soil fertilization, but it is little used because of its insoluble form. The main objective of this study is to investigate TRP effects on rhizobia associated with the multipurpose leguminous tree *Sesbania sesban* grown on a sandy soil, poor in phosphorus and not sterilised. The experiment included treatments with and without TRP and was conducted during 105 days. At the end, 114 nodules have been collected and analysed by PCR/RFLP of 16S-23S intergenic spacer. Sixteen different RFLP profiles corresponding to different genomic groups of rhizobia have been detected. Five were dominant and present in both treatments. Five groups appear only in treatments without TRP whereas the six others are only in nodules of plants with TRP, suggesting a different capacity of natural phosphates solubilization by these strains.

1. Introduction

Compared with the other major nutrients, phosphorus (P) is by far the least mobile and is, after nitrogen (N_2), the most limiting factor for plant growth (Bieleski, 1973; Vance et al., 2000). The bioavailability of soil inorganic phosphorus in the rhizosphere varies considerably with plant species and soil conditions. Tropical and subtropical soils are often extremely phosphorus-deficient, mainly due to high phosphorus sorption capacities. Importance of P in plants nutrition has been extensively reported. In particular, P is needed for plant growth, nodule formation and development on legumes, and synthesis of ATP (source of energy necessary for the cleavage and reduction of N_2 into ammonia), each process being vital for biological nitrogen fixation (Waidyanatha et al., 1979; Islam et al., 1980). Leguminous trees required P to assure a good growth and a better N_2-fixation, and their effectiveness in soils improving may be hindered by a P deficiency (Giller and Cadisch, 1995). P fertilization is often necessary to circumvent phosphorus deficiency. In Mali, imported P fertilizers are expensive, and the locally produced Tilemsi rock phosphate (TRP) deposits supply the farmers with a cheaper alternative (Bationo et al., 1997). This low cost insoluble phosphate has demonstrated is usefulness in agroforestry systems (Bâ and Guissou, 1996; Bâ et al., 2001; Babana and Antoun, 2005).

The shrub leguminous Sesbania sesban is an important agroforestry species (Odee et al., 2002) which is often used as cover tree by farmers (Desaeger and Rao, 2001) and as green manure (Giller, 2001). In some dry lands of West Africa, *S. sesban* is indiscriminately used as timber wood and building, as fodder for livestock and also as fertilizer. This fallow species, like other legumes, may form symbiosis with wide indigenous rhizobial populations (Odee et al., 1995), but is among the least promiscuous when compared to other tropical trees like *Leucaena leucocephala* and *Gliricidia sepium* (Bala and Giller, 2001) and nodulated most effectively only with its homologous strains (Odee et al., 2002). Its N_2-fixation capacity may be about of 20 kg N^{-1} ha^{-1} (Kang et al., 1999; Ndoye and Dreyfus, 1988). Previous studies showed the lack of compatible rhizobia and of nodulation of *S. sesban* in different African, Asian and South American soils (Bala et al., 2003a, b). However, *S. sesban* presents a low response to inoculation by rhizobia and arbuscular mycorrhizal fungi (Ndoye and Dreyfus, 1988; Habte and Manjunath, 1991). It has been shown that P deficiency is an important constraint to the use of inoculation technology to increase *S. sesban* productivity in the Ethiopian highlands ecosystem for increased and sustainable crop-livestock productivity (Haque et al., 1996). However reports

* Corresponding author. Tel.: +223 66 81 12 44.
E-mail addresses: ousmane_sacko@yahoo.fr, osacko@ml.refer.org (O. Sacko), iiyattara@yahoo.fr (I.I. Yattara), lahbibmessaoud@yahoo.fr (M. Lahbib), Marc.Neyra@ird.fr (M. Neyra).
[1] Present address: Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpelier, France.

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about phosphorus often focus on its impact on nutrition and/or trees growth, with the aim of having a good yield. Ndiaye et al. (2009) for example showed that natural rock phosphate can significantly increase the shoot biomass and mineral (N, P and K) content of *S. sesban* seedlings. On the other hand, there is little information about micro-organisms behaviour in soil in the presence of such important nutrient as phosphorus. A better knowledge of phosphorus-micro-organisms interaction may therefore be a major trump for a sustainable growth of this tree.

The global aim of our study was to explore the effects that phosphorus may have on the rhizobial behaviour in *S. sesban* rhizosphere, in particular under the influence of Tilemsi rock phosphate.

2. Materials and methods

2.1. Soil sampling

The soil used in this experience has been taken in the ISRA (Institut Sénégalais des Recherches Agricoles) station in Nioro (Senegal), 13°44' N and 15°47' W, with 700 mm annual rainfall. Soil sample was collected between 0 and 25 cm depth. It is a sandy soil with neutral pH and 23.40 ppm available P. It contains 2.06% clay, 0.62% thin limon, 7.74% rough limon, 49.2% thin sand, 36.8% rough sand, 2.04 mg kg^{-1} soil total C, 0.14 mg kg^{-1} soil N, 106 ppm total P. The pH (H_2O) and (KCl) are, respectively, 7.23 and 5.63. The soil has been crushed and passed through a 1 mm sieve.

2.2. Fertilization and experimental design

The rock phosphate used as fertilizer (TRP) originated from Bourem, in the Tilemsi valley (Mali). It is one of the most soft and receptive phosphate in West Africa (Truong et al., 1978). It is used as pulverized form with 30% P_2O_5. TRP, actually commercialized, presents 0.007% of solubility in water. Its chemical contents are reported in Table 1. The experience has been carried out during 105 days in Laboratoire Commun de Microbiologie IRD/ISRA/UCAD in Dakar (Senegal). TRP has been applied and mixed to 1 kg of soil per bag to a final concentration of 50 mg P kg^{-1}, corresponding approximately to 150 kg P_2O_5 ha^{-1}. Each treatment (with or without TRP) was repeated 15 times.

2.3. Plant material

S. sesban seeds were scarified into 98% H_2SO_4 for one hour. They were after plentifully rinsed out with sterile distilled water, soaked overnight into last rinsed water and transferred on medium agar during 2 days at 28 °C temperature. Pre-germinated seeds had been planted out in plastic bags at the rate of two seedlings per bag. After a week, only one seedling was preserved into each bag. Seedlings in bags have been put into greenhouse in ambient conditions and plants were watered daily.

Table 1
Chemical composition of different natural rock phosphates from West Africa (after Truong et al., 1978).

Rock phosphates (country of origin)	Total content (%)		Solubility (% of total P_2O_5)			CO_3/PO_4 substitution
	P_2O_5	CaO	Citrate	Citric acid	Formic acid	
Arli (Burkina Faso)	30.8	47.6	5.4	19.2	38.7	0.098
Kodjari (Burkina Faso)	27.16	44.8	6.1	18.8	37.1	0.093
Tahoua (Niger)	34.5	44.8	8.3	19.3	34.0	0.112
Taiba (Senegal)	37.8	44.8	5.0	19.8	38.7	0.098
Tilemsi (Mali)	30	43.1	10.4	29.7	47.3	0.210
Hahotoe (Togo)	35.4	36.4	4.3	19.1	36.7	0.088
Gafsa (Tunisia)	30.2	31.9	20.5	37.8	78.6	0.254

2.4. Bacterial molecular characterization

One-hundred and fourteen nodules (58 on plants growing on soil without TRP, 56 with TRP) have been collected randomly at the end of the experiment (105 days after seedling). They were surface sterilised by immersion in 3.3% (w/v) $Ca(OCl)_2$ for 3 min, and rinsing in sterile water. This was followed by a second immersion in 96% ethanol for 2–3 min and rinsing in sterile water. From this stage the nodules were manipulated aseptically. Each nodule was crushed in 300 μl of sterile water with plastic pestle sterilised in 96% ethanol in a 1.5-ml Eppendorf tube 150 μl of 2× CTAB/PVPP buffer sterilised in 96% ethanol in a 1.5-ml Eppendorf tube 150 μl of 2× CTAB/PVPP buffer (0.2 M Tris–HCl, pH 8; 0.04 M EDTA pH 8; 2.8 M NaCl; 4% w/v CTAB (hexadecyltrimethylammonium bromide); 2% w/v PVPP (polyvinylpolypyrrolidone)) was added to 150 μl of crushed nodule for DNA extraction. The homogenate was incubated at 65 °C for 60 min and centrifuged for 10 min at 15,000 × *g* to remove cellular debris. Supernatant was then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) and centrifuged for 15 min at 15,000 × *g*. DNA from the aqueous phase was purifed from phenol with 300 μl of chlorophorm isoamyl alcohol (24:1 v/v) and centrifuged for 15 min at 15,000 × *g*. Supernatant was centrifuged one more time for 5 min. DNA from the aqueous phase was precipitated overnight at −20 °C with the addition of 0.1 volume of sodium acetate and 2.5 volumes of absolute ethanol. The samples were centrifuged for 30 min at 13,000 rpm at +4 °C. The resulting DNA pellet was washed with 70% v/v ethanol by centrifugation for 15 min at 13,000 rpm at +4 °C, vacuum dried, and solubilized in 20 μl of ultrapure water. The purity and the quantity of DNA extracted were estimated by spectrophotometry (Pharmacia Biotech) in the range 200–340 nm. Bacterial genomic DNA was extracted from 1.5 ml of stationary phase bacterial cultures grown in YM (Yeast extract Mannitol medium). Cells were pelleted by centrifugation and resuspended by homogenisation in one volume of 1× CTAB/PVPP buffer. Total genomic DNA was recovered and purified as described above for crushed nodule DNA. PCR amplification of 16S-23S rDNA spacer region was performed using two primers: FGPS1490-72 (5'-TGCGGCTGGATCCCCTCCTT-3') (Normand et al., 1996), and FGPL132-38 (5'-CCGGGTTTCCCCATTCGG-3') (Ponsonnet and Nesme, 1994). PCR was carried out in 25 μl reaction volume containing 50 ng of pure total DNA extract, one dried bead (Ready-to-Go PCR beads, Pharmacia Biotech) containing 1.5 U of Taq polymerase, 10 mM Tris–HCl, (pH 9 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP and 1.0 μM of each primer. PCR amplification was performed in GeneAmp PCR System 2400 (Perkin Elmer) thermal cycler adjusted to the following temperature profile: initial denaturation at 95 °C for 5 min; 35 amplification cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension 72 °C for 1 min; and final extension at 72 °C for 3 min. After electrophoresis of 3 μl on a 1% (w/v) agarose gel in TBE buffer (1.1 w/v Tris–HCl; 0.1% w/v $Na_2EDTA \cdot 2H_2O$; 0.55% w/v boric acid), the gel was stained for 30 min in an aqueous solution of ethidium bromide (1 μg/ml) and photographed under UV illumination with Gel Doc (BIO-RAD) software. Restriction fragment analysis of 16S–23S intergenic spacer region was performed on aliquots (6–10 μl) of PCR products digested with restriction endonucleases *Msp*I as specified by the manufacturer (Amersham Pharmacia Biotech) with an excess of enzyme (10 U per 20 μl reaction volume) for 2 h. Restricted DNA was analysed by horizontal electrophoresis in 2.5% (w/v) agarose MetaphorR (FMC BioProducts, Rockland, Marine USA). Electrophoresis was carried out at 80 V for 3 h in 11 × 14-cm gels. Gel was stained and photographed as described above.

Table 2
Number of nodules in the different IGS groups, with or without TRP.

IGS groups	Without TRP	With TRP
I	18	15
II	14	12
III	5	1
IV	8	16
V	1	
VI	6	4
VII		2
VIII		1
IX		1
X		2
XI		1
XII	1	
XIII	1	
XIV		1
XV	2	
XVI	2	
Total	58	56

2.5. Statistical analysis

The data were analysed as unordered rows by columns contingency tables using the Likelihood ratio test implemented by the StatXact statistical analysis software package (CYTEL Software, Cambridge, MA), with IGS RFLP groups in rows and frequency of each IGS type in columns.

3. Results and discussion

A total of 114 nodules have been analysed for the two treatments (56 with and 58 without TRP). Sixteen different *Msp*I RFLP profiles have been discriminated (Table 2). The more frequent are showed in Fig. 1. Several authors have demonstrated the discriminating power of PCR-RFLP analysis of 16S–23S IGS regions for studying natural bacterial diversity and grouping genetically related strains (Navarro et al., 1992; Jensen et al., 1993; Laguerre et al., 1996; Doignon-Bourcier et al., 1999; Krasova-Wade et al., 2003; Diouf et al., 2007). It appears therefore that a remarkable diversity exist among rhizobia nodulating *S. sesban* in a same sandy Senegalese soil. This observation confirmed those of Odee et al. (1995) in Kenya, of Wolde-Meskela et al. (2004) in Ethiopia and of Sharma et al. (2005) in the semi-arid Delhi region where different species of *Sinorhizobium* nodulating *S. sesban* dominate. This large rhizobial diversity has already been shown in West African soils for other legume species such as cowpea (Krasova-Wade et al., 2003), *Pterocarpus erinaceus* and *Pterocarpus lucens* (Sylla et al., 2002), *Acacia seyal* (Diouf et al., 2007), *Acacia senegal* (Sarr et al., 2005; Fall et al., 2008). Despite of this diversity, a group of five profiles (I, II, III, IV and VI) is present in more than 85% of all the nodules that have been analysed, and are encountered in both treatments (Table 2). Even more, two profiles (I and II) are dominant, with half of the observed profiles, bringing to light a clear competitiveness for nodulation in such conditions, whatever the addition or not of TRP. On the other hand, it is interesting to observe that eleven profiles are found only in one treatment, and not in the other. Six profiles (VII to XI, and XIV) are encountered only in presence of TRP, whereas five profiles (V, XII, XIII , XV and XVI) are only in nodules of plants without TRP (Table 2). The exact *P* value (0.044) indicates that the null hypothesis can be statistically rejected and that there is altogether a significant difference in the genetic nodule population structure linked to the treatment with or without TRP. However this difference is slight, and is mainly due to two of the dominant profiles (IGS groups III and IV) and to the small groups specific to each treatment. It would be interesting to precise if these specific profiles correspond to closely related strains, and if they are strongly different from one treatment to the other. If yes it could mean that the presence of TRP really influence strains behaviour and induce significant changes in population structure. It is well known that rhizobia are often good phosphate-solubilizing bacteria (Abd-Alla, 1994; Abril et al., 2007; Taiwo and Ogundiya, 2008), but that differences exist between strains. Rosas et al. (2006) for example have shown that *Sinorhizobium meliloti* strain 3DOh13 solubilized iron and phosphate while *Bradyrhizobium japonicum* strain TIIIB was a poor phosphate solubilizer. The solubilization ability may be dependant on the form of P: from a total of 446 rhizobial isolates tested for P solubilization by the formation of visible dissolution halos on agar plates, 198 (44% of the isolates)

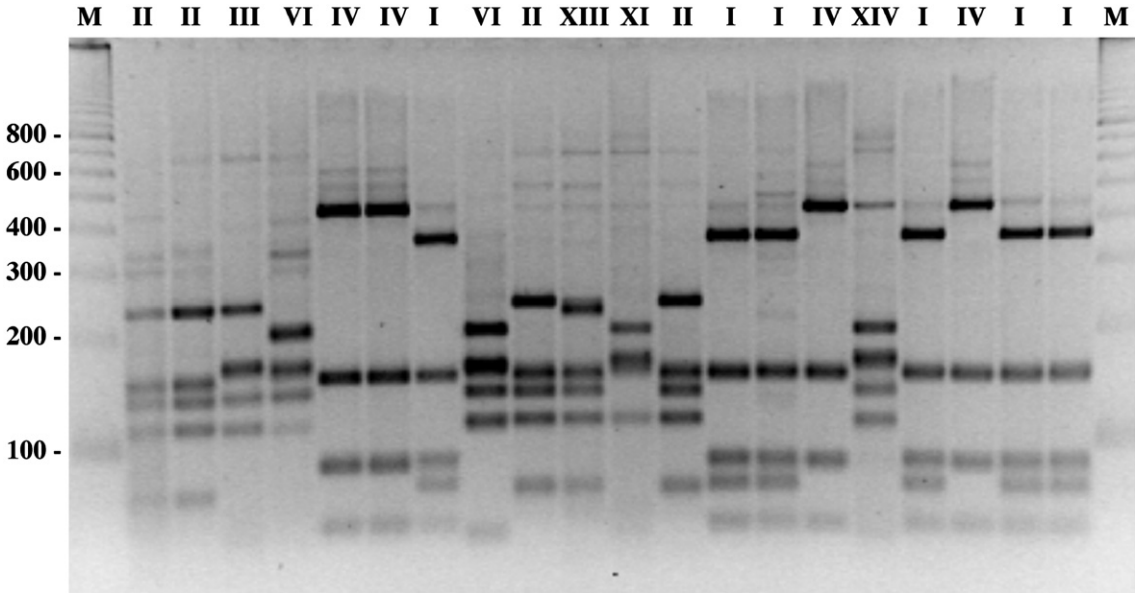

Fig. 1. Major restriction patterns of PCR-amplified 16S–23S rDNA IGS of crushed nodules DNA of *S. sesban*, obtained with *Msp*I. Lanes M, 100 bp DNA size marker (Pharmacia Biotech).

solubilized $Ca_3(PO_4)_2$ and 341 (76%) inositol hexaphosphate (Alikhani et al., 2006). The differences that were observed may be the result of a differential ability to solubilize TRP.

In conclusion, it appears that a great diversity exists among strains of rhizobia able to nodulate *S. sesban* in a given soil. Some of them are dominant for competition for nodulation. The addition of Tilemsi rock phosphate induce significant modifications of rhizobial populations. However the differences are slight and must be confirmed by further analyses. TRP has no effects on most of the major IGS groups encountered, but can have on the other hand an impact on the behaviour of minority groups. It could be due to a difference of capacity to solubilize phosphates, which would deserve to be studied, in particular in the optics of the inoculation with these strains, if they turn out besides good nitrogen fixers in symbiosis with *S. sesban*.

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