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Diversity of Rhizobia Nodulating *Crotalaria* spp. from Senegal

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Abstract

A total of 117 rhizobia strains was isolated from root nodules of nine *Crotalaria* spp. Nodules were collected at eight geographical sites in Senegal. Growth in yeast mannitol agar (YMA) revealed that rhizobia strains of *Crotalaria* spp. include both fast and slow-growing strains. Host-specificity shows two groups of specificity in *Crotalaria* spp. tested: group I includes *C. glaucoides*, *C. perrottetii* and *C. podocarpa* which were effectively nodulated only by fast-growing strains, and group II includes *C. comosa*, *C. goreensis*, *C. hyssopifolia*, *C. lathyroides*, *C. ochroleuca*, *C. retusa* which were effectively nodulated only by slow-growing strains. Slow-growing rhizobia have a broad host range and nodulated *Crotalaria* of group II and *Acacia albida* and *Indigofera microcarpa*, while fast-growing strains nodulated only *Crotalaria* spp. None of the strains effectively nodulated *Sesbania rostrata* or *Acacia raddiana*. Numerical analysis of whole cells proteins profiles of these strains obtained by SDS-PAGE shows that slow-growing strains are related to

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Bradyrhizobium japonicum and fast-growing strains are not related to any known reference strains and constituted a new group of rhizobia.

Keywords: *Crotalaria* spp., rhizobia, growth, host-specificity, SDS-PAGE

1. Introduction

Crotalaria is a genus of tropical legume widespread in Africa and Madagascar (Polhill, 1982) recovered mostly in humid soils. This genus has about 550 species in Africa and Madagascar including 63 species in West Africa (Polhill, 1982) and 33 species in Senegal (Berhaut, 1976). *Crotalaria* species are annual shrubs very useful in agriculture (Magingo, 1992), as cover plants in plantations, green manure or in intercropping system. They are also a source of forage and protein for livestock. Among the 550 species, 136 species are known to be nodulated with indigenous soil rhizobia (Allen and Allen, 1981; de Faria et al., 1989) and to fix atmospheric nitrogen. They present great potentialities for soil amelioration in tropical arid and semi-arid areas (Polhill, 1982; Magingo, 1992).

In Senegal, incorporation of *Crotalaria* species in agricultural system is quite unknown. However, they have a potential for use in senegalese cropping systems, especially in fallow for a rapid restoration of soil fertility. Thus, rhizobia must be isolated and characterized for strategic use of elite ones as inoculants.

Specific reports about rhizobia associated with *Crotalaria* spp. are very few. The only information about these rhizobia were obtained by a few studies on tropical isolates which included only a few strains from *Crotalaria* spp. In these studies *Crotalaria*'s strains were designated as *Bradyrhizobium* spp. (Date, 1976; Van Rossum et al., 1995; Gao et al., 1994; Giller and Wilson, 1991).

The aim of this study was to establish a collection of indigenous rhizobia associated with native *Crotalaria* species and to characterize them by different methods such as growth, host-range and whole cells proteins.

2. Material and Methods

Fresh and firm root nodules were collected from naturally occuring *Crotalaria* species in eight geographical sites in Senegal (Bel-Air, Grand-Yoff, Djilor, Fanghote, Ferlo, Kabrousse, Kaparan, Kolda). Rhizobial strains were isolated from these nodules using standards procedures (Vincent, 1970). Strains were grown on YMA at 28°C and time required for the first visible colony to appear was recorded (Graham et al., 1991; Moreira et al., 1993).

Table 1. List of *Crotalaria* isolates

Strains	Growth on YMA	Plant of isolation	Geograph. origin
ORS 1860, ORS 1917, ORS 1933, ORS 1937, ORS 1938, ORS 1954, ORS 1955, ORS 1970, ORS 1991, ORS 1992, ORS 2057, ORS 2058, ORS 2059, ORS 2060, ORS 2061, ORS 2062, ORS 2063, ORS 2064, ORS 2065, ORS 2066, ORS 2067, ORS 2068, ORS 2070, ORS 2071, ORS 2073, ORS 2102	fast	*C. podocarpa*	Bel Air
ORS 1923, ORS 1924, ORS 1925	fast	*C. perrotteti*	Ferlo sud
ORS 1926, ORS 1939	fast	*C. perrotteti*	Kolda 1
ORS 1927, ORS 1928, ORS 2038, ORS 2039, ORS 2041, ORS 2042, ORS 2043, ORS 2044, ORS 2030, ORS 2032, ORS 2099	fast	*C. perrotteti*	Kaparan
ORS 2031, ORS 2033, ORS 2035, ORS 2036, ORS 2037, ORS 2092, ORS 2093, ORS 2094, ORS 2095, ORS 2097	fast	*C. perrottetii*	Djilor
ORS 1936	fast	*C. glaucoïdes*	Bel Air
ORS 2023, ORS 2024, ORS 2076	fast	*C. glaucoïdes*	Kaparan
ORS 2026, ORS 2027, ORS 2045, ORS 2046, ORS 2047, ORS 2049, ORS 2050, ORS 2051, ORS 2052, ORS 2053, ORS 2055, ORS 2056, ORS 2072, ORS 2100, ORS 2101	fast	*C. glaucoïdes*	Djilor
ORS 1801, ORS 1862, ORS 2034, ORS 2074, ORS 2075, ORS 2089, ORS 2090, ORS 2091	slow	*C. goreensis*	Kaparan
ORS 1811	slow	*C. goreensis*	Kabrousse
ORS 2088	slow	*C. goreensis*	Djilor
ORS 1935	slow	*C. goreensis*	Kolda
ORS 1810	slow	*C. lathyroides*	Kabrousse
ORS 1869, ORS 2108	slow	*C. lathyroides*	Kaparan
ORS 1813	slow	*C. hyssopifolia*	Kabrousse
ORS 1814, ORS 1815, ORS 1816	slow	*C. hyssopifolia*	Fanghote
ORS 1819	slow	*C. retusa*	Kabrousse
ORS 1863, ORS 1864, ORS 1865, ORS 1866, ORS 1867, ORS 1868, ORS 1929, ORS 2104, ORS 2105, ORS 2106, ORS 2107, ORS 2109	slow	*C. comosa*	Kaparan
ORS 1969, ORS 1971	slow	*C. retusa*	Kolda
ORS 2021, ORS 2022, ORS 2077, ORS 2078, ORS 2079, ORS 2080, ORS 2081, ORS 2082, ORS 2083, ORS 2084, ORS 2085, ORS 2086, ORS 2087	slow	*C. retusa*	Grand-Yoff (Dakar)

Table 2. Host specificity of *Crotalaria* spp. rhizobia from Senegal. Fast-growing.

Fast-growing	1955	1860	1917	1933	1924	1925	1926	1928	1936	1937	1938	1939	1954	1927
Plants of isolation	*Cpodo*	*Cpodo*	*Cpodo*	*Cpodo*	*Cperrot*	*Cperrot*	*Cperrot*	*Cglauc*	*Cglauc*	*Cpodo*	*Cpodo*	*Cperrot*	*Cpodo*	*Cperrot*
Origin	BelAir	BelAir	BelAir	BelAir	FerloS	FerloS	Kolda1	Kaparan	BelAir	BelAir	BelAir	Kolda1	BelAir	Kaparan
Host-plants														
Group I														
C. podocarpa	E	E	E	E	E	I	e	E	E	E	E	E	E	e
C. glaucoides	E	E	E	E	E	e	e	E	E	E	E	E	E	E
C. perrotteti	e	E	E	E	HE	e	e	E	E	E	e	E	e	e
Group II														
C. goreensis	o	o	o	o	o	o	o	o	o	o	o	I	I	o
C. comosa	o	o	o	o	o	o	o	o	o	o	o	o	o	o
C. hyssopifolia	o	o	o	I	I	o	o	o	I	I	I	o	I	o
C. lathyroides	o	I	I	I	I	I	I	I	I	I	I	I	I	I
C. ochroleuca	I	o	o	o	o	o	o	I	o	I	o	o	I	I
C. retusa	I	o	I	I	I	o	I	I	I	o	o	o	I	I
A. albida	o	o	o	o	o	o	o	o	o	o	o	o	o	o
I. microcarpa	o	o	o	o	o	o	o	o	o	o	o	o	o	o
A. raddiana	I	I	I	I	I	I	I	I	I	I	I	I	I	I
S. rostrata	o	o	o	o	o	o	o	o	o	o	o	o	o	o

HE = very highly effective >600 nmoles; E = highly effective: 350–600 nmoles; e= effective: 100–350 nmoles; I = ineffective <100nmoles; o = no nodulation; ps = pseudonodules.

Table 2. Continued. Slow-growing.

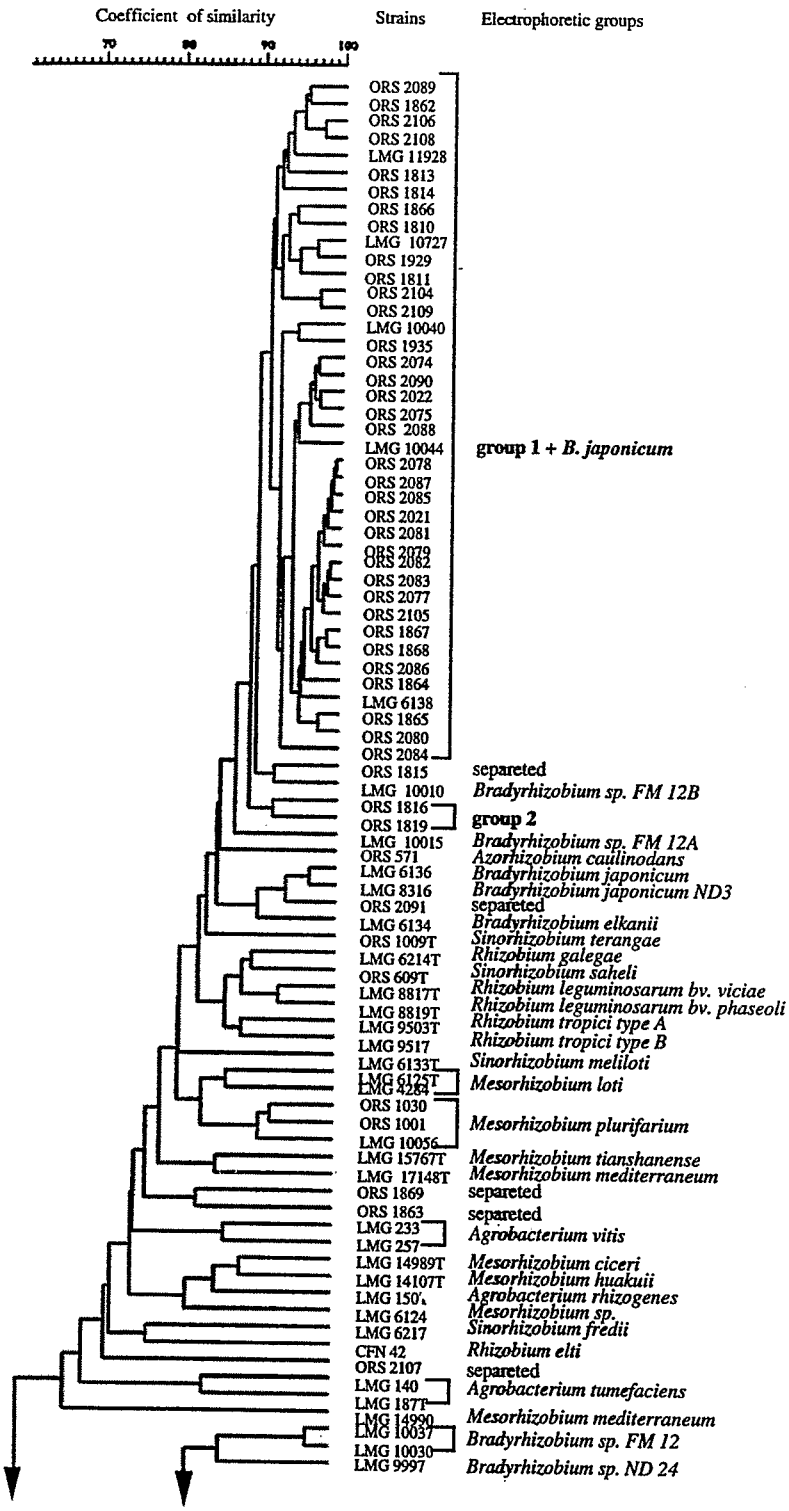
Slow-growing	1935	1816	1810	1929	1811	1815	1814
Plants of isolation	*Cgoreen*	*Chysso*	*Clathyr*	*Ccomo*	*Cgoreen*	*Chysso*	*Chysso*
Origin	Koldal	Fangh	Kabrou	Kaparan	Kabrou	Fangh	Fangh
Host-plants							
Group I							
C. podocarpa	o	o	o	o	o	o	o
C. glaucoides	o	o	o	o	o	o	o
C. perrotteti	o	o	o	o	o	o	o
Group II							
C. goreensis	e	e	e	e	o	o	o
C. comosa	HE	HE	e	e	e	e	o
C. hyssopifolia	E	e	HE	HE	e	e	HE
C. lathyroides	E	HE	E	E	E	HE	e
C. ochroleuca	e	E	e	HE	e	E	e
C. retusa	e	E	HE	HE	E	E	e
A. albida	E	e	E	E	E	E	e
I. microcarpa	HE	e	HE	E	E	E	e
A. raddiana	I	o	ps	ps	ps	ps	o
S. rostrata	I	o	I	I	o	o	o

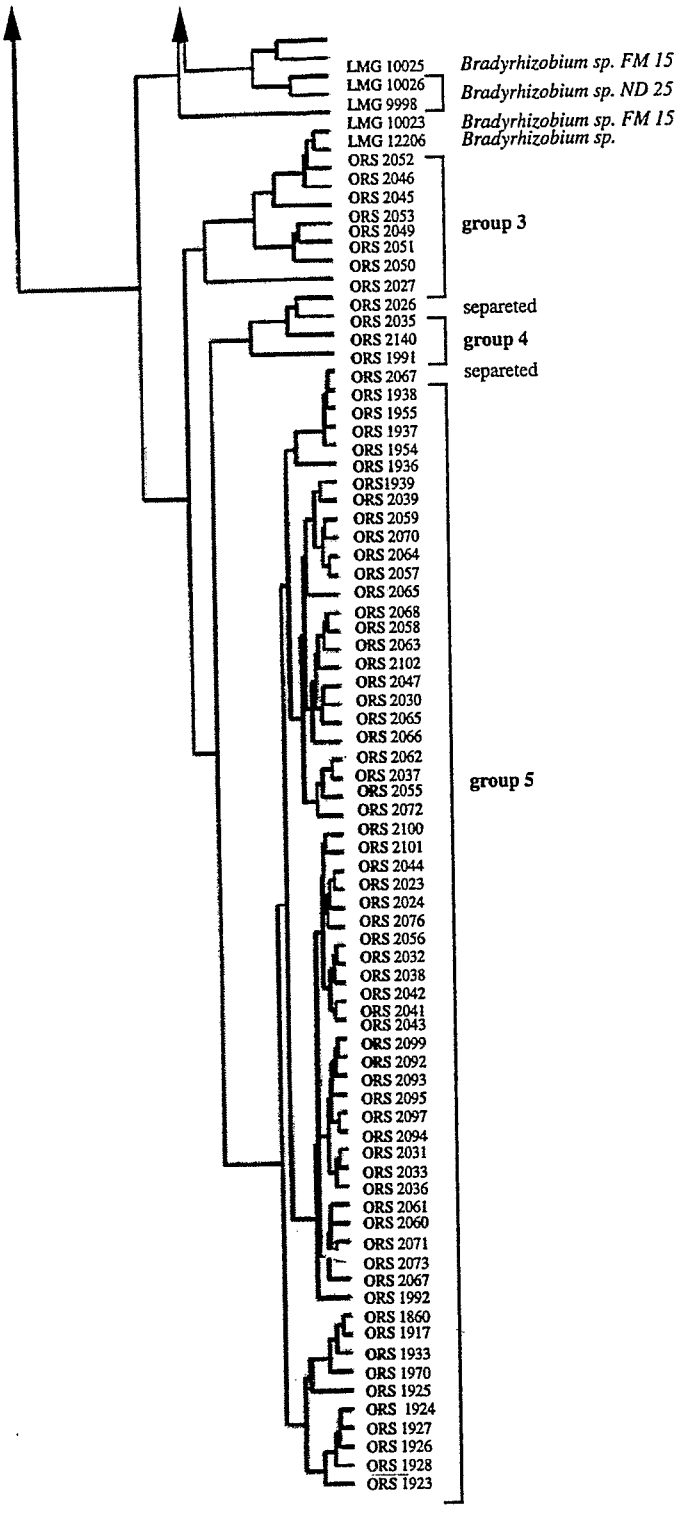
HE = very highly effective >600 nmoles; E = highly effective: 350–600 nmoles; e= effective: 100–350 nmoles; I = ineffective <100nmoles; o = no nodulation; ps = pseudonodules.

Twenty one isolates were tested for nodulation with nine *Crotalaria* species (*C. comosa, C. glaucoides, C. goreensis, C. hyssopifolia, C. lathyroïdes, C. ochroleuca, C. perrottetii, C. podocarpa, C. retusa*) and with *Acacia raddiana, Faidherbia albida* (syn. *Acacia albida*), *Sesbania rostrata* and *Indigofera microcarpa*. Plants were cultivated in Gibson tubes (Gibson, 1963) containing Jensen nutritive medium and placed at 28°C in a growth-chamber. The ability of the isolates to effectively fix nitrogen with these legumes was assessed by acetylene reduction assay (Hardy et al., 1973). Nodulated root system were

See the following pages for Fig. 1.

Figure 1. Schematic dendrogram showing the relationship among the SDS-PAGE whole cell proteins of *Crotalaria* strains and references of *Rhizobium, Brayrhizobium, Azorhizobium, Mesorhizobium, Sinorhizobium,* and *Agrobacterium*. The dendrogram is based on mean correlation coefficient values (r) which were grouped by unweighted average pair group method.


Coefficient of similarity
70
80
90
100
Strains
Electrophoretic groups
ORS 2089
ORS 1862
ORS 2106
ORS 2108
LMG 11928
ORS 1813
ORS 1814
ORS 1866
ORS 1810
LMG 10727
ORS 1929
ORS 1811
ORS 2104
ORS 2109
LMG 10040
ORS 1935
ORS 2074
ORS 2090
ORS 2022
ORS 2075
ORS 2088
LMG 10044
group 1 + B. japonicum
ORS 2078
ORS 2087
ORS 2085
ORS 2021
ORS 2081
ORS 2079
ORS 2082
ORS 2083
ORS 2077
ORS 2105
ORS 1867
ORS 1868
ORS 2086
ORS 1864
LMG 6138
ORS 1865
ORS 2080
ORS 2084
ORS 1815
separeted
LMG 10010
Bradyrhizobium sp. FM 12B
ORS 1816
ORS 1819
group 2
LMG 10015
Bradyrhizobium sp. FM 12A
ORS 571
Azorhizobium caulinodans
LMG 6136
Bradyrhizobium japonicum
LMG 8316
Bradyrhizobium japonicum ND3
ORS 2091
separeted
LMG 6134
Bradyrhizobium elkanii
ORS 1009T
Sinorhizobium terangae
LMG 6214T
Rhizobium galegae
ORS 609T
Sinorhizobium saheli
LMG 8817T
Rhizobium leguminosarum bv. viciae
LMG 8819T
Rhizobium leguminosarum bv. phaseoli
LMG 9503T
Rhizobium tropici type A
LMG 9517
Rhizobium tropici type B
LMG 6133T
Sinorhizobium meliloti
LMG 6125T
LMG 4284
Mesorhizobium loti
ORS 1030
ORS 1001
Mesorhizobium plurifarium
LMG 10056
LMG 15767T
Mesorhizobium tianshanense
LMG 17148T
Mesorhizobium mediterraneum
ORS 1869
separeted
ORS 1863
separeted
LMG 233
LMG 257
Agrobacterium vitis
LMG 14989T
Mesorhizobium ciceri
LMG 14107T
Mesorhizobium huakuii
LMG 150
Agrobacterium rhizogenes
LMG 6124
Mesorhizobium sp.
LMG 6217
Sinorhizobium fredii
CFN 42
Rhizobium elti
ORS 2107
separeted
LMG 140
LMG 187T
Agrobacterium tumefaciens
LMG 14990
Mesorhizobium mediterraneum
LMG 10037
LMG 10030
Bradyrhizobium sp. FM 12
LMG 9997
Bradyrhizobium sp. ND 24


LMG 10025
LMG 10026
LMG 9998
LMG 10023
LMG 12206
ORS 2052
ORS 2046
ORS 2045
ORS 2053
ORS 2049
ORS 2051
ORS 2050
ORS 2027
ORS 2026
ORS 2035
ORS 2140
ORS 1991
ORS 2067
ORS 1938
ORS 1955
ORS 1937
ORS 1954
ORS 1936
ORS1939
ORS 2039
ORS 2059
ORS 2070
ORS 2064
ORS 2057
ORS 2065
ORS 2068
ORS 2058
ORS 2063
ORS 2102
ORS 2047
ORS 2030
ORS 2065
ORS 2066
ORS 2062
ORS 2037
ORS 2055
ORS 2072
ORS 2100
ORS 2101
ORS 2044
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ORS 2024
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ORS 2056
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ORS 2043
ORS 2099
ORS 2092
ORS 2093
ORS 2095
ORS 2097
ORS 2094
ORS 2031
ORS 2033
ORS 2036
ORS 2061
ORS 2060
ORS 2071
ORS 2073
ORS 2067
ORS 1992
ORS 1860
ORS 1917
ORS 1933
ORS 1970
ORS 1925
ORS 1924
ORS 1927
ORS 1926
ORS 1928
ORS 1923
Bradyrhizobium sp. FM 15
Bradyrhizobium sp. ND 25
Bradyrhizobium sp. FM 15
Bradyrhizobium sp.
group 3
separeted
group 4
separeted
group 5

incubated with 10% (v/v) acetylene in a closed bottle of known volume. After 30 min of incubation, gas phase samples are analysed by gas chromatography to measure the concentration of accumulated ethylene according standard procedures (Hardy et al., 1973). The acetylene reduction activity (ARA) was expressed as nanomoles of ethylene produced per hour per plant. Classification of strains in effectiveness was obtained as: very highly effective (HE) >600 nmoles; highly effective (E): 350–600 nmoles; effective (e): 100–350 nmoles; and ineffective (I) <100 nmoles.

Whole cells protein extracts from the isolates were prepared and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using few modifications to the procedure of Laemmli (1970) as described by Kiredjian et al. (1986). Electrophoretic patterns were grouped by numerical analysis using Pearson correlation coefficient (Dupuy et al., 1994) and compared to reference strains belonging to genera *Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium, Mesorhizobium, Agrobacterium* and other *Bradyrhizobium* sp. defined by Moreira et al. (1993) and Dupuy et al. (1994).

3. Results and Discussion

A collection of 117 rhizobial strains was established (Table 1). These isolates include fast and slow-growing strains for which first colonies appeared at 48 h and more than 72 h, respectively. Fast-growing strains were isolated from *C. glaucoides, C. perrottetii* and *C. podocarpa,* and slow-growing strains from *C. comosa, C. goreensis, C. hyssopifolia, C. lathyroides, C. ochroleuca,* and *C. retusa.*

According to the host-range study, the isolates and their *Crotalaria* hosts can be divided into two groups of specificity (Table 2): Group I includes *C. glaucoides, C. perrottetii,* and *C. podocarpa* which were effectively nodulated with fast-growing strains. Group II includes *C. comosa, C. goreensis, C. hyssopifolia, C. lathyroides, C. ochroleuca,* and *C. retusa* which were effectively nodulated with slow-growing strains. *Acacia albida* and *Indigofera microcarpa* were belonged to this nodulation group.

Rhizobial strains of *Crotalaria* spp. were highly specific: fast-growing strains effectively nodulate only their origin plants (*Crotalaria* species of group I) while slow-growing strains were promiscuous and effectively nodulate not only *Crotalaria* species of group II but also others tropical legumes such as *Acacia albida* (woody legume) and *Indigofera microcarpa* (herbaceous legume). No effective nodulation was observed on *Sesbania rostrata* and *Acacia raddiana* which were specifically nodulated by fast-growing rhizobia belonging to genus *Azorhizobium, Mesorhizobium* and *Sinorhizobium.*

The taxonomic position of all isolates was studied by SDS-PAGE analysis.

Dendrogram (Fig. 1) shows 5 electrophoretic groups at a level of similarity of 90%. Groups 1 and 2 include only slow-growing rhizobia and the wild type strain of *B. japonicum* (LMG 6138). Groups 3–5 were only fast growing and not related to any known species. Fast and slow-growing strains was not related. Electrophoretic groups are similar to those obtained by host-range analysis. According to their taxonomical position, fast-growing strains constitued a new group of rhizobia. *Crotalaria* rhizobia show a great diversity by SDS-PAGE (5 groups) and no correlation was found between electrophoretic groups and geographical origin.

Our results show first two types of *Crotalaria* rhizobia according to growth. Host specificity in *Crotalaria* spp. has for the first time been shown and is different from earlier reports which indicate that these species was promiscuous (Date, 1976; Giller et Wilson, 1991). Distinctions among *Crotalaria* rhizobia were demonstrated by protein analysis, which revealed a separate group. All *Crotalaria* strains presented previously were related to *Bradyrhizobium japonicum* (Gao et al., 1994; Van Rossum et al., 1995).

This study (first in genus *Crotalaria*) revealed that *Crotalaria* strains were fast and slow-growing and these two groups were not related either by host range or by SDS-PAGE analysis. The slow-growing strains are species of *Bradyrhizobium* while the fast-growing are apparently not related to any known rhizobial species.

Other techniques like DNA-DNA hybridization and 16S rDNA sequencing will be further used to elucidate the taxonomical position of fast-growing strains.

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