Enzyme polymorphism of *Azorhizobium* strains and other stem- and root-nodulating bacteria isolated from *Sesbania rostrata*

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SUMMARY

Relationships between bacterial groups nodulating *Sesbania rostrata* were evaluated through examination of electrophoretic polymorphism of esterases and metabolic enzymes. The following conclusions were drawn: (i) the differentiation of two genomic species within *Azorhizobium* strains and a group of non-identified strains (probably *Rhizobium*) was strongly supported by enzyme electrophoresis; (ii) esterases were more electrophoretically polymorphic than metabolic enzymes, since 35 and 11 electrophoretic types, respectively, were detected within the 57 strains studied; (iii) strains isolated from stem or root nodules were genetically very similar and could not be differentiated; (iv) six *Azorhizobium* strains isolated from plants growing in saline soils could not be grouped separately from the other strains, which might be attributed to the adaptation of azorhizobia to epiphytic conditions; and (v) a comparative study of esterase patterns of azorhizobia showed that strains isolated in the Philippines probably originated in northern Senegal, but did not reveal a clear separation between strains originating from northern and central Senegal.

Key-words: Nitrogen, Enzyme polymorphism, *Azorhizobium;* Stem- and root-nodulating bacteria; Electrophoretic types, *Sesbania rostrata.*

INTRODUCTION

The tropical legume *Sesbania rostrata* generally behaves as a wild annual plant in periodically flooded soils. The study of bacteria able to produce N_2-fixing nodules, both on the stems and on the roots of *S. rostrata,* led Dreyfus *et al.* (1988) to propose a new genus *Azorhizobium,* quite distinct from the genera *Rhizobium* and *Bradyrhizobium,* and containing one species, *A. caulinodans* (type strain ORS 571). These bacteria were unusual in that they fixed atmospheric nitrogen both symbiotically and *ex planta* (Elmerich *et al.,* 1982). DNA-rRNA hybridization studies placed *Azorhizobium* closer to *Xanthobacter* and *Bradyrhizobium*

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than to *Rhizobium* and *Agrobacterium*. In addition to azorhizobia, two strains isolated from root nodules of *S. cannabina* and *S. grandiflora,* respectively, formed effective nodules on both roots and stems of *S. rostrata,* but did not fix N_2 in culture. The DNA-rRNA hybridization results showed that these strains belonged to the *Rhizobium-Agrobacterium* complex (Dreyfus *et al.,* 1988). More recently, Rinaudo *et al.* (1991) analysed the genetic diversity of 191 strains isolated from stem and root nodules of *S. rostrata* grown in different geographical areas in Senegal and in the Philippines, by examining DNA base composition, DNA-DNA hybridizations and the ability to fix nitrogen in symbiosis with *S. rostrata* and/or in the free-living state. The following conclusions were drawn: (i) most of the isolates (184 strains) belonged to the genus *Azorhizobium;* (ii) the seven remaining strains exhibited very low levels of DNA binding with *Azorhizobium* strains and probably belonged to the genus *Rhizobium,* since they had G+C values between 59 and 63 mol %, were fast-growing and did not fix N_2 under free-living conditions; (iii) the *Azorhizobium* strains could be divided into two groups: genomic species 1, constituting the major group (175 strains), corresponds to *A. caulinodans* since it contains type strain ORS 571, while genomic species 2 contains only nine strains.

The DNA-DNA hybridization method, which measures the relatedness of entire genomes, is relatively unaffected by small variations in DNA sequences (Schleifer and Stackebrandt, 1983). Other methods such as protein gel electrophoresis, DNA restriction fragment length polymorphism or multilocus enzyme electrophoresis, must be used in order to distinguish differences between closely related organisms, such as azorhizobia, where most strains constitute a narrow cluster which can be grouped into a single species, *A. caulinodans.*

The aim of this study was to evaluate the differences between and within the bacterial groups found by DNA-DNA hybridization within the *S. rostrata* nodulating strains and to examine the possible correlation between the genetic diversity of strains and environmental factors by comparing the electrophoretic profiles of esterases and metabolic enzymes. Multilocus enzyme electrophoresis is probably one of the best approaches in large-scale studies to estimate the genetic diversity and structure of related populations (Selander *et al.,* 1986). It has recently been used to study the genetic population structure of soil bacteria involved in symbiotic associations with plants, namely the *Rhizobiaceae* (Engvild and Nielsen, 1984; Young, 1985; Young *et al.,* 1987; Pinero *et al.,* 1988; Martinez-Romero *et al.,* 1991) and *Frankia* strains (Gardes *et al.,* 1987; Prin *et al.,* 1991). The present study is the first report on genetic diversity among bacterial symbionts of *S. rostrata,* assessed by multilocus enzyme electrophoresis.

MATERIALS AND METHODS

Bacterial strains

The 57 bacterial strains included in this investigation are listed in table I. They were isolated from stem and root nodules of *S. rostrata* plants located in various geographical areas in Senegal and The Philippines. All of the strains were selected from the collection of strains previously studied by Rinaudo *et al.* (1991). Fifty strains were found to belong to the genus *Azorhizobium:* 41 strains belong to genomic species 1, which corresponds to *A. caulinodans,*

αNA = α-naphthyl acetate.
αNB = α-naphthyl butyrate.
αNP = α-naphthyl propionate.
βNA = β-naphthyl acetate.
βNB = β-naphthyl butyrate.
βNP = β-naphthyl propionate.
DIA = diaphorase.
ET = electrophoretic type.
GDH = glutamate dehydrogenase.
HBD = hydroxybutyrate dehydrogenase.
IA = indoxyl acetate.
LDH = lactate dehydrogenase.
MDH = malate dehydrogenase.
MF = relative mobility value.
NI strains = non-identified strains.

Table I. Origin of the strains tested and DNA relatedness.

Serial no.	Strain Registry no.	Origin	Isolated from	% Reassociation with labelled DNA from strain ORS 571 (*)	% Reassociation with labelled DNA from strain SD 02 (*)
Azorhizobium strains:					
Genomic species 1 *(A. caulinodans)*					
1	ORS 571	North Senegal	stem nodule	100	47
2	SV 01	"	"	91	—
3	SV 12	"	"	101	—
4	SV 17	"	"	97	—
5	SV 27	"	"	98	—
6	SV 31	"	root nodule	87	—
7	SV 33	"	"	87	—
8	BD 05	"	"	92	—
9	RT 01	"	stem nodule	88	—
10	RT 06	"	"	91	—
11	RT 10	"	"	104	51
12	RT 13	"	"	87	—
13	RT 15	"	root nodule	84	—
14	FY 01	"	stem nodule	93	—
15	FY 10	"	"	81	—
16	FY 12	"	"	88	—
17	FY 18	"	"	90	—
18	FY 29	"	root nodule	89	48
19	SG 01	Central Senegal	stem nodule	97	—
20	SG 03	"	"	96	—
21	SG 10	"	"	94	—
22	SG 21	"	"	97	—
23	SG 23	"	"	83	—
24	SG 26	"	root nodule	85	—
25	DP 03	"	stem nodule	88	—
26	DP 04	"	"	92	—
27	DP 07	"	"	97	—
28	DP 13	"	"	92	—
29	DP 23	"	root nodule	81	—
30	KL 03	"	stem nodule	94	—
31	KL 05	"	"	80	—
32	KL 08	"	"	96	—
33	KL 14	"	"	90	—
34	KL 17	"	root nodule	92	59
35	SD 01	"	stem nodule	104	—
36	SK 02	"	"	99	—
37	IRG 10	The Philippines	"	92	—
38	IRG 13	"	"	100	—
39	IRG 22	"	"	93	—
40	IRG 23	"	root nodule	100	—
41	IRG 42	"	stem nodule	96	—
Genomic species 2					
42	SG 05	Central Senegal	stem nodule	44	79
43	SG 06	"	"	46	94

(continued on next page)

Serial no.	Strain Registry no.	Origin	Isolated from	% Reassociation with labelled DNA from strain ORS 571 (*)	strain SD 02 (*)
44	SG 09	"	"	51	76
45	SG 19	"	"	51	83
46	SG 22	"	"	48	89
47	SG 25	"	"	50	90
48	SG 28	"	root nodule	47	92
49	SD 02	"	stem nodule	53	100
50	SD 04	"	"	44	86
NI strains:					
51	RT 09	Northern Senegal	stem nodule	4	0
52	RT 11	"	"	2	−1
53	RT 12	"	"	0	2
54	RT 14	"	root nodule	4	0
55	DP 21	Central Senegal	"	0	6
56	KL 13	"	stem nodule	−2	4
57	TAL 674	NifTal Culture Collection	"	4	3

(*) From: Rinaudo *et al.* (1991): ORS 571, type strain of genomic species 1 *(A. caulinodans)*; SD 02, type strain of genomic species 2.

and 9 strains to genomic species 2. The seven other strains probably belong to the genus *Rhizobium* as previously indicated. They will be designated here as NI strains (non-identified strains).

Preparation of bacterial extracts

Isolates were grown for 24 h on an orbital shaker at 28°C in 50 ml of yeast extract-lactate medium (Rinaudo *et al.*, 1991). Cells were harvested by centrifugation at 8,000 *g* for 10 min at 4°C, then washed twice in Tris 0.075 M, glycine 0.06 M, pH 8.7 buffer. After resuspension in 1.5 ml of the same buffer, the bacteria were sonicated (ultrasonificator Vibra Cell Bioblock Scientific, Illkirch, France, catalog no. 72441, with microtip) for 3 min at a 15 % pulse, sonic power of 2, with ice cooling. The sonicated suspension was centrifuged at 20,000 *g* for 20 min at 4°C, and the supernatant stored at −80°C.

Electrophoresis

Horizontal slab gel electrophoresis was performed in composite polyacrylamide-agarose gels using a discontinuous Tris-glycine buffer pH 8.7, and a constant voltage (7 V/cm) until the bromophenol blue marker had run 13 cm (Uriel, 1966; Goullet and Picard, 1985a). The esterases were stained according to Uriel (1961) using α-naphthyl acetate (αNA), β-naphthyl acetate (βNA), α-naphthyl propionate (αNP), β-naphthyl propionate (βNP), α-naphthyl butyrate (αNB), β-naphthyl butyrate (βNB) and indoxyl acetate (IA) as substrates. The following five metabolic enzymes were assayed: glutamate dehydrogenase (GDH), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), hydroxybutyrate dehydrogenase (HBD) and diaphorase (DIA). GDH was stained by the method of Baptist *et al.* (1969), MDH and LDH according to Siciliano and Shaw (1976), HBD according to Selander *et al.* (1986) and DIA as indicated by Pasteur *et al.* (1987).

The electrophoretic relationships between the strains were established by multiple replicate runs in which each of the enzyme bands was compared side by side on the same gel. Extracts of independent cultures of several strains were compared under the same conditions. Each band was characterized by the relative mobility value (MF) which is the running distance of the band as a percentage of the distance of the dye front. The electrophoretic type (ET), which is the combination of electromorphs (mobility variants of each enzyme), was determined for each strain.

Numerical analysis

For both kinds of enzymes (esterases and metabolic enzymes), the genetic distance between each possible pair of strains was estimated using the Jaccard similarity coefficient (Sneath and Sokal, 1973). For each pair-wise calculation, this procedure involves taking the sum of effective matches and dividing it by the total possible number of matches. A matrix was constructed with the genetic distance values for all pair-wise combinations of strains, and cluster analysis was done by the unweighted pair-group method with averages (UPGMA) (Sneath and Sokal, 1973; from Li, University of Texas, Houston).

RESULTS

Characterization of enzyme bands

Twenty-four esterase types were identified among the 57 tested strains based on their spectrum of hydrolytic activity toward the seven tested substrates (table II). They were numbered E_1 to E_{24}. For example, E_{24} is defined as the esterase able to hydrolyse only βNA among the seven tested substrates. One enzyme, defined by its hydrolytic activity, can be present as one or several of a number of variants with regard to their electrophoretic mobility. Esterase types E_1, E_4, E_{14}, E_{19}, E_{22} and E_{23} gave from 1 to 3 variants (bands) depending on the strains used (table III). For one given strain, the combination of the detected esterase types with their electrophoretic mobility represent the ET.

MDH, HBD, LDH and DIA gave 1 to 3 bands by strain, whereas GDH gave only 1 band (table IV).

Enzyme polymorphism among the strains

Esterases

The patterns of esterase banding differed both in the number of bands, type of enzymes and their migration distance (fig. 1). The combination of the type of esterases and their electrophoretic variations enabled the identification of 26, 4 and 5 different ET in genomic species 1, genomic species 2 and NI strains, respectively (table III). Some ET were present more frequently than others (ET 3 and 6 of genomic species 1; ET 27 of genomic species 2). Twenty-six among the 35 ET described were represented by only one strain.

Metabolic enzymes

The electromorphs obtained with the five metabolic enzymes allowed for the identification of 3 ET in genomic species 1, and 4 ET in genomic species 2 and in the NI strains (table IV). Most of the strains of genomic species 1 (36 out of 41 strains) were characterized by ET 1. Six out of the 9 strains of genomic species 2 were characterized by ET 4.

Numerical analysis

Esterases

Genotypic diversity among the 26 ET of genomic species 1 of *Azorhizobium* strains was very high (fig. 2). ET 3, 8, 11, 13 and 9 formed a cluster at a level of about 0.57, which was joined by a second cluster of four ET (ET 19, 23, 12 and 10) at a level of 0.64. The remaining 17 ET of genomic species 1 fell into single lineages or clusters diverging at depths from about 0.78 to 0.84.

Genomic species 1 and 2 were joined at a level of 0.96.

Genotypic diversity among NI strains resulted in five ET with pair-wise genetic distances comprised between 0.75 and 0.86. These strains joined the *Azorhizobium* groups at levels of 0.98 and 0.99.

Metabolic enzymes

Genotypic diversity was much less pronounced with metabolic enzymes than with esterases, especially in genomic species 1 of *Azorhizobium*: ET 1 and 3 formed a cluster at a level of 0.09, which was joined by a single strain (ET 2) at a level of 0.33 (fig. 3).

Table II. Identification of the 24 types of esterase (E_1 to E_{24}) detected in the 57 strains tested: each type is characterized by its spectrum of hydrolytic activity toward 7 substrates.

Substrate(*)	E_1	E_2	E_3	E_4	E_5	E_6	E_7	E_8	E_9	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{15}	E_{16}	E_{17}	E_{18}	E_{19}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
αNA	+	−	+	+	−	+	+	+	+	−	−	+	+	−	−	−	+	+	−	−	−	−	−	−
αNP	+	+	+	+	+	+	+	+	−	+	+	+	−	+	−	−	−	−	+	+	−	−	−	−
αNB	+	+	+	−	+	+	+	−	−	+	−	−	−	+	+	−	−	−	+	−	+	−	+	−
IA	+	+	−	+	+	+	−	−	+	−	+	−	+	−	−	+	+	−	−	−	−	−	−	−
βNA	+	+	−	+	+	−	−	+	+	−	+	−	+	−	+	+	−	−	−	−	−	−	−	+
βNP	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	−	+	−	+	−	+	−	−
βNB	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	+	−	−

(*) αNA = α-naphthyl acetate; αNP = α-naphthyl propionate; αNB = α-naphthyl butyrate; IA = indoxyl acetate; βNA = β-naphthyl acetate; βNP = β-naphthyl propionate; βNB = β-naphthyl butyrate.

Table III. Electrophoretic mobilities (MF) of the esterase types detected among the strains tested.

Strain no.	ETno.	E_1	E_2	E_3	E_4	E_5	E_6	E_7	E_8	E_9	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{15}	E_{16}	E_{17}	E_{18}	E_{19}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Azorhizobium strains:																									
Genomic species 1 *(A. caulinodans)*																									
1	1	47	20	—	57/68	—	—	—	—	—	—	—	—	—	10	70	—	—	52	50/55	—	—	—	—	—
2,7	2	20/47	—	—	57/70	—	—	—	—	—	—	—	—	—	7	—	—	—	52	50/55	—	—	—	68	—
3,4,37,38, 39,40,41	3	47	20	—	57/68	—	—	52	—	—	—	—	—	—	10	—	—	—	—	—	—	—	—	55/70	—
5,6	4	20/47	—	—	57/68	—	—	—	—	—	—	—	—	—	10	—	—	—	52	50/55	—	—	—	70	—
8	5	47	20	—	68	—	—	—	—	—	—	—	—	57	—	—	—	—	52	50	—	—	—	—	—
9,14,19,20, 21,22	6	47/58	20	—	68	—	—	50	—	—	60	—	—	—	—	—	—	—	—	63	—	—	—	—	—

10	7		20	52	57/70	—	—	—	—	—	—	—	—	—	7	—	—	—	—	—	—	—	—	—	—	—
11	8	47	20	—	57/68	—	—	52	—	—	—	—	—	—	10	—	—	—	—	—	—	—	—	70	—
12	9	20	—	—	57/68	—	—	—	—	—	55	—	—	—	10	—	—	—	—	—	—	—	—	70	—
13	10	47	20	—	52/68	—	—	—	—	—	55	—	—	—	7	—	—	—	—	—	57	—	—	70	—
15	11	47	20	—	57/68	—	—	—	—	—	55	—	—	—	7/50	—	—	—	—	—	—	—	—	—	—
16,17	12	47	20	—	57/68	—	—	—	—	—	—	—	—	—	7	—	—	—	—	—	—	—	55	50	—
18	13	47	20	—	57/68	—	—	—	—	—	55	—	—	—	10	—	—	—	—	—	—	—	—	—	—
23	14	20/47/57	—	55	68	—	—	—	—	—	—	—	—	—	15	—	—	—	—	—	—	—	59/73	—	—
24	15	20/47	—	55	60/68	—	—	—	—	—	—	—	—	—	15	—	—	—	—	—	—	—	—	—	—
25,26	16	20/47	—	55	63/70	—	—	—	—	—	57	—	—	—	7	—	—	—	—	66	—	—	73	—	—
27	17	20/47	—	—	68	—	—	—	—	50	—	—	—	—	7	—	—	—	—	52	—	—	—	—	—
28	18	47	20	—	70	—	—	—	—	50	—	—	—	—	7	—	—	—	—	66/94	—	55	—	—	—
29	19	47	20	—	57/68	—	—	—	—	—	—	—	50	—	7	—	—	—	—	—	—	—	—	73	—
30	20	47/57	15	—	68	—	—	—	—	—	—	—	52	—	—	—	—	—	—	—	12	55	—	—	—
31	21	42	20	—	68	—	—	47	—	—	—	—	—	57	7	—	—	—	—	—	50	—	—	—	—
32	22	47	20	—	64/70	—	—	—	—	—	—	—	—	—	7/55/57	—	—	—	—	66	—	—	73	—	—
33	23	47	20	—	52/68	—	—	—	—	—	—	—	—	—	7	—	—	—	—	—	—	—	73	—	—
34	24	47	—	—	57/68	—	—	—	—	—	—	—	52	—	10	—	20	—	—	—	—	55	70	—	—
35	25	47/55	20	—	57/70	—	—	—	—	—	—	—	—	—	7	—	—	—	—	66	—	—	—	—	—
36	26	47	20	59	50/66	—	—	—	—	—	—	—	—	—	7	—	—	—	—	63	—	—	—	—	—
Genomic species 2																											
42,43,44, 45,46,47	27	40	20	—	50/66	—	—	—	76	—	—	15	—	—	—	—	—	—	—	47/52/60	—	—	—	—	—
48	28	40	25	—	50/66	15	—	—	76	—	—	—	—	—	—	—	—	—	—	47/63	—	—	—	—	—
49	29	40	15	—	52/66	—	—	—	—	—	—	—	—	—	—	—	—	—	—	57	—	—	—	47/50	19
50	30	47	15	—	52/66	—	—	—	76	—	40	—	—	—	—	—	—	—	—	57	—	—	—	50	19
NI strains:																											
51,52	31	—	—	42/54	—	—	—	—	—	—	—	47	—	—	—	—	—	—	—	—	—	—	—	—	—
53	32	30/50/58	—	44	—	—	4	—	—	—	—	—	—	—	—	—	—	—	—	54	—	—	—	—	—
54	33	58	—	35/44	—	83	—	—	—	—	—	—	—	—	—	—	—	28	—	—	—	—	—	—	—
55,56	34	55	—	42	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
57	35	58	—	40	—	—	—	—	—	—	—	—	—	—	61	—	—	—	—	—	—	—	—	—	—

Table IV. Electrophoretic mobilities (MF) of malate-, glutamate-, hydroxybutyrate- and lactate-dehydrogenases and diaphorase, among the 57 strains tested.

Strain no.	ET no.	MDH	GDH	HBD	LDH	DIA
Azorhizobium strains:						
Genomic species 1 *(A. caulinodans)*						
All of the strains except strains 8, 10, 12, 18, 23	1	40/42/45	39	38/43	38/40	35/47
8	2	42/45	39	43	40	35/47
10, 12, 18, 23	3	40/42/45	39	38/43	38/40/42	35/47
Genomic species 2						
42, 43, 44, 45, 46, 47	4	36/45	39	34/43	34/36	35/43
48	5	36/45	39	34/43	34/36	35/51
49	6	36/45	39	34/36/43	34/36	35/51
50	7	36/45	39	34/36/43	34/36/38	35/43
NI strains:						
51, 52, 55, 56	8	25	34	43	32	49
53	9	25/30	61	38	30	42
54	10	28	38	49/51	44/52	43
57	11	25	38	49	58/60/62	53

The four ET of genomic species 2 constituted a cluster at a level of 0.26, which diverged from genomic species 1 at a level of 0.72.

ET 8 and 9 and ET 10 and 11 detected in NI strains joined the *Azorhizobium* groups at levels of 0.94 and 0.98, respectively.

DISCUSSION

Electrophoretic enzyme polymorphism was used to evaluate relationships between the bacterial groups nodulating the tropical legume *Sesbania rostrata*. Cluster analysis of genetic distances confirmed our previous results based on DNA-DNA hybridization: (i) the differentiation of two genomic species within *Azorhizobium* strains, and (ii) the separation of *Azorhizobium* strains and NI strains. Such a discrimination was particularly obvious with metabolic enzymes (fig. 3). Furthermore, the present study showed evidence that esterases are more electrophoretically polymorphic than metabolic enzymes, since 35 and 11 ET were respectively detected with the former and the latter enzymes within the 57 strains studied: even very closely related strains could often be distinguished by the esterases system. Similar observations have previously been made for strains of *Aeromonas* (Picard and Goullet, 1985), *Enterobacter* (Goullet and Picard, 1986), *Providencia* (Goullet and Picard, 1985b) and *Yersinia* (Goullet and Picard, 1988).

The 41 strains of genomic species 1 were characterized by 26 ET with esterases and by three ET with metabolic enzymes. These strains are more than 81 % related to type strain ORS 571 by DNA-DNA hybridization (table I), illustrating the fact that enzyme polymorphism is more sensitive to small differences between strains than is the DNA-DNA hybridization method. Similar results had been obtained with

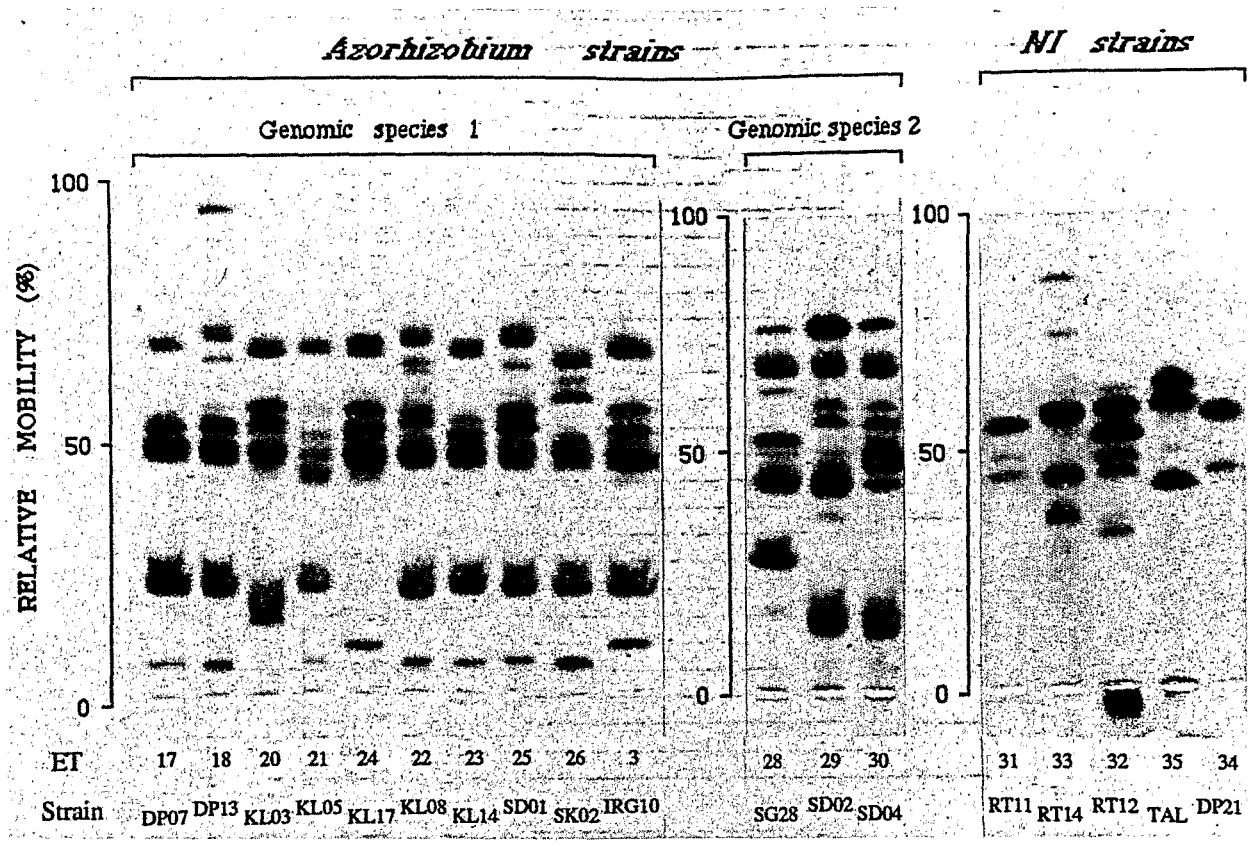

Fig. 1. Electrophoretic profiles (after horizontal acrylamide-agarose gel electrophoresis) of α-naphthyl propionate esterase, among strains chosen to represent different electrophoretic types.

other closely related strains such as *Neisseria* species (Chun *et al.*, 1985), or *Frankia* strains (Gardes *et al.*, 1987). The nine strains of genomic species 2 were characterized by four ET with both enzyme systems, six isolates having identical patterns. These four ET diverged at distances from 0.46 to 0.76 with esterases, but only from 0.10 to 0.26 in the metabolic enzymes. The latter enzymes were less electrophoretically polymorphic. The NI strains constituted the most genetically variable group, since they were characterized by ET diverging at distances higher than 0.75 and 0.83 with esterases and metabolic enzymes, respectively.

Our previous results based on the analysis of a large collection of strains isolated from *S. rostrata* showed that the type of nodule (stem or root nodules) was not correlated with the differentiation of *Azorhizobium* strains into two genomic species, since 90 and 92 % of strains that were isolated from stem and root nodules, respectively, belonged to genomic species 1. Moreover, all of the strains were able to nodulate both the stems and the roots of *S. rostrata*. The enzyme results obtained in the present study, confirm that the population of strains isolated from root and stem nodules are genetically very similar, and provide some extra information. Esterase analysis indicates that there is no ET grouping within the root-nodulating strains, and there are examples of root and stem isolates that share the same ET fingerprint (ET 2, 3 and 4).

Six strains tested in this study were isolated from plants growing in saline soils, namely

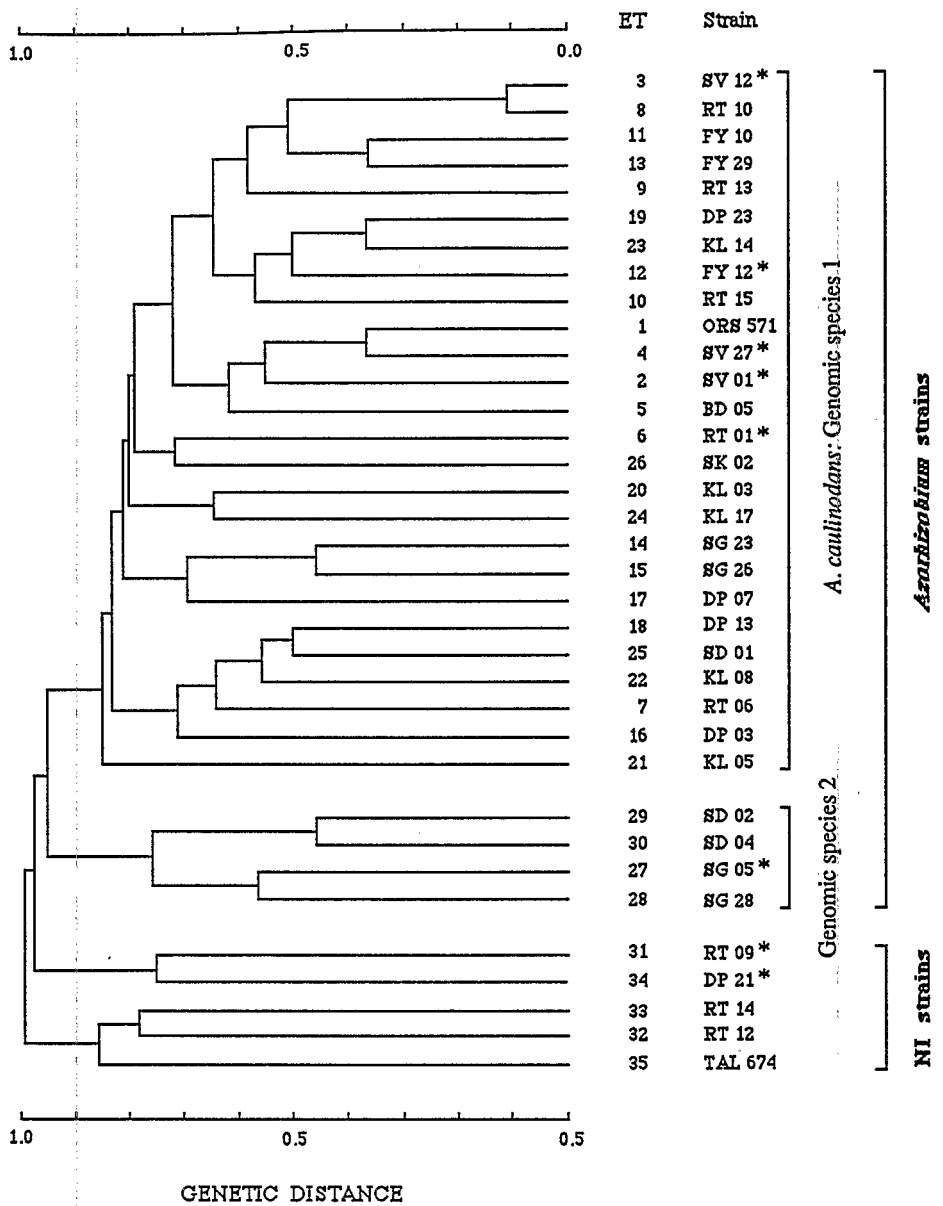

Fig. 2. Genetic relatedness among the 35 ET of the 57 tested strains, based on detectable electrophoretic allelic variations at 24 enzyme loci corresponding to esterases (data from table III).

The asterisks indicate that other strains with the same ET are included in table III. A representative strain is listed for each ET.

Boundoum (strain 8) and Diouroup (strains 25, 26, 27, 28 and 29), and appeared indistinguishable from the other strains: the corresponding ET (ET 4, 16, 17, 18 and 19) were widespread among the ET of the *Azorhizobium* strains belonging to genomic species 1. According to Adebayo *et al.* (1989), *Azorhizobium* strains are particularly well adapted to epiphytic growth and survival on leaves or flowers of *S. rostrata*. Such an adaptation may result in a large proliferation of stem-nodulating strains due to environmental factors such as wind, rain or insects, and

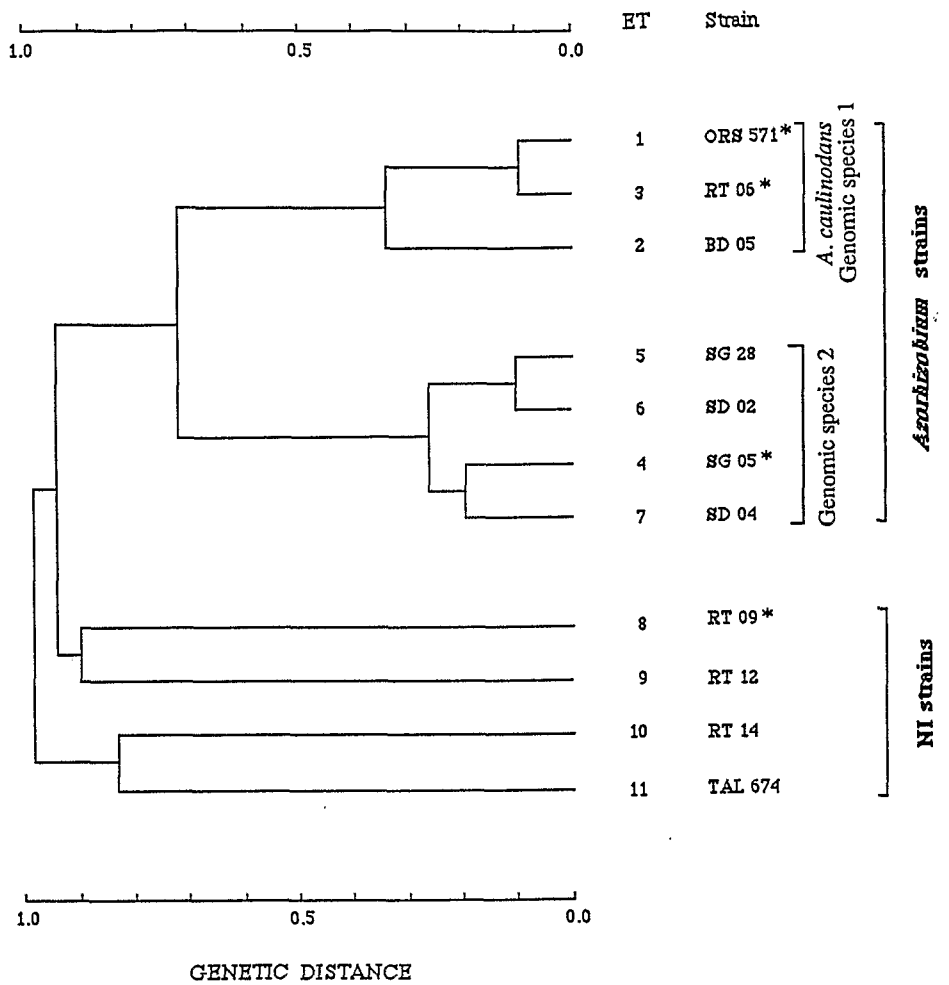

Fig. 3. Genetic relatedness among the 11 ET of the 57 tested strains, based on detectable electrophoretic alleic variations at 5 enzyme loci: MDH, GDH, HBD, LDH and DIA (data and abbreviations from table IV).

The asterisks indicate that other strains with the same ET are included in table IV. A representative strain is listed for each ET.

could possibly explain their presence in saline environment, since stem nodules are produced on an aerial portion of the plant where the effect of adverse soil conditions may be lower than at the root level. There is no data available on the tolerance of *Azorhizobium* to salinity. However, Arunin *et al.* (1988) observed that the growth of *S. rostrata* plants cultivated in saline rice soils in Thailand was enhanced both in flooded and upland conditions, when inoculated with *Azorhizobium* strain ORS 571.

We analysed a large number of strains originating from various geographical areas, which enabled us to examine the possible correlation between the genetic diversity of strains and their geographic origin. In our previous study based on DNA-DNA hybridizations (Rinaudo *et al.*, 1991), we noticed that azorhizobia of genomic species 2 were restricted to one geographic area (two stations of central Senegal, Sandiara and Senghor). In contrast, azorhizobia of genomic species 1 originated from all sta-

tions in Senegal and The Philippines. These strains appeared to be very closely related by the DNA-DNA hybridization method. The esterase system was much more sensitive and allowed us to discriminate between them. The five strains from The Philippines had exactly the same esterases pattern (ET 3) as strains SV 12 and SV 17 from northern Senegal (table II). Such a result supports the hypothesis of Ladha *et al.* (1988) suggesting that azorhizobia isolated from The Philippines might have been introduced as seed contaminants from Senegal. Some additional comments on the distribution of strains of genomic species 1 originating from northern and central Senegal can be made. Cluster analysis with the esterase system resulted in a dendogram in which ET were classified according to increasing genetic distances (fig. 2). One can observe that azorhizobia from northern Senegal belong to clusters or lineages situated at the top of the dendogram (ET 3, 8, 11, 13, 9, 12, 10, 1, 4, 2, 5 and 6), except for strain RT 06 (ET 7), whereas azorhizobia from central Senegal belong to the next ET (ET 26, 20, 24, 14, 15, 17, 18, 25, 22, 16, 21, 29, 30, 27 and 28), except for strains DP 23 (ET 19) and KL 14 (ET 23). However, there is no clear separation enabling us to conclude that strains fall into two genetically distinct groups reflecting geographic origin. Except for a few strains belonging to genomic species 2, azorhizobia constitute a homogeneous group.

Polymorphisme enzymatique de souches de *Azorhizobium* et d'autres bactéries isolées de nodules de tiges et de racines de *Sesbania rostrata*

Les relations existant entre les différents groupes bactériens capables de noduler *Sesbania rostrata* ont été évaluées par analyse du polymorphisme enzymatique d'estérases et d'enzymes métaboliques. Les conclusions de cette étude sont les suivantes: (1) la différenciation des souches de *Azorhizobium* en deux espèces génomiques et d'un groupe de bactéries non encore identifiées (appartenant probablement au genre *Rhizobium*) a été confirmée par les deux systèmes enzymatiques; (2) les estérases sont plus polymorphes que les enzymes métaboliques: 35 et 11 types électrophorétiques ont été respectivement identifiés parmi les 57 souches étudiées; (3) les souches isolées de nodules de tige ou de racines sont génétiquement très proches et n'ont pu être différenciées; (4) les six souches de *Azorhizobium* isolées de plantes se développant sur sols salés ne se sont pas distinguées des autres souches, ce qui peut être attribué au fait que les *Azorhizobium* sont adaptés aux conditions épiphytes; et (5) une étude comparative des profils d'estérases de souches de *Azorhizobium* a montré que les souches isolées aux Philippines sont probablement originaires du Nord Sénégal, mais n'a pas permis d'établir une séparation nette entre souches de *Azorhizobium* provenant du nord ou du centre du Sénégal.

Mots-clés: Azote, Polymorphisme enzymatique, *Azorhizobium;* Bactéries des nodules de tiges et de racines, Electrophorétypes, *Sesbania rostrata.*

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