Isolation and evaluation of strains of Rhizobium *collected from centres of diversity in South America*

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The selection of superior strains of *Rhizobium* is one of the key steps in improving the nitrogen fixation capacity of legume plants. A major source of variability which can be exploited in strain evaluation programmes is the genetic heterogeneity among wild strains of rhizobia which have evolved in the centres of diversity of the host plant. The collection of nodules, isolation of strains and their evaluation in symbiosis with groundnuts are described. Some of the variability in response to temperature and host genotypes is discussed to demonstrate the wealth of potential variability available among these strains.

INTRODUCTION

TROPICAL SOILS often lack available nitrogen. Exploitation of the legume-*Rhizobium* symbiosis can provide an important, highly economic source of available nitrogen for crops grown in tropical soils.

The selection of appropriate strains of *Rhizobium* is the first key step in a successful legume-*Rhizobium* symbiosis. Many strains of *Rhizobium* present in the soil may fix insufficient nitrogen for maximum crop yields. The presence of nodules on a legume does not necessarily mean that nitrogen is being fixed since rhizobia can induce nodule formation but then fail to fix nitrogen efficiency. If the bacteria do not fix enough nitrogen for high yields, then the most economic method of providing additional nitrogen for the crop is through the use of more effective strains of *Rhizobium*.

EXPLOITING THE POTENTIAL OF THE *RHIZOBIUM*

ROUGHLEY (1980) concluded that the genetic heterogeneity among wild strains of *Rhizobium* makes selection from such populations the most profitable first step in the improvement of nitrogen fixation. Strains of *R. trifolii* have been selected for tolerance to cold (GIBSON, 1977) and for greater competitive ability (GIBSON *et al.*, 1976) while cowpea rhizobia have been selected for tolerance to high temperatures (DAY *et al.*, 1978).

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Nodule collection and strain isolation

Isolation of rhizobia from native legumes is the most practical method of obtaining a heterogeneous population of strains that usually vary both in nitrogen-fixing potential and response to environmental factors.

Although our recent experience has been with groundnuts (*Arachis hypogaea* L.) which are infected by rhizobia of the cowpea cross-inoculation group, the principles and techniques can be adapted to any tropical legume. The genus *Arachis* is native to South America (GREGORY *et al.*, 1973). It ranges geographically from the equator near the mouth of the Amazon to 34 S near the Rio de la Plata in Uruguay. From the Atlantic coast the genus ranges westward to the Parana River and the eastern foothills of the Andes. It is found in broken forest to open grasslands of 'Pantanal' and 'Campo', in 'Caatinga', 'Parque', 'Chaco' and 'Cerrado'. The habitats of *Arachis* plants range from semi-arid regions to areas that receive 2000 mm or more of rain per year. Their distribution covers a great diversity of climatic and ecological conditions which are frequently extreme.

Nodules from plants of the genus *Arachis* were obtained by germplasm collecting expeditions of 1976 77, 1979 and 1980 sponsored by the International Board for Plant Genetic Resources and several South American countries and led by W. C. Gregory (N. C. State University, Raleigh) and C. E. Simpson (Texas A&M University, Stephenville). These collectors placed nodules in previously prepared 7·5 ml plastic vials containing anhydrous calcium chloride covered with a cotton plug. Strains were isolated from these nodules (*Table 1*) and are being evaluated for their nitrogen-fixing ability and response to environmental factors (SCHNEEWEIS *et al.*, 1978). The importance of collecting rhizobia was not recognized until late in the *Arachis* collecting programme, thus no rhizobia were obtained from large areas of South America where *Arachis* was collected. Although additional collections are being made, numerous areas with diversified climatic and ecological conditions have yet to be covered.

Strains isolated from these nodules were identified as *Rhizobium* through nodulation tests since this is the only certain method of identifying rhizobia. Several test plants other than the actual host can be used in determining the nodulating capacity of a particular strain (STOWERS and ELKAN, 1980). Siratro (*Macroptilium atropurpureum*) grown in 180 ml urine specimen bottles capped with plastic bags is used in our laboratory to identify rhizobia of the cowpea group.

Greenhouse evaluation

The effectiveness of a rhizobial strain must be measured in plant tests. Generally two or more diverse groundnut hosts are used in initial testing. Preliminary assessment of the nitrogen-fixing ability of strains of *Rhizobium* is conducted in modified Leonard jars (WACEK and BRILL, 1976) in the greenhous. The jars and a 1:1 sand:vermiculite medium are autoclaved before use to prevent contamination.

Groundnut seeds of each genotype are surface-sterilized by soaking in calcium hypochlorite solution (61 g /litre) for 10 min followed by rinsing with sterile water five times. The groundnuts are then pregerminated in sterilized vermiculite and placed at a depth of 25 mm in the medium in the jars. Before

Table 1. Origin of Rhizobium *isolates collected from South American root nodules*

Collection No.	*NCSU Strain No.*	*Host plant*	*Country of origin*	*Soil description*
1	NC 1.2 NC 1.3 NC 1.8 NC 1.10 NC 1.12 NC 1.15	*Arachis* species	Brazil	Light sandy soil
3	NC 3.1 NC 3.2	*A.* species	Brazil	Red black soil weathered from limestone
6	NC 6	*A.* species	Argentina	—
7	NC 7.1 NC 7.2	*A. diogoi* Hoehne	Brazil	Reduced grey argillaceous soil and calcareous gravel
22	NC 22.4	*A. helodes*	Brazil	Brown sand
23	NC 23.2 NC 23.3 NC 23.4	*A.* species	Brazil	Calcareous
43	NC 43.2 NC 43.3	— (No collection information) —		
56	NC 56 NC 56.1 NC 56.2	*A* species	Argentina	Dark alluvial gravel
59	NC 59.2	*A.* species	Argentina	Alluvial red silty sand
62	NC 62	*A.* species	Argentina	Deep light brown alluvial sand
70	NC 70 NC 70.1	*A.* species	Bolivia	Light brown alluvial clay-loam-gravel
71	NC 71	*A.* species	Bolivia	Light red alluvial sandy loam
77	NC 77.3	*A. hypogaea* cv. 'Colorado Chico del Palmar'	Bolivia	Light red or brown sandy loam-alluvium
83	NC 83 NC 83.1 NC 83.2	*A.* species	Bolivia	Light brown sandy loam
92	NC 92	*A. hypogaea* cv. 'Overo Colorado Blanco'	Bolivia	Dark alluvial loam
93	NC 93.1	*A.* species	Bolivia	Deep alluvial light brown tan or light sand
120	NC 120 NC 120.1 NC 120.2	*A.* species	Paraguay	Deep white sand
123	NC 123 NC 123.1	*A.* species	Paraguay	Deep light colored sand
134	NC 134 NC 134.1	*A.* species	Paraguay	Light sand
136	NC 136	*A.* species	Paraguay	Brown sandy soil
138	NC 138.1 NC 138.2 NC 138.3	*A. hypogaea* cv. 'Overo'	Bolivia	—

Collection No.	*NCSU Strain No.*	*Host plant*	*Country of origin*	*Soil description*
144	NC 144.1	*Phaseolus* species	Bolivia	Deep red clay
	NC 144.2			
146	NC 146.1	*A. hypogaea* cv. 'Overo'	Bolivia	
	NC 146.2			
150	NC 150.7	*A. hypogaea* cv. 'Palido'	Bolivia	Light brown sandy loam
151	NC 151.4	*A. hypogaea* cv. 'Sara Mani'	Bolivia	Medium heavy, dark brown
178	NC 178	*A.* species	Brazil	Red iron-gravel and boulders 'cerrado'
181	NC 181	*A.* species	Brazil	Brown sandy gravel loam
216	NC 216.5			

covering the seed, a 10 ml suspension of the proper rhizobial strain (about 10^9 cells/ml) is added aseptically to the seed for all treatments except for an uninoculated control where sterile culture medium alone is added to the seeds. A nitrogen control (10 ml of a 1 mg N/ml solution of NH_4NO_3 applied three times during the test) is also included. The seed and inoculum are then covered with sand. The jars are watered through the glass tube into the bottom storage jar. The distilled water moves up through a 6 mm thick nylon wick into the media in the upper jar. Treatments are generally replicated five times with plots arranged in a randomized block design in the greenhouse. Nutrient solution (150 ml) is added twice during the growing period. The nutrient solution consisted of Bond's stock salt mixture supplemented with zinc, molybdenum and cobalt micronutrients. After 50 days of growth, the plants are harvested. Plant colour is rated on a scale of 1 to 3 with 1 = yellow and 3 = green. Nitrogenase activity is measured for the root system of each plant using acetylene reduction methodology. Nodules are counted and removed so that nodule mass can be determined. The roots and plant tops are dried and weighed. In earlier tests, tops were ground for determination of nitrogen using the Kjeldahl technique; however, this is not always done since dry weight and nitrogen content were highly correlated.

Evaluation of South American strains

Forty two strains isolated from nodules collected in South America were tested for their ability to form an effective symbiosis with the cultivar 'NC 2' in the greenhouse using the procedures previously described. Several of the strains were as effective as the control inoculant 176A34 (*Table 2*) suggesting that this new *Rhizobium* germplasm may be useful in developing inoculants for improved groundnut cultivars.

Nine rhizobial strains included five South American isolates were tested in symbiosis with the host genotypes 'NC 4' and 'Argentine' as previously described.

The mean for the nitrogen control and the mean for the strains were superior to the mean for the uninoculated control (*Table 3*). Strains CB 756, NC 123, NC 6, 8B4 and 32H1 produced significantly larger ($P<0.01$) larger plants than the nitrogen control, indicating that these strains were efficient in nitrogen fixation.

Table 2. Results of greenhouse nodulation test of South American Rhizobium *isolates;* Arachis hypogaea *(cv. NC 2) was the test host*

Strain No.	*Plant colour**	*Nodule number†*	*$CN_2(C_2H_2)$ fixed µM C_2H_4/plant/h*
1.2	1·0	1·0	1·308
1.8	1·0	0·5	0·104
1.10	1·0	1·0	2·172
1.12	2·0	1·5	0·742
1.15	1·0	1·0	0·000
3.1	3·0	2·5	16·774
3.2	2·5	2·5	14·838
6.0	3·0	2·0	0·057
7.1	1·5	2·0	7·898
7.2	2·0	3·0	5·494
22.4	1·5	2·0	1·658
23.2	1·5	3·0	6·880
23.3	1·5	3·0	6·057
23.4	1·5	2·5	2·984
42.2	1·0	2·5	0·000
43.3	3·0	2·5	7·854
56.0	3·0	3·0	18·608
56.1	3·0	2·5	11·808
56.2	2·5	3·0	19·144
62.0	1·5	2·0	4·766
68.3	1·0	1·5	0·000
70.0	3·0	2·0	10·089
70.1	3·0	2·5	12·890
71.0	1·5	3·0	17·557
77.3	1·5	0·5	0·000
83	3·0	2·0	5·924
83.1	3·0	2·5	8·452
83.2	2·5	2·0	6.228
92.0	2·0	2·5	19·809
93.1	1·5	1·5	5·953
99.2	1·5	3·0	2·510
120.0	1·0	2·5	0·122
120.1	1·0	2·5	0·088
120.2	1·0	3·0	0·078
123.0	2·5	2·0	3·105
123.1	3·0	2·5	5·304
134.0	3·0	2·0	4·910
134.1	2·5	2·5	10·082
146.1	2·0	3·0	17·768
146.2	3·0	3·0	18·896
170.0	3·0	2·5	9·070
181	2·5	1·5	5·271
176A34‡	2·5	2·5	14·736
LSD ($P=0·05$)	0·9	2·0	6·976
($P=0·01$)	1·2	2·7	9·359

* Plant colour code: 1 = yellow, 2 = green, 3 = dark green.
† Nodule number code: 0 = 0, 1 = 1–10, 2 = 11–100, 3 = over 100.
‡ Strain 176A34 isolated from *Vigna unguiculata* from The Nitragin Co., Milwaukee, WI.

Table 3. Means for nitrogen-fixing traits for South American and other isolates of Rhizobium

Treatment	*Plant colour**	*Nodules plant*	*$N_2(C_2H_2)$ fixed*†	*Nodule mass (mg)*	*Specific activity*‡	*Dry wt of plant (g)*
CB 756§	3·0	88·5	[illegible]·87	109	18·33	3·45
10D§	1·0	190·7	0·37	90	6·37	2·39
NC 71	2·2	249·7	2·74	118	41·18	2·32
NC 123	2·9	97·7	1·29	86	11·91	3·41
NC 150.7	2·0	133·4	2·44	122	19·98	2·60
NC 151.4	2·1	224·2	3·89	187	22·07	2·62
NC 6	2·8	135·0	1·38	121	11·87	3.68
8B4§	2·8	181·0	2·19	111	21·59	3·63
32H1	2·8	103·3	1·92	126	18·54	3·79
Uninoculated control	1·0	2·3	0·00	2	0·00	1·62
Nitrogen control	2·9	2·4	0·14	4	3·76	2·99
LSD (P=0·05)	0·2	50·5	0·68	41	7·00	0·93
(P=0·01)	0·3	66·9	0·90	54	9·30	1·23

* Rated with 1 = yellow, 2 = green, 3 = dark green.
† μM C_2H_4/plant/h.
‡ $N_2(C_2H_2)$ fixed/nodule mass (g).
§ Strain CB 756 isolated from *Macrotyloma africanum* from CSIRO, Australia; strain 10D isolated from peanuts from ICRISAT, Hyderabad, India; strain 8B4 isolated from *Arachis glabrata* from the Nitragin Company.

Table 4. Means for nitrogen-fixing traits for Rhizobium *isolates from South America*

Treatment	*Plant colour**	*Nodules per plant*	*$N_2(C_2H_2)$ fixed*†	*Dry weight of plant (g)*
NC 3.1	2·5	137·1	4·76	2·79
NC 56.2	2·5	195·9	3·35	3·16
NC 70.1	2·5	122·3	3·09	3·75
NC 92	2·3	176·1	3·29	2·28
NC 146.1	2·2	172·4	4·45	2·96
NC 178	2·2	112·3	3·35	3·38
NC 134.1	2·4	100·8	2·54	2·51
RP 182-13‡	2·7	99·8	3·59	3·42
32H1 (Control strain)	2·6	89·6	3·11	3·36
Uninoculated control	2·2	18·4	0·25	1·73
Nitrogen control	2·4	36·2	0·46	3·56
LSD (P=0·05)	*NS*	42·5	1·19	1·01
(P=0·01)		56·4	1·58	1·31

* Rated with 1 = yellow, 2 = green, 3 = dark green.
† μM C_2H_4/plant/h.
‡ Strain RP 182-13 isolated from groundnuts from North American Plant Breeders, Princeton, IL.
NS denotes no significant differences.

Nine additional rhizobial strains (seven South American isolates plus two commercial strains) were tested in a second study using the same host genotypes. The nitrogen control mean plant dry weight and the mean of the strains were significantly higher than that of the uninoculated control (*Table 4*). No strain had a significantly higher mean plant dry weight than the

Table 5. Mean effect of strains of Rhizobium *on traits indicative of nitrogen fixation*

Strain	*Nodule No.*	*Nodule weight (mg)*	*$N_2(C_2H_2)$ fixed**	*Specific activity*†	*Plant dry weight (g)*
NC 7.2	158	244·8	2·261	9·154	7·1
NC 123	98	103·4	1·487	12·614	8·9
NC 181	93	94·4	1·683	15·498	9·6
NC 70.1	111	132·5	1·815	16·308	9·8
NC 56.2	132	201·6	2·380	12·095	7·0
32H1	84	114·1	1·626	13·672	8·2
LSD (*P* = 0·05)	59	98·4	1·094	*NS*	2·5
(*P* = 0·01)	78	130·6	1·453		3·3

* μM C_2H_4/plant/h.
† $N_2(C_2H_2)$ fixed/nodule weight (g).
NS denotes no significant differences.

Table 6. Temperature × strain means for $N_2(C_2H_2)$ fixed and plant weight

Trait	*Temperature*	*Strain*					
		NC 7.2	*NC 123*	*NC 181*	*NC 70.1*	*NC 56.2*	*32H1*
Plant weight (g)	30/26	9·0	8·8	11·0	9·6	6·5	8·8
	26/22	5·4	8·9	8·2	10·0	7·5	7·6
$N_2(C_2H_2)$ fixed	30/26	1·985	1·382	1·395	1·742	1·793	1·222
(μM C_2H_4/plant/h)	26/22	2·512	1·592	1·971	1·888	2·967	1·996

nitrogen control. However, some strains were superior in all respects except plant colour to the uninoculated control and several strains (NC 70.1, NC 178, NC 134.1 and RP 182–13) differed in no respect from the control strain 32HI.

Field evaluation

We are now in the process of testing some of these isolates in the field and we have developed a formal procedure for field evaluation of rhizobial strains (ELKAN *et al.*, 1980). In field studies conducted on soils supporting large populations of endemic rhizobia, single strain isolates significantly influenced nodulation, nitrogenase activity and plant dry weight (WYNNE *et al.*, 1981). Strains both less and more effective than the naturally occurring rhizobial population have been observed.

Response to temperature

Five rhizobial strains isolated from nodules collected at different elevations in South America were tested at 30/26 and 26/22°C day–night temperatures in symbiosis with the groundnut cultivar NC 4. The plants were grown in modified Leonard jars as described for greenhouse testing. Nodulation, $N_2(C_2H_2)$ fixed, specific activity and plant dry weight were all significantly affected by the use of different rhizobial strains. Strain NC 7.2 produced the greatest nodulation; however, it had the lowest specific activity of any strain tested (*Table 5*). Strains NC 181 and NC 70.1 gave the greatest specific activity (not significant) and produced significantly heavier plants than two other strains. There was a significant temperature × strain interaction for $N_2(C_2H_2)$ fixed and plant dry weight but not for nodulation (*Table 6*). Plants inoculated

Table 7. Mean squares from analysis of variance of nitrogen-fixing traits for strain evaluation study

Source	df	Plant colour	Nodule No.	Nitrogenase activity (C_2H_4 h plant)	Nodule mass	Dry wt plant
Total	108					
Block	4	0·029	7631·0*	38·728**	0·0059	1·7069
Host	1	0·390*	104867·3**	38·728**	0·1590**	96·6952**
Treatment	10	5·407**	65439·2**	14·067**	0·0290**	4·8064**
N sources	2	10·553**	192516·7**	30·795**	0·1110**	9·7841**
Uninoculated control v all others	1	17·183**	212158·2**	36·294**	0·1240**	19·4785**
N salts v *Rhizobium*	1	2·404**	211882·1**	31·514**	0·1200**	0·0932
Among strains	8	4·134**	33179·7**	9·808**	0·0083**	3·5719**
Host by treatment	10	0·064	11461·6**	3·164**	0·0071**	1·2454
Host by N sources	2	0·0261	12034·1*	3·522**	0·0173**	0·4648
Host by (uninoculated control v others)	1	0·0342	14860·4*	4·672**	0·0212**	0·0075
Host by (N salts v *Rhizobium*)	1	0·1312	11623·8*	3·066*	0·0170**	0·9218
Host by strain	8	0·0735	11315·8**	3·060*	0·0044*	1·4399
Error	83	0·0575	2571·7	0·466	0·0017	0·8700

Table 8. Means of hosts by strains for nitrogen-fixing traits of strain evaluation test

Host (cv.)	Treatment	Plant colour	Nodule number	Nitrogenase activity	Nodule mass	Dry weight of plant
Argentine	CB 756	2·9	55·2	0·91	0·052	1·95
	10D	1·0	104·0	0·00	0·047	2·07
	NC 71	2·1	155·4	1·24	0·046	1·23
	NC 123	2·9	60·3	0·96	0·066	2·27
	NC 150.7	2·0	145·2	2·12	0·102	2·26
	NC 151.4	2·1	194·4	2·14	0·105	1·72
	NC 6	2·7	99·4	1·09	0·087	2·79
	8B4	2·6	173·0	1·77	0·072	2·63
	32H1	2·6	71·4	1·36	0·074	2·59
	Uninoculated control	1·0	4·6	0·00	0·003	0·70
	Nitrogen control	2·8	0·0	0·00	0·000	1·75
NC 4	CB 756	3·0	121·8	2·82	0·166	4·94
	10D	1·0	277·4	0·75	0·134	2·71
	NC 71	2·2	344·0	4·24	0·190	3·40
	NC 123	3·0	135·2	1·60	0·108	4·33
	NC 150.7	2·0	121·6	2·76	0·143	2·94
	NC 151.4	2·0	254·0	5·64	0·270	3·51
	NC 6	2·8	170·6	1·66	0·156	4·57
	8B4	3·0	189·0	2·60	0·151	4·63
	32H1	3·0	135·2	2·49	0·178	4·99
	Uninoculated control	1·0	0·0	0·00	0·000	2·53
	Nitrogen control	3·0	4·8	0·27	0·007	4·22
	LSD ($P=0·05$)	0·3	63·8	0·86	0·052	1·17
	($P=0·01$)	0·4	84·7	1·14	0·069	1·56

with strain NC 7.2 were almost twice as heavy at 30/26°C than at 26/22°C while plant dry weight produced by the remaining strains was almost equal at both temperatures. These data suggest that there is considerable genetic heterogeneity among these strains of *Rhizobium*. The variability existing in this strain collection should make it a valuable reservoir for future selection studies.

EFFECT OF HOST ON STRAIN PERFORMANCE

We have found that the nitrogen fixing ability of a strain of *Rhizobium* is significantly influenced by the host groundnut cultivar (WYNNE *et al.*, 1980).

Nine rhizobial strains were recently tested in symbiosis with two diverse groundnut genotypes, NC 4 and 'Argentine'. NC 4 is a Virginia type (subsp. *hypogaea* var. *hypogaea*) and 'Argentine' is a Spanish type (subsp. *fastigiata* var. *vulgaris*). The strains produced a significant effect on all parameters (*Table 7*). The host × strain interaction was significant for nodulation and $N_2(C_2H_2)$ fixed. Most strains produced significantly more nodules in symbiosis with NC 4 (*Table 8*). Differences in magnitude of response of several strains for $N_2(C_2H_2)$ fixed also depended upon the host genotype resulting in a significant interaction. These and other data (WYNNE *et al.*, 1980, 1981) suggest that the host cultivar must be considered in strain evaluation.

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