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Increasing Nitrogen Fixation of the Groundnut by Strain and Host Selection

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As the cost of nitrogen fertilizer derived from fossil fuels continues to rise, biological nitrogen fixation will become more important for the continued productivity of agricultural crops. In the immediate future successful increases in biological nitrogen fixation most likely will come from improvements in the symbiotic fixation by legumes.

Groundnuts (*Arachis hypogaea* L.), since they are grown on about 18 million hectares in 82 countries, should contribute to the increase in dinitrogen fixed. Increases in dinitrogen fixed by groundnuts can be accomplished by the selection of more effective strains of *Rhizobium* and/or selection of more efficient host plants.

Selection of Effective Strains of *Rhizobium*

Groundnuts are a member of the cowpea cross-inoculation group. Rhizobia isolated from a diverse group of legumes are capable of nodulating groundnuts (Burrill and Hansen 1917; Walker 1928; Carroll 1934; Raju 1936; Mostafa and Mohmoud 1951; Berenyi 1962; Rajagopalan and Sadasivan 1964; Doku 1969; Gaur et al. 1974a). Not all rhizobial strains are equally effective in fixing nitrogen in symbiosis with groundnuts (Allen and Allen 1940; Collins 1943; Erdman 1943; Berenyi 1962; Denarie 1968; Vidyasekaran et al. 1973; Dadarwal et al. 1974; Weaver 1974). Therefore, effective rhizobial strains should be identified and used to inoculate groundnuts to ensure an effective symbiosis (Erdman 1943; Schiffmann 1961; Schiffmann and Lobel 1970; Gaur et al. 1974a; Lopes et al. 1974; Weaver 1974; Burton 1976). However, to be useful, effective strains must survive in and colonize the soil into which they are introduced. Unfortunately many effective strains have been unable to survive and colonize under field conditions (Schiffmann 1961; Shimshi et al. 1967; Schiffmann and Alper 1968b; Pant and Iswaran 1970; Gaur et al. 1974b; Iswaran and Sen 1974; Kumara Rao et al. 1974). Introduced strains which survive must also be able to compete for infection sites on the root with less effective native strains. Frequently introduced strains are not very competitive (Berenyi 1962; Denarie 1968).

Significant responses to inoculation with effective strains have largely been restricted to controlled conditions (Chomchalow 1971) and field experiments on virgin groundnut soils (Duggar 1935b; Collins 1943; Schiffmann 1961; Berenyi 1962; Shimshi et al. 1967; Schiffmann and Alper 1968a,b; Denarie 1968; Burton 1976). When inoculation with effective strains has been successful, the result has been increased yields of fruit, plant dry matter, nodulation, percentages of large fruits and seeds, and nitrogen content of foliage and seed.

The collection, identification, and use of superior strains of rhizobia should be an integral part of a groundnut research program. Inoculants produced from superior strains specifically tailored to the local environment should be used where nodulation is not adequate for good growth and high yields. These inoculants should also be used where nodulation appears adequate but a growth response to nitrogen fertilizer is observed since this suggests that the strains producing the nodules may not be efficient.

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Nodule Collection, Strain Isolation, and Maintenance

Strains of *Rhizobium* for groundnuts are maintained at several international and national centers such as ICRISAT, USDA, Beltsville, Maryland, USA, and NifTAL, University of Hawaii, USA. Because of the limited research on the groundnut-*Rhizobium* symbiosis, only a few of the available strains have been tested with groundnuts. For example, the USDA *Rhizobium* culture collection catalog (1979) lists 16 strains for groundnuts of which only four are recommended for symbiotic effectiveness. The NifTAL catalog (1978) recommends five strains and lists 11 additional strains that are effective with groundnuts.

Because of the limited number of recommended strains of *Rhizobium* and the lack of knowledge about their performance in symbiosis with the groundnut germplasm growth in North Carolina, we concluded that one of the principal goals of our program should be to isolate and identify effective rhizobial strains.

Initially we obtained strains of rhizobia from other researchers. Additional strains of *Rhizobium* were isolated from nodules collected from centers of diversity for the genus *Arachis* in South America. The nodules were obtained from *Arachis* collecting expeditions sponsored by IBPGR and led by W. C. Gregory (North Carolina State University, Raleigh) and C. E. Simpson (Texas A & M University, Stephenville). After sampling, the nodules were placed in 7.5 ml plastic vials containing anhydrous calcium chloride, covered with a cotton plug, and mailed to our laboratory in North Carolina. After the nodules were received from South America, they were rehydrated in sterile water for 4 hours at 5 C. They were then aseptically dissected, and a nichrome wire was used to streak some of the tissue on yeast extract mannitol agar in previously poured petri plates. Cultures were incubated at 28 C and examined daily for raised mucoid colonies typical of rhizobia. These colonies were restreaked until pure cultures were obtained. Using these techniques, 234 bacterial isolates representing 78 germplasm collections were obtained from nodules collected in 1976–77 (Tables 1, 2). Additional strains are now being isolated from nodules collected in 1979 and the spring of 1980.

The *Rhizobium* culture collection is maintained in screw-capped tubes containing yeast mannitol agar and stored at 5 C. These cultures are maintained in duplicate with one set being the working collection. In addition to agar slants, the mother collection is also preserved on porcelain beads with silica gel and in lyophilized form. These latter two techniques limit loss of culture viability with minimal risk of mutation or contamination. Transfers of the *Rhizobium* collection are provided upon request to interested investigators. Additional cowpea strains from the diverse environments where groundnuts are grown need to be collected.

Evaluation of Effectiveness of Strains

We use siratro (*Macroptilium atropurpureum*) for preliminary screening of isolates. This small-seeded legume is grown in the growth chamber in 30 ml serum bottles capped with plastic bags. The roots are examined for nodulation after 21 days. Strains capable of nodulating siratro are increased for testing with groundnuts. This further evaluation of these rhizobia is usually completed in stages. We first test rhizobia in the greenhouse with two host genotypes of different origin. Those strains that are effective in greenhouse studies must then be tested in the field in competition with indigenous rhizobia.

Greenhouse Evaluation

Argentine (Spanish type) and NC-4 (Virginia type), two cultivars representing the two subspecies of groundnuts, are used as host plants (Wynne et al. 1980). Plants are grown in modified Leonard jars similar to those proposed by Wacek and Alm (1978). The jars and a 2:1 sand:vermiculite medium are autoclaved before use to prevent contamination.

Seeds of each genotype are surface-sterilized by soaking in calcium hypochlorite solution (10 g in 150 ml water) for 10 min followed by rinsing with sterile water five times. The groundnuts are then pregerminated in sterilized vermiculite and placed 25 mm into the medium in the jars. Before covering the seed, a 1 ml suspension of the proper rhizobial strain (approximately 10^9 cells/ml) is added aseptically to the seed for all treatments except for an uninoculated control where sterile culture medium alone was added.

Table 1. Origin of *Rhizobium* isolates collected from *Arachis* species growing in South America.

Coll No	Isolated from	Lat	Long	Elevation (m)	Soil description	Date collected
1	*Arachis* sp	19 02'S	56 39'W	80–100	Light sandy soil	7 Dec 1976
3	*Arachis* sp	20 22'	56 58	100–200	Red, black soil weathered from limestone	11 Dec 1976
6	*Arachis* sp	25km W of Mburucuya				26 Dec 1976
7	*A diogoi* Hoehne	17 40'	57 45'	0–100	Reduced argillaceous soil and calcareous gravel	6 Dec 1976
22	*A helodes*	16 03'	57 13'	170	Brown sand	17 Dec 1976
23	*Arachis* sp				Calcareous origin	17 Dec 1976
56	*Arachis* sp	24 04'	65 24'	1565	Dark alluvial gravel	30 Mar 1977
59	*Arachis* sp	23 04'	63 53'	350–400	Alluvial reddish sand	2 Apr 1977
62	*Arachis* sp	22 51'	63 56'	350	Sandbank of alluvium	4 Apr 1977
70	*Arachis* sp	21 41'	63 45'	1000	Light brown, alluvial clay-loam-gravel	8 Apr 1977
71	*Arachis* sp	21 41'	63 44'	870–1000	Light red alluvial sandy loam	8 Apr 1977
83	*Arachis* sp	20 17'	63 28'	900	Light brown sandy loam	14 Apr 1977
93	*Arachis* sp	17 19'	63 18'	350	Deep sand in "matorral"	20 Apr 1977
99	*Arachis* sp	15 44'	63 05'	250	Brown to gray alluvial soil	27 Apr 1977
120	*Arachis* sp	26 22'	57 05'	ca 65	Deep white sand banks	16 June 1977
123	*Arachis* sp	25 23'	57 16'	ca 175	Light colored sand	17 June 1977
134	*Arachis* sp	22 15'	57 28'	ca 210	Light sand	24 June 1977
136	*Arachis* sp	22 23'	56 27'	ca 220	Brown sandy soil	24 June 1977
178	*Arachis* sp	21 34'	57 15'	ca 225	Red iron-gravel and granite boulders-"cerrado"	29 June 1977
181	*Arachis* sp	21 30'	57 01'	ca 350	Brown sandy gravel loam	29 June 1977

Table 2. Origin of *Rhizobium* isolates collected from South American groundnuts.

Coll. No.	Isolated from	Area collected	Soil description	Date collected
77	Colorado Chico del Palmar (red seeds)	Cototo, 10 km E of Villa Montes		11 Apr 1977
92	Overo Colorado Blanco (grande)	Saavedra, Sta. Cruz	Dark black loam	19 Apr 1977
138	Overo	Puento de Mataral, Sta. Cruz		25 Apr 1977
146	Overo	Valle Abajo, Mairana		28 Apr 1977
150	Palido	Teneria — Aiquile, dept. Cochabamba	Brown sandy loam	29 Apr 1977
151	Sara Mani	Mesa Rancho — Aiquile	Medium heavy, dark brown	29 Apr 1977

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 area. The distilled water moves through a 6 mm thick nylon wick up into the media in the upper jar. Treatments are 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 Pond's Stock salt mixture supplemented with zinc, molybdenum and cobalt micronutrients. After 50 days of growth, the plants are harvested. Plant color is rated on a scale of 1–3 with 1 = green and 3 = yellow. 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 root and plant tops are dried, weighed, and the tops are ground for determination of nitrogen using the Kjeldahl technique.

We have evaluated several strains of *Rhizobium* in the greenhouse for their ability to fix nitrogen. Rhizobial strains have been found to significantly influence plant color, plant dry weight, nodule number, nodule mass, percent nitrogen, total nitrogen, and nitrogenase activity (Table 3). Strains often perform differentially on the two host genotypes giving a significant host × strain interaction for traits indicative of their nitrogen fixing ability. This strain-host specificity suggests that rhizobial strains must be screened in symbiosis with diverse hosts or with the host genotype with which they are going to be used.

Total nitrogen accumulated is the best measure of the efficiency of a rhizobial strain. However, both plant color and plant dry weight are significantly correlated with total nitrogen suggesting that the measurement of these two traits is sufficient in evaluating strain efficiency in greenhouse studies (Table 4). These traits can be utilized by researchers with limited resources to screen rhizobia for effectiveness in symbiosis with local groundnut genotypes.

From the more than 100 unique isolates that we have evaluated in the greenhouse, we have identified several strains that fix more nitrogen than the commercial strains used as checks. Although additional testing is needed, these strains are now available to other rhizobiologists interested in cowpea rhizobia.

Field Evaluation of Rhizobia

After greenhouse evaluation, we test the nitrogen fixing ability of rhizobial strains in the field in the presence of endemic rhizobia (Elkan et al. 1980). In a preliminary study we evaluated strains previously tested in the greenhouse for their nitrogen fixing ability in the field in order to compare field and greenhouse test results. Nine strains of *Rhizobium* in symbiosis with 48 genotypes were tested in a field previously planted to groundnuts.

The rhizobial strains applied in a water suspension significantly influenced nodulation (0.05 level of probability) and nitrogen fixed as measured by nitrogenase activity (0.01 level). When averaged over the 48 host genotypes, the greatest nodulation was produced by strain 176A34 (Table 5). Strains 176A22 and 3G4b4 also produced significantly more nodules than

Table 3. Strain and control means for nitrogen-fixing traits for greenhouse trials.

Strain	Plant		Nodule		N content plant	Total N plant	Nitrogenase activity
	Color[a]	Weight (g)	No	Mass (g)	(%)	(mg)	(μmol C_2H_4 hr per plant)
				Study 1			
176A22	1.67ab[b]	3.12ab	58.9b	.120ab	3.69a	93.0a	6.68abc
3G4b4	2.11bcd	2.00de	25.0cd	.059c	2.82cd	42.9c	3.81d
42B2	2.30cde	1.80e	58.5b	.094bc	2.91cd	38.9c	5.08cd
3G4b21	2.11bcd	2.47cd	31.9cd	.062c	3.20bc	64.5b	5.64bcd
32H1	1.78bc	2.59bc	56.8b	.098abc	3.63ab	73.7b	5.30bcd
3G4b5	2.40de	2.01e	22.1d	.056c	2.76d	42.1c	3.70d
176A34	1.80bc	2.56cd	43.6bc	.085bc	3.25abc	67.8b	7.67ab
32Z3	2.67def	1.99e	32.9cd	.107abc	2.23e	34.2c	4.54d
3G4b20	1.33a	3.65a	80.1a	.140a	3.69a	107.3a	8.32a
Control	3.00ef	1.56e	0e	0d	1.59f	17.3d	0e
				Study 2			
32H1	1.11a	2.84a	37.3bc	.068c	3.69a	94.1a	4.49bc
29C2	1.80b	2.15bc	6[illegible].1a	.110a	2.74b	51.4c	7.60a
3I6N18	2.56c	1.42d	47.7ab	.045bcd	1.24d	15.0d	0.08d
3G4b9a	2.57c	1.35cd	0.7f	.001e	1.33d	14.0d	0d
3I6N9	2.56c	1.70bcd	53.6a	.072ab	1.21d	17.2d	0.09d
3G4b10	2.44c	1.98bcd	16.0de	.029de	1.73cd	31.5d	1.48d
RP182-13	1.10a	2.78a	52.3ab	.073ab	3.52a	88.4a	5.51ab
3I6N22	1.70b	2.24abc	28.7cd	.050cd	3.23a	62.6bc	4.14bc
3G4b19	1.80b	2.30ab	47.4ab	.062bc	3.61a	74.0ab	6.03ab
Control	2.20c	1.49cd	7.1e	.014de	1.97c	23.7d	2.74cd

a Rating 1 = green and 3 = yellow

b Means with different letters are significantly different (.05 α level) according to t test

Table 4. Correlation coefficients for nitrogen-fixing traits in two greenhouse studies.

Trait	Study	Trait: Plant weight	Nodule number	Nodule mass	Percent nitrogen	Total N_2	Nitrogenase activity
Plant color[a]	1	.93**	.84**	.75*	.95**	.95**	.89**
	2	.93**	.44	.54	.94**	.97**	.78**
Plant weight (g)	1	—	.79**	.54	.83**	.99**	.83**
	2	—	.45	.61	.90**	.97**	.73*
Nodule number	1	—	—	.89**	.83**	.82**	.75*
	2	—	—	.92**	.33	.39	.47
Nodule mass (g)	1	—	—	—	.89**	.67*	.68*
	2	—	—	—	.49	.54	.68*
% Nitrogen of plant	1	—	—	—	—	.81**	.57*
	2	—	—	—	—	.97**	.86**
Total N_2 of plant (g)	1	—	—	—	—	—	.86**
	2	—	—	—	—	—	.78**

a Rating 1 = green and 5 = yellow

* ** Indicates simple correlation coefficients significant at 0.05 and 0.01 levels of probability

Table 5. Mean nodulation rating and nitrogenase activity for strains of *Rhizobium* and an uninoculated control for field grown groundnuts.

Strain	Nodulation rating[a]	Nitrogenase activity[b]
3G4b20	2.81	39.1
176A34	3.10	38.7
176A22	3.07	35.1
3G4b5	3.06	35.8
3G4b4	3.07	31.0
3G4b21	2.98	32.8
42B2	3.06	33.8
32H1	2.93	35.7
32Z3	3.04	31.0
Control	2.89	32.5
LSD (0.05)	0.17	3.0

a Rated 156 days after planting with 1 = little and 5 = heavy nodulation

b μmol C_2H_4 plant per hr. Mean over 48 genotypes sampled 91, 95, 100, 119 and 127 days after planting

the inoculated controls which were inoculated with the naturally occurring strains. The greatest nitrogenase activity, however, occurred with strain 3G4b20. Strains 176A34, 3G4b5 and 32H1 also had significantly higher nitrogenase activity than the naturally occurring strains. Conversely, strains 3G4b4 and 32Z3 had slightly but insignificantly lower nitrogenase activity than the endemic strains.

The nitrogenase activity for the rhizobial strains when applied to Florigiant, a Virginia cultivar that is grown on most of the acreage in North Carolina, was determined for five sampling dates (Table 6). All plots of Florigiant including the uninoculated control were heavily nodulated. Six of the nine strains, however, had slightly higher mean nitrogenase activity than the naturally occurring strains (control), although only strain 3G4b21 was significantly better than the control. These data indicate that some strains are able to compete for infection sites and are more effective than naturally occurring strains. Strain 42B2 successfully competed for infection sites but produced less effective nodules than the naturally occurring strains. This strain was also ineffective in greenhouse evaluations.

Nitrogenase activity measured in the greenhouse for these same strains in symbiosis with a Spanish and Virginia cultivar was correlated (r = 0.73*) with mean nitrogenase activity

Table 6. Nitrogenase activity (μmol C_2H_4 hr per plant) for strains of *Rhizobium* and an uninoculated control for peanuts of cv Florigiant for five sampling dates.

Strain	Days after planting 91	95	100	119	127	Mean
3G4b20	49	70	64	69	28	56
176A34	45	59	61	49	20	45
176A22	52	60	68	65	30	55
3G4b5	49	52	74	61	32	54
3G4b4	58	50	73	48	30	52
3G4b21	55	71	69	74	33	60
42B2	44	47	45	49	18	41
32H1	38	50	71	66	39	53
32Z3	37	56	56	62	26	47
Control	48	51	54	64	26	49
Mean	47.4	55.6	63.6	60.6	28.3	51.1

LSD (0.05) Sampling date 8
LSD (0.05) Strain 12

in the field. This indicates that screening of rhizobial strains in the greenhouse is effective as a preliminary predictor of rhizobial strain performance in the field.

A similar field study conducted during 1979 involved 10 rhizobial strains and two groundnut cultivars, Florigiant and Spantex, (Spanish type). The strains significantly influenced nodulation, nitrogenase activity and plant weight but not yield of fruit (Table 7). A significant (0.05 level) genotype - strain interaction was found for plant weight, indicating that rhizobial strains were not equally effective for the two groundnut cultivars (Table 8). These results strongly suggest that the host genotype must be considered in rhizobial strain selection.

These field data are not very dramatic but they are very encouraging. Considering that soil fertility and endemic rhizobial populations are high enough to produce high yields, results under these test conditions can be translated into spectacular yield increases on nitrogen deficient soils with ineffective or low rhizobial populations.

Identification of Cowpea Miscellany Subgroups

The identification of effective strains of rhizobia through plant tests in the field and greenhouse is slow and tedious. It would be advantageous if it were possible to identify a subgroup of the cowpea rhizobia whose primary host cultivar is

Table 7. Strain and control means for yield and nitrogen-fixing traits for field-grown groundnuts.

Strain treatment	Nodule[a] Number	Dry wt (mg)	Nitrogenase activity (μmol C_2H_4 plant per hr)	Shoot weight (g)	Yield per plant (g)
Control (endemic strain)	396abc	475bcd	1.67abc	54.1abc	718a
Nitrogen control	276c	370d	1.17c	59.7abc	646a
32H1	427abc	442bcd	1.51abc	62.5ab	726a
CB 756	334bc	378cd	1.87a	56.3abc	562a
3G4b5	485abc	503a-d	1.70abc	53.9abc	562a
3G4b21	593a	727a	1.88a	70.2a	695a
NC 146 1	642a	625abc	1.44abc	54.0abc	541a
NC 92	584a	642ab	1.78ab	53.6abc	634a
NC 7 1	553ab	465bcd	1.28bc	51.9bc	654a
NC 3 1	458abc	488a-d	1.65abc	53.1bc	597a
NC 71	437abc	322d	1.62abc	44.5c	542a
NC 56	396abc	501a-d	1.94a	61.4ab	650a
176A22	545ab	538a-d	1.64abc	55.7abc	595a
NC 120	500abc	494a-d	1.67abc	67.6ab	686a

a Nodule number, weight, and shoot weight are means for plants sampled 59 and 166 days after planting. Nitrogenase activity, 59 days after planting, and fruit weight, 166 days after planting, are means for a single sampling date.
Means with different letters are significantly different at 5% level according to Duncan's multiple range test.

Table 8. Ranking of strains of *Rhizobium* as they affect plant weight for a Spanish and Virginia groundnut cultivar.

Rank	Florigiant	Spantex
1	32H1	NC 120
2	CB 756	3G4b21
3	NC 146 1	NC 56
4	NC 3 1	32H1
5	NC 7 1	176A22
6	3G4b21	CB756
7	3G4b5	NC 146 1
8	NC 120	3G4b5
9	NC 92	NC 3 1
10	NC 56	NC 71
11	176A22	NC 92
12	NC 71	NC 7 1

the groundnut or a specific cultivar-group of groundnuts.

We have adapted for use with *Rhizobium* sp two DNA-DNA hybridization techniques which allow us to rapidly determine the genetic relationship of isolates within this group of rhizobia. Isolates from individual legume cultivars nodulated with cowpea miscellany rhizobia are being compared using these hybridization techniques to determine if there is (or are) any subgroups of cowpea rhizobia favoring groundnuts. Conversely, if this host-isolate interaction is determined to be truly wide spectrum, we hope to determine if the more ineffective and effective isolates can be genetically grouped. This study is in the preliminary stages. The genetic diversity of this group of bacteria is apparent from the DNA-DNA hybridizations. Early evidence points to the possibility that bacteria more efficient in nitrogen fixation with groundnuts are identifiable. If the presence of subgroups (or subspecies) is confirmed, then it would be relatively easy to identify subgroups of the cowpea miscellany, thus reducing the need for plant tests. This work is continuing and is being expanded in our laboratory.

Selection of Efficient Host Plants

A second approach to increasing nitrogen fixed by groundnuts, which is applicable regardless of whether plants are well nodulated by native rhizobia or whether inoculation is required for adequate nodulation, is to develop host genotypes that are more efficient in fixing nitrogen. Although symbiotic nitrogen fixation has been studied for almost a century, little effort has been given to increasing nitrogen fixation in legumes through breeding. Enhancing the nitrogen fixing process in a leguminous crop through breeding requires (1) ample genetic variability, (2) an understanding of the genetic control of the process, (3) a technique for measuring the desired trait indicative of nitrogen fixation, and (4) a breeding strategy to efficiently utilize the variation.

Genotypic Variation in Nitrogen Fixing Traits

Differences in nodulation for groundnut genotypes in the field were first reported by Duggar (1935a). Duggar (1935c) found that a runner (ssp *hypogaea*) genotype developed larger and more nodules than a Spanish (ssp *fastigiata*) genotype. Inoculation with a single strain of *Rhizobium* increased nodulation of the Spanish line but not of the runner. Albrecht (1943) observed significant increases in fruit yield of a Spanish line when inoculated with three single strains of *Rhizobium* and a commercial inoculum, while a runner type did not respond to any of the treatments.

Burton (1976) found differences in nitrogen accumulation among peanut cultivars grown in the greenhouse with fixation being the only nitrogen source. Inoculated with single strains of *Rhizobium* isolated from plants of four genera, cv Florunner, a Virginia type, was consistently higher in nitrogen content than the Spanish cultivars, Comet, Starr and Spantex.

We have found host plant differences in nodulation and nitrogen fixing activity in both greenhouse and field studies (Wynne et al. 1978). Genotypic differences in nodulation by the native rhizobia found in North Carolina groundnut fields have also been observed. The nodulation for 48 genotypes shown in Table 9 is typical of the response observed in North Carolina. Virginia types such as cvs Florigiant, Va-72R, NC-5 and NC-6 are more heavily nodulated than Spanish or Valencia types such as cvs Spanhoma, Spantex, Starr, Argentine, Tennessee Red or New Mexico Valencia.

Table 9. Genotypic means for nodulation in North Carolina field study during 1977 at the Upper Coastal Plain Research Station.[a]

Genotypes	Nodulation X	Genotypes	Nodulation X
1 Starr	1.45	25 PI 221068	4.45
2 Tamnut 74	2.25	26 PI 241633	2.45
3 Georgia 255	2.60	27 PI 158850	3.00
4 G-169	2.45	28 PI 158852	3.25
5 EM 12	1.65	29 Spanhoma	2.25
6 Florunner	2.75	30 Spantex	2.40
7 Florigiant	4.90	31 Dixie Spanish	2.85
8 Va 72R	4.85	32 Starr	2.20
9 Early Bunch	4.30	33 Argentine	2.10
10 UF 714021	3.90	34 Schwarz 21	2.05
11 NC 6	3.75	35 Chico	2.05
12 Tifrun	3.65	36 PI 337396	3.10
13 A 69	4.10	37 PI 261954	2.85
14 UF 75102	3.00	38 PI 261955	3.00
15 Va 70-64	4.50	39 Tennessee Red	2.80
16 NC 5	4.35	40 N. M. Valencia	3.15
17 NC 2	3.45	41 PI 275743	2.20
18 NC-Fla 14	3.35	42 PI 275744	3.05
19 NC 4	3.90	43 PI 275078	2.50
20 PI 268837	3.00	44 Greg #182	1.30
21 PI 313946	3.50	45 Greg #190	1.15
22 PI 313947	3.80	46 71 SAN 290	2.10
23 PI 313950	4.45	47 71 SAN 291	1.90
24 *A. monticola*	2.80	48 NC 3033	3.25

[a] Nodulation rated 156 days after planting with 1 = little and 5 = high

Nodulation for the genotypes listed in Table 9 was measured at a single harvest date near maturity. We have found estimates of nitrogen fixing traits at a single harvest may not be reliable as an indicator of the relative performance of a genotype. For example, when eight cultivars were sampled four times during the growing season for their nitrogen fixing ability, both cultivars and harvest dates significantly influenced nodulation and nitrogenase activity (Table 10). The date × cultivar interaction was also significant for all three traits. The two genotypes from the ssp *fastigiata*, Tennessee Red and Spanhoma, had lower mean nodule number, nodule weight, and nitrogenase activity when averaged over all harvest dates. Florigiant, the predominant cultivar in North Carolina, had the highest mean nodule number, nodule weight, and nitrogenase activity when averaged over all sampling dates. However, the ssp *fastigiata* cultivars were not lowest at all sampling dates nor was Florigiant highest for all sampling dates. These and other unpublished data suggest that selection of a genotype based on a single evaluation of nitrogen fixing ability during the growing season may not always identify the superior genotype.

The seasonal pattern of nodulation and $N_2(C_2H_2)$ fixed was similar in this and other field studies. The pattern is better illustrated from bimonthly sampling dates for the cultivars Florigiant and Argentine (Figs. 1 and 2). Nodulation increased during the growing season until a peak was reached 84 days after planting for Argentine and 98 days after planting for Florigiant. Plants of Florigiant averaged 1470 nodules compared to 908 for Argentine at the time of maximum nodulation. Nitrogen fixation increased during the growing season until a peak was reached 84 days after

Table 10. Means for dates by cultivars for nitrogenase activity and nodulation (Lewiston, North Carolina, 1978).

Cultivar	Sampling date (days after emergence)			
	28	56	84	117
	Nitrogenase activity (μ moles C_2H_4 plant per hr)			
Tennessee Red	4.21	15.59	24.66	17.01
Spanhoma	3.35	13.79	21.69	13.44
Florunner	3.86	23.04	37.33	24.69
B1	1.77	6.04	28.87	27.62
Florigiant	5.51	18.83	35.12	34.57
NC 4	1.76	15.59	41.55	26.66
NC 6	3.93	20.13	33.99	34.18
Early Bunch	4.68	17.64	29.56	24.51
	Nodulation (nodules/plant)			
Tennessee Red	200.6	666.0	923.1	857.6
Spanhoma	162.1	492.4	613.0	494.0
Florunner	21[illegible]9	771.9	1380.3	1051.8
B1	151.5	460.9	1315.9	950.1
Florigiant	178.3	771.3	1440.3	1445.1
NC 4	202.9	600.7	1273.9	1659.0
NC 6	187.2	948.5	1265.8	1302.5
Early Bunch	254.0	909.5	1320.4	1266.7
	Nodule weight (g/plant)			
Tennessee Red	0.20	0.75	1.09	1.13
Spanhoma	0.26	0.60	0.90	0.80
Florunner	0.23	1.26	2.37	1.45
B1	0.27	0.57	1.55	1.44
Florigiant	0.21	1.11	2.90	2.77
NC 4	0.41	0.87	2.10	2.50
NC 6	0.46	1.28	2.34	1.78
Early Bunch	0.45	1.21	1.47	1.37

planting. Nitrogen fixed decreased after this date. Maximum $N_2(C_2H_2)$ values exceeded 70 μmol C_2H_2 plant per hr for Florigiant compared to less than 50 for Argentine. Unfortunately, total nitrogen analyses have not been completed and the relationship of nodulation and $N_2(C_2H_2)$ values to total nitrogen are not known. The rapid increase in nitrogen fixation during the growing season corresponds to the time of fruit formation and filling, and the decrease corresponds to maturation.

These data suggest that a single evaluation of the nitrogen fixing ability of a genotype using acetylene reduction if taken during the period of peak activity might be effective for preliminary screening of genotypes for nitrogen fixation. However, selection using total nitrogen accumulated or dry matter accumulated through

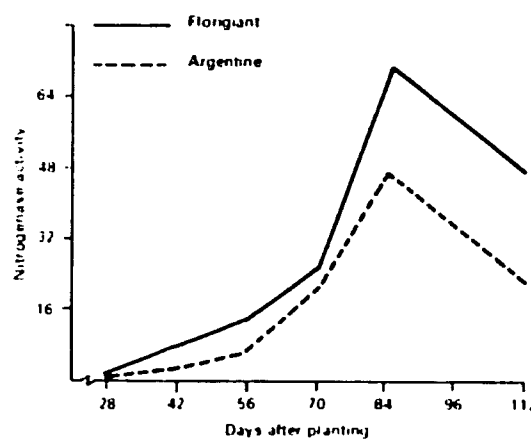

Figure 1. Means of cultivars for nitrogenase activity (μmol C_2H_4 plant per hr) over harvest dates (Clayton 1978).

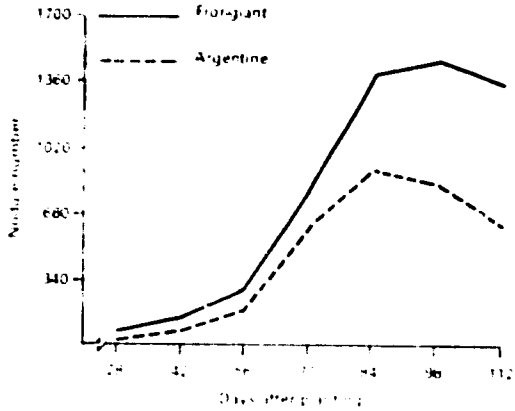

Figure 2. Means of cultivars for nodulation (nodule number plant) over harvest dates (Clayton 1978)

the growing season is almost certain to be more effective than $N_2(C_2H_2)$ measured once or even several times during the growing season. However, several $N_2(C_2H_2)$ measurements during the growing season might be useful in choosing parents. Two lines producing similar amounts of nitrogen but having different peak periods of activity as determined by acetylene reduction might produce transgressive segregates.

Quantitative Genetics of Nitrogen Fixation

We have investigated the genetic control of nitrogen fixation using a diallel cross in the greenhouse and a population of late generation lines in the field.

Early Generation: Greenhouse

The F_1 generation of a diallel cross of 10 cultivars from South America was evaluated in an analysis of gene action for traits related to nitrogen fixation. Hybrid progenies were significantly different for all traits (Table 11). General combining ability, which is usually indicative of additive gene action, was significant and greater than specific combining ability for nodulation, $N_2(C_2H_2)$ fixed, plant weight, nitrogen content and total nitrogen. Correlations between parental and general combining ability effects were nonsignificant for all traits, so simple evaluation of lines for nitrogen fixing capacity in the greenhouse may not identify superior parents for use in breeding programs.

Late Generation: Field

Thirty F_4 generation lines from a Virginia × Spanish cross were grown at two field sites in order to determine the genetics of traits indicating nitrogen fixation for a population of late generation lines. The Virginia parent (NC-6), a high yielding Virginia cultivar, is well nodulated by the indigenous rhizobial strains in North Carolina, while the Spanish parent (922) is poorly nodulated. Plot means for $N_2(C_2H_2)$ fixed, nodule number, nodule weight and dry weight of plant were analyzed considering locations and entries as random effects. Although the study when completed will consist of several sampling dates over years, parental and line means for one sampling date 90 days after planting suggest that sufficient variability exists for progress from selections in this population (Table 12). The Spanish line is lower in nodulation, has less nitrogenase activity and smaller plant weight than the Virginia parent. The range of means for the 30 lines generally equal or exceed the range of the parents. Genotypic variance was estimated from the variance among the means for the 30 late generation lines. These estimates were used to estimate heritability as follows:

$$h^2 \approx H = \frac{\hat{\sigma}_G^2}{\hat{\sigma}_G^2 + \frac{1}{sr\ell}\left[\sigma_E^2 + S_G^2 + Sr\sigma_{LG}^2\right]}$$

Where: $\hat{\sigma}_G^2$ = estimate of genotypic variance,

σ_E^2 = estimate of error variance, and

$S = 2$, $r = 3$ and $\ell = 2$.

Heritability estimates ranged from 0.45 for nodule number to 0.80 for $N_2(C_2H_2)$ fixed (Table 13). These estimates indicate that selection for traits indicative of nitrogen fixation should be effective for this population. Selections will be made and the response to selection for nitrogen fixing traits and the effect of selection for nitrogen fixation on productivity will be determined.

An Alternative Breeding Strategy

It has been suggested that the nitrogen fixation in the field may be limited to a large extent by the availability of photosynthate. Enrichment of the atmosphere in the foliar canopy of

Table 11. Mean squares from the diallel analysis of characters indicative of nitrogen-fixing ability.

Source	df	Nodule number	Nodule mass (mg)	Nitrogenase activity (μmol C_2H_4 plant per hr)	Plant weight (g)	Nitrogen content (%)	Total nitrogen (mg plant)
Blocks	3	4742**	64505**	73.85**	7.85**	0.5965**	5780**
Genotypes	98	1477**	10030**	6.47**	1.92**	0.1418**	2190**
Parents	9	726	8543	6.09	0.60	0.1508	534
Hybrids	88	1562**	10132**	6.58**	2.08**	0.1423**	2378**
General Combining Ability	9	4216**	26950**	17.10**	3.72**	0.4910**	3327**
Specific Combining Ability	35	1533**	263**	4.68	1.97**	0.0699	2252**
Maternal	9	1433**	16796**	3.74	2.32**	0.2197**	3674**
Reciprocal	35	941	5964	6.50*	1.69	0.1052	1954**
Parents vs hybrids	1	723	14423	0.08	0.27	0.0143	479
Error	280	742	4825	3.97	0.53	0.0845	619

Table 12. Parental means and range of means for single sample 90 days after planting of groundnut parents and F_4 generation population.

Identity	Means over locations			
	Nodule number	Nodule weight (g)	Nitrogenase activity (μmol C_2H_4 plant per hr)	Plant dry weight (g)
Spanish parent (922)	255	0.245	1.783	20.3
Virginia parent (NC 6)	474	0.518	4.028	29.9
F_4 lines	152–391	0.156–0.453	1.112–4.103	12.7–29.5

Table 13. Heritability estimates for nitrogen fixation traits for late generation lines from cross of Spanish and Virginia lines sampled 90 days after planting.

Trait	Estimate
Nodule number	0.45
Nodule mass (dry wt plant in g)	0.63
N_2 (C_2H_2) in μmol C_2H_4 plant per hr	0.80
Dry weight shoot (g)	0.74

groundnuts with 1500 ppm carbon dioxide during daylight hours increased nitrogen fixation, measured by the acetylene reduction assay, by 60% and also increased plant growth and nodulation supposedly because of larger amounts of available photosynthate (Hardy and Havelka 1976; Havelka and Hardy 1976). Diurnal studies and shading experiments at ICRISAT (Dart 1977) and leaf removal studies at North Carolina (unpublished) also suggest that the rate of nitrogen fixation is governed by the availability of photosynthate. Once the groundnut plant enters the reproductive stage the nodules, as a photosynthate sink, must compete not only with the growing vegetative parts, but also with the developing fruit. Hardy et al. (1971) reported that over 90% of the total amount of nitrogen fixed occurred during the period of fruit formation and maturation with rate of fixation entering an exponential phase at or about the time of pegging. Since variation in photosynthetic rates and net photosynthate accumulation has been demonstrated for groundnuts (McCloud et al. 1977, Pallas and Samish 1974; Pallas 1973; Williams et al. 1975; Emery et al. 1973), it should be possible to select photosynthetically superior genotypes which fix more nitrogen.

Preliminary analysis of several field studies conducted in North Carolina indicates that there is adequate variability in net photosynthetic efficiency (biological yield) to select genotypes that are more efficient in accumulation of photosynthate (Ball et al. 1979). Furthermore,

we have found biological yield (total dry matter), economic yield (fruit) and nitrogen fixed $N_2(C_2H_2)$ to be positively correlated (r .48**–.72** with 38 df). This suggests that groundnut breeders can indirectly select for greater nitrogen fixation by selecting for biological and or economic yield. Thus selection for economic yield may be an effective method of increasing nitrogen fixation when groundnuts are well nodulated by native rhizobia. This hypothesis is presently being tested in studies at North Carolina.

Summary

The atmospheric nitrogen fixed by groundnuts can be increased dramatically by the selection and use of effective strains of *Rhizobium* if the groundnut plants are poorly nodulated or nodulated with ineffective strains. Because of a significant genotype · strain interaction, the host genotype must be considered in strain selection. Strains can be selected after they have shown broad adaptation in symbiosis with a number of diverse host genotypes or they may be selected in symbiosis with the single host genotype to be grown.

Sufficient variability exists for selection of host genotypes with greater nodulation and greater nitrogen fixing potential. Preliminary estimates of heritability for late generation lines from a Virginia · Spanish cross suggest that selection should be effective for traits indicative of nitrogen fixation. However, since biological yield and economic yield appear to be correlated with nitrogen fixation, it may be possible to select for higher nitrogen fixation by selecting for biological yield and or economic yield.

References

Albrecht, H. R. 1943. Factors influencing the effect of inoculation of peanuts grown on new peanut lands. Proceedings of the Soil Science Society of America 8: 217–220.

Allen, O. N. and Allen, E. K. 1940. Response of the peanut plant to inoculation with rhizobia with special reference to morphological development of the nodules. Botanical Gazette 102: 121–142.

Ball, S. T., Wynne, J. C., and Isleib, T. G. 1979. Net photosynthetic efficiency and partitioning of photosynthate in peanut cultivars (Abs.). Proceedings of the American Peanut Research and Education Association 11: 43.

Berenyi, N. 1962. Some influences of nitrogen fertilization and seed inoculation with rhizobia on the growth and nitrogen fixation of peanuts. M. S. thesis, Dept. of Soil Science, North Carolina University, Raleigh, USA.

Burrill, T. J. and Hansen, R. 1917. Is symbiosis possible between legume bacteria and nonlegume plants? Illinois Agricultural Experiment Station Bulletin 202.

Burton, J. C. 1976. Pragmatic aspects of the *Rhizobium* leguminous plant association. Pages 429–446 *in* W. E. Newton and Nyman, C. J. (ed.), Proc. of the 1st International Symposium on Nitrogen Fixation. Volume 2. Washington State University Press, Pullman.

Carroll, W. R. 1934. A study of *Rhizobium* species in relation to nodule formation on the roots of Florida legumes. I. Soil Science 37: 117–134.

Chomchalow, S. 1971. The effectiveness of introduced *Rhizobium* strains on 'Rayong' peanut. Thailand Journal of Agricultural Science 4: 85–94.

Collins, W. O. 1943. Preliminary report on legume inoculation studies. Soil Science Society of American Proceedings 8: 221–222.

Dadarwal, K. R., Singh, C. S., and Subba Rao, N. S. 1974. Nodulation and serological studies of rhizobia from six species of *Arachis*. Plant Soil 40: 535–544.

Dart, Peter. 1977. Advisory group meeting on the potential use of isotopes in the study of biological dinitrogen fixation. The ICRISAT Microbiology Program, Hyderabad, India. November 21–25.

Denarie, J. 1968. Inoculation des legumineuses a Madagascar. Ann. Agron. 19: 473–496.

Dowd, E. V. 1969. Host specificity among five species in the cowpea crossinoculation group. Plant Soil 30: 126–128.

Duggar, J. F. 1935a. The nodulation and other adaptations of certain summer legumes. Journal of the American Society of Agronomy 27: 32–37.

Duggar, J. F. 1935b. The effect of inoculation and fertilization on the Spanish peanuts and root nodule numbers. Journal of the American Society of Agronomy 27: 128–133.

DUGGAR, J. F. 1935c. Nodulation of peanut plants as affected by variety, shelling of seed and disinfection of seed. Journal of the American Society of Agronomy 27: 286–288.

ELKAN, G. H., WYNNE, J. C., SCHNEEWEIS, T. J., and ISLEIB, T. G. 1980. Field inoculation of peanuts with single strain isolates of *Rhizobium*. Peanut Science (in review).

EMERY, D. A., WYNNE, J. C., and RICE, P. W. 1973. Can reproductive efficiency in cultivated peanuts be improved? Oleagineux 28: 399–403.

ERDMAN, L. W. 1943. New developments in legume inoculation. Soil Science Society of America Proceedings 8: 213–216.

GAUR, Y. D., SEN, A. N., SUBBA RAO, N. S. 1974a. Problem regarding groundnut (*Arachis hypogaea* L.) inoculation in tropics with special reference to India. Indian Academy of Science Proceedings 40(5): 562–570.

GAUR, Y. D., SEN, A. N., and SUBBA RAO, N. S. 1974b. Promiscuity in groundnut *Rhizobium* association. Zbl. Bakt. Abt. II: 129–369.

HARDY, R. W. F., and HAVELKA, U. D. 1976. Photosynthate as a major factor limiting nitrogen fixation by field-grown legumes with emphasis on soybean. Pages 421–439 *in* P. S. Nutman (ed.), Symbiotic Nitrogen Fixation in Plants IBP7. Cambridge University Press, Cambridge, Maryland, USA.

HARDY, R. W. F., BURNS, R. C., HEBERT, R. R., HOLSTEN, R. D., and JACKSON, E. K. 1971. Biological nitrogen fixation: Pages 561–590 *in* A key to world protein. Plant Soil. Sp. Vol.

HAVELKA, U. D., and HARDY, R. W. F. 1976. $N_2(C_2H_2)$ fixation, growth, and yield response of field-grown peanut (*Arachis hypogaea* L.) when grown under ambient and 1500 ppm CO_2 in the foliar canopy. Page 72 *in* Agronomy Abstracts.

HAWAII, UNIVERSITY OF. 1978. Catalogue of strains from the NifTAL *Rhizobium* collection (abbreviated). Paia, Maui, Hawaii.

ISWARAN, V., and SEN, A. 1974. Some studies on the groundnut *Rhizobium* symbiosis. Zentralbl. Bakteriol Parasit Infektionskr Hyg. Zweite Naturwiss Abstracts 129(5): 477–480.

KUMARA RAO, J. V. D., SEN, A., and SHEN[illegible] T. 1974. Inhibition of groundnut *Rhizobium* in Indian soils. Indian Academy of Science Proceedings 40(5): 535–539.

LOPES, E. S., LOVADINI, L. A. C., MIYASAKA, S., IGUE, T., and GIARDINI, A. R. 1974. Selection of strains of *Rhizobium* species for peanuts (*Arachis hypogaea* L.) and *Galactia striata* (Jacq.). Urban. Bragantia 33: CV-CX.

MCCLOUD, D. E., DUNCAN, W. G., and MCGRAW, R. L. 1977. Physiological aspects of peanut yield improvement. Page 90 *in* Agronomy Abstracts.

MOSTAFA, M. A., and MAHMOUD, Z. M. 1951. Bacterial isolates from root nodules of *Zygophyliaceae*. Nature (Lond.) 167: 446–447.

PALLAS, J. E., Jr. 1973. Diurnal changes in transpiration and daily photosynthetic rate of several crop plants. Crop Science 13: 82–84.

PALLAS, J. E., Jr. and SAMISH, Y. B. 1974. Photosynthetic response to peanut. Crop Science 17: 478–482.

PANT, S. D., and ISWARAN, V. 1970. Survival of groundnut *Rhizobium* in Indian soils. Mysore Journal of Agricultural Science 4: 19–25.

RAJAGOPALAN, N., and SADASIVAN, T. S. 1964. Some aspects of root nodulation in tropical legumes. Current Science 33: 197–202.

RAJU, M. S. 1936. Studies on the bacterial-plant groups of cowpea, *Cicer* and *Dhaincha*. I. Classification. Abl. Bakt. Abt. II 94: 249–262.

SCHIFFMANN, J. 1961. Field experiments on inoculation of peanuts in northern Negro soils. Israel Journal of Agricultural Science 11: 151–158.

SCHIFFMANN, J., and ALPER Y. 1968a. Effects of *Rhizobium*-inoculum placement on peanut inoculation. Experimental Agriculture 4: 203–208.

SCHIFFMANN, J., and ALPER Y. 1968b. Inoculation of peanuts by application of *Rhizobium* suspension into the planting furrows. Experimental Agriculture 4: 219–226.

SCHIFFMANN, J., and LOBEL R. 1970. Haemoglobin determination and its value as an early indication of peanut *Rhizobium* efficiency. Plant Soil 33: 501–512.

SHIMSHI, D., SCHIFFMANN, J., KOST, Y., BIELORAI, H., and ALPER, Y. 1967. Effect of moisture regime on nodulation of inoculated peanuts. Agronomy Journal 59: 397–400.

USDA, Cell Culture and Nitrogen Fixation Lab. 1979. USDA — Beltsville *Rhizobium* culture collection catalogue. Beltsville, Maryland, USA.

VIDHYASEKARAN, P., BALARAMAN, K., DEVEEGASUNDARUM, M., and RANGASWAMI, G. 1973. Relationship between virulence and auxin production by *Rhizobium* isolates from groundnut. Current Science 42:2) 66–67.

WACEK, T. J., and ALM, D. 1978. Easy-to-make Leonard jar. Crop Science 18: 514–515.

WALKER, R. H. 1928. Physiological studies on the nitrogen fixing bacteria of the genus *Rhizobium*. Iowa Agricultural Experiment Station Bulletin 113.

WEAVER, R. W. 1974. Effectiveness of rhizobia forming nodules on Texas grown peanuts. Peanut Science 1: 23–25.

WILLIAMS, J. H., WILSON, J. H., and BATE, G. C. 1975. The growth and development of four groundnut (*Arachis hypogaea* L.) cultivars in Rhodesia. Rhodesian Journal of Agricultural Research 13: 131–144.

WYNNE, J. C., ELKAN, G. H., MEISNER, C. M., SCHNEEWEIS, T. J., and LIGON, J. W. 1980. Greenhouse evaluation of strains of *Rhizobium* for peanuts. Agronomy Journal 72: 645–649.

WYNNE, J. C., ELKAN, G. H., SCHNEEWEIS, T. J., ISLEIB, T. G., PRESTON, C. M., and MEISNER, C. A. 1978. Increasing nitrogen fixation of the peanut. Proceedings of the American Peanut Research and Education Association 10: 22–29.