PULSE MICROBIOLOGY

Institute Seminar

PULSE MICROBIOLOGY AT ICRISAT
- Past, present and future

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7 July, 1981

PULSE IMPROVEMENT PROGRAM
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INDIA.

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The Pulse Microbiology programme has inevitably concentrated on the Rhizobium-legume symbiosis. This involves not only the ecology of the Rhizobium, and of the legume,but the formation, development and decline of the symbiotic system with each cycle of plant growth. Clearly other micro-organisms are involved in the Rhizobium ecology and are also relevant to the initiation and development of the symbiosis - the most notable being the mycorrhizal fungi. As far back as 1924, Jones referred to the "tripartite" association, legumes - rhizobia - mycorrhizae. Mycorrhizae are now attracting the sort of interest which Rhizobium attracted 30 years ago. While I look forward to collaboration in studies of the mycorrhizae with the pulses in the future, this presentation will be confined to the Rhizobium-legume symbiosis.

THE ROOT NODULE BACTERIUM

Classification of Rhizobium and host-Rhizobium specificity

It is important to attempt to place the Rhizobium-legume association in context. There are many, apparently beneficial, microorganism-plant associations recorded but the root nodule bacterium Rhizobium only nodulates members of the family Leguminosae - with one recorded exception (Parasponia, Ulmaceae). Our knowledge of the extent of nodulation within the Leguminosae however is not complete. Of upto 12,000 species, only about 1200 had been examined upto the mid 50's and of those about 9% apparently bore no nodules (Allen and Allen, 1961). The most recent testing has redressed this balance and the majority have been examined but 9% are still claimed not to form nodules (Corby, 1980) (Table 1).

Not only is the base of knowledge incomplete but the most popular classification developed 50 years ago is based on specific names derived from the major temperate hosts studied to that time (Fred, Baldwin and McCoy, 1932). This classification, which has still to be replaced, illustrates the limited importance attached to the tropical species and clearly shows the emphasis on temperates. There were six "species" whose main hosts are shown in Table 2.

It is evident even from a listing of the hosts so far studied that the "cowpea miscellany" houses the majority of the legumes. In fact the predominance of relatively "primitive", open pollinated, legumes in the group and the considerable degree of cross infection or "promiscuity" has been interpreted as indicating that the cowpea Rhizobium is the ancestral type of Rhizobium (Norris, 1956). While the classification has remained continually under criticism, and there are many published data showing cross infection between the groups, it remains the popularly used description of Rhizobium.

TABLE 1

Incidence of nodulation in Leguminosae (from Corby, 1980)

	Sub-family		
	Mimosoideae	Caesalpinoidae	Papilionaceae
		(% of species)	
Reported positive	95	42	100*
Reported conflictingly	0	11	0*
Reported negatively	1	30	0*
Not reported	4	17	0*
Total genera/sp.	59/2830	152/1900	4[illegible]/7000**

*"It seems safe to assume that most species will be found to nodulate", (Corby, 1980)

** By difference from totals quoted in Willis.J.C., (1955) "A dictionary of the flowering plants and ferns", Cambridge University Press.

TABLE 2

Classification of root nodule bacteria

Species	Group names	Host genera and species
1. *Rhizobium meliloti*	Medic	*Medicago, Melilotus, Trigonnella*
2. *Rh. trifolii*	Clover	*Trifolium*
3. *Rh. leguminosarum*	Pea	*Lathyrus, Lens, Pisum, Vicia*
4. *Rh. phaseoli*	Bean	*Phaseolus* (part only)
5. *Rh. lupini*	Lupin	*Lupinus, Ornithopus*
6. *Rh. japonicum*	Soybean	*Glycine*
7. *Rh.* Sp.	Cowpea	*Acacia, Albrus, Albizzia, Alysicarpus, Andira, Arachis, Baptisia, Cajanus, Canavalia, Clianthus, Crotalaria, Cytisus, Cyamopsis, Derris, Desmodium, Dolichos, Enterolobium, Erythrina, Genista, Hardenbergia, Hymenaea, Indigofera, Inga, Kennedya, Lespedeza, Lonchocarpus, Mucuna, Parkia, Phaseolus* (part) *Piscidia, Pithecellobium, Platylobium, Pongamia, Pterocarpus, Pueraria, Pultenaea, Stylosanthes, Stizolobium, Tephrosia, Ulex, Vigna, Voandzeia.*
	Strain-specific	Species of: *Amorpha, Amphicarpa, Caragana, Cicer, Coronilla, Dalea, Lotus, Onobrychis, Robinia, Sesbania, Strophostyles.*

The classification also coincides to some extent with a division in rate of growth of the cells - the cowpea group generally being slow growers (5-9 days) in culture and all the remainder except R. lupini being fast growers (2-5 days) However even this classification has been shown here at ICRISAT to be open to question as fast and slow growers have been found in both pigeonpea and chickpea.

The ICRISAT legumes fit into two groups of the classification. Pigeonpea and groundnut rhizobia belong to the cowpea group and will generally cross-infect. There has been insufficient study of our local strains to determine whether the two hosts may benefit from the same strains but some evidence of interaction has been found in relation to effectiveness and infectiveness.

There is however some local evidence that pigeonpea may not be as promiscuous as some other legumes nodulating with the cowpea group. Estimates of numbers of rhizobia in the presence of other organisms can only be reliably made on the basis of nodule formation on plants inoculated with various amounts of the carrier. Thus a dilution series is prepared and estimates made by using Most Probable Number estimates. Siratro (Macroptilium atropurpureum) is a small, pasture plant traditionally used for detection of cowpea rhizobia as it grows and nodulates well in test tubes under axenic conditions. However when estimates were made from soils from ICRISAT and Hissar with both siratro and pigeonpea as the trap host, many more of the Hissar isolates were able to nodulate siratro than pigeonpea (Table 3). In a study at ICRISAT of a total of 30 isolates from Mung, Indigofera, Sesbania, groundnut and Aeschynomene, all nodulated siratro but only 4 (ex Sesbania and groundnut) nodulated pigeonpea.

In view of the interest of breeders in genera related to Cajanus and the probability that they may well be used in future breeding programmes, it is important to establish the compatibility of pigeonpea strains with Atylosia and Rhynchosia. The first of these tests is just complete - a range of species of each genes was inoculated with one effective pigeonpea strain. Nodules only formed on 7 of the 8 Atylosia species and 6 of the 9 Rhynchosia. The potential significance of compatibility is well illustrated in two examples with soybean. In Nigeria the Indonesian-type soybeans nodulate with natural cowpea rhizobia but U.S. varieties require selected (U.S.) strains to form nodules. The decision must be made whether to use the Indonesian type (and perhaps lower yields) and avoid nodulation problems or accept, at this stage, the necessity for inoculation of all varieties from the U.S.

Caldwell (1966) found that the soybean cv. Hardee was ineffectively nodulated by some commonly used Rhizobium strains. In Australia where the soybean breeding programmes have been based on U.S. varieties it was found that Hardee with many desirable attributes, did not form nodules at all with the particular inoculant strain CB1809 sold in Australia. In the University of Sydney breeding programme, the decision was made to screen all advanced lines for compatibility with the strain CB1809 and, if nodules are not formed, to select nodulating isolines. This was considered preferable to changing the single recommended Rhizobium strain.

TABLE 3

Soil populations of cowpea *Rhizobium* (MPN/g dwt soil) when tested on siratro and pigeonpea

Soil	Numbers of rhizobia nodulating	
	Siratro	Pigeonpea
Kashmir	190,000	3,270
Hissar (1)	3,400	60
Hissar (2)	4,300	0
Maharashtra	43,000	90
ICRISAT	19,300	19,300

The situation with chickpea appears much more clear-cut. Gaur and Sen (1979) recently tested 71 chickpea strains against 88 species of legume, and 287 strains from 52 of these legumes against chickpea (Table 4). The only cross infections recorded were by 18 out of the 71 chickpea rhizobia, which nodulated Sesbania bispinosa and S. sesban, and one isolate from S. bispinosa which ineffectively nodulated chickpea. At ICRISAT none of 172 chickpea strains nodulated Sesbania setaria. Cross infection between Cicer species has not yet been examined but is necessary if wild Cicer are to be incorporated into breeding programmes.

Naturally occurring rhizobia

Populations of infective rhizobia are present in soils where commercial crop or pasture legumes are commonly grown. The extent to which spread of rhizobia can accompany development of a crop is well illustrated in Australia-Medicago and Trifolium species are naturalized throughout the temperate farming areas of Australia and where they are growing they are generally nodulated. There are no known indigenous legumes which have related effective rhizobia so the rhizobia obviously spread with the species. However there remain many areas where these legumes have not become naturalized - almost certainly due to nutrient limitations - and the rhizobia are also absent. In contrast subtropical most pasture legumes introduced into Australia in the last 30 years nodulate naturally without inoculation - almost invariably with "cowpea group" rhizobia already present on native legumes.

In 1976 information was collected at a number of the villages under study by ICRISAT, on the presence of nodules in pigeonpea. Clearly rhizobia were present although it is certain that no inoculum had ever been used in the villages (Table 5).

However nodule numbers per plant were generally low. Generally at ICRISAT Centre, there are high numbers of cowpea rhizobia in both Vertisols and Alfisols. There were however 5 out of the 15 Vertisol fields where numbers were considered low i.e. < 1000/g. In 3 of 4 fields examined rhizobia were found to a depth of 160 cm (Table 6).

The situation is different with chickpea. In Vertisols on ICRISAT between 10^2 and 10^4 rhizobia per gram soil are present in the top 10 cm while in adjacent Alfisols the populations are almost non-existent. Interestingly once introduced into these soils they persist at least for the next two seasons. Populations of soils from Hissar, Parbhani and Gwalior have ranged from < 100 to 10,000/g soil. ICRISAT staff have observed farmers' fields in Madhya Pradesh and Andhra Pradesh where sown chickpea were not nodulated.

Rhizobium infections on both pigeonpea and chickpea tend to be low under paddy and numbers can decline after introduction with the host. The reduction in numbers would seem to be a direct effect of waterlogging and lack of oxygen.

It is important to note that all these observations refer to presence of rhizobia on nodulation - this does not per se ensure that associations formed will be effective in fixation.

TABLE 4

Specificity of chickpea and its *Rhizobium* (adapted from Gaur & Sen 1979)

Source of isolates	No. of host species	No. of isolates checked	No. of isolates nodulating chickpea*	No. of chick-pea isolates nodulating *Sesbania***
Group				
Rh. meliloti	5	26	0	0
Rh. trifolii	5	26	0	0
Rh. leguminosarum	5	29	0	0
Rh. phaseoli	2	16	0	0
Rh. lupin and *lotus rhizobia*	3	21	0	0
Rh. japonicum	1	5	0	0
Rh. sp. (cowpea)	62	75	0	0
" " *Atylosia scarabaeoides*		5	0	0
" " *Cajanus cajan*		5	0	0
" " *Rhynchosia minima*		1	0	0
" " *Sesbania bispinosa*		8	1	8
Cicer arietinum		71	71	18
		298		

*4 varieties: Chaffa, C235, L144 and N853. ***S. bispinosa* and *S. sesban*

TABLE 5

Nodulation of intercropped pigeonpea in VLS villages, Kharif, 1977.

Site and Soil	Mean no. nodules/plant (% damaged by insects)		
	25 days	40 days	70 days
Aurupalle			
Shallow black 1	6 (4)	4 (0)	7 (24)
Shallow black 2	4 (0)	7 (17)	11 (17)
Shallow red	14 (0)	21 (5)	23 (11)
Dokur			
Shallow red	13 (2)	9 (8)	20 (7)
Shallow red	7 (2)	10 (5)	74 (4)
Shallow red	6 (0)	11 (2)	14 (7)
Shallow red	7 (12)	11 (11)	15 (9)
Kanzara			
Medium black	29 (2)	34 (10)	38 (15)
Medium black	20 (6)	46 (19)	20 (65)
Medium black	32 (5)	32 (14)	33 (19)
Medium black	37 (20)	49 (11)	33 (20)

TABLE 6

Populations of cowpea group rhizobia (MPN/g dry soil) at different depths of 2 Alfisol and 2 Vertisol fields, ICRISAT.

Soil depth (cm)	Alfisol		Vertisol	
	Field A	Field B	Field C	Field D
0-5	2,000	33,900	1,700	250,000
5-10	20,400		1,500	
20-30	105,000	9,300	5,900	81,000
50-60	46,800	300	590	43,700
100-110	16,600	48	37	1,100
150-160	2,100	0	42	630

Effectiveness of fixation of naturally occurring populations

Within successful associations there is a range of effectiveness i.e. associations which fix different quantities of nitrogen under low nitrogen conditions.

To sample the strains present in the soil populations one can collect from nodules of particular host grown in the soil or from nodules of a "trap" host. In the latter case isolates are often obtained after serial dilutions of soils are added to trap hosts grown in test tubes. In our case nodules were always selected from the ultimate positive dilution probably selecting the most commonly occurring strain. Selected by this means, isolates from both pigeonpea and chickpea have shown a quite wide range of effectiveness with a particular host perhaps not surprising with pigeonpea with its associations with the cowpea group but less expected with chickpea with its specific rhizobia and relatively recent use in this environment.

The potential importance of the effectiveness of a local population is obvious. The presence of ineffective strains for a particular host minimizes the potential fixation by that host and introduction of an effective strain is essential.

Another aspect deserves emphasis - all selected inoculant strains were originally isolated from fields. Although the era of genetic manipulation is upon us, so far we have confined our work here to isolates collected from natural situations. This probably applies to all commercially utilized Rhizobium strains in the world. These are generally the best selections - they do not have magical properties and are unlikely to be "wonder" strains.

Selection of Rhizobium strains

The first criterion for selection of inoculant strains is effectiveness in nitrogen fixation with the anticipated host(s) generally under controlled conditions. Field evaluation must then ultimately be made. At ICRISAT, initial isolations were tested with plants grown in sterilized media in bottle-jar assemblies or in pots, then selections were examined in unsterilized soil in pots, and finally evaluated in the field. Using plant response as the measure, a number of strains of fairly consistent superior performance for a fairly narrow range of host genotypes has been selected, but it cannot be claimed that we understand the attributes contributing to this relative superiority or know whether these strains are effective with a wide range of cultivars.

Using one variety of pigeonpea the correlation between testing techniques has not been good. Strains performing well in the glasshouse sometimes fail to do so in one field or soil while being successful in another. Further interactions confuse the picture. An example of a strain x year (= environment ?) interaction of pigeonpea was that studied in field B-9 in 1978-79 and 1979-80. In experiments where 7 strains were common to the tests in consecutive years the four which gave significantly increased yield in 1978-79 were not significantly different from the control in 1979-80 but were significantly poorer than a fifth strain which was the only one to significantly increase yields beyond the control in that year (Table 7). Similar results have been obtained with chickpea.

It is evident that we need to ascertain whether the inoculant strains are in fact forming nodules. While a positive growth response can reasonably be interpreted as successful nodulation, the absence of such a measurable response may be due to either failure of the strain to infect, or its inferiority to native strains.

An important criterion of Rhizobium performance is competitive ability - an attribute quantified essentially by rating how successful a strain is in forming nodules when other strains are present. The ability of a strain to "compete" is in part dependent on the host - the data in Table 8 illustrate this with Vigna species inoculated with 2 strains singly and in a mixture (Thompson unpublished data). CB756 was very successful as a sole strain inoculum on 3 of the 4 species while CB1015 was successful on only 2. Neither strain was markedly successful on adzuki bean. With the two strains mixed CB756 dominated the infections in cowpea and adzuki bean but CB1015 was most 'competitive' with mung bean.

Responses to inoculation on ICRISAT

Of 11 experiments conducted with and without inoculation on ICRISAT with chickpea, two have shown significant responses in yield. One of these was following paddy (Table 9). I find the low proportion surprising in view of the specifidity of chickpea rhizobia, and the distance of Hyderabad from the traditional chickpea growing areas of India.

A similar rate of success has been achieved with pigeonpea on ICRISAT. Of 12 experiments, two in different parts of the same field in successive years, demonstrated significant responses in yield (Table 7), although other criteria of nodulation showed some responses in other fields in other years (Thompson et al, 1980).

As pigeonpeas have presumably been cultivated in this region of India for centuries a response is an interesting phenomenon. As we have used "improved" varieties it may be evidence of host x strain specificity.

The continuing reports of significant responses to nodulation of pigeonpea on Indian research stations (Rewari et al, 1980) are equally surprising - during 1978-79, 8 of 18 experiments showed significant responses in grain yield.

I would submit that the likelihood of obtaining significant yield responses to legumes on research stations where effective inocula have been used, and where soil nitrogen levels are likely to be high, is low. It is likely tobe comparable to, and as rewarding as, attempting to demonstrate phosphate responses by cereals on the precision fields of ICRISAT.

TABLE 7

Relationship between grain yields of pigeonpea inoculated with 7 *Rhizobium* strains in consecutive years on the same field.

(correlation between years r = 0.17)

	1978-79	(kg/ha)	1979-80
IHP 35	1750*		1440
IHP 147	1720*		1700
IHP 71	1680*		1530
IHP 195	1590*		1630
IHP 24	1560		1640
IHP 100	1540		1830*
IHP 229	1340		1520
Uninoculated	1370		1430

*Significantly superior to uninoculated control ($P < 0.05$)

TABLE 8

The success of inoculant strains in nodulating a range of *Vigna* spp. in the field at Tamworth, NSW.

Host	Cowpea *V. unguiculata*		Mung bean *V. radiata*		Black gram *V. mungo*		Adzuki bean *V. angularis*	
	% of nodules identified as:							
Inoculation treatment	CB756	CB1015	CB756	CB1015	CB756	CB1015	CB756	CB1015
Uninoculated	6	8	2	32	5	46	23	7
CB756	76[a]	0	67[a]	6	84[a]	1	30	8
CB1015	9	4	0	86[b]	0	87[b]	24	4
CB756 + CB1015	52[a]	0	9	77[b]	30[a]	49	54[a]	1

χ^2 Analyses a = significant increase of CB756 over uninoculated control.

b = significant increase of CB1015 over control.

TABLE 9

Yield of chickpea after paddy

Treatment	Dry matter (kg/ha)	Grain yield (kg/ha)
Control	1483	1090
Inoculated + N*	2391	1762
Inoculated	2679	1801
SE ±	161	123

*Calcium ammonium nitrate added at rate of 150 kg N/ha

THE LEGUME HOST

To improve the efficiency of the symbiotic relationship more emphasis has been placed on the variation within the bacterial component, very often with little consideration given to the host.

In chickpea and pigeonpea the nodule begins from an infection via the root hair and the plant responds positively by providing the bacteria-filled cells with meristematic tissue to invade. As in all legumes the nodule becomes an integral part of the plant. Photosynthate is provided, N_2-diffuses from the soil atmosphere via the outer parenchymatous cells into the bacteroids and is reduced, and the products, as combined nitrogen, are transported from the root to be assimilated by the plant. While nodules have a higher % N than leaves (e.g. cowpea 5-7% compared with leaves 2.5-4.5%) (Eaglesham et al, 1977), the nodule does not accumulate N. These authors showed that at any one time even when nodules were contributing ca 96% of the total N they only contained 7% of the total N in the plant.

Although infection is obviously dependent on the presence of bacteria, the process of nodule formation is soon controlled by the host. Until the meristematic tissue is formed, and leghaemoglobin is produced the bacteria are parasitic. Full symbiosis follows, but the N produced is obviously governed by the size of the sink, which is the plant itself. Fixation in a nodule continues until external damage occurs to the nodule or it degenerates as a result of stresses (temperature, high or low moisture, nitrate or stage of development of the host). This degeneration is therefore also largely a reflection of the hosts' influence.

An interesting hypothesis which has not proved to be universally acceptable was that the legume host could select effective strains from a mixed population. Robinson (1969) found evidence of this with Trifolium pratense and other workers have supported this finding (e.g. Masterson and Sherwood, 1974). However other workers have failed to find supporting evidence e.g. Gibson et al (1975) not only found that natural populations of rhizobia under Trifolium subterraneum in Australia were of varying effectiveness, as we have found here with pigeonpea and chickpea, but the mean effectiveness of the population of a region were as low as 62% of that of a standard effective strain. This does not suggest strong selection pressure for effectiveness.

Even when we ostensibly "compare Rhizobium strains" for efficiency or effectiveness, we in fact evaluate in terms of measurements made on the host. Similarly nodule tissue is quantified by weight of the nodules, not by enumeration of the rhizobia.

In examining the place of superior N-fixation in plant breeding much of the emphasis has been on selection of plants with greater amounts of nodule tissue (e.g. Mytton 1978) working with Trifolium repens. Latterly Zary et al (1978) have selected cowpea plants for superior N-fixation as measured by acetylene reduction - a direct measure of nitrogenase activity. P.D. Graham of CIAT (personal communication) selects Phaseolus vulgaris on superior plant vegetative growth under controlled conditions. Wynne et al (1980) also consider.

that leaf dry weight is a suitable parameter on which to base selection of groundnuts.

While nodulation characteristics are heritable, and can often be improved by appropriate selection, nodulation is more variable in outcrossing than self-pollinated species (Gibson, 1980). There is no doubt that variability exists between germplasm lines of both chickpea (self-pollinated) and pigeonpea (an outcrossing species)(Tables 10 and 11). Wide ranges of nodule number, nodule weight and nitrogenase activity have been found and seem suitable criteria for selection. Under field conditions these criteria have been found to be correlated well with yield in chickpea (Table 12) but, apparently because of difficulties of finding nodules on pigeonpea, correlations are poor with this species (Table 13). It is clearly important to choose reliable selection criteria which themselves are measures of fixation or are well correlated with such measures.

If only N is limiting in the test situation then a measure of the total N taken up by the plant may well serve as a simple selection criterion (unfortunately such a criterion is wholly destructive). Grain yield has been traditionally used by breeders and under the same low N conditions is likely to be a useful criterion. In fact the breeder testing under N-stress may well be selecting for N-fixation. However under these circumstances it is arguable that yield of dry matter at flowering or some other stage more closely related to the cessation of fixation may well be a more meaningful criterion e.g. in pigeonpea variety ICP-1 nitrogen uptake ceased reached a maximum at 130 days on black soil and was almost maximal on red soil, in the absence of applied N (Saxena and Sheldrake, 1977). As fixation is likely to have finished by this stage selection may be best made at this point.

The host and Rhizobium interaction raises real issues in relation to the field of plant breeding. Rhizobium workers in this field advocate collaboration with breeders and cite the separate genetic control of capacities to infect and fix nitrogen as justifying simultaneous selection for both characters (Mytton, 1978). Selection for general effectiveness, requiring the accumulation into one genotype of sufficient genetic information for it to react very effectively with a range of genetically different partners, is likely to be slow (Mytton, 1978).

If on the other hand the attempt is made to breed rigid specificity into a legume we must assume that the partnership can always be established. Not only is this a mammoth task (Gibson, 1980) but is probably only realistic where the range of useful genotypes is narrow - this in turn may infer that we are dealing with a very restricted environment, or where natural effective rhizobia do not occur. The latter is currently the situation in Australia with soybean, and in one major breeding programme, screening of all advanced selections was against the one strain used in the tightly controlled Australian inoculant industry. The extreme of this viewpoint is that of Caldwell and Vest (1977) wh suggested selection of hosts resistant to infection by the inefficient strains of a region.

In Nigeria where local soybean lines nodulate well with local strains of rhizobia, introduced U.S. varieties do not. In contrast to the above approaches, the IITA soybean breeding programme proposes to incorporate the promiscuity of the local lines into high yielding improved cultivars (IITA 1978)

TABLE 10

Range of symbiotic parameters and yield of chickpea cultivars sown in the field at ICRISAT, Rabi 1976-77

Character	Days after planting	Range of values
Nod. Number	25-30	4-48
	45-50	10-76
	70-75	1-20
Nod. Weight (mg/plant)	25-30	0.3-55
	45-50	2-105
	70-75	1-195
Top Weight	25-30	-
	45-50	0.7-6.2
	70-75	1.8-39.2
Grain yield (g/pl)		1.9-23.5

TABLE 11

Range of symbiotic characteristics in the 110 pigeonpea crossing-block entries used at ICRISAT. (25 days after planting; Rainy season 1977; Alfisol)

Character	Range
Nodule number	6.7 - 37.8
Nodule weight (mg/plant)	9 - 55
N_2-ase activity	
$\mu M C_2H_4$/plant/h	1.1 - 11.3
$\mu M C_2H_4$/nodule/h	65 -565
Shoot weight (mg/plant)	380 -1400
Root weight (mg/plant)	38 - 185

TABLE 12

Correlations between N_2-fixation parameters and yield of chickpea 61 days after planting

	Nodule wt.	N_2ase/pl.	Grain yield
Nod. No.	.788***	.778***	.761***
Nod. Wt.		.763***	.813***
N_2ase/Pl.			.668**

Significant at 1% * Significant at 0.1% n=20

TABLE 13

Correlations (r) between criteria measured in inoculation experiments on a Vertisol field at ICRISAT in consecutive years (pigeonpea)

	30 days after sowing			At grain harvest	
	Nodule wt/ plant	N_2-ase/ plant	Shoot Wt/plant	Plant wt/ha	Grain wt/ha
1978-79					
Nodule no/plant	.411	.300	.053		[illegible]
Nodule wt/plant	1	-.092	.480		-.233
N_2-ase/plant		1	.208		.339
Shoot wt/plant			1		.437
1979-80	40 days after sowing			At grain harvest	
Nodule no/plant	.813*	.345	-.473	-.232	.603*
Nodule wt/plant	1	.713*	-.409	.166	.327
N_2-ase/plant		1	.073	-.383	-.298
Shoot wt/plant			1	-.445	-.615***
Harvest total wt/ha				1	.960***

*Correlation significant ($P<0.5$) *** Correlation siginficant ($P<0.001$)

This particular situation is unusual and does not to my mind justify the across-the-board acceptance of the principle if we know that we can provide strains superior to the local strains for the particular host.

In recent discussions within the pulse programme there was general, but not unanimous acceptance of the principle that all experimental studies on ICRISAT controlled fields, including breeding material, should be inoculated. While the opponents reasonably object to imposition of use of single strains which allow for host x strain interactions, the proponents equally reasonably claim that the natural populations differ between fields and generally cover a wide range of effectiveness. A compromise has been suggested and may well be mutually acceptable - the use of mixed effective strain inocula. Such a decision involves reconsideration of a practice which has been consistent within the pulse microbiology programme since its inception - viz. use of single strain inocula.

THE ENVIRONMENT

I do not propose to attempt an exhaustive list of environmental factors affecting the symbiosis but to emphasize a few particular points which may be relevant to ICRISAT and its crops.

Nutrients

As pointed out by Munns and Mosse (1980) "the nutritional requirements of legumes resemble those of other plants except that their potential for [nitrogen fixation] creates special demands notably for molybdenum and cobalt, but also for copper, phosphate and zinc". In fact Byth (1979) pointed out that the question of mineral interaction is inseparable from biological symbiotic N-fixation.

The requirements for zinc for the symbiosis are virtually unknown (Edwards, 1975) but some data (Demetrio et al, 1972) suggest N-fixation may be adversely affected by low levels. It is therefore a debatable point whether the top dressings of zinc applied to Vertisols on ICRISAT are removing an important variable from our studies.

There has been considerable study of the effect of inorganic nitrogen on the symbiotic system and there is no doubt that infection is reduced by NO_3 (e.g. Munns, 1968). This is also illustrated in data from ICRISAT with pigeonpea (Table 14). However there is also evidence of benefit to early growth and nodulation (e.g. Dart and Wildon, 1970). A 20 kg/ha dosage of N is recommended for grain legume sowing in many states of India although it is difficult to find critical data supporting this recommendation.

Iron is specifically required by N_2-fixing systems being an essential component of the two enzymes comprising nitrogenase (Klucas et al, 1968). The evidence of iron chlorosis in chickpea at Hissar recently emphasises the marginal nature of the Fe levels in those soils - of particular interest however is that some lines are much more susceptible than others. We have no evidence of direct effects on nodulation or N-fixation of chickpea in this situation. In one study at ICRISAT plants nodulated in the presence of low Fe but nodules degenerated prematurely.

TABLE 14

Effect of fertilizer nitrogen on nodulation, nitrogenase activity and top growth of pigeonpea cv. ICP-1 in Alfisol, Rainy season, 1977.

Fertilizer N applied (kg/ha)	Nodule No.	Nod.Dry wt (mg)	N_2-ase activity $\mu M C_2H_4/h$ per plant	per g Nod. dry wt.	Shoot dry wt (g)
			20 days after planting		
0	17	19	3.65	459	.28
20	12	8	1.69	282	.35
100	9	5	0.51	205	.33
C.D. at 5%	5	N.S	0.52	158	N.S
			60 days after planting		
0	39	351	21	77	18.8
20	36	344	18	54	24.8
100	42	369	18	53	28.3
C.D. at 5%	N.S	N.S	N.S	N.S	5.9

Seasonal and physical environment

Temperature effects are manifest in every aspect of growth and with the nodulated legume they are particularly important. Modification of soil temperature is possible by mulching and no doubt occurs in the mixed or intercropping system, but the potential for amelioration is usually minimal and escape is the most readily employed technique. Even then with the confounding of temperature with other seasonal variables makes the effect of individual components difficult to separate.

This confounding of temperature, especially with moisture requires greatly increased inputs to clarify the factors governing nodulation and nodule activity. Not only is this important in the normal growing seasons but assumes ever greater significance with proposed changes in sowing times (e.g. early chickpea, rabi sown pigeonpea). Some recent observations (below) illustrate this and emphasize the need for collaborative field studies especially with Physiologists Studies in controlled environment where all variables are measured are required to separate those effects. It is hoped that the proposed collaborative studies between the University of Reading and ICRISAT on chickpea can assist in this.

With pigeonpea and chickpea at ICRISAT interest by breeders and physiologists in modification of sowing date inevitably involves consideration of temperature effects. With the collaboration of the ICRISAT breeders it was possible in 1980 to examine nodulation of a range of chickpea lines sown in late September, rather than at the normal time in late October. The results were dramatic - fixation of the early sown crops reached a rapid peak followed on equally rapid decline so that fixation had finished by the time the normally sown crop had formed its nodules. The current explanation of these data is that, although nodules were able to form, the symbiotic system broke down when soil temperature exceeded 35°C. These results agree with those of Minchin et al (1980) who showed marked reduction of nitrogenase activity at 30°/18° under controlled conditions compared with 22°/18°. Similar results have been reported by Dart et al, 1976.

In contrast pigeonpea sown by Dr.Chauhan in April, 1981 with soil temperatures up to 50° C nodulated well and the nodules were active while the soil temperature ranged 25-45°C.

The influence of soil moisture on nodulation and N fixation is one of the most neglected areas of studyin relation to environment although it is obviously very relevant to the ICRISAT crops. Sheldrake and Saxena (1979) showed that numbers of chickpea nodules decreased during the normal Hyderabad rabi season when moisture was declining. Studies by the Microbiology section have shown a benefit from irrigation on nodule quantity, fixation and final yield. We do not know whether irrigation of pigeonpea during the rabi season would promote continuous fixation of nodules. There is also a very marked detrimental effect of soil desiccation on nodule formation by clover even though the plant is surviving and the *Rhizobium* populations are not adversely affected (Worrall and Roughley, 1976).

There is currently considerable emphasis on the sowing of pulses after paddy. Not only does this involve understanding of the role of the moisture on the plant but it is particularly significant in relation to rhizobia. The decline in numbers of rhizobia in paddy soils after flooding strongly suggests adverse effects of waterlogging.

Salinity tolerance varies between species of legume but it is suggested that the tolerance of the host for nodulation may be lower than that of the rhizobia themselves (Parker et al, 1977). There is also room for selection amongst rhizobia. A chickpea Rhizobium strain selected for salinity tolerance at ICRISAT performed outstandingly under similar conditions in the Sudan.

Cultural practices

Within ICRISAT there has been considerable study of particular techniques of growing some of our mandate legumes e.g. the broadbed and furrow technique on Vertisols with sorghum and pigeonpea.

To date we have no information on the effect of such procedures on root development or nodule formation, activity, persistence compared with more common farmer operations.

The Indian farmer commonly uses simple implements providing shallow cultivation which (a) does not greatly affect the spatial relationships of the profile as far as distribution of nutrients and organic matter, and (b) does not change the condition of the subsoil especially in relation to root penetration. How significant is periodic deep ripping as used at ICRISAT in allowing root and nodule development to depths not so readily tapped without ripping? Is it relevant to the farmers situation ? Is there any point in attempting to study Rhizobium ecology or residual nitrogen, in soils which receive high inputs of nutrients and such thorough, periodic mixing ?

Inevitably a serious study of N-fixation by legumes should not only attempt to understand the net inputs and residual effects but the spatial aspects of these inputs. Presumably because of the obvious and dramatic changes which arose from land clearing to allow agriculture in many parts of Africa there has been considerable emphasis on nitrogen profiles (e.g. Greenland, 1975). However although continually cropped lands of India are unlikely to show such wide ranges the possibility of leaching and subsequent recovery by deep rooting plants is particularly relevant to understanding of the performance of pigeonpea. There would seem to be a place for the study of nitrogen profiles in India.

MEASUREMENT OF NITROGEN FIXATION

The measurement of nitrogen fixation, both in short and long term, is essential to our understanding of the contribution of the legume and its place in any cropping system. Direct and indirect methods are used.

Net nitrogen uptake of a legume based on Kjeldahl determinations using a non-legume control

At ICRISAT up to half the nitrogen take up by pigeonpea is calculated to have been fixed in one experiment (Table 15). The maximum figure established was 69.4 kg N/ha fixed by cv. T-7.

With chickpea no comparable significant results have been obtained at ICRISAT although safflower, maize and wheat have all been used as non-legume controls.

The non-nodulating legumes (already available in groundnut and in soybean) is potentially a very valuable tool as a control in such studies. It is for this reason that we always watch for the occurrence of such plants in pigeonpea and chickpea.

Effect of nitrogen fertilizer

The results of N fertilizer applications in India to both chickpea and pigeonpea have been variable. Saxena and Sheldrake (1980) cite conflicting results while also pointing out that effects could be observed on nodule or plant growth without being reflected in final yield. Such effects are not uncommon in other non-legume crops where early obvious responses can interact with moisture to cause a depression in yield.

In our own data significant yield increases were obtained for pigeonpea to dressings of 20 kg N/ha on an Alfisol and 200 kg N on a Vertisol (Table 16). The commonly recommended Indian practice of applying 20 kg N/ha to pulses has not been sufficiently studied. The effect appears to be synergistic (cf. Dart and Wildon, 1970) where the early provision of N overcomes the short period of N starvation but there can be early adverse effects on nodulation (Table 15). There is a real need to establish whether N starvation occurs especially in warm environments where nodulation is very rapid and nitrate levels may be already high at the beginning of the rainy season.

If a small dressing fails to produce a response, but a high dressing does so this is usually interpreted as an indication of sub-optimal fixation. This may well have been the case in the Vertisol in Table 14. If on the other hand the response obtained to large doses of applied N is similar to that provided by a starter dose it is commonly assumed that nitrogen fixation is apparently adequate under those conditions. However absence of a response merely indicates that nitrogen supply is not limiting and does not necessarily indicate that fixation could not be improved. This attitude is evident in the discussion by Hawtin et al (1980) in regard to chickpea - "nitrogen fixation is normally adequate, at least for present yields, as there are few reports of positive responses to N fixation".

TABLE 15

Total N uptake and fixation by some pigeonpea cultivars on an Alfisol at ICRISAT Centre, rainy season 1977

Cultivar	Maturity (days)	N yield kg/ha Plant + Root + Nodules	N yield kg/ha Fallen plant parts	Total N-up take	Balance against sorghum (N-fixed)
Prabhat	115	57.7	11.4	69.1	+ 4.4
Pant A-3	115	63.1	8.5	71.6	+ 6.9
UPAS-120	125	76.3	15.5	91.8	+ 27.1
T-21	130	91.4	16.5	107.9	+ 43.2
BDN-1	130	93.6	24.6	118.2	+ 53.5
No.148	150	102.2	17.6	119.8	+ 55.1
JA-275	170	60.2	17.7	77.9	+ 13.2
ICP-7035	170	80.0	21.0	101.0	+ 36.3
ICP-7065	175	79.6	28.1	107.7	+ 43.0
T-7	215	112.8	21.3	134.1	+ 69.4
NPWR-15	240	99.6	14.7	114.3	+ 49.6
Sorghum	175	64.7	0	64.7	

TABLE 16

Effect of fertilizer nitrogen on grain yield of pigeonpea cv. ICP-1 in Vertisol and Alfisol (rainy season)

Fertilizer N applied (kg/ha)	Vertisol (1979)		Alfisol (1977)	
	Shoot Dry Wt.	Grain yield	Shoot Dry Wt.	Grain yield
	(kg/ha)			
0	8725	1834	2564	850
20	8425	1885	3147	970
200	10929	2234	3560	970
C.D. at 5%	1033	305	401	105

Residual effect of the legumes on a subsequent non-legume crop

A recent study on a Vertisol at ICRISAT showed that the beneficial effect of sole crop pigeonpea on the yield of a subsequent maize crop was equated with an N application of 35-40 kg/ha (Figure 1). This estimate shows remarkable agreement with previous estimates by Sheldrake and Narayanan (1979) that leaf fall and roots could contribute 30-35 kg/ha to the soil. The N removed by the initial pigeonpea crop was greater than that removed 17kg/ha by the sole sorghum crop grown at the same time. Thus the total net N input in this experiment was over 50 kg assuming pigeonpea and sorghum take up similar amounts of mineral N from the soil.

With chickpea results are not so clearcut. Experiments at ICRISAT of similar design to the pigeonpea experiment did not show any consistent effects.

As might be expected in view of the large masses of nodules formed, groundnut has been favoured as a rotation crop in Uganda - McWalter and Wimble, (1976).

In the world literature on beneficial residual effects of tropical and sub-tropical legumes, the majority of favourable responses have been with forages. Data are now appearing showing beneficial residual effects of temperate grain crops such as lupin, but the sub-tropical and tropical grain crops have been least rewarding in this respect.

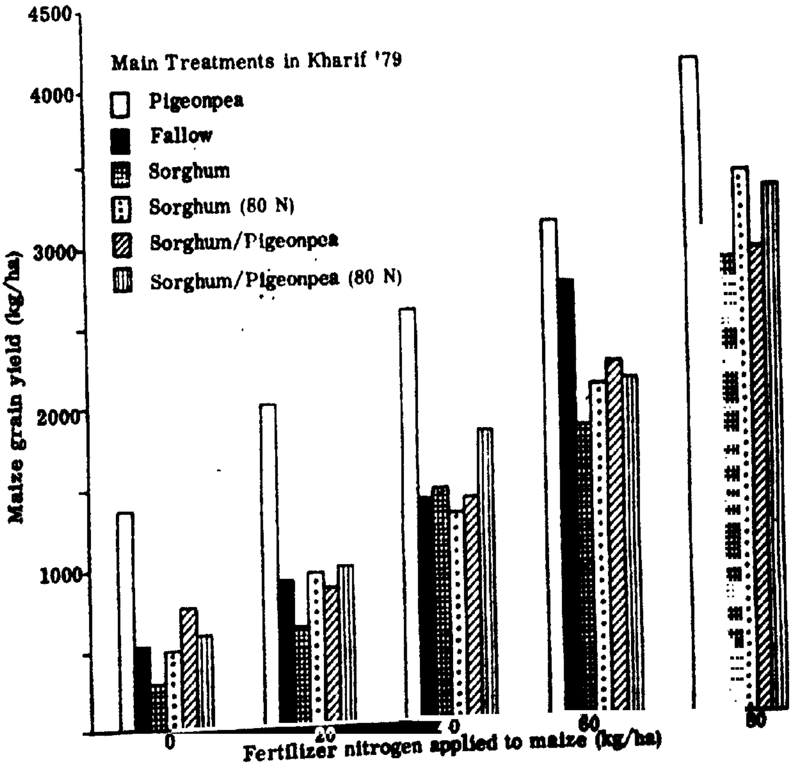
Giri and De (1980) found that yields of pearl millet receiving no N fertilizer but following pigeonpea at Delhi were equivalent to those where 30 kg N/ha was applied. They ranked groundnut, cowpea, pigeonpea and mung beans in order of benefits to subsequent crops. Saxena and Tilak (1975) demonstrated a significant residual effect of inoculated soybean on subsequent wheat yield. On the other hand, Jones' (1974) demonstration of benefit from groundnut was accompanied by evidence that cowpea did not benefit the subsequent maize crop.

Acetylene reduction technique

This technique, depending on the ability of nitrogenase enzyme to reduce C_2H_2 at a rate related to its ability to reduce N_2 has been used extensively since its first implementation in the late 1960's. It is a test which only gives a measure of current activity and requires digging and incubation of the nodules in an atmosphere of C_2H_2 for ½-1 hour.

The technique has provided valuable insights into the fixation pattern of the pulses. Probably because of the greater ease of recovery of chickpea nodules, some very striking effects are evident. Seasonal profiles of activity at Hyderabad and Hissar are strikingly different in both magnitude and duration of activity, fixation is short lived at ICRISAT while it continues throughout the winter at Hissar. Perhaps more importantly the rates of fixation during early growth differ markedly between Hyderabad and Hissar.

FIGURE 1



RESIDUAL EFFECT OF PIGEONPEA ON GRAIN YIELDS OF MAIZE, (Kharif 1980).

The data were collected at H.A.U., Hissar under conditions commonly followed by farmers, i.e. adequate prior moisture and declining temperatures. When chickpea grown at Hyderabad is irrigated regularly similar rates and duration of fixation can also be induced (Figure 2).

Nitrogenase activity of chickpea early in the life of the plant grown in the field at ICRISAT can be well correlated with nodule quantity and with final grain yield.

With pigeonpea the situation is very different although fixation also declines after flowering. During the active period, specific activity (activity/unit mass of nodules) is certainly higher than chickpea although it is arguable whether there is more N fixed per unit area. The problem with pigeonpea is that recovery of nodules is difficult and, perhaps not surprisingly, correlations between mean nitrogenase activity at 30-40 days and final dry matter or grain yields are poor. The relationships of these parameters will need to be established under conditions where nodules are recoverable (e.g. under controlled conditions) but there is clearly a need for alternative procedures which do not involve excavation.

Biochemical techniques

In 1977 Matsumoto et al showed that the long-recognized ureides (allantoin and allantoic acid) were commonly associated with nodulation in soybean, and current evidence suggests that the ureides are probably the major metabolite into which gaseous N_2 is converted in the nodule. Thus there is an opportunity to measure N fixation indirectly by assay of the ureide content.

At ICRISAT, Dr.Matsumoto found however that the ureides were only of significance in one of the mandate legumes, viz. pigeonpea. The nitrogen of bleeding sap from the hypocotyl region can contain over 50% of the total soluble nitrogen as ureides with the remainder as NO_3 (presumably absorbed from the soil), and amino acids plus amides (source yet to be established).

While the technique is obviously attractive, much more study is necessary to develop it as a reliable measure of N_2-fixation and currently Dr.Ann Mary Mariadass is working on this programme as a Research Fellow.

The source of the amides and amino-acids (soil or fixation) and the level of ureide production from other pathways must be determined. The correlation between concentration of ureides in xylem sap exuded from decapitated plants and that found in different plant parts is poor but is being further investigated.

However, the possibility of obtaining a reliable estimate of fixation for field grown pigeonpea remains; the incentive to develop such an assay certainly remains. We clearly need an alternative to excavation of nodules, which in any case has not proved rewarding.

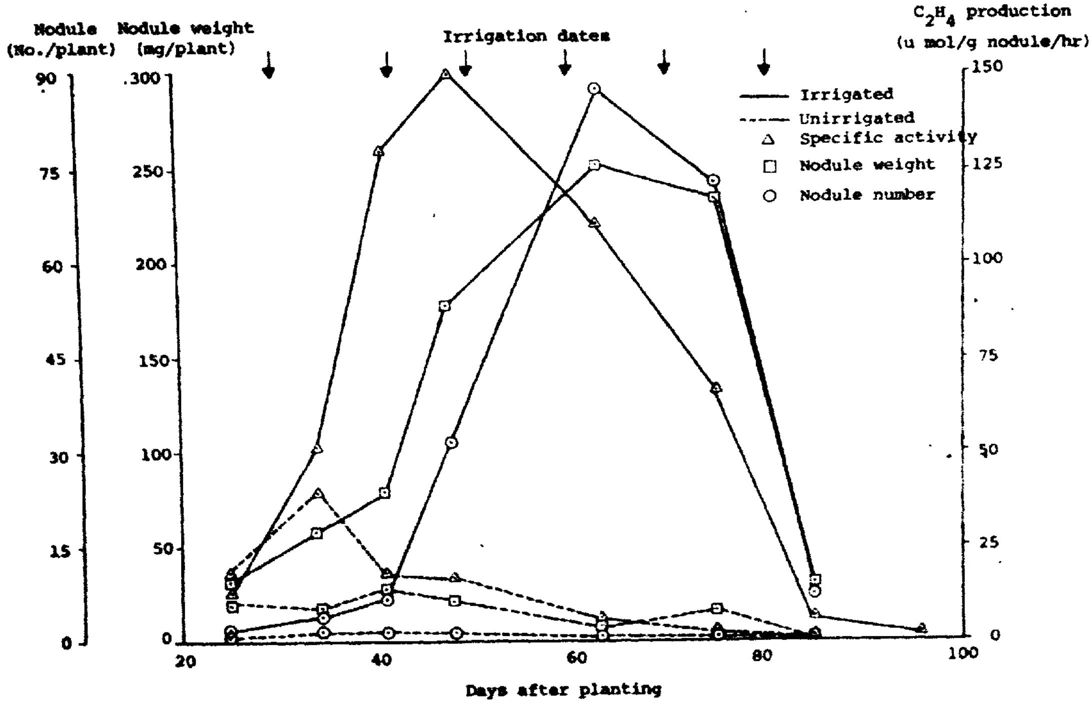


FIGURE. 2: NODULATION AND N_2-FIXATION WITH AND WITHOUT IRRIGATION IN A VERTISOL BY CULTIVAR 850-3/27

Use of ^{15}N

^{15}N usage in studying of nitrogen fixation is based on availability to the legume of gaseous N_2 and soil/fertilizer N which differ in ^{15}N content. This is most readily studied by using ^{15}N enriched soils (i.e. by adding enriched fertilizer) although ^{15}N can be used under controlled conditions (natural abundance of ^{15}N is 0.366% of atmospheric N, so that 5 atom % excess is in fact 5.366% ^{15}N).

The simplest procedure involves examination of the N taken up by a legume and a non-nitrogen fixing plant, usually a non-legume, supplied with a small amount of ^{15}N fertilizer and calculating the differential amounts of ^{15}N present. Fried and Middleboe (1977) proposed the following:

$$\text{amount fixed} = \frac{\text{atom \% } ^{15}\text{N excess in legume crop}}{\text{atom \% } ^{15}\text{N excess in reference crop}} \times \text{total N in legume crop}$$

This assumes that the $^{15}N:^{14}N$ ratio of the mineral N taken up by the two crops is the same.

Another approach is to use the A value concept (Fried and Broeshart, 1975) which involves the assumption that a plant confronted by two sources of a nutrient will take up the nutrient in proportion to the amounts available from the two sources. This enables one to ensure that the non-fixing control has an adequate N supply so that it explores a similar volume of soil to the legume.

$$\text{A value} = \frac{\text{\% nitrogen derived from soil}}{\text{\% nitrogen derived from fertilizer}} \times \text{rate of N applied}$$

The N_2 fixed thus = A value (fixing system) - A value (non-fixing system) x fraction fertilizer N (fixing system) use.

A field experiment with ^{15}N conducted on pigeonpea in 1980 at ICRISAT was to utilize these concepts.. As the soil was an Alfisol, castor was used as the non-fixing control. The plants grew poorly in 1980 so we propose to repeat the experiment in 1981.

While enrichments of soils by ^{15}N fertilizers are commonly of the order of > 1 atom percent excess ^{15}N, there is some evidence that lightly labelled soils may be of use to screen breeding materials for N_2-fixing ability (Kohl and Shearer, 1981). The major limitation is that the rate of mineralisation of ^{15}N enriched organic matter should be constant during the experiment so that the $^{15}N:^{14}N$ ratio in inorganic N released during the experiment is constant. Such equilibration following enrichment with fertilizer may take a number of years. With equilibrated soils, screening can be done with a few plants with selection in favour of those with least ^{15}N.

The particular advantage of the ^{15}N technique based on whole plant sampling is that it is an integrated measure and does not suffer from the "one off" limitation of the acetylene reduction technique. The ^{15}N technique can be used on the vegetative material at final harvest and seeds saved for further utilisation.

INOCULANTS

Preparation

The majority of legume inoculants on sale in the world are prepared by impregnation of a finely ground carrier, commonly peat, but in India, lignite. The quality of the inocula is the most readily defined in terms of numbers of suitable nodule bacteria per unitquantity of carrier. Unless the product is prepared by impregnation of a pure culture into a sterile sealed package of carrier, the proliferation of contaminants is such that a reliable plate count is not possible. Regrettably, Indian inoculants whether produced by Universities or private companies, are generally not prepared in this fashion and the necessary control procedures involving serial-dilution/plant-infection tests are also not utilized. The result is that quality control is poor and the quality of inoculants on sale to farmers (and available between institutions for All India coordinated trials) is poor (Figure 3).

In order to service ICRISAT experiments our own inoculants are currently produced using irradiated peat imported from Australia. As collaborating scientists from Indian institutions provide us with pure bacterial cultures, we have undertaken to store them by freeze drying, return them when required and to prepare peat-based cultures of those strains required for use in All India studies.

Meanwhile studies have been initiated to,

(a) attempt to simplify methods of broth production using simple metal fermentors rather than expensive glass flasks and shakers,

(b) test alternative carriers,

(c) develop suitable means of sterilizing finely ground carrier in the sealed packet before impregnation.

Because the impregnated peat is moist,and needs to remain so,waterproof packaging is necessary. For the same reasons the package must be effectively sealed. The packing material of choice is plastic - polyethylene most commonly used. However this is not heat resistant and as autoclaving is a successful means of sterilizing the package before impregnation, polypropylene has proved useful.

Recently, Dr.Goswami of Punjab Agricultural University spent some time on these studies and some preliminary storage data including Dr.Goswami's first tests are presented (Table 17).

FIGURE 3

QUALITY OF INDIAN INOCULANTS

ISI Standards A = 10^7/g upto expiry
B = 10^8/g at manufacture

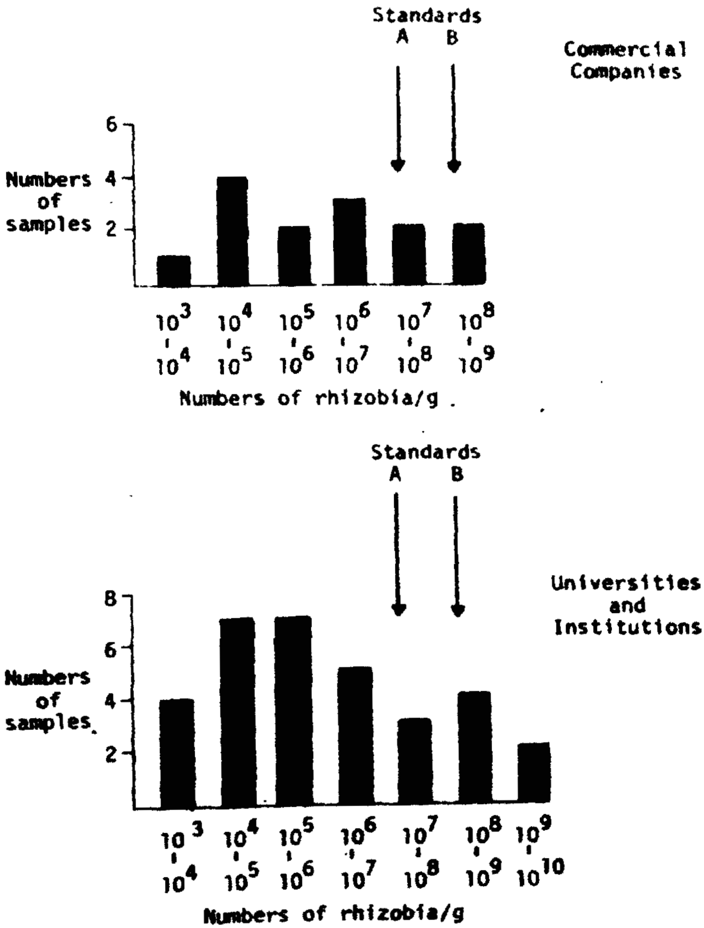


TABLE 17

Preliminary (1980-81) data

Survival of *Rhizobium* in various carriers

Sterility of carrier		Log. No. rhizobia*/g moist carrier			
		Sterile**		Non-sterile	
Time from impregnation		**2 weeks**	**8 weeks**	**2 weeks**	**8 weeks**
Carrier					
Australian peat (neutralised)	(pH 7.4)	9.49	8.91	-	-
Ooty Peat (neutralised)	(pH 7.6)	9.34	9.24	8.84	8.24
Lignite (neutralised)	(pH 6.1)	8.26	8.66	8.84	8.08
Press mud	(pH 8.0)	7.48	5.87	< 4.5	< 4.68
Charcoal	(pH 8.1)	8.51	8.34	8.26	7.24

*Each figure is the geometric mean of populations of 4 strains (1 pigeonpea, 2 groundnut and 1 chickpea)

**All except Australian peat sterilized by autoclaving.

Meanwhile ICRISAT staff have been invited to assist in redrafting the Indian Standards for inoculants to include more efficient counting techniques involving serial-dilution/plant-infection techniques, and use of identification procedures. There is little doubt that unless strict control measures are implemented in India the whole concept will lose credibility with the farmers.

Use of inoculants

Production techniques throughout the world generally reflects the fact that inoculants are normally applied to seeds, because this is the easiest way to introduce them into the soil.

Problems however arise from this practice - (a) some seeds are not easily inoculated (the obvious example is groundnut where the seed coat is so easily damaged in the process, and soybean is also readily damaged by use of adhesive solutions), (b) where the use of harmful insecticides or fungicides is practiced (e.g. groundnut, Phaseolus vulgaris and Pisum sativum) added rhizobia can rapidly die, (c) sowing under dry conditions especially with small seed which cannot be deeply buried (and so escape high temperatures), (d) the risk with some species that a proportion of the inoculum can be carried up on the seed coat at germination.

Brockwell et al (1980) have shown that application of inoculant directly to the soil either by implantation of "solid" inocula (i.e. normal inocula carried on inert material such as sand) or "liquid inocula" (i.e. normal inocula suspended in water and sprayed into the soil near the seed) both likely to givebetter responses with pasture seeds than seed inoculation, when adverse conditions exist.

Already groundnut microbiologists are utilizing alternative inoculation procedures at ICRISAT. Similarly there are situations where pulses are sown under adverse conditions, especially the dry sowing of pigeonpea in Vertisols. It is not difficult to anticipate that successful introductions of chickpea in farmers' fields in South India may also be dependent on dry sowings in anticipation of late Kharif rains for germination. On ICRISAT the preference for use of ridges rather than flat cultivation may be unfavourable to survival of rhizobia due to less favourable temperature and/or moisture relationships.

Evaluation of success of inoculants

Inoculant strains ideally are chosen for their ability to infect the host under a range of conditions and again ideally, to provide the maximum plant response in terms of N-fixation.

In fact they are often chosen essentially on their effectiveness with the host and there may be no measure of their ability to infect the host. Ultimately this ability must be tested.

With all its faults associated with nodule size etc. the most common criterion of ability to infect is to determine the proportion of nodules due to the inoculant strain. This in turn involves recognition and identification.

Identification of inoculant strains

Serology has been most publicised tool for recognition of rhizobia from nodules for many years (e.g. Vincent, 1941). Its application ranges from the simple agglutination tests (mixing rabbit-based antisera with an unknown in a tube) through gel diffusion (where recognizable bands are formed where differing substances meet within agar containing antisera and antigen) immuno-flourescence which results from conjugation of immunoglobulin with a flourescent dye visible under the microscope, to ELISA (enzyme linked immunosorbent assay) (Reddy, 1980) where a colour reaction can, in the ultimate use of the technique, be automatically read and the results quantified and recorded by electronic equipment. Unfortunately with these methods cost is positively related to speed of determination.

Rabbits are normally used for production of antisera and facilities for the housing are currently almost complete at ICRISAT. For most of the techniques a pure culture of an isolate for each nodule is necessary - this is time consuming and facility-saturating as it is not unreasonable to consider 40 nodules/plot as a minimum sample. With adequately sized nodules and a well-developed technique, direct nodule squashes are possible, and in fact preferable, with immunofluorescence, but the technique must be well developed by the operating laboratory - without a culture of the nodule forming organism there are no second chances.

Antibiotic resistant strains are readily selected as mutants by subjecting a normal Rhizobium population to growth on an antibiotic-containing medium. Commonly selection of the mutants is made at a concentration in the agar at 100-200 ug/ml although some antibiotics are particularly prone to produce mutants of reduced effectiveness (Schwinghamer, 1967). Mutants resistant to streptomycin and spectiomycin are commonly selected. When used as inocula the identity of this strain can be readily confirmed by growth of a nodule isolate on agar containing the relevant antibiotic(s). This technique (introduced by Obaton, 1971) was employed by Bonyong Toomsan to identify a streptomycin resistant inoculant strain of chickpea Rhizobium in a field inoculation trial at ICRISAT. Success of the inoculant was closely related to the population of natural rhizobia present in the soil. To date similar studies have not been made with pigeonpea at ICRISAT.

Inherent antibiotic resistance is a concept utilized by Josey et al (1978). It was found with R. leguminosarum that reasonably consistent growth reactions could be measured by particular strains inoculated on agar incorporating low levels (5-30 µg/ml) of different antibiotics. Several levels of a range of suitable antibiotics are selected so that they each only adversely affect some strains of rhizobia but not others. The ability or otherwise of a single strain or isolate to grow on each of the test plates is recorded and the final result constitutes a genetic "fingerprint". The antibiotic resistance profile isolates of a population of unknowns can potentially be used to classify them.

This potential ability to classify unknons is particularly appealing as a means of determining whether changes occur in fields between years.

The first results were encouraging: they successfully placed the known inoculant strain (a mutant resistant to a high level of antibiotic) into 1 or 2 groups depending on the number of "errors" allowed and subdivided the remaining population of 473 isolates into 119 classes, many of which only contained one strain.

The technique is not without its problems and further development at this stage is in the hands of Dr.Eden Bromfield of Rothamsted who is attempting refinements as part of a collaborative project with ICRISAT sponsored by British Overseas Development Administration. Strains from both pigeonpea and chickpea are being studied.

Phage typing. The technique of identifying bacteria by their reaction to a range of viral bacteriophages has been known for a long time but has remained essentially of academic interest to date with Rhizobium. Our collaborating laboratory, Rothamsted Experimental Station in U.K. is currently investigating its use with isolates and soils from ICRISAT.

THE FUTURE

Short-term objectives (1-2 years)

(a) To develop and establish suitable methodology for:

i) identification of Rhizobium for study of field performance of inoculants,

ii) measurement of nitrogen fixation,

iii) manufacture of high quality legume inoculants with Indian materials,

iv) screening of germplasm and breeders material (in collaboration with breeders)

(b) To study recognized constraints to nodulation and fixation with existing methodology:

i) effect of insect damage of nodules on N fixation by pigeonpea,

ii) effect of nodulation on incidence of disease (in collaboration with Pulse Pathology)

iii) effect of time of sowing on N fixation - to date these studies have utilized sowings made by physiologists (pigeonpea) and breeders (chickpea). Implicit in this study is measurement of the effects of temperature and moisture. Studies may employ glasshouse experimentation using temperature baths depending on the degree of involvement of the University of Reading Plant Environment Laboratory with whom ICRISAT is currently proposing to collaborate.

iv) effect of dry sowing of inoculated seed and of introduction of granular or liquid inoculants separate from the seed before sowing, on survival of rhizobia,

v) examination of compatibility of close relatives of both pulses to their respective rhizobia,

vi) determination of heritability of nodulation.

(c) To examine the response of pulses sown into farmers' fields and low input areas of ICRISAT. These sowings have already been made at Aurupalle and ICRISAT with pigeonpea.

Studies on farmers' fields. There are a various reasons for this approach:

i) in principle any agricultural development should be tested in the fields of the farmer whom we are committed to assist. This particularly applies when the conditions under which their development has been made differ from those of the farm. Reed et al (1980) stated that surveys of over 1000 fields of pigeonpea across 13 states of India from 1975 to 1980 have indicated that over 80% of this crop is grown. with few or no purchased inputs. Chickpea is probably grown under somewhat more favourable conditions but inputs are again relatively low.

Thus we are interested whether inoculation can provide a response in any of the measurable criteria relevant to N-fixation.

ii) a major input into any research programme is the definition of new problems. If our inoculation experiments fail we shall need to determine why. To do this we shall have to return to the laboratory, to devise new procedures but ultimately again we must return to the farm. One may reasonably anticipate that nutrient deficiencies may be revealed.

I wish to make it perfectly clear that these studies are simple experiments and not demonstrations.

In addition I submit that there is a need for opportunities to work in villages other than those we are fortunate enough to be associated with through the Vilage Level Studies. There is currently a strong bureaucratic wall separating us from the farmers of India. While we accept its existance there is no reasonable justification for our claim to be serving the problems of the farmer of the SAT.

Mid-term objectives (1-3 years)

To utilize developed methodology to assist in,

i) selection of desirable *Rhizobium* strains of versatility in relation to hosts and environments of India (and other countries ?).

ii) measurement of N fixation and inputs under normal farming conditions.

iii) screening, selection and breeding for high fixation levels in collaboration with breeders,

iv) advising manufacturers on techniques of inoculant manufacture.

Long-term objectives (1-10 years)

(a) To develop collaborative long term rotation experiments on ICRISAT under conditions relevant to the farmer with particular emphasis on nitrogen balance determinations,

(b) Collaborative studies on N balances in all cropping systems,

(c) To develop breeding strategies in collaboration with the breeders to maximise N fixation by breeders material,

(d) To include in the programme, and integrate with the Rhizobium studies, the currently proposed studies of mycorrhizae being initiated in the Cereal microbiology programme,

(e) To provide usable technology for inoculant manufacture and usage in all countries relevant to the ICRISAT programme.

ACKNOWLEDGEMENTS

The scientists, Dr.O.P.Rupela and Dr.J.V.D.K.Kumar Rao have been responsible for all of the ICRISAT Pulse Microbiology studies and deserve to be especially acknowledged both for their work and for their assistance in enabling me to become familiar with it. The sterling services of a very valuable band of technical, laboratory and field assistants has made the work possible and I thank them for their contribution. The names of visiting scientists who have worked in the pulse programme have been introduced in the relevant parts of the text.

Finally I thank Dr.Peter J. Dart, foundation Pulse, Groundnut, Millet and Sorghum Microbiologist to whose energies I must attribute the initial establishment of a wide ranging programme, for whose continued interest and involvement I must give thanks and whose foresight enables us to plan the next phase of our studies with adequate facilities.

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