

## STUDIES ON INOCULATION OF COVER LEGUMES FOR IMPROVING NITROGEN FIXATION

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### SUMMARY

*Many rhizobial strains were screened for their nodulating ability against Pueraria, Centrosema, Calopogonium and Desmodium spp. using seedlings grown in nutrient agar. Pueraria and Calopogonium spp. formed effective nodules and fixed nitrogen in sterile sand culture with most of the strains that produced nodules on them in nutrient agar.*

*Inoculation of seeds with some effective strains improved growth of Pueraria in pots but not in the field. This is probably due to natural populations of rhizobia in the soil which were able to successfully compete with the introduced strains in nodulating on the legume. There was evidence that indigenous rhizobia themselves formed effective nodules and fixed nitrogen.*

### INTRODUCTION

The main advantage of legumes over naturals as ground covers lies in the ability of the former to fix atmospheric nitrogen. But although legumes have been traditionally cultivated as covers under rubber their ability to fix nitrogen has not received sufficient attention. A study of the improvement of covers by enhancing their ability to fix nitrogen is important considering the high cost of inorganic nitrogenous fertilizers. It is also important to get maximum benefits from legume covers, which are expensive to maintain.

Jeevaratnam (1957 ; 1959) initiated a programme of rhizobial screening but did not find consistent improvement in growth of covers from artificial seed inoculation. However, following his work, the Rubber Research Institute of Ceylon supplied rhizobial cultures to estates on request, for the inoculation of legume seeds. This practice has been discontinued since about 1963. The Rubber Research Institute of Malaysia has supplied rhizobial cultures from 1938 (Beeley, 1938) and recently claimed yield increases up to 200 kg/ha following artificial inoculation (International Rubber Digest, 1976), but there is no experimental evidence that artificial inoculation *per se* was responsible for this. Seed inoculation has been recommended as a routine measure in North Sumatra following observations of meagre and ineffective natural nodulation (Brandt, 1973). On the other hand, some recent pot culture experiments done at the Rubber Research Institute of India (Mathew *et al.*, 1974) revealed that even without inoculation *Pueraria* receiving optimum doses of P, K and Mg fixed 6 times more nitrogen than the controls in the first 3½ months and 4 times more in a period of 7½ months. Some 265 kg/ha nitrogen were fixed in the presence of optimum nutrient levels; this is with indigenous soil rhizobia. It thus appears that a need for effective rhizobia as well as nutrients can be major constraints to optimum nitrogen fixation by covers.

If artificial inoculation can increase nitrogen fixation by legume covers this should be a very effective and cheap method of improving the nitrogen status in rubber soils. This paper examines the performance of some recent introductions of inoculant strains with cover legumes under local conditions.

## EXPERIMENTAL AND RESULTS

*Screening of rhizobial strains in agar-deeps*

In order to be able to screen a large number of strains rapidly for nodulation, Gibson's (1963) method of partly enclosed culture of plants on seedling agar was attempted, but was found unsuitable for two reasons. First, especially with *Pueraria*, roots dipped in the liquid nutrient medium decayed, apparently due to lack of aeration. Second, fungal and bacterial contaminations were excessive and often interfered with the growth of the plants. Therefore, the method had to be suitably modified. Fifty ml conical flasks were filled to 5 cm below the brim with  $\frac{1}{4}$  strength seedling agar medium (Jensen, 1942). After the seedling agar solidified a 1 cm deep layer of yeast mannitol agar was poured over it and inoculated with the test strain. A few days later, when well grown colonies of the rhizobium had formed, a layer of plain agar, 1 cm deep, was poured over the yeast mannitol agar layer and two germinating seeds were placed on it. The opening of the flask was covered with aluminium foil. At the stage when plumules were touching the foil, one of the seedlings from each flask and the foil were removed and the neck of the flask plugged with sterile cotton wool so that the cotyledons and shoot tip were outside. All operations were done under aseptic conditions. The flasks were then left in the greenhouse in a box so that the roots were not exposed to light. Plants were examined for nodulation 14 - 18 days later at which stage absorption of moisture by the roots had shrunk the agar revealing the nodules especially those around the collar. This made examination of nodules quite convenient.

Using this technique several strains of rhizobia obtained from Rothamsted Experimental Station, U.K., and from the Nitragin Company, U.S.A., were screened for nodulation against *Pueraria phaseoloides*, *Calopogonium mucunoides*, *Centrosema pubescens* and *Desmodium ovalifolium* (Table 1).

TABLE 1 : NODULATING ABILITY OF SOME COVER LEGUMES WITH SELECTED RHIZOBIAL STRAINS

Rhizobial Strain	<i>Pueraria</i>	<i>Calopogonium</i>	<i>Centrosema</i>	<i>Desmodium</i>
Rothamsted 3404				—
„ 3405				—
„ 3406				—
„ 3807	+			
„ 3808	—			
„ 3816	+			
„ 3824 (CB 756)	+	+	—	—
„ 3826	—	+	+	
„ 3830	—	+	—	
„ 3831	—			
„ 3877 (CB 1024)	+	+	—	—
„ 3915	—	+	—	
'Nitragin' EL 29	+	+		
„ LX 146	+	+		

+ Nodules, — No Nodules

Whereas *Pueraria* and *Calopogonium* formed nodules with many of the strains tested, *Centrosema* and *Desmodium* did not, and these appear to be rather more strain-specific although all these legumes belong to the same cross-inoculation group, viz., the cowpea miscellany, the members of which are generally promiscuous in their nodulation habit.

#### Screening in Leonard Bottle-Jar Assemblies

Tests in agar-deeps reveal only whether or not a test strain nodulates a particular legume. Therefore, to ascertain the most effective strains, a comparison of the efficiency of strains was done against *Pueraria* and *Calopogonium*. Sterilized Leonard assemblies were used as described by Vincent (1970) with 1/5 strength Jensen's (1942) nutrient medium (pH 6.8). Seeds after acid treatment to break dormancy were inoculated using agar cultures, and 40% gum arabic (pH 6.5) as an adhesive. Each seed was smeared with about 10,000 bacterial cells. The seeds for control bottles were coated only with gum. Fifteen seeds were planted in each bottle and after the seedlings emerged the bottles were placed in a greenhouse. One week later, plants were thinned down to four per bottle. The nitrogen controls were given 70 ppm N, as ammonium nitrate, 12 days after sowing. Plants were harvested 6 weeks after sowing and total dry matter, shoot nitrogen content and dry weight of nodules were estimated.

The differences between treatments were highly significant with respect to all three parameters assessed for both *Pueraria* and *Calopogonium* (Table 2).

TABLE 2 : EFFECT OF SOME RHIZOBIAL STRAINS ON GROWTH, NODULATION AND NITROGEN CONTENT OF *PUERARIA* AND *CALOPOGONIUM*

Treatment (Rhizobial strain)	<i>PUERARIA</i>			<i>CALOPOGONIUM</i>		
	Plant dry wt* (g)	Nodule dry wt* (g)	Shoot nitrogen (%)	Plant dry wt* (g)	Nodule dry wt* (g)	Shoot nitrogen (%)
Rothamsted 3807	0.56	0.001	1.36	—	—	—
„ 3816	1.57	0.133	3.19	—	—	—
„ 3824 (CB 756)	1.56	0.128	3.45	1.79	0.084	2.63
„ 3877 (CB 1024)	1.38	0.134	3.46	1.22	0.052	2.51
„ 3826	—	—	—	1.74	0.073	2.86
„ 3830	—	—	—	1.36	0.071	2.31
„ 3915	—	—	—	0.74	0.019	1.28
'Nitragin' EL 29	1.52	0.152	3.11	1.74	0.082	2.53
„ LX 146	1.22	0.101	3.23	1.82	0.076	2.88
No Nitrogen (Control I)	0.47	0	1.27	0.71	0	1.13
70 ppm Nitrogen (Control II)	2.05	0	2.89	2.01	0	2.84
L.S.D. 5%	0.31	0.028	0.39	0.34	0.018	0.43
L.S.D. 1%	0.56	0.050	0.70	0.61	0.032	0.77

\*Values are for 4 plants

All strains except 3807 were effective with *Pueraria* and produced significant increases in growth, nodulation and leaf nitrogen content as compared with the uninoculated control. LX 146 appeared to be comparatively less effective. The nitrogen controls produced greater dry matter than with even the most effective strains but had less nitrogen in the shoots than those inoculated with some of the effective strains.

Strain 3915 was not effective with *Calopogonium* and 3877 and 3830 appeared to be only semi-effective. The behaviour of strain 3830 was rather exceptional; it nodulated well but did not increase growth or fix as much nitrogen as the more effective strains.

The results also confirm that nodulation in agar deeps is not a sufficient criterion of effectiveness of a strain.

#### *Evaluation in pot experiments*

The more promising strains from Leonard Assembly tests were next tested in soil in pots with *Pueraria* as the host legume. Pots (9" deep, 9" diameter) were filled with soil collected from a field in Dartonfield with about 90% naturals and the balance *Pueraria* and *Desmodium*, both moderately nodulated. The pots received a basal dressing of fertilizer as 300 kg/ha saphos phosphate, 150 kg/ha muriate of potash, 100 kg/ha dolomite, 8 kg/ha copper sulphate, 15 kg/ha zinc sulphate and 400 g/ha sodium molybdate. The fertilizers were mixed into the first few inches of soil. Fifty acid-treated and inoculated seeds, as described before, were sown per pot and three weeks later the number of plants were thinned down to 6 per pot. Nitrogen as urea was applied to the appropriate pots at this stage at 20 or 40 kg N/ha. Pots were arranged in a randomised block design and left in the open well separated from each other to avoid cross contamination. Each treatment was replicated 6 times. Plants were harvested three months after sowing and total growth, nodulation, % effective nodules and % leaf nitrogen were estimated (Table 3).

TABLE 3 : EFFECT OF INOCULANT STRAINS OR NITROGEN ON GROWTH, NODULATION AND LEAF NITROGEN CONTENT OF *PUERARIA* IN POTS

Inoculant strain/N - level		Plant dry wt (g/pot)	Nodule dry wt (g/pot)	Effective nodules (%)	Leaf nitrogen (%)
Rothamsted	3816	42.4	1.56	76.0	3.83
"	3824 (CB 756)	49.2	1.91	78.7	3.88
"	3877 (CB 1024)	44.5	1.83	80.0	3.93
' Padukka Isolate '		38.8	1.67	82.0	3.83
' Nitragin ' EL	29	41.8	1.63	78.7	3.88
"	LX 146	36.0	1.52	82.7	3.76
No Inoculant (No N)		32.9	1.56	86.7	3.99
"	20 kg N/ha	44.5	1.69	78.0	3.89
"	40 kg N/ha	60.9	1.62	86.0	3.94
L.S.D. 5 %		5.2	(N.S.)	(N.S.)	(N.S.)
L.S.D. 1 %		9.3			

The results show that inoculation with all strains except LX 146 and added nitrogen significantly increased plant growth. But % leaf nitrogen, nodulation and % effective nodules were not affected by inoculation or added nitrogen. It is unusual that nitrogen did not suppress nodulation. Profuse nodulation and high percentage of effective nodules and high leaf nitrogen content in the uninoculated control treatment suggest that indigenous rhizobia are also active in fixing nitrogen though probably not as effectively as the inoculant strains.

### Field evaluations

The final test of any inoculant strain is its performance in the field. Therefore 4 strains viz., Rothamsted 3824, 3877, EL 29 and LX 146, nitrogen at 40 kg/ha and a control (no inoculation) were tested out at 6 sites located in five soil series viz., Parambe, Agalawatta, Homagama, Matale and Boralu (Silva, 1969), in a randomised block experiment, with six replicates of each treatment. Each plot consisted of a row of plants 2 m long, and plots were separated from one another by a 2m vacant strip with a drain in the middle as a precaution against cross contamination. As a further precaution all experiments were laid on flat land. Basal applications of fertilizer, seed inoculation and other experimental details were as for the pot experiment already described. Plants were thinned down to 15 per plot three weeks after sowing and nitrogen was applied to the appropriate plots at this stage.

Experiments at two sites had to be abandoned due to natural hazards.

Plants were harvested 3½ months after sowing; dry weight of plants, nodule weight, % effective nodules and % leaf nitrogen were determined. The results for one site which is typical of all sites are given in Table 4. Differences between treatments are not significant for any of the parameters suggesting that none of the inoculant strains improved fixation under field conditions. As in the pot experiment, good nodulation, growth and percentage leaf nitrogen in the uninoculated controls suggest active fixation by indigenous *rhizobia*.

TABLE 4 : EFFECT OF FOUR RHIZOBIAL STRAINS ON GROWTH, NODULATION AND LEAF NITROGEN CONTENT OF *PUERARIA* IN THE FIELD

Treatment (Rhizobial strain)	Plant dry wt (g/plot)	Nodule dry wt (g/plot)	Effective nodules (%)	Leaf nitrogen (%)
Rothamsted 3824 (CB 756)	200.3	1.61	69.0	3.06
" 3877 (CB 1024)	212.5	1.41	49.0	3.24
'Nitragin' LX 146	148.4	1.14	50.8	2.64
" EL 29	231.3	1.64	48.0	3.52
No Inoculant	186.9	1.47	38.0	3.15
" + 40 lb N/ac	292.8	1.11	39.5	4.24
	N.S.	N.S.	N.S.	N.S.

## DISCUSSION AND CONCLUSIONS

It is to be concluded that strains that were effective under laboratory conditions failed to improve nitrogen fixation in association with *Pueraria* in the field, and indigenous rhizobia themselves were effective. This implies that either the introduced strains did successfully nodulate the legume in the field but were no more effective than the indigenous strains, or that the latter successfully competed with the alien strains at nodulation masking the effect of the alien strains. Competition from natural populations of rhizobia appears to be the more plausible reason because *Pueraria* nodulates freely in local soils. However this question can be resolved only by assessing the extent of nodulation by the inoculant strains in competition with native strains. Such studies done elsewhere (Date, 1974) have shown that when indigenous strains are present in the soil only a small percentage of the nodules may be formed by the inoculant strain.

Soil pH is a further factor that is likely to affect nitrogen fixation by inoculant strains in local soils. The strains that were tested have been isolated from soils less acidic than the local soils. The soil pH at the four sites from which data were collected ranged from 4.4 to 4.8. However, although this pH may not be optimal it is known that cowpea type rhizobia are tolerant of acid conditions and can effectively nodulate in acid soils (Norris, 1967).

Why there was a response to inoculation in the pot experiment but not in the field also needs explanation. Rhizobial density is greatest in the first few inches of the soil. Disturbing the soil during potting destroys the spatial relationship of rhizobia in the original soil and could "dilute" out the cells in the entire soil mass. This may have given a greater chance for introduced strains to nodulate in pots as against in the field.

The invasiveness of a strain in the field would depend on its ability to colonize the soil and rhizosphere rapidly and to form nodules in competition with natural populations. These attributes are more likely to be found in effective selections from indigenous populations than in foreign strains. It therefore appears that a programme of isolating and screening from local populations might be more fruitful than screening foreign strains for effectiveness under local conditions. These studies are now being carried out at the Rubber Research Institute of Sri Lanka.

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