

EFFECTS OF INOCULUM SIZE AND PHOSPHORUS APPLICATION ON NODULATION AND N₂ FIXATION IN COWPEA (*Vigna unguiculata* (L.) walp) USING ¹⁵N METHODOLOGY.

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Abstract

In a greenhouse study at the Institute of Agricultural Research and Training (IAR&T), Ibadan, Nigeria, isotope dilution method was used to evaluate the effect of varying inoculum sizes with and without phosphorus (P) application on nodulation and N₂ fixation of cowpea (*Vigna unguiculata* (L.) Walp) cv. Ife Brown. Different inoculum sizes (concentrations) were formulated by severally diluting a re-suspended (2.4×10^8 cells/ml) solution of *Bradyrhizobium* spp strain IR_c 27 and mixing with known (1.5kg) weight of soil; phosphorus was applied to the appropriate pots. The control pots were neither treated with *Bradyrhizobium* spp nor P. Seeds of Ife Brown and a maize reference crop were sown after treatment application.

Significant ($P \leq 0.05$) interaction effects of inoculum size and P application were obtained on nodulation, dry matter yield, percentage nitrogen derived from atmosphere (% Ndfa), nitrogen yield, amount of nitrogen fixed separately but in varying degrees. The inoculum size of 3.2×10^5 cells/g soil plus P appeared to be most beneficial in terms of cowpea N₂ fixation (proportion and amount) but on nodule number and dry matter yield variables, 10^4 cells/g soil plus P was found appropriate.

Therefore, in order to ensure high inoculation success, a range between the two inoculum sizes plus P application (20 ppm) is suggested. Phosphorus application caused significant variable increases in % Ndfa, amount of N fixed and N yield only when inoculum size higher than 10^3 cells/g soil was used, depicting that it was feasible to improve inoculation success through increasing inoculum concentration.

INTRODUCTION

Apart from the effectiveness of a *Rhizobium* strain and its compatibility with a host legume, the method of its application on the legume seed and edaphic factors, the strain population used is another potent factor influencing inoculation success. According to William (1983) and Mulongoy and Ayanaba (1986), competition for nodulation by inoculant strains can be enhanced through inoculation of the soil with high rhizobial populations and repeated inoculum application (Benbrahim *et al.*, 1992). Roughley (1982) indicated that the effects of adverse environmental conditions can be ameliorated through the use of high rhizobial populations. Effect of low pH in an acid soil was ameliorated through the use of a high population of *Rhizobium* in such a soil (Robson and Loneragan, 1970).

Amarger (1981) found that when two or more rhizobia were competing for infectible sites in a rhizosphere, most of the nodules found will be due to the most populous strain. The findings of Brockwell *et al.*, (1988) showed that the degree of nodulation by inoculant strain is dependent on the size of resident *Rhizobium* population.

There is, however, no agreement among scientists as regards the optimum inoculant size that may most enhance competitiveness, persistence and overall inoculation success. However, Roughley (1980) indicated that with sparse indigenous soil rhizobia, 100 inoculant rhizobia per seed might ensure that most nodules are formed by the inoculant strain. Date (1988), however, suggested 100,000 rhizobial cells per seed as being adequate with high soil indigenous rhizobia. In a NifTAL and FAO Bulletin (1984), a range of 1,000 - 10,000 rhizobia per seed was recommended, depending on seed size. On the other hand, Brockwell (1981) showed that while a population of 1000 resident rhizobia per gram of soil reduced the chances of successful legume inoculation to a low level, in contrast, 50 resident rhizobia of soil was found to eliminate inoculation response (Thies *et al.*, 1991). The workers showed a positive correlation between the number of indigenous rhizobia and the competitive ability of inoculated strains.

A conducive soil environment is a must for inoculation success. Thus, amelioration of a poor edaphic condition should be carried out. Application of fertilizer to improve soil nutrient status is one way of removing potent barriers capable of impairing success of inoculation (Vincent, 1980). Adequate supply of certain nutrient elements especially starter N and P are known to facilitate legume symbiotic N₂ fixation (Sprent, 1978; Abbot *et al.*, 1979). Phosphorus has been identified as the most critical element for nodulated grain legume nutrition in the tropics; (Webster and Wilson, 1980; Friesen *et al.*, 1980). Application of P has been found to be vital also for tree legumes (e.g. *Acacia saligna*) establishment (Benbrahim *et al.*, for cowpea nodulation and N₂ fixation (Mulongoy, 1985). Though required in a low amount (Rhode, 1981), application of P has been found to increase cowpea growth in height and nodulation. With copious application of P, Vose and Jones (1963) were able to reduce the depressing effects of Al-ions and Mn-ions in nodulation of inoculated grain legumes, leading to increases in nodule number and weight (Ssali, 1986). As regards the rate of P application, Balasubramanian *et al.* (1980) stimulated increased nodule number and weight by applying 30kgP/ha, but Rao and Mader (1975) were able to obtain enhanced nodule mass with 45kgP/ha. The objective of the study reported here was to evaluate the effects of varying inoculum rates and P application on N₂ fixation of cowpea, *Vigna unguiculata* cv. Ife Brown.

Materials And Method

A Bradyrhizobium sp. (cowpea miscellany) IRc 27 was obtained from the culture stock of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. A yeast extract mannitol (YEM) broth-culture of the strain was incubated on a mechanical shaker for 7 days at 28°C, later centrifuged at 500g for 20 minutes and re-suspended in sterile distilled water to give a cell density of 2.4×10^9 /ml, as measured by dilution plate count.

Muriate of potash (60%K) and single super phosphate (18%P) were applied to all pots at 15ppmKCl and 15ppmP, respectively.

Treatments:

Two factors were involved in this study, namely

1. Inoculum

Four (4) concentrations (inoculum sizes) and the control. The following cell concentrations which constituted the levels of the factor (inoculum) were obtained after several dilution exercises on the re-suspended 2.4×10^8 cells/ml:

Inoculum concentration 0	-	no inoculation.
Inoculum concentration 1	-	3.2×10^3 cells\ g soil.
Inoculum concentration 2	-	3.2×10^4 cells\ g soil.
Inoculum concentration 3	-	3.2×10^5 cells\ g soil.
Inoculum concentration 4	-	3.2×10^6 cell \ g soil.

The indigenous rhizobial population determined by the plant infection technique was 2.4×10^3 cells\ g soil.

II. Phosphorus application :

The levels were 0 and 20ppmP.

Ten (10) treatment combinations were obtained; replicated four times using RCB design. The fixing crop was cowpea (*Vigna unguiculata*) cultivar Ife Brown, while maize (*Zea mays.*) variety TZB -SR was used as the non - fixing reference crop. Soil inoculation, seed sowing and N fertilization;

A composite soil sample was obtained from several cores obtained from a field which was previously under a secondary bush. The soil (1.5kg per pot) were inoculated (mixed thoroughly) with the appropriate inoculum concentrations (levels) and P. The application of the treatments were made according to the treatment labels on the pots. Seeds (4/pot) of the fixing and non-fixing crops were sown in pots as appropriate. The seedlings were later thinned to two per pot two weeks after planting (WAP), followed by application of N fertilizer (10ppmN as labelled sulphate of ammonia 10% atom excess 15_N in solution (25ml/pot) to the two crops. The physical, chemical and biological properties of the soil used are contained in Table 1.

Harvesting was done 42 days after planting (dap) for determination of nodule number and dry matter (80°C for 48h) yield (DMY). The nitrogen content of the plant was determined by Kjeldahl digestion method (Eastin, 1978) and the atom % 15_N excess by emission spectrometry (Fiedler and Proksh, 1975). The amount of nitrogen fixed was calculated using 15_N isotope dilution equation of Fried and Middelboe (1977). Statistical analysis was by ANOVA and treatment comparisons were made using the least significant difference (LSD) method (Steel and Torrie, 1960).

Table 1.
Physical, Chemical and Biological Properties of the Soil Used

Properties	Values
pH(1:) Soil/H ₂ O ratio	6.80
Organic Carbon (%)	0.75
Total N (%)	0.09
Sand (%)	80.00
Silt (%)	13.00
Clay (%)	7.00
Exchangeable bases (Meg/100g):	
Ca	2.35
Mg	1.57
K	0.19
CEC	4.49
Available P (ppm)	4.54 (Bray 1)
Resident <i>Rhizobium</i> population	2.4 x 10 ³ cells/g soil (Plant infection technique).

RESULTS AND DISCUSSION

Nodulation:

Results of nodulation in response to inoculum concentration and applied P are presented in Table 2. Irrespective of whether P was applied or not, increasing inoculum rate resulted in greater nodulation of the crop showing that rhizobial population is a potent factor that could influence inoculation success. However, application of P (20 ppm) promoted higher nodulation in general. In a similar experiment, Wagner *et al.*, (1978) and Balasubramanian *et al.*, (1980) reported increase in nodulation with application of 15 ppmP and 17 ppmP respectively. Though, the rate was not specified, Ssali (1986) obtained increased nodulation of grain legume with inoculation. A comparable degree of nodulation was observed between using a lower inoculum rate of 10⁴ cells/g with P. and when a higher rate of 10⁶ cells/g soil was used without P. Application of P therefore appeared to have resulted in inoculum use economy. In this experiment, reliance only on indigenous rhizobial population (2.4 x 10³ cells/g soil) for nodulation resulted in a lower number of nodules/plant than when inoculation was done suggesting that inoculation is vital when native rhizobial population is low. In an earlier study by Daramola (1989), inoculation was found to promote crown and total nodule numbers, respectively, in a soil containing about 2000 rhizobia per g soil. However, Brockwell (1981) observed reduced legume inoculation success with as low as 1000 resident rhizobia per gram soil.

Table 2
Cowpea Nodulation Response to Application of Varying Inoculum (rhizobial) Populations with and without P

Inoculum Size	OP	Module 20ppmP	Number per plant Inoculum size (mean)
0	2.83	6.33 ^c	4.58 ^c
3.2 x 10 ³	6.00 ^d	8.00 ^{cb}	7.00 ^b
3.2 x 10 ⁴	8.33 ^{cb}	8.67 ^b	8.5 ^b
3.2 x 10 ⁵	6.67 ^{cb}	6.33 ^c	6.50 ^{bc}
3.2 x 10 ⁶	8.50 ^b	13.66 ^a	11.08 ^a
P application (mean)	6.47 ^b	8.60 ^a	
		Lsd (0.05)	%CV
Applied P	=	1.99	= 35
Inoculum size	=	2.29	= 26
Interaction	=	2.00	= 16

Means followed by same letter (row and column) are not significantly different.

The contrast could be explained by differentials in soil properties other than biological nature of the soil.

Dry Matter Yield (DMY):

Application of P (20 ppm) stimulated dry matter yield (shoot) of plants whether or not the soil was inoculated (Table 3). Considered across inoculum rates used in this experiment, P application (20 ppm) gave significantly ($P \leq 0.05$) higher DMY. A significantly higher cowpea DMY ($P \leq 0.05$) was obtained with 15 ppm by Daramola (1989) compared to the OP treatment. In his work, Rhode (1981) obtained enhanced growth in cowpea with a small (though not specified) amount of P application. Generally, increasing inoculum dose with and without P application led to increases in DMY. In most cases, however, with P application (20 ppm), the percentage increases recorded with each successive increase in inoculum size over the no - inoculum were more substantial. For instance, compared to the no - inoculum treatment, 14% and 2% increases in DMY with P (20 ppm) and without P, respectively were obtained for the same 10⁵ cells/g soil inoculum dose. The increase was found to be significant at 5% level. Though, inoculation status was not stated, Sharma *et al.* (1974) reported increases in DMY of cowpea with 13 ppm P. Across the inoculum sizes in this study, we obtained a 22% DMY increase due to P use (Table 3).

TABLE 3

Cowpea Shoot Dry Matter Yield Response to Application of Varying Inoculum (rhizobial) Population with and without P

Inoculum size	OP	20ppmP	Inoculum size (mean)
0	0.82 ^d	1.00 ^{bc}	0.91 ^b
3.2 x 10 ³	0.86 ^{bcd}	1.02 ^{bc}	0.91 ^b
3.2 x 10 ⁴	0.88 ^{bcd}	1.10 ^b	1.02 ^{ab}
3.2 x 10 ⁵	0.84 ^{cd}	1.14 ^{ab}	0.99 ^b
3.2 x 10 ⁶	1.06 ^{bc}	1.30 ^a	1.18 ^a
P application (mean)	0.92 ^b	1.12 ^a	
		Lsd (0.05)	%CV
Applied P	=	0.86	11.1
Inoculum size	=	0.18	15.0
Interaction	=	0.17	9.5

Means followed by same letter (row and column) are not significantly different.

With inoculum rate of 10⁴ cells/g soil plus P, DMY was about 25% greater than when P was not applied (Table 3). Also, following P application, plant DMY for 10⁴ cells/g soil inoculum rate was significantly greater ($P < 0.05$) than 10⁵ cells/g soil without P. A similar comparison showed that statistically same amounts of dry matter were recorded for 10⁴ cells/g soil plus P was significantly ($P < 0.05$) different compared to either 10⁵ or 10⁶ cells/g soil with P. Thus with P, 10⁴ cells/g plus soil could be regarded as an optimum rate for DMY in this study in which the soil used contained a low (2400 cells/g soil) indigenous rhizobial population. Although the rate of application of inoculum was not stated, Roughley (1980) obtained enhanced DMY in a legume when the resident rhizobial population was low.

Results obtained in this study also showed that P application was capable of promoting enhanced dry matter accumulation with a much lower inoculum dose than when P was not applied. Earlier workers found that in the tropics, P was a critical element in legume nutrition (Webster and Wilson, 1980) and in growth, nodulation and N₂ fixation (Friesen *et al.*, 1980; Mulongoy, 1985). N₂ Derived from Atmosphere:

Phosphorus application to the soil, whether inoculated or not, enhanced the capacity of the plant to fix higher %N₂ from the atmosphere (Table 4). With and without P application.

TABLE 4

Proportion of N derived from Atmosphere (%Ndfa) by Cowpea Treated with Varying Inoculum Strain Populations and Two P Levels.

Inoculum size	OP	20ppmP	Inoculum size (mean)
0	45.30 ^{ef}	48.52 ^e	44.90 ^c
3.2 x 10 ³	44.86 ^f	48.02 ^e	46.44 ^c
3.2 x 10 ⁴	52.78 ^d	58.76 ^{bc}	55.77 ^b
3.2 x 10 ⁵	57.12 ^c	62.39 ^a	59.76 ^a
3.2 x 10 ⁶	60.88 ^{ab}	62.72 ^a	61.80 ^a
P application (mean)	52.19 ^a	56.08 ^a	
	Lsd (0.050)	%CV	
Applied P	NS	13.0	
Inoculum size	3.42	5.3	
Interaction	3.32	4.0	

Means followed by the same letter (row and column) are not significantly different.

Increasing greater %N₂ was derived from the atmosphere beyond 10³ cells/g soil inoculum dose; the increases being more marked with P application. These results indicate that legume could be induced to derive greater N₂ from the atmosphere by inoculating the soil with superior rhizobial especially when combined with P application.

Irrespective of P application, %Ndfa by plants was similar with inoculum doses of 10⁵ to 10⁶ cells/g soil. However, either of the two doses gave significantly higher %Ndfa compared to an inoculum dose of 10⁴ cells/g soil. Therefore, for economic expediency the inoculum dose of 10⁵ cells/g soil with or without P obviously recommends itself because beyond this dose, no significant proportion of N₂ was fixed from the atmosphere. Results of this trial show that with P, significantly ($P \leq 0.05$) greater proportion of N₂ was derived from the atmosphere by the legume which received 10⁵ cells/g soil (Table 5) than without P application. For example, with and without P, inoculum sizes of 10⁴, 10⁵ and 10⁶ cells/g soil separately resulted in significantly higher amount of N-fixation compared to 10³ cells/g soil. However, compared to O P application, applying 20 ppm P resulted in significantly higher amount of fixation for either 10⁴ or 10⁵ inoculum dose (Table 5). Inoculum dose beyond 10⁵ cells/g soil led to a decrease in the amount of N fixed with and without P, the decline becoming significant when inoculation dose of 10⁶ cell/g soil was applied along with P. This finding presents a further proof that P application can promote inoculum economy.

TABLE 5

Amount of N₂ Fixed in Cowpea Inoculated with varying Inoculum strain Populations and two P Levels.

Inoculum size	OP	20ppmP	Inoculum size (mean)
0	13.81 ^d	15.84 ^d	14.82 ^c
3.2 x 10 ³	13.76	15.68 ^d	14.73 ^c
3.2 x 10 ⁴	21.47 ^c	26.45 ^b	23.96 ^b
3.2 x 10 ⁵	25.72 ^b	29.22 ^a	27.47 ^a
3.2 x 10 ⁶	25.38 ^b	26.78 ^b	26.08 ^{ab}
P application			
(mean)	20.03 ^a	22.79 ^a	
	Lsd (0.05)		%C ^{1/2}
Applied P	=	NS	28.0
Inoculum size	=	2.47	10.0
Interaction	=	2.35	6.4

Means followed by same letter (row and column) are not significantly different.

Considered either as inoculum x P interaction effect or inoculum effect alone, the dose of inoculum 10⁵ cells/g soil gave the greatest amount of N₂ fixed compared to any of the other doses used. The amount of N₂ fixation under 10⁴ cells/g soil with P was even found to be greater (by 3 and 4% respectively) than the amount fixed under either 10⁵ or 10⁶ cells/g soil without P. This underscores the synergistic role of applying P to inoculated legume (Table 5). The synergistic effect of inoculation and P application even became significant at as low 10⁴ cells/g soil in the soil used for this study which contained 2400 rhizobia/g soil. Thus, our data, like that of Mulongoy (1985), showed the importance of P in N₂ fixation of a grain legume such as cowpea. It has also been stressed by Sprent (1978) and Abbot *et al.*, (1979) that besides starter N, P application is crucial to symbiotic N₂ fixation.

Nitrogen Yield:

Increases in N yields were obtained due to increases in inoculum size with and without P application (Table 6). Increases in plant N yield were also obtained with inoculation by Abbot *et al.*, (1979). The increases in N yield became significant (P ≤ 0.05) starting with 10⁴ cells/g soil with and without P.

TABLE 6**N Yield of Cowpea Inoculated with Varying Inoculum Strain Populations and two P Levels (mg/plant)**

Inoculum Size	OP	20ppmP	Inoculum size mean
0	30.50 ^c	32.67 ^c	31.67 ^c
3.2 x 10 ³	30.67 ^c	32.67 ^c	31.58 ^c
3.2 x 10 ⁴	40.67 ^b	45.00 ^a	42.83 ^b
3.2 x 10 ⁵	44.33 ^a	46.83 ^a	45.58 ^a
3.2 x 10 ⁶	41.67 ^b	42.67 ^b	42.17 ^b
P application			
(mean)	37.57 ^a	39.97 ^a	
	Lsd (0.05)	%V	
Applied P	= NS	16.4	
Inoculum size	= 2.50	5.4	
Interaction	= 2.88	4.4	

Means followed by same letter (row and column) are not significantly different.

Irrespective of P application, N yield was greatest when inoculum size used was 10⁵ cells/g soil. Increasing inoculum dose from 10³ to 10⁶ cells/g soil led to significant ($P \leq 0.05$) losses in N yield under the two P regimes (Table 6). Application of P to the legume with 10⁴ cells/g soil gave as much N as when compared to 10⁵ cells/g soil dose without P (Table 6). These findings show that through P application, greater N yield is possible with lower inoculum dose.

However, with applied P, N yield of the plant were similar for 10⁴ and 10⁵ cells/g soil, respectively, suggesting that an optimum dose lies between 10⁴ and 10⁵ cells/g soil for N yield. Assessed across inoculum doses application of P resulted in about 15% increase over the O P treatment, showing the importance of P for increased N yield of cowpea. The need for P by nodulated legume has been emphasized by Kang and Nangju (1983). Also, considered across P application 10⁵ cells/g soil gave significant higher N yield compared to the other inoculum doses. Employing a higher dose of 10⁶ cells/g soil led to 8% reduction in N yield which was found to be significant (Table 6).

CONCLUSION

Inoculating the soil with the lowest inoculum dose (10³ cells/g soil) in this experiment with or without P application gave virtually same values for the N variables obtained from the uninoculated plants (Tables 4, 5 and 6). However, increasing the dosage to 10⁴ cells/g soil led to significant increase in these variables. These results underscore the importance of employing high inoculum population in order to obtain positive results from inoculating soil containing indigenous rhizobia. Compared to O P application, applying 20 ppm P to a

soil inoculated separately with 10^4 and 10^6 cells/g soil resulted in significant enhancements in both percent and amount of N_2 fixed by the plant from the atmosphere. Nodule number of plants with 10^6 cells/g soil was lower relative to when 10^4 cells/g soil dose was used, even though both the percent and amount of N_2 fixed were significantly higher for the former dosage. These findings show that nodule number is not a good index of legume ability to fix nitrogen. Thus, for these variables, the best inoculum size x P interaction proved to be 3.2×10^4 cells/g soil Plus P. For every inoculum dose used, P application promoted higher DMY compared to when P was not applied. Considering all the variables assessed as indices of inoculation success; a range of inoculation size 10^4 to 10^6 cells/g soil, particularly with P application, is recommended.

The findings in this trial indicate that with and without P application, there is a threshold rhizobial population below which a legume - *Rhizobium* symbiosis would not be fully expressed. The indigenous population in the soil used appeared to have fallen below this threshold, hence the responses obtained to inoculation. Phosphorus application promoted inoculation success in terms of N_2 fixation and N yield at a lower inoculum dose compared to when no P was applied.

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