PN-AAX-470

FINAL REPORT

TITLE: COMPETITION BETWEEN RHIZOBIAL INOCULANTS AND INDIGENOUS RHIZOBIA

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AWARD NUMBER: 83-CRSR-2-2320

PROJECT PERIOD: 6/1/83 through 1/31/87

OBJECTIVES

To quantitatively determine the competitiveness of rhizobial inocula to each other and to indigenous soil rhizobia in order to assess whether the kinetic model so derived can be used to predict the success or failure of Superior strain inocula in the field.

RESULTS AND DISCUSSION

The following publications and manuscript are enclosed. These studies were all supported and so acknowledge by this grant.

1. G. A. El Hassan, B. S. Hernandez and D. D. Focht. 1986. Comparison of Hup trait and intrinsic antibiotic resistance for assessing rhizobial competitiveness axenically and in soil. App. and Env. Microbiol. 51:546-551.

2. G. A. El Hassan and D. D. Focht. 1986. Comparison of inoculant and indigenous rhizobial dinitrogen fixation in cowpeas by direct nitrogen-15 analyses. Soil Sci. Soc. Am. J. 50:923-927.

3. M. Poth, J. S. LaFavre and D. D. Focht. 1986. Quantification by direct ^{15}N dilution of fixed N_2 incorporation into soil by Cajanus cajan (Pigeon pea). Soil Biol. Biochem. 18:125-127.

4. B. S. Hernandez, M. Poth and D. D. Focht. 1987. Increased effectiveness of competitive rhizobia strains upon inoculation of Cajanus cajan. Appl. Environ. Microbiol. (Revised manuscript returned to Editor after review and conditional acceptance.)

Publication #1 involves two parts using 6 different inocula strains with Cajanus cajan: (1) an axenic study using different ratios of two different inocula to assess their competitiveness against one another; (2) the use of four of these competitive strains (P 132, IHP 147, 401, 11A1) against indigenous soil rhizobia in green house studies. The study shows that in axenic culture, competitiveness of dual strains conforms to a fractile distribution plot, so that infectivity indices can be calculated. The P 132 was the best competitor and had the highest infectivity index among all tested. The greenhouse studies were consistent with the axenic studies: P 132 was the best competitor.

Publication #2 results from a study built upon the work of the earlier study except that the cultivar (Vigna unguiculata) was grown in large pots containing ^{15}N enriched soil. Different inocula densities were used for the same 6 strains employed in Publication #1. This study also showed that P 132 was the best competitor, gave the highest yield response, and fixed the most N. Presumptive evidence was presented to show that P 132 was also a good survivor, having been inoculated two

years before and still dominating over the other strains and indigenous rhizobia.

Publication #3 is not directly involved in the objectives of the project, but is a tangential "spin-off". This work shows that about the same amount of N is added to the soil by N_2 fixation in Cajanus cajan as in the aerial portion of the plant.

Publication #4 represents a field lysimeter study with ^{15}N enriched soil employing the same four inoculant strains used in the previous publications. This study shows that all strains are more competitive than the indigenous rhizobia and give better yield responses and higher BNF. Again P 132 was clearly the superior competitor and gave the highest yields. Unlike the other 3 inoculants, it formed 100% of all nodules on the plant.

These results clearly establish that P 132, a strain isolated from Panama, is superior to all other "superior" strains [1HP147 (ICRISAT), 401 (Cornell), 22A1, A26, A28 (Nitragin)] tested. The results obtained from axenic in vitro studies were useful in predicting success for P 132 in greenhouse and field studies. This isolate is clearly the best one yet demonstrated for superior competitiveness and performance in Cajanus cajan and Vigna unguiculata.

XN-NAX-470-A

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1986, p. 546–551
0099-2240/86/030546-06$02.00/0
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Vol. 51, No. 3

Comparison of Hup Trait and Intrinsic Antibiotic Resistance for Assessing Rhizobial Competitiveness Axenically and in Soil

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Received 12 November 1985/Accepted 10 December 1985

The competitiveness of dual-strain inocula of cowpea rhizobia for nodulation of *Vigna unguiculata* (L.) Walp. was studied axenically between one slow-growing strain (P132, HP147, 401, or 22A1) and one fast-growing strain (176A26 or 176A28) at logarithmic inoculum ratios ranging from 10^3 to 10^{-3}. Nodule infectivity was determined by multiple intrinsic antibiotic resistance, since both fast-growing strains were sensitive. Different hydrogen uptake (Hup) efficiencies of dual-strain inocula allowed for the comparison of an indirect rapid method. Infectivity data based on antibiotic resistance and Hup efficiency were fit to linearized fractile plots of log-normal distributions to determine C_{AB} (percent infectivity at a 1:1 inoculum density) or I_{50} (inoculum ratio at 50% infectivity). The slow growers were always better competitors and had I_{50} values which ranged from 7 to 160,000 and C_{AB} values which ranged from 62 to 97%. P132 was the best competitor of all those tested. Antibiotic resistance and Hup efficiency methods were in agreement with 401 (Hup^-) and 176A26 (Hup^+), but the Hup efficiency method overestimated the I_{50} index with 22A1 (Hup^-) and 176A28 (Hup^+). The competition of each of the four slow-growing strains with indigenous rhizobia was examined in *Cajanus cajan* from three tropical soils. Nodule infectivity for all strains ranged from 42 to 96%, and P132 was the best competitor in all the soils. Hup efficiency overestimated infectivity by about 2-fold when Hup^+ inocula (P132 and HP147) were used but underestimated infectivity by more than 100-fold when Hup^- inocula (401 and 22A1) were used. Although the Hup trait has limited quantitative usage axenically, it is only qualitative in soil competition studies and can only be used with Hup^+ inocula.

The methods most commonly used for the identification of inoculum strains from nodules are immunofluorescence (3), immunodiffusion (4, 22), and antibiotic resistance (1, 9, 10, 16). These methods are all very time-consuming. A more rapid and less tedious procedure for assessing nodule infectivity between competing strains would be to measure specific differences in nodule metabolism. One such metabolic function that is directly pertinent to nitrogenase activity is the hydrogen uptake (Hup) trait (17). La Favre and Focht (11) showed that differences in Hup activity between inoculum strains and indigenous soil rhizobia were consistent with infectivity determinations from nodule typing by antibiotic resistance. Yet, to our knowledge, no attempt has been made to determine if the Hup trait can be used to assess nodule infectivity.

There has been considerable interest in fast-growing cowpea rhizobia because they are easy to work with and are suitable for genetic manipulation and large-scale production of inoculants. Consequently, we chose to evaluate the competitiveness between fast and slow growers as well as between Hup^+ and Hup^- rhizobia. Nicol and Thornton (15) stated that a faster-growing strain would be the better competitor for nodulation, but Franco and Vincent (5) found just the opposite. Trinick et al. (19) found that the fast-growing rhizobium strain NGR-234 was a better competitor against slow-growing rhizobia at 23 to 25°C but found that the opposite was true at the more realistic tropical temperature of 26 to 30°C.

We therefore undertook this study to determine if the Hup trait can be used as a marker for the identification of competing strains in both axenic and nonaxenic studies and to examine the competitive nodulation abilities of fast- and slow-growing rhizobia.

MATERIALS AND METHODS

Rhizobium strains. Strain 22A1, a Hup^- slow grower (doubling time [t_d], 13 h) was obtained from J. C. Burton of Nitragin Co., Milwaukee, Wis., and was originally isolated from *Vigna unguiculata* (L.) Walp. The sources and physiological characteristics of strains 176A26 (t_d, 1.7 h), 176A28 (t_d, 4.6 h), 401 (t_d, 11 h), P132 (t_d, 16 h), and HP147 (t_d, 30 h) have been described previously (7).

Media and inoculants. The maintenance medium for all the strains was yeast extract-mannitol agar. It was used as a solid medium either on petri plates or in tubes as slants to maintain the strains. Glutamate-yeast extract was used as a solution medium to grow cells for inocula in axenic studies, and yeast extract-mannitol was used for the preparation of inoculants for soil studies. Both media have the following mineral salts composition per liter of water: 1.0 g of KH_2PO_4, 1.0 g of K_2HPO_4, 0.2 g of NaCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of yeast extract, 0.5 mg of $FeSO_4$, and 1.0 mg of $CaCl_2$. In addition, glutamate-yeast extract contains 5.0 g of glutamate (monosodium salt), and yeast extract-mannitol agar contains 1.0 g of KNO_3, 10.0 g of mannitol, and 15.0 g of agar. The pH was adjusted to 7.0 with NaOH before autoclaving at 121°C and 18 lb/in^2 for 15 min.

Inocula were prepared by growing rhizobia in sidearm flasks (250 ml) containing 25 ml of glutamate-yeast extract or yeast extract-mannitol medium. Flasks were placed on rotary shakers (100 rpm) and incubated at 28°C until an optical density (at 525 nm) of 0.70 was obtained.

Axenic plant cultures. Cowpeas [*V. unguiculata* (L.)

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Walp., var. California no. 5 blackeye] were grown in culture tubes (38 by 200 mm). The tubes were filled to 2 cm with gravel and then fine vermiculite (washed twice with deionized water), and a watering tube (8 mm in diameter) was inserted into the culture tube on top of the gravel to facilitate the addition of N-free nutrient solution and water (21). The tubes were saturated with N-free nutrient solution, covered with aluminum foil, and autoclaved for 30 min at 121°C. Autoclaved gravel (1 cm) was added to the top of the tube to minimize contamination and reduce evaporation.

Cowpea seeds were surface sterilized in 0.1% $HgCl_2$ for 4 min and then rinsed in 10 changes of sterile distilled water before they were planted aseptically in the culture tubes, one seed per tube. Extra tubes were planted to ensure that a sufficient number of healthy plants would be available prior to the start of the experiment. The aluminum cover was retained on top of the tube until plant emergence. At that time, each plant was inoculated with a 2-ml mixture of glutamate-yeast extract broth cultures containing 1 ml of each of two competing strains in the following inoculum ratios: 10^3, 10^2, 10^1, 10^0, 10^{-1}, 10^{-2}, and 10^{-3}. Gravel (~1 cm) added after inoculation, and sterilized nutrient solution and water were added alternately to the culture tubes through the watering tube as needed. Four replicate tubes for each inoculum strain and four uninoculated controls were used. The tubes were placed in a randomized block design on a rack which was covered on all sides with wood boards to protect the roots from light. The temperature in the greenhouse was maintained at between 21 and 27°C. A mercury lamp was installed in the greenhouse to add supplemental lighting, since the experiment was performed during the winter months. Plants were harvested 6 weeks after planting for the determination of acetylene reduction rates, H_2 evolution rates, dry mass, and nodule occupancy.

Soil plant cultures. Pigeon pea [*Cajanus cajan* (L.) Millsp., var. 64–26] seeds were inoculated separately with P132 (Hup^+), HP147 (Hup^+), 401 (Hup^-) and 22A1 (Hup^-) and placed in pots containing 3.5 kg of soil. The method of inoculation and the Bayano soil used in this study have been described previously (7a). The other two tropical soils used in this study were a fine, mixed, hyperthermic Typic Hapludult (Tocumen) of pH 5.4 and a fine, mixed, hyperthermic Ultic Haplorthox (Los Lotes) of pH 4.2. All pots, including the uninoculated control, were replicated four times and treated with PO_4^{-3}-P (75 kg · ha^{-1}), Ca^{+2} (909 kg · ha^{-1}), and MoO_4^{-2} (3.2 kg · ha^{-1}) to alleviate mineral deficiencies and acidity. Plants were grown in greenhouses at the University of Panama for 60 days, after which determinations of dry mass, H_2 production, acetylene reduction activity, and nodule occupancy were made.

Acetylene reduction and hydrogenase assays. Hup efficiency in axenic and soil systems was determined with the formula of Schubert et al. (18), whereby percent relative efficiency = [1 −(H_2 evolved/C_2H_2 reduced)] × 100. Plant shoots were cut off, and roots were removed from tubes. Vermiculite or soil was gently washed from the roots, and the entire root system of each plant was placed in a widemouthed Erlenmyer flask (250 ml) containing a moistened filter paper. Each flask was closed with a rubber stopper containing a glass tube and serum cap for gas sampling purposes. Each flask was incubated for 1 h and subsequently sampled for H_2 by withdrawal of a 10-ml sample, which was then stored in a VACUTAINER (Becton Dickinson Vacutainer Systems, Rutherford, N.J.) bottle for not more than 3 days prior to analysis by gas chromatography. Several preliminary experiments with known amounts of H_2 indicated no loss when the samples were stored under these conditions. A 25-ml quantity of air was removed from each flask and replaced with a 25-ml injection of acetylene. Each flask was incubated for 25 min, whereupon a sample was removed and analyzed by gas chromatography for ethylene. Gas-chromatographic analyses for H_2 and ethylene have been described elsewhere (7a, 11).

The fractional efficiency of each of the competing strains was determined with the following equation: $f_B = (C - A)/(B - A)$ where f_B = the fraction of activity due to strain B, B = the efficiency of strain B, A = the efficiency of strain A, and C = the observed efficiency of the mixture.

Nodule typing from axenic plant cultures. Roots were washed free of vermiculite, and 15 nodules were randomly selected from each replicate tube and pooled together to yield 60 nodules per determination. The nodules were surface sterilized by immersion in 95% ethanol for 30 s and then treated with 0.2% $HgCl_2$ (acidified with a 5-ml · $liter^{-1}$ HCl solution) for 4 min. The nodules were then rinsed in five changes of sterile distilled water. Further handling of the nodules was done under aseptic conditions. Each nodule was cut in half with a razor blade that was sterilized prior to each cutting by immersion in ethanol followed by flaming. Each half was pressed onto an agar plate (yeast extract-mannitol agar) containing the following concentrations of antibiotics to which the four slow-growing strains were resistant: rifampin (60 μg · ml^{-1}) and streptomycin (200 U · ml^{-1}), HP147; kanamycin (60 μg · ml^{-1}) and tetracycline (60 μg · ml^{-1}), P132; and penicillin (200 U · ml^{-1}) and streptomycin (200 U · min^{-1}), 401 and 22A1. The plates were incubated at 28°C, and results were recorded after 5 to 7 days of incubation. All media contained 20 μg of cycloheximide^{-1} (Calbiochem-Behring, La Jolla, Calif.) to inhibit the growth of fungal contaminants.

Nodule typing from soil plant cultures. Preliminary studies were conducted to determine the concentrations of three antibiotics which permitted the growth of the four inoculant strains used for assessing competitiveness against indigenous rhizobia. P132, HP147, 401, and 22A1 were resistant to the following combined concentrations in yeast extract-mannitol agar: nalidixic acid (100 μg · ml^{-1}), erythromycin (50 μg · ml^{-1}), and rifampin (100 μg · ml^{-1}). 176A26 and 176A28 were not used in this study because they were sensitive to the levels of antibiotics needed to suppress the growth of indigenous rhizobia. The preparation and methodology for nodule typing were identical to those described above except that 10 nodules were randomly selected from each replicate pot and pooled together to yield 40 nodules per determination.

RESULTS AND DISCUSSION

There were no significant differences in shoot dry matter between axenic and nonaxenic inoculated cultures. The greatest differences in dry matter occurred in soils, in which the following yields (grams · $plant^{-1}$) and ranges were observed: Los Lotes (2.58 to 2.72), Tocumen (3.87 to 4.29), and Bayano (8.48 to 8.80). Only with the axenic treatments were the yields greater in inoculated (1.13 ± 0.15 g · $plant^{-1}$) than in uninoculated (0.73 ± 0.081 g · $plant^{-1}$) cultures.

If a 1:1 mixture of two different rhizobial strains produced equal infectivity, the ratio of population density to infectivity would be arithmetic and normally distributed. This is obviously not the case, as shown by others (1, 16, 19) who formulated equations which described competitiveness as a logarithmic function. Thus, populations which are lognormally distributed should be transformable to the fractile

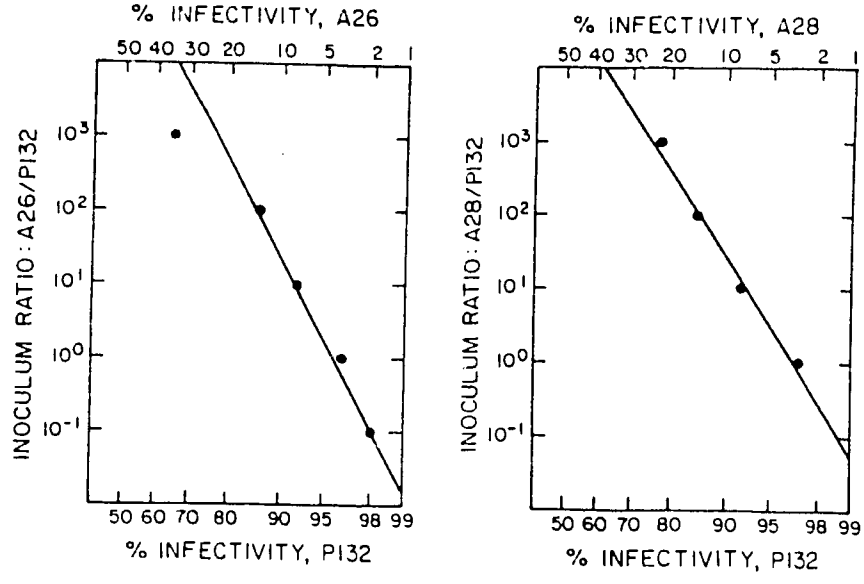

FIG. 1. Nodule infectivity as a function of dual-inoculum ratios of strain P132 versus 176A26 and 176A28. Each point was derived from 60 samples.

plots shown in Fig. 1 to 4. The midpoint (50%) representing the ratio of the dual-inoculum density which produces equal infectivity of nodules is referred to as the I_{50} index. Others (1, 20) have used the C_{AB} index, which represents the frequency of infectivity of two different strains present in equal quantities. This index can be found from the fractile plots in Fig. 1 to 4 where the line intersects the 10^0 inoculum ratio. Further details regarding transformations of log-normally distributed populations to fractile plots can be found elsewhere (6).

Since the data generally conform to the linearized transformations shown in Fig. 1 to 4, the I_{50} index can be determined among highly competitive strains; such a determination would otherwise be empirically impossible. P132 was clearly the best competitor against the two fast-growing strains. The I_{50} values of 1.6×10^5 and 6.3×10^4 against 176A26 and 176A28, respectively, were orders of magnitude greater than the I_{50} values attained by the other three slow-growing strains (HP147, 22A1, and 401) against the same fast-growing strains. In no instance were the fast growers the dominant competitors.

Although it has been inferred that growth rate might be an important criterion for competitiveness in nodulation (15), the results reported here do not support this concept. In all cases, the two fast-growing strains 176A26 and 176A28 required 7 and 1.6×10^5 more cells, respectively, per slow-growing competitor to effect an equal infectivity (I_{50}) of the plant. Thus, the observation by Trinick et al. (19) that the rate of growth on laboratory media did not always correlate with better competitive ability was consistent with our own.

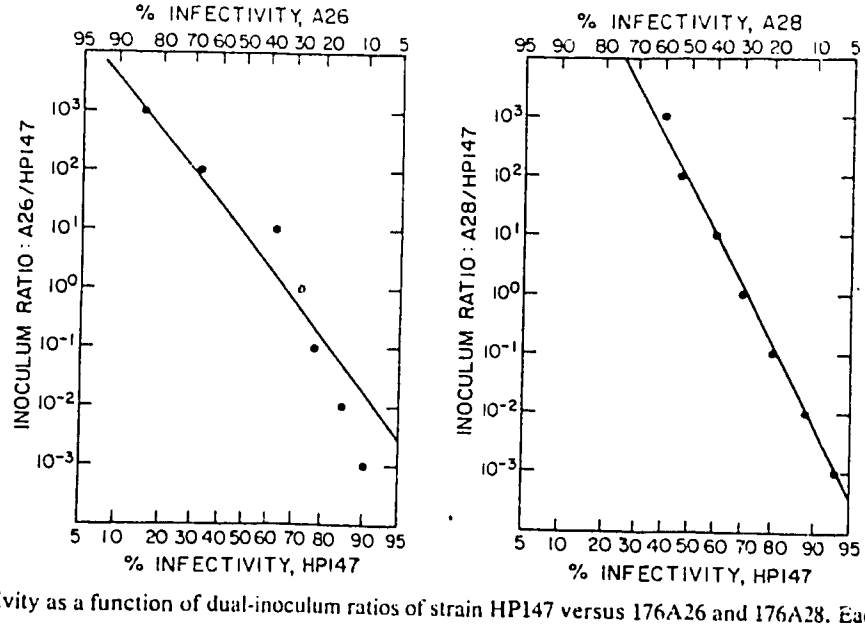

FIG. 2. Nodule infectivity as a function of dual-inoculum ratios of strain HP147 versus 176A26 and 176A28. Each point was derived from 60 samples.

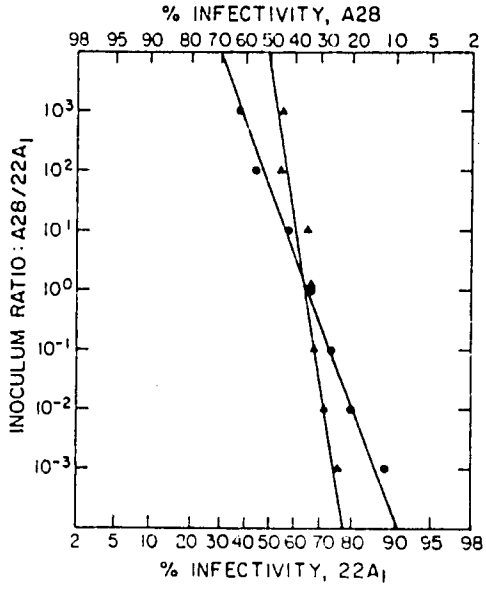

FIG. 3. Comparison of nodule infectivity with dual-inoculum ratios of strains 22A1 and 176A28 as determined by antibiotic resistance (●) and Hup efficiency (▲). Antibiotic resistance points were derived from 60 samples, and Hup efficiency points were derived from 3 samples.

Franco and Vincent (5), who used an agar test tube method, also found that slow growers were better competitors than fast growers.

The dominance of P132 as a competitor was also demonstrated in all three soils tested (Table 1). It occupied 68, 87, and 96% of all the nodules typed from the Bayano, Los Lotes, and Tocumen soils, respectively. The recoveries were numerically higher than those found with any of the other three slow-growing strains; in only two comparisons

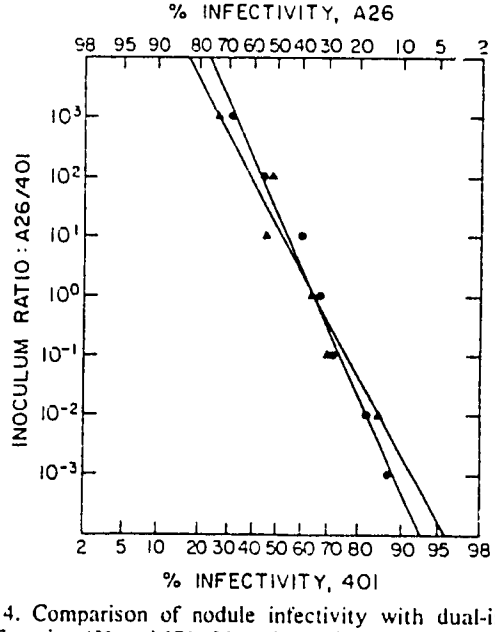

FIG. 4. Comparison of nodule infectivity with dual-inoculum ratios of strains 401 and 176A26 as determined by antibiotic resistance (●) and Hup efficiency (▲). Antibiotic resistance points were derived from 60 samples, and Hup efficiency points were derived from 3 samples.

TABLE 1. Frequency of inoculum strain infectivity in nodules of *C. cajan* as determined by selective multiple antibiotic resistance

Soil	% of nodules infected by inoculum strain[a]:				
	None	P132	HP147	401	22A1
Bayano	0.02a	0.68d	0.42b	0.48b	0.54bc
Tocumen	0.02a	0.[illegible]of	0.89ef	0.77de	0.79de
Los Lotes	0.00a	0.87ef	0.66cd	0.78de	0.77cd

[a] Numbers followed by the same letter are not significantly different ($P \leq 0.05$) among columns and rows by multiple paired comparisons of normalized binomial distributions (14).

were these values statistically the same (Table 1, Tocumen HP147 and Los Lotes 401).

It is unclear whether inoculum density has any effect upon competition between strains. Since the number of viable cells will drastically be reduced as a function of time (2) (i.e., from inoculation to infection), the I_{50} will also be dependent upon the survival of inoculum strains. Bohlool and Schmidt (3) showed that the percentage of *R. japonicum* 110 nodules declined from 85 to 55 to 41% in 0 to 10 to 30 days, respectively, indicating that the initial population density was reduced by the time infection occurred. Thus, depending on differences in survival rates between two competing populations, the true I_{50} value at the time of infection may be higher or lower than the indices reported here. Ireland and Vincent (8) reported that both relative numbers and differences in competitive ability among strains are important in determining which rhizobium strain will form nodules on the legume host. Moreover, Weaver and Frederick (20) indicated that a high inoculum density may shorten the time of nodulation if the soil population is below a certain level, even though the total nodule weight formed on the plant later may not increase. On the other hand, Means et al. (13) reported that competition was often found to be independent of relative numbers of rhizobium strains, since strain 76, which induced chlorosis, was responsible for the formation of 85% of the nodules formed on soybeans even though it constituted as little as 1% of the population of the seed-applied inoculum.

The Hup trait was not as accurate as antibiotic resistance in determining nodule infectivity. Although there was good agreement between the axenic mixture of 401 and 176A26 (Table 2 and Fig. 4), the agreement between the axenic mixture of 22A1 and 176A28 was poor (Table 2 and Fig. 3). Coincidentally, the C_{AB} values (65 and 35%, respectively) were the same for both mixtures and both methods. Since the Hup trait is theoretically applicable only when the indigenous rhizobia have Hup efficiencies significantly different from those of the inoculum strains, only limited comparisons could be made between the two methods. With the Hup$^+$ strains in the Bayano soil, infectivities as deter-

TABLE 2. Infectivity index (I_{50}) in axenic plant culture tubes as determined from nodule typing by intrinsic antibiotic resistance

Inoculum strains	Infectivity index[a]
P132 and 176A26	160,000
P132 and 176A28	63,000
HP14. and 176A28	70
HP147 and 176A26	7
22A1 and 176A28	50 (7,000)
401 and 176A26	25 (16)

[a] Numbers in parentheses were determined by the Hup efficiency method.

TABLE 3. Percent relative Hup efficiency as determined from root nodules of *C. cajan* previously inoculated with rhizobia

System	% Hup efficiency for inoculum strain[a]:				
	None	P132	HP147	401	22A1
Axenic		100a	91a	55c	56c
Bayano soil	82b	98a	91a	77b	84b
Tocumen soil	96a	99a	98a	95a	95a
Los Lotes soil	97a	98a	97a	98a	99a

[a] Numbers followed by the same letter are not significantly different ($P \leq 0.05$) among columns and rows by Duncan's multiple range test.

mined by antibiotic resistance and Hup efficiency (Tables 1 and 3) were, respectively, 68 versus 89% (P132) and 42 versus 100% (HP147). There was even less agreement between the two methods when the Hup$^-$ strains were considered. The respective infectivities as determined by antibiotic resistance and Hup efficiency were as follows: strain 401, 48 versus 23% (Bayano soil), 77 versus 2% (Tocumen soil), and 78 versus 0% (Los Lotes soil); strain 22A1, 54 versus 0% (Bayano soil), 79 versus 2% (Tocumen soil), and 77 versus 0% (Los Lotes soil).

In soil, the Hup method overestimated the competitiveness of Hup$^+$ inoculum strains and underestimated the competitiveness of Hup$^-$ inoculum strains. Although it is possible that the more active Hup$^+$ nodules are a sink for exogenous H_2 produced by the Hup$^-$ nodules of the root system, we were never able to detect Hup in flasks incubated with Hup$^+$ nodules. D. J. Arp (personal communication) has observed that only crushed nodules take up H_2. Moreover, axenic mixtures of Hup$^-$ and Hup$^+$ nodules should give consistently higher infectivity frequencies when determined by Hup efficiency than when determined by antibiotic resistance. No such relationship was noted between 22A1 and 176A28 or between 401 and 176A26. Let us consider that a third member of the H_2 metabolic community may be present on the surfaces of nonaxenic nodules but not axenic nodules. Since it is not possible to remove the rhizoplane microflora from the entire root system, net measurements of H_2 consumption would always be greater than measurements of intranodule H_2 consumption alone. Therefore, the infectivity of Hup$^+$ inocula would be overestimated, whereas the infectivity of Hup$^-$ inocula would be underestimated. It has in fact been well established that H_2-oxidizing bacteria colonize the nodule in its rhizosphere in soil and that their numbers and activity are directly proportional to an H_2 gradient and inversely proportional to the Hup efficiency of the rhizobial endophyte (12). In light of these observations, it should come as no surprise that the agreement between the Hup efficiency and antibiotic resistance methods in determining nodule infectivity was poorer when inocula were Hup$^-$ than when they were Hup$^+$.

Nodule typing by antibiotic resistance is very tedious and time-consuming and requires 2 to 3 h for typing 50 to 60 nodules from a single treatment of four plants. By comparison, Hup efficiency requires only 40 min of analysis by gas chromatography (10 min per plant with four replicates) for a combined assay of ethylene and H_2. Thus, the ease and rapidity with which results are obtained with the Hup method make this a potentially useful screening tool for competition studies of rhizobia. However, the method can only be used for soil competition studies when Hup$^+$ inocula are used to challenge indigenous rhizobia, which have significantly lower Hup efficiencies. Until the contribution of H_2 metabolism from other bacteria in the rhizoplane can be determined or eliminated during the assay, the Hup method can only provide circumstantial evidence of competitive success.

ACKNOWLEDGMENTS

This work was supported in part by grant 83-CRSR-2-2320 from USDA/SEA/AID. G.A.E.H. is grateful for financial assistance received from the Democratic Republic of the Sudan during this project.

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Comparison of Inoculant and Indigenous Rhizobial Dinitrogen Fixation in Cowpeas by Direct Nitrogen-15 Analyses

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XN-AAX-470-B

Reprinted from the *Soil Science Society of America Journal*
Volume 50, no. 4, July–August 1986
677 South Segoe Rd., Madison, WI 53711 USA

Comparison of Inoculant and Indigenous Rhizobial Dinitrogen Fixation in Cowpeas by Direct Nitrogen-15 Analyses[1]

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ABSTRACT

Soil that contained ^{15}N enriched organic matter (0.461 % ^{15}N) was used to determine competitiveness of six strains at different logarithmic inoculum densities against indigenous rhizobia and against a previous surviving inoculant (strain P132). Analyses of N content of plant tissues by direct ^{15}N technique showed that cowpeas (*Vigna unguiculata* L. Walp.) were capable of deriving 60 to 98% of shoot N from N_2 fixation. The two fast-growing strains (176A26 and 176A28) were poorer competitors and fixed less N_2 compared to the other slow-growing strains. Inoculum density had no effect upon yield response of cowpeas, but inoculation with strains P132, 401, and 22A1 effected greater seed yield, shoot dry matter, total N, and percentage of N derived from fixation (86–98%) than other strains and the uninoculated control (60–73%). By contrast, N_2 fixation and yield parameters in inoculated cowpeas were not significantly different from inoculated controls that contained residual P132 from a previous inoculum study. The higher hydrogen uptake (Hup) efficiency of nodules containing residual P132 (98 ± 2%) facilitated presumptive identification of P132 (100% ± 0 Hup efficiency axenically) as the surviving and infecting inoculant strain since nodules infected by indigenous rhizobia had lower Hup efficiencies (88 ± 2%).

Additional Index Words: ^{15}N isotopes, *Vigna unguiculta* (L.) Walp., Hup^+, Hup^-, competition.

ElHassan, G.A., and D.D. Focht. 1986. Comparison of inoculant and indigenous rhizobial dinitrogen fixation in cowpeas by direct nitrogen-15 analyses. Soil Sci. Soc. Am. J. 50:923–927.

SUPERIOR STRAIN INOCULA should give higher rates of biological nitrogen fixation (BNF) and a subsequently larger amount of plant N derived from this process than from the soil. Specific quantitative methods of assessing how much N is derived from N fixation and from soil N can only be determined with ^{15}N isotopes. There has consequently been considerable interest recently in the use of ^{15}N for determination of BNF. The isotope dilution method involves the use of ^{15}N fertilizer (NH_4^+, NO_3^-, or urea), and the comparison of its uptake between a nodulating and non-nodulating isoline of soybeans [*Glycine max* (L.) Merr.] (Diebert et al., 1979). Using this methodology, it has generally been shown that about equal amounts of plant N are derived from both sources (soil and BNF) with soybeans (Ham, 1978; Rennie et al., 1982; Rennie and Kemp, 1983). There are two major problems, however, with the indirect ^{15}N dilution method. First, the assumption that nodulating and nonnodulating isolines derived the same amount of N from the soil and fertilizer N has never been verified, and in fact may be inaccurate (Crisswell et al., 1976). Second, nonnodulating isolines of cowpea rhizobia are unknown, and cowpeas [*Vigna unguiculata* (L.) Walp.] are always nodulated in soil, presumably because cowpea rhizobia are ubiquitous and because cowpea is less host-specific than soybean.

In a recent study, LaFavre and Focht (1983) determined N_2 fixation in pigeon peas (*Cajanus cajan* Millsp.) by using a new direct ^{15}N method in which the soil organic N was enriched in ^{15}N as a result of a previous ^{15}N isotope study (Focht and Stolzy, 1978). Their results showed that 91 to 94% of plant N was derived from N_2 fixation with all unfertilized treatments; this supported Quilt and Dalal's (1979) contention that pigeon pea could derive most of its N from fixation in low N-containing soils. Thus, the use of an ^{15}N enriched soil would permit the direct comparison of BNF in cowpea among different strain inocula.

Cowpea is native to Africa, yet it is also grown extensively in Latin America, the southern USA, and southeast Asia. This crop is tolerant to both high temperature and periods of drought (Rachie, 1974). Because it fixes N, cowpeas are also very important crops in the less developed countries (LDCs) for N_2 addition in rotations with other grain and forage crops. However, information is still scanty regarding the need for inoculation of this crop in the LDCs.

Many studies have shown that competition among rhizobia is a major practical limitation to establish-

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ment of superior N_2 fixing inoculant strains in nodules (Amarger and Lobreau, 1982; Bohlool and Schmidt, 1973; Weaver and Frederick, 1974; Johnson et al., 1965; Boon-Kerd et al., 1978). However, Kremer and Peterson (1982) reported that inoculant strains were apparently able to compete effectively with indigenous rhizobia for nodule sites—particularly the cowpea rhizobia, which occupied the majority of the nodules despite the presence of indigenous rhizobia in soil ($>10^3$ g^{-1}).

Because farmers in the LDCs cannot afford to inoculate on a year-to-year basis, it would be desirable to know if superior inoculum strains could survive in soil for several years and compete with less effective indigenous rhizobia. The following study was thus undertaken to (i) evaluate the competition of inocula strains against indigenous rhizobia; (ii) to assess the survival and competitiveness of a highly effective strain (P132) that had been inoculated in soil 2 yr ago; and (iii) to measure yield parameters and the amount of N derived from BNF and from the soil by using direct ^{15}N methodology.

MATERIALS AND METHODS

Soil

This study was done in the same soil used by laFavre and Focht (1983) for growing pigeon peas. The soil was comprised of a 1:1 (w/w) mixture of a San Emigdio and Greenfield soil, both of which were sandy loams (mixed, thermic Typic Xerothents) used and described previously by Focht and Stolzy (1978). Uninoculated soil and soil inoculated with P132 from that study were examined to determine the success of inocula strains against indigenous rhizobia and to assess the survival and competitive ability of P132 2 yr after its addition to this soil. All soil contained ^{15}N enriched organic matter (0.461% ^{15}N; total N of 0.042%) as determined by Kjeldahl digestion (Bremner and Mulvaney, 1982) and ^{15}N analysis (Bremner, 1965).

In order to accomplish this study, two experiments were designed: I, inocula strains vs. indigenous rhizobia; II, inocula strains vs. indigenous rhizobia plus P132, which was inoculated 2 yr earlier. We will refer to them in the text as Exp. I and II.

Rhizobium Strains

Strains 176A26, 176A28, and 22A1 (isolated from cowpea) were obtained from Dr. J. C. Burton of Nitragin Co., Milwaukee, WI. Strain 401 (isolated from cowpea) was obtained from Dr. M. Alexander, Cornell Univ. Strain P132 (isolated from cowpea) was obtained from B. C. Hernandez, Univ. of Panama. Strain HP147 (isolated from pigeon pea) was obtained from Dr. P. Dart, ICRISAT, India. HP147, 401, 22A1, 176A26, and 176A28 were used in both experiments, while P132 was used only in Exp. I. Physiology of these strains has been described elsewhere (Hernandez and Focht, 1984; ElHassan et al., 1986).

Media

Maintenance medium for all strains was yeast-extract mannitol agar (YEMA). It was used as a solid medium either for petri plates or in tubes as slants to maintain strains. However, glutamate yeast extract (GYE) was used as a liquid medium for growing cells because generation times were shorter, and maximum cell densities were greater than with YEM. Both media have the following mineral salts composition per liter of water: 1.0-g KH_2PO_4, 1.0-g K_2HPO_4, 0.2-g NaCl, 0.1-g $MgSO_4 \cdot 7H_2O$, 1.0-g yeast-extract, 0.5-mg $FeSO_4$, and 1.0-mg $CaCl_2$. In addition, GYE has 5.0-g glutamate (monosodium salt), and YEMA has 1.0-g KNO_3, 10.0-g mannitol, and 15.0-g agar. The pH was adjusted to 7.0 with NaOH before autoclaving at 121°C and 1.2×10^5 Pa (18 psi) for 15 min.

The cells were grown in 25 mL of GYE medium on a rotary shaker (100 rpm) at 28°C for all sources of inocula.

Inocula

Inocula were prepared by growing rhizobia in side-arm flasks (250 mL) containing 25mL of GYE medium. Flasks were placed on rotary shakers (100 rpm) and inoculated at 28°C until an optical density (525 nm) of 0.70 was obtained.

Inocula for Exp. I contained (cells mL^{-1}): 10^9, 3×10^8, 10^8, 3×10^7, 10^7, 3×10^6, 10^6, 3×10^5, 10^5, 3×10^4, 10^4, 3×10^3, 10^3, 3×10^2, and 10^2. Cell concentrations from 3×10^8 to 10^2 were made with sterile distilled water starting with a 10^9 cells mL^{-1} inoculum. With Exp. II, only the 10^9 cells mL^{-1} inoculum was used, since this experiment was not intended to compare inoculum densities with indigenous rhizobia, but rather to determine if P132 competed favorably with inoculum strains at the highest inoculum density.

Plant Growth and Analyses

Two-gallon plastic nursery pots, 21-cm tall and 21 and 17 cm in diameter at the top and bottom, respectively, with four drainage holes, were lined with polyethylene bags and filled with 14.5 kg ^{15}N-labeled soil. Nine tensiometers were installed randomly among the pots to monitor the water potential. The ceramic cup was placed below the soil where the highest root activity was expected.

Cowpea, variety California no. 5 Blackeye, was used in these experiments. Seeds were surface sterilized in 0.1% $HgCl_2$ for 4 min and then rinsed in 10 changes of sterile distilled water. Seeds were planted, four per pot, and plants were thinned to one per pot, 10 d after planting, to retain seedlings of the most uniform growth. Each remaining seedling was inoculated by delivering 5 mL of glutamate-yeast extract broth culture to a hole dug next to the seedling.

Treatments for Exp. I included 6 strains × 15 cell concentrations (as mentioned previously) × 4 reps + 5 control plants (not inoculated). Experiment II was not designed to include different inoculum densities: it consisted of five inoculated and one uninoculated (residual P132) treatments, each replicated four times. Treatments were placed in a randomized block design. Tensiometers were checked daily, and pots were filled to the top with deionized water when the tensiometer reading exceeded 0.004 MPa. Temperature in the greenhouse was maintained between 24 and 27°C.

After completion of acetylene reduction assays (ARA) and hydrogenase assays, pods were harvested at the end of the experiments, dried at 60°C in a forced-draft oven, and subsequently shelled to remove the seeds. Total dry pod, pea, shoot, and root weights were determined separately. The N concentration in leaf, pod, and pea was determined by a micro-Kjeldahl procedure (Bremner and Mulvaney, 1982).

Acetylene Reduction and Hydrogenase Assays

At 60 d after planting, plant shots were cut off and roots removed from soil for later analyses. The soil was gently washed from the roots, and the entire root system of each plant was placed in a wide-mouth Erlenmyer flask (250 mL) containing a moistened filter paper. The flasks were closed with a rubber stopper containing a glass tube and serum cap for gas sampling purposes. Each flask was incubated for 1 h and subsequently sampled for H_2. Thereafter, 25 mL of air was removed from the flasks, and replaced with a 25-mL

Table 1. Comparison of yield and N_2-fixation parameters in cowpea as affected by competition between inocula strains and indigenous (control) rhizobia.

Inoculum strain	ARA†	% relative efficiency‡	Shoot dry weight	Nodule dry weight	Pod + seed dry weight	Pod no.
	nmol plant^{-1} s^{-1}		g plant^{-1}			
P132	4.20ab*	97a	17.5a	1.3b	9.5a	6.3a
22A1	3.50abc	88b	17.6a	1.5a	9.8a	6.4a
176A26	3.42abc	97a	17.9a	1.5a	10.3a	6.0a
176A28	3.03abc	97a	17.6a	1.4ab	10.1a	6.2a
HP147	2.69bc	95a	17.0a	1.4ab	9.9a	5.6a
401	2.33c	ND	17.1a	1.4ab	10.4a	6.1a
Control	4.50a	88b	15.5b	0.5c	6.2b	5.6a

* Numbers followed by the same letter within columns are not significantly different at $p = 0.05$ by Duncan's Multiple Range Test; ND = not determined.

† Acetylene reduction assay, nmol C_2H_4 plant^{-1} s^{-1}.

‡ $\left[1 - \frac{\text{nmol } H_2 \text{ plant}^{-1}\text{ s}^{-1}}{\text{nmol } C_2H_4 \text{ plant}^{-1}\text{ s}^{-1}}\right] \times 100.$

injection of acetylene. The flask was incubated for 25 min, whereupon a sample was removed and analyzed by gas chromatography for ethylene.

Hydrogen gas was determined on a Varian 3700 gas chromatograph equipped with a thermal conductivity detector, using a molecular sieve 5A column (7.0-m by 1.6-mm i.d.) at 50°C with N_2 as carrier gas at a flow rate of 15 mL min^{-1}. Ethylene was determined on a Varian 3700 gas chromatograph equipped with a flame ionization detector, using a porapak T column (1-m by 1.6-mm i.d.) with N_2 carrier gas at a flow rate of 25 mL min^{-1} at 80°C.

Hydrogen uptake (Hup) efficiency was determined using the definition of Schubert et al. (1977), whereby

$$\% \text{ relative efficiency} = (1 - H_2 \text{ evolved}/C_2H_2 \text{ reduced})\ 100\,.$$

This method was used for presumptive identification of inoculum strains in nodules against indigenous rhizobia when the two had significantly different Hup efficiencies. This method has been described in detail elsewhere (ElHassan et al., 1986).

^{15}N Determination

The ^{15}N content of plant tissue samples was determined by the method of Bremner (1965). Specifically, plant tissue N was converted to NH_4^+ by micro-Kjeldahl digestion and recovered by subsequent distillation. Ammonium was converted to N_2 by oxidation with Na hypobromite in Rittenberg tubes. The ^{15}N-isotope of N_2 was determined on a Finnigan 4000 mass spectrometer according to previous methodology (LaFavre and Focht, 1983).

RESULTS

The yield parameters, ARA and percent relative efficiency are shown in Tables 1 and 2. All inoculated treatments except 401 and HP147 gave no significant difference in ARA compared to the uninoculated (indigenous rhizobia) control. The uninoculated treatment and 22A1 had lower percent relative efficiencies compared to the other treatments (Table 1). The lower relative efficiencies of 22A1 and indigenous rhizobia were expected since they are Hup$^-$. The inoculated treatments showed highly significant increase in shoot, nodule, pod, and seed dry weights (Table 1, 3) over the uninoculated treatment, but there was no difference in pod number between the treatments.

Table 2. Comparison of yield and N_2-fixation parameters in cowpea as affected by competition between inocula strains and indigenous rhizobia, which include P132 from a previous inoculum study.

Inoculum strain	ARA†	% relative efficiency‡	Shoot dry weight	Nodule dry weight	Pod + seed dry weight	Pod no.
	nmol plant^{-1} s^{-1}		g plant^{-1}			
HP147	2.50a*	99a	16.3a	1.0a	7.4a	3.3b
401	3.81a	98ab	15.6a	0.9a	7.3a	3.7b
22A1	4.00a	96ab	17.0a	1.1a	7.1a	3.3b
176A26	3.31a	98ab	16.7a	1.0a	7.2a	4.0b
176A28	4.22a	96b	16.3a	1.1a	8.4a	3.3b
Control (res. P132)	3.11a	98ab	16.6a	1.0a	7.0a	5.8a

* Numbers followed by the same letter within columns are not significantly different at $p = 0.05$ by Duncan's Multiple Range Test; ND = not determined.

‡ Acetylene reduction assay, nmol C_2H_4 plant^{-1} s^{-1}.

‡ $\left[1 - \frac{\text{nmol } H_2 \text{ plant}^{-1}\text{ s}^{-1}}{\text{nmol } C_2H_4 \text{ plant}^{-1}\text{ s}^{-1}}\right] \times 100.$

Table 3. Comparison of harvest indices in cowpea as influenced by competition between inocula strains and indigenous (control) rhizobia.

Inoculum strain	Shoot + pea + pod			Shelled peas		
	Dry weight	N	N	Dry weight	N	N
	g plant^{-1}	g kg^{-1}	g plant^{-1}		g kg^{-1}	g plant^{-1}
P132	27.0a*	0.21ab	0.58ab	7.4ab*	0.36a	0.27ab
HP147	26.9a	0.23a	0.61ab	8.6a	0.38a	0.33a
22A1	27.4a	0.22ab	0.60a	9.0a	0.38a	0.34a
401	27.5a	0.18abc	0.49ab	9.0a	0.37a	0.33a
176A26	28.2a	0.19abc	0.54ab	9.1a	0.36a	0.33a
176A28	27.7a	0.17bc	0.47bc	7.9ab	0.35a	0.27ab
Control	21.7b	0.16c	0.35c	5.5b	0.35a	0.19b

* Numbers followed by the same letter within columns are not significantly different at $p = 0.05$ by Duncan's Multiple Range Test.

By contrast, when the inocula treatments were compared against the uninoculated control, which contained P132 from a previous inoculum trial (LaFavre and Focht, 1983), there was no significant difference in ARA as well as percent relative efficiency among all treatments (Table 2). There was also no significant difference in shoot, nodule, pod, and seed dry weights and pea yields between inoculated and uninoculated (residual P132) treatments (Tables 2 and 4). However, pod number was significantly higher in the uninoculated (residual P132) treatment than inoculated treatments (Table 2).

The percent of plant N derived from N_2 fixation can be calculated from the following equation.

$$\% \text{ N from fixation} = \left[\frac{\%^{15}\text{N plant} - \%^{15}\text{N soil}}{\%^{15}\text{N air} - \%^{15}\text{N soil}}\right] \times 100\,.$$

The atom % ^{15}N of the soil organic N_2 was 0.461%[3]

[3] The use of the total average ^{15}N content of the soil Kjeldahl N and the possible errors due to nonuniform labeling makes this estimate conservative, as discussed in more detail by LaFavre and Focht (1983).

Table 4. Comparison of harvest indices in cowpea as influenced by competition between inocula strains and indigenous (control) rhizobia, which includes P132 from a previous inoculum study.

Inoculum strain	Shoot + pea + pea			Shelled peas		
	Dry weight	N	N	Dry weight	N	N
	g plant⁻¹	g kg⁻¹	g plant⁻¹		g kg⁻¹	g plant⁻¹
HP147	23.8a*	0.23a	0.55a	6.4a	0.37a	0.24a
401	22.9a	0.24a	0.55a	6.5a	0.38a	0.25a
22A1	24.1a	0.23a	0.55a	6.2a	0.37a	0.23a
176A26	23.9a	0.24a	0.58a	7.2a	0.39a	0.27a
176A28	24.7a	0.24a	0.59a	6.4a	0.37a	0.24a
Control (res. P132)	23.6a	0.25a	0.59a	7.0a	0.39a	0.27a

* Numbers followed by the same letter within columns are not significantly different at $p = 0.05$ by Duncan's Multiple Range Test.

Table 5. Direct comparison of N_2-fixation in cowpea by ^{15}N analyses among inoculum and indigenous (control) rhizobia

Inoculum strain	Atom % ^{15}N	SD	% N fixed†	N_2 fixation‡
				g N plant⁻¹
P132	0.378bc*	±0.016	86ab	0.50ab
HP147	0.389ab	±0.022	73bc	0.45bc
22A1	0.368c	±0.010	98a	0.63a
401	0.376bc	±0.015	88ab	0.45ab
176A26	0.382a	±0.010	70c	0.38c
176A28	0.392a	±0.001	70c	0.33c
Control	0.399a	±0.013	60c	0.21c

* Numbers followed by the same letter within columns are not significantly different at $p = 0.05$ by Duncan's Multiple Range Test.
† Determined from shoot + pod + pea ^{15}N, i.e., % N fixed = [(% ^{15}N plant − % ^{15}N soil)/(% ^{15}N air − % ^{15}N soil)] × 100.
‡ Determined from % N fixed from (†) and total plant N from shoot + pod + pea (Table 3).

Table 6. Direct comparison of N_2-fixation in cowpea by ^{15}N analyses among inoculum and indigenous (control) rhizobia, which includes P132 from a previous inoculum study.

Inoculum strain	Atom % ^{15}N	SD	% N fixed†	N_2 fixation‡
				g N plant⁻¹
HP147	0.385ab*	±0.003	78a	0.43a
401	0.378b	±0.006	86a	0.47a
22A1	0.380ab	±0.009	83a	0.46a
176A26	0.389a	±0.008	72a	0.43a
176A28	0.387ab	±0.003	75a	0.42a
Control (res. P132)	0.379ab	±0.007	84a	0.52a

* Numbers followed by the same letter within columns are not significantly different at $p = 0.05$ by the Duncan's Multiple Range Test.
† Determined from shoot + pod + pea ^{15}N, i.e., % N fixed = [(% ^{15}N plant − % ^{15}N soil)/(% ^{15}N air − % ^{15}N soil)] × 100.
‡ Determined from % N fixed from (†) and total plant N from shoot + pod + pea (Table 4).

and the atom % ^{15}N of the air was 0.366%. The percent of plant N derived from fixation ranged from 98 (22A1 inoculated) to 60% (uninoculated). Moreover, there was no statistical difference in N derived from fixation between plants inoculated with fast-growing strains (176A26 and 176A28) and the uninoculated treatment (Table 5). However, the % N derived from fixation of slow-growing strains P132, 401, and 22A1 (86–98%) was significantly higher than the two fast-growing strains 176A26 and 176A28 (60–70%) (Table 5). Because there was no correlation with yield response of cowpeas among inoculation densities of all strains, these data are not shown.

DISCUSSION

Acetylene reduction activity reported in this study was somewhat comparable to what was reported by Zablotowicz and Focht (1981) for cowpeas under greenhouse conditions. The yield parameters, shoot dry weight, nodule mass, pod dry weight, and seed weight, showed a highly significant difference between inoculated and uninoculated treatments, which indicated that the indigenous rhizobia were less effective competitors (Table 1). Similar studies with cowpeas by Anonymous (1971) showed the superiority of inoculant strains over indigenous rhizobia. Furthermore, Moawad and Bohlool (1984) also showed that strains B_{213} and B_{215} outcompeted indigenous rhizobia and formed most of the nodules in *Leucaena leucocephala*. In contrast, several investigators reported that the inoculation with highly effective strains of *Rizobium japonicum* do not always result in soybean yield increases in areas where they are grown. This is due partly to the failure of the inoculum strain to compete with resident rhizobia (Kosslak et al., 1983; Ham et al., 1971; Kvien et al., 1981; Johnson et al., 1965; Caldwell and Vest, 1970; Bohlool and Schmidt, 1973) and also to the greater specificity of strain recognition in soybeans than in cowpeas.

The direct ^{15}N technique indicated that cowpeas have a capacity of deriving 60 to 98% of their N from N_2 fixation (Tables 5 and 6). These results were higher than those reported by Rennie et al. (1982) and Rennie and Kemp (1983) for soybeans, which derive approximately 50% of their plant N from N_2 fixation and lower than what was reported by LaFavre and Focht (1983) and Quilt and Dalal (1979) for pigeon peas, which derive nearly all their N from N_2 fixation. Obviously, differences in total and available soil N as well as crops would account for the differences between these studies. The estimates of total N_2 fixed (Tables 4 and 5) include only the above ground portion of the plant. Since recent ^{15}N studies with pigeon pea (Poth et al., 1986) establish that an equal or greater amount of N is fixed into the soil as into the shoot biomass, our estimates of N_2 fixation are conservative.

Recently, ElHassan et al. (1986) showed that the Hup trait—though far less quantitative than multiple antibiotic resistance—could be used for presumptive identification of nodule infectivity in soil when the inoculum strain had a significantly higher Hup efficiency than the indigenous soil rhizobia. They also observed that P132 was the most competitive of the same six strains studied herein in axenic Leonard jars and in three different tropical soils. Although it is not possible to ascertain the competitiveness of other inocula strains against residual P132 in soil by use of the Hup trait, comparison of Hup efficiencies between the uninoculated soils from Exp. I and II is relevant. Strain P132 in axenic plant culture has a Hup efficiency of 100 ± 0%. (We have never observed H_2 evolution from this isolate.) Nodules from the soil used in Exp. I, from which P132 was absent, had a Hup efficiency of 88 ± 2%. Nodules from soil used in Exp. II, which was inoculated with P132 2 yr hence, but treated identically as the uninoculated soil from Exp.

I experimentally and temporally (with respect to storage at ambient temperatures), had a Hup efficiency of 98 ± 2%. Clearly, it is far more tenuous to dismiss the differences in Hup efficiencies and in N content (Tables 3 and 4) of the two controls as a series of fortuitous events than it is to assign these differences to a direct result of cause and effect.

There was no correlation between yield response and inoculum density with any of the inoculum strains. These results were contrary to those of Ireland and Vincent (1968), Weaver and Frederick (1972) and Bromfield and Jones (1980), all of whom demonstrated that competitive success of inoculum strains was largely dependent on their number to the indigenous soil rhizobia. However, in those studies, the indigenous rhizobia were obviously much better competitors than the ones studied herein. The poor competition of indigenous rhizobia in the same ^{15}N enriched soil was also noted previously by LaFavre and Focht (1983).

In summation, three important points can be made from this study. First, the two fast-growing strains fixed less N_2 compared to three of the slow-growing strains. Second, N_2 fixation and yield parameters of cowpeas were significantly greater with all inoculum strains than with uninoculated control, yet were unaffected by inoculum density. Third, strain P132 was also a good survivor in soil that had been inoculated 2 yr earlier. The latter two points may be of direct agronomic importance to LDCs where cowpeas constitute an important food source inasmuch as inoculation with superior strains not only may ensure initial success, but may not be necessary each succeeding year if the strain is a good survivor and competitor.

ACKNOWLEDGMENTS

This work was supported in part by a grant from USDA/SEA/AID 83-CRSR-2-2320. The senior author is grateful for financial assistance received from the Democratic Republic of the Sudan while working on this project.

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XN-AAX-470-C

Soil Biol. Biochem. Vol. 18, No. 1, pp. 125–127, 1986
Printed in Great Britain

0038-0717/86 $3.00 + 0.00
Pergamon Press Ltd

SHORT COMMUNICATION

QUANTIFICATION BY DIRECT ^{15}N DILUTION OF FIXED N_2 INCORPORATION INTO SOIL BY *CAJANUS CAJAN* (PIGEON PEA)

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(*Accepted* 4 *July* 1985)

Significant amounts of N_2 fixed by legume crops are undoubtedly incorporated into the soil, but specifically how much? The total N_2 fixed by a particular legume includes not only the fixed N_2 in the harvested portion of the aboveground biomass, but also the fixed N_2 in the leaves and stems returned to the soil, as well as that in the roots, nodules and root exudates. Most estimates of N_2 fixation are based solely on the aboveground plant material, and the N contribution to the soil is generally ignored (Nutman, 1976; La Rue and Patterson, 1981). Both the N balance (difference) and the ^{15}N fertilizer dilution techniques employ non-fixing plants as controls. The controls are used to account for the uptake of unlabeled soil N and make analysis of soil for total N and atom% ^{15}N unnecessary (Rennie *et al.*, 1978; La Rue and Patterson, 1981; Witty, 1983). This avoidance of soil N analysis is understandable since the techniques available for soil N analysis, when coupled with spatial variability, make it difficult to measure increases in a large and variable pool of N.

The difference between the natural abundance of soil ^{15}N and atmospheric N_2 atom% ^{15}N (the delta technique) can be used to measure the incorporation of fixed N_2 into soil (Amarger *et al.*, 1979), but is fraught with difficulties, including the analytical problems of measuring small differences. Other problems include variations in soil atom% ^{15}N with depth (Steele *et al.*, 1981), and location in a field. The task is complicated further by the enrichment of ^{15}N during plant decomposition (Turner *et al.*, 1983) and plant N uptake (Kohl and Shearer, 1980). These factors combine to make it difficult using the delta technique to discern the amount of N a legume crop adds to the soil.

To quantify the amount of fixed N_2 incorporated into the soil, we used a soil which had been enriched with ^{15}N. The soil had been in lysimeters which were heavily fertilized with $(^{15}NH_4)_2SO_4$ for 2 yr between 1973 and 1975 (Focht and Stolzy, 1978). The soil was removed from the lysimeters during the summer of 1981. Over the years the ^{15}N applied had become relatively uniformly incorporated into the soil organic matter as verified when the soil was used, in a greenhouse study, to directly estimate N_2 fixation by *Cajanus cajan* (pigeon pea) via analysis of plant material for ^{15}N enrichment (La Favre and Focht, 1983a). Using ^{15}N labeled soil has several advantages over the use of ^{15}N enriched fertilizer. The ^{15}N fertilizer procedure relies on comparing the subject plant with some nonfixing control. This is essential since there are three sources of N in a labeled fertilizer N_2 fixation experiment: the fertilizer, the soil and the atmosphere. The control plant is used to correct for plant N derived from the soil. A modified isotope dilution calculation is then performed (Boddey *et al.*, 1984; Rennie, 1984; Ledgard *et al.*, 1985). With labeled soil this is unnecessary since the principles of isotope dilution can be directly applied (i.e. no control plant is necessary) to account for fixed N incorporated into the plant (Chalk *et al.*, 1983; La Favre and Focht, 1983a) as well as the soil. The analysis of the soil used by La Favre and Focht (1983a) to grow pigeon pea affords an opportunity to quantify the amount of fixed N_2 which is incorporated into the soil by direct ^{15}N dilution and is the topic of this report.

The system used to grow pigeon pea has been described by La Favre and Focht (1983a). The plants were grown in PVC columns (30 cm dia × 90 cm) in ^{15}N enriched soil (86 kg-soil per column). This soil was low in N (0.042%) and had demonstrated a very low denitrification potential (Focht and Stolzy, 1978). Columns for all treatment were amended with $CaSO_4 \cdot 2H_2O$, 0.96 g; $Ca(H_2PO_4) \cdot 2H_2O$, 2.04 g; and K_2SO_4, 4.46 g. A range of rhizobia strains with different N_2 fixation efficiencies (H_2 uptake, Schubert and Evans, 1976), were used to inoculate the plants. In addition, there were N fertilized and unfertilized controls which were not inoculated but were nodulated by inefficient indigenous soil rhizobial strains. Each treatment was replicated five times, and the plants were grown in the greenhouse for 225–252 days. At the end of the experiment the shoots were harvested, and the soil removed from each column. Coarse root material was removed and the soil was air dried and stored. Soils were sieved (<2 mm) before chemical analysis. Two samples from each column were then analyzed for total N and ^{15}N content. Total soil N was determined by Kjeldahl analysis (Bremner and Mulvaney, 1982). The ^{15}N content of the soil was determined by mass spectrometry following digestion and steam distillation (La Favre and Focht, 1983a).

The soil ^{15}N enrichment determined from stored unplanted soil samples was 0.458 atom% ^{15}N, a value which does not differ significantly (two-tailed t-test, $\alpha = 0.05$) from that determined by La Favre and Focht (1983a). The significantly reduced atom% ^{15}N values (two-tailed t-test) for the soil after the *C. cajan* final harvest indicates a substantial soil N input (Table 1). The input is due to N_2

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Table 1. Comparison of fixed N_2 incorporation into the aboveground crop (*Cajanus cajan*) and soil as directly measured by ^{15}N dilution

Rhizobium[1] strain	Soil N[2]			N_2-fixation[4]	
	atom% ^{15}N	%Nf	(mg N kg^{-1} soil)	Crop (kg ha^{-1})	Soil (kg ha^{-1})
P132	0.433 ± 0.006	27.2	423 ± 17	134	148
P148	0.429 ± 0.003	31.5	430 ± 10	115	179
HP147	0.432 ± 0.010	28.3	418 ± 12	96	153
401	0.434 ± 0.005	26.1	428 ± 14	105	144
None, −N	0.431 ± 0.012	29.3	423 ± 13	67	160
None, +N	0.423 ± 0.005	ND[3]	439 ± 16	ND[3]	ND[3]

[1]Uninoculated plants were nodulated by indigenous soil rhizobia.
[2]Atom% ^{15}N measured by mass spectrometry. Mean ± SD are given for atom% and total N determinations ($n = 5$). All atom% ^{15}N are significantly less than the enrichment before planting of 0.458 ± 0.012 atom% ^{15}N ($\alpha = 0.05$). %Nf is the percent soil N derived from fixation as defined by equations in the text.
[3]Not determined since the addition of fertilizer N (3.0 g-N $plant^{-1}$, as $[NH_4]_2SO_4$) precludes ^{15}N estimation of dinitrogen fixed.
[4]Assuming a plant density of 15,000 ha^{-1}. Crop values measured by direct ^{15}N dilution by La Favre and Focht (1983a).

fixation except in the fertilized treatment in which case the reduced atom% ^{15}N would be from a combination of N fertilizer and N_2 fixation.

The dilution of the originally enriched soil-N (0.458 atom% ^{15}N) by atmospheric-N (0.366 atom% ^{15}N) can be represented mathematically as (Rennie *et al.*, 1978; Hauck, 1982),

$$(X)(0.458\%) + (Y)(0.366\%) = (X + Y)(Z), \qquad (1)$$

where X is the amount of indigenous soil N, Y is the amount of soil N derived from fixation, and Z is the observed atom% ^{15}N of the soil after the *C. cajan* was harvested. The fraction of soil N derived from fixation (Nf) would then be,

$$Nf = Y/(X + Y).$$

Since this is a ratio, the absolute values of X and Y do not have to be determined to solve for Nf. We can employ an algebraic substitution of $X = 1$ in equation (1), and then solve for a corresponding value of Y, and from Y subsequently calculate the percent of soil N derived from fixation (%Nf) as follows:

$$Y = (0.458\% - Z)/(Z - 0.366\%)$$

$$\%Nf = [(Y)/(Y + 1)](100).$$

This allows the determination of %Nf solely from soil atom% ^{15}N values for soils before planting and after harvesting.

All treatments had N incorporations from fixation which amounted to at least 26% of the total soil N (Table 1). Using these figures in conjunction with the Kjeldahl determinations of total soil N, the amount of N derived from fixation and incorporated into the soil was calculated for a crop of 15,000 plants ha^{-1}. This allows comparison with the N derived from fixation incorporated into the above ground biomass (Table 1). Surprisingly, in every case the incorporation of biologically fixed N into soil was greater than the amount of fixed N incorporated into the above ground plant biomass as measured directly by ^{15}N dilution (La Favre and Focht, 1983a). Sheldrake and Narayanan (1979) found that the root systems of pigeon peas contained 10 kg N ha^{-1}. Using their estimate to account for root N still leaves the N addition to the soil greater than the N addition to the plant in every case.

The estimate of fixed N incorporated into the soil includes N from unrecovered and senescent nodules, root exudates, and fine roots. There is also the possibility that some small fraction of the N added to soil was from N_2 fixation by hydrogen-oxidizing bacteria. Hydrogen-oxidizing bacteria populations develop in the rhizosphere of low efficiency *Rhizobium* strains (i.e. 401) using the H_2 evolved from root nodules for growth (La Favre and Focht, 1983b). The H_2 oxidizer *Xanthobacter autotrophicus* is a known N_2 fixer (Aragno and Schlegel, 1981).

The value for plant biomass fixed N does not include the input from the abscissed leaves and flowers, which were not recovered in this study. In the field abscissed leaves and flowers would be incorporated into the soil. This tends to make our estimate for N incorporation into soil from N_2 fixation by pigeon pea conservative.

Acetylene reduction measurements indicate that the average total N fixation was 45% greater than could be accounted for by isotope dilution measurements of fixed N in the aboveground crop (La Favre and Focht, 1983a). Our soil analyses account for the remaining fraction of the total N_2 fixed, as measured by C_2H_2 reduction, by showing that the remainder was incorporated into the soil.

The magnitude of the soil N addition measured in this study would be virtually impossible to measure in typical agriculturel soils. This is understandable since it represents a relatively small N addition in comparison to the large soil N pool. Attempts to measure the addition in the field would be further confounded by spatial variability. We were able to measure the incorporation of N_2 fixed by pigeon pea into this soil only because of the low initial content of soil N and the high soil ^{15}N enrichment which had equilibrated over a period of years. Our results clearly demonstrate that, in this low N soil, the amount of N incorporated into the soil from N_2 fixation by pigeon pea is at least equal to the N incorporated into the aboveground plant biomass.

Acknowledgements—This research was supported by grants to D. Focht from the United States Department of Agriculture, Science and Education Administration and the United States Department of State—Agency for International Development. We thank F. Sutherland and R. Collins for technical assistance.

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XN-AAX-470-

INCREASED EFFECTIVENESS OF COMPETITIVE RHIZOBIA STRAINS UPON INOCULATION OF *CAJANUS CAJAN*

B. S. Hernandez,[1] M. Poth,[2] and D. D. Focht[3]

ABSTRACT

A field study was conducted in lysimeters containing ^{15}N enriched soil to determine the effects of 4 competitive rhizobial strains upon yield parameters of pigeon peas (*Cajanus cajan*). The greatest differences observed were in seed yields: strain P 132 effected the highest (121 ± 20 g/plant), and the control (indigenous rhizobia) effected the lowest (43.9 ± 8 g/plant). With the exception of seeds and pods, the dry matter weight was not different. Although there appeared to be no effect of inoculum strains on the fractional content of N derived from biological nitrogen fixation (BNF) when the total plant biomass was considered, strains P 132 and 401 partitioned more of the N derived from fixation into seeds and leaves than the other strains. Because the seeds comprised the major portion of plant N, more total N and more derived from BNF (about half) were found in plants inoculated with P 132, while the smallest amount was found in the uninoculated controls. P 132 was also the best competitor with respect to indigenous rhizobia, and accounted for all the nodules found from the plants in which it was inoculated.

KEY INDEX WORDS: ^{15}N, nitrogen fixation, legumes, tropical soils, pigeon peas

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INTRODUCTION

Cajanus cajan L. Millsp. (pigeon peas) are commonly nodulated by the cowpea miscellany group of rhizobia, which are indigenous in tropical soils. Nitrogen fixation is often limited if the natural population is ineffective at promoting high yields. Thus, it may be necessary to replace the existing rhizobia by introducing more effective strains, but this is possible only if the inoculum strain can compete successfully against indigenous rhizobia. Although several investigators have been mainly concerned with the ability to compete for nodule sites (2,4,11), the capacity to fix a significant amount of nitrogen is a criterion that is equally as important.

In a recent greenhouse study with ^{15}N, El Hassan and Focht (7) showed that three of six rhizobial strains were good competitors, by comparison to the indigenous rhizobia, and also increased seed yield, shoot dry matter, total N and percentage N derived from fixation. Although ^{15}N has been used to measure N_2 fixation in legumes, few studies have evaluated the importance of strain effects on biological nitrogen fixation. Only two ^{15}N studies performed in the greenhouse, have alluded to the possible importance of strain effect upon partitioning of nitrogen to and within the plant (7,12).

The following study was undertaken to determine 1) if superior inocula strains (with respect to indigenous rhizobia) were competitive under field conditions; and 2) if different rhizobial strains can influence the plant partitioning of biologically fixed dinitrogen.

MATERIALS AND METHODS

Rhizobium Strains. Sources and physiological characteristics of rhizobial strains P 132, IHP 147, 401, and 22A1 have been described previously (7,10). The four strains were intrinsically resistant to rifampicin (150 μg/ml), erythromycin (100 μg/ml), and nalidixic acid (150 μg/ml).

Media and Inoculant Preparation. Strains were maintained on yeast extract mannitol (YEM) agar slants (10). Liquid cultures were grown in side arm flasks (250 ml) containing 50 ml of YEM broth on a rotary shaker platform (125 rpm) at 28°C to an optical density of 0.6 (525 nm). Seeds of Cajanus cajan (L.) Millsp. var. 64-2b (pigeon peas) were inoculated with a peat-based inoculant which was prepared by mixing 25 g of sterile peat (Nitragin Co.) with 100 ml of a liquid culture to obtain a final rhizobial count of 10^8/g peat.

Chemicals. Antibiotics (rifampicin, nalidixic acid, and erythromycin) were obtained from Sigma Chemical Co., St. Louis, MO. $(^{15}NH_4)SO_4$ (99.07 atom. % ^{15}N) was obtained from Biorad, Richmond, CA.

Field Studies. Cajanus cajan was grown at the field station of the University of Panama, Tecumen, R.P. Plants were grown in 55-gallon lysimeters, which had a surface area of 0.196 m, a depth of 100 cm, and a drain at the bottom. Each lysimeter contained one plant, and the distance between plants was 50 cm. The soil contained in the lysimeters was classified as a mixed, hyperthermic Tropecatic Haplorthox. ^{15}N enriched wheat straw (10 atom % ^{15}N) from a previous study (9) was

added to the soil three years prior to the field study to give an organic ^{15}N enrichment of 0.400%. At planting, $^{15}(NH_4)_2SO_4$ was added in sufficient amount to increase the enrichment to 0.430% ^{15}N. Treatments consisted of inoculated (P 132, IHP 147, 22A1, and 401) and uninoculated (control) plants, which were each replicated 4 times. Eucalyptus sp. was used as a non-leguminous control plant to measure the available ^{15}N content of the soil. Plants were grown to maturity and plant parts (leaves, pods, seed, and shoot) were harvested separately and dried at 68°C for a period of 48 hours. Nitrogen contents of plant material were determined by the procedures of Bremner and Mulvaney (3). ^{15}N mass spectrometric analyses of plant and soil samples were determined by Isotope Services, Inc. (Los Alamos, NM).

Identification of Rhizobial Strain in the Nodule. Antibiotic typing of nodules was used to determine the rhizobial strain present in the nodules. After the plants were harvested, the roots were dug up and attached nodules were collected and washed free of soil. Replicates within each treatment were pooled together, and the nodules were prepared for typing by surface sterilization in 95% ethanol (30s), followed by treatment with 0.2% acidified HgCl for 4 min and rinsing in sterile distilled water. Each nodule was crushed with a sterile glass rod, and the resulting bacteroid suspension was streaked on YEMA plates containing erythromycin (50 μg/ml), rifampicin (100 μg/ml) and nalidixic acid (100 μg/ml). Cultures were incubated at 28°C for 6 days. Infection by the inoculum strain was noted by growth on the plates.

RESULTS

The atom % ^{15}N of Kjeldahl digests of soil was 0.410 ± 0.004 for 25 samples of duplicated analyses. The atom % ^{15}N from the 4 samples of Eucalyptus leaves (0.437 ± 0.027) and that from 4 samples of shoots (0.424 ± 0.027) were not significantly different and were pooled together to use the value of 0.431 ± 0.021 for the available N content of the soil. The fractional content of N derived from biological nitrogen fixation (BNF) was determined by the isotope dilution procedure of McAuliff et al. (14). Further discussion of this procedure can be found in the recent review by Chalk (5).

There was no difference in dry matter yields of leaves and stems among inoculum strains (Table 1), but there was considerable difference in seed yield between the uninoculated and inoculated plants. The % N content was unaffected by the inoculum strain (Tables 1 and 2). The most significant difference effected by inoculation was upon seed yield (Table 1), which was highest with P 132 (121 g/plant) and lowest with the control. Consequently, a significantly greater amount of total N among inoculum treatments was noted: P 132 was the highest, and the uninoculated control was the lowest (Table 2). Nitrogen fixation was also significantly higher in plants inoculated with P 132.

Although there was no inoculum effect upon ^{15}N enrichment of the total plant, there was a difference in ^{15}N content among the plant components (Table 3). This difference was most pronounced with strain P 132,

which had the highest proportion of N derived from BNF (i.e., the lowest ^{15}N enrichment in the most active parts of the plants, namely the leaves and seeds). The partitioning effect with strain 401 was less pronounced but still statistically significant. Despite the lack of high statistical significance ($P < 0.05$) with strains 22A1 and IHP 147, the F values are statistically higher than the control at $P < .10$.

Rhizobia strain inoculations were successful as evidenced by the high proportion of antibiotic resistant rhizobia in the nodules (Table 4). Although all nodules were not recovered due to senescence, sloughing, and mechanical disruption after harvest, more intact nodules were found among P 132-inoculated plants than among others. P 132 was the most competitive strain since it formed all of the nodules typed (Table 4).

DISCUSSION

Although all strains competed successfully in comparison to the indigenous rhizobia, not all were equally effective. This is clearly shown by comparing P 132 and 22A1. While 22A1 performed well as a competitor, it did not fix as much nitrogen or effect as great a seed yield as P 132. The superiority of strain P 132 in terms of both competitiveness and effectiveness has also been shown in previous experiments (7,8).

Strains P 132, and to a lesser extent 401, were able to influence the partitioning of fixed nitrogen to the seeds and leaves. The partitioning of fixed N_2 to the more valuable and important parts of the plant by rhizobium strains should be an important selection criteria.

Strain P 132 provided approximately half of the plant nitrogen, while strains 401, 22A1, and IHP 147 fixed considerably less. The quantity of nitrogen fixed by P 132 is comparable to that reported for soybeans (16), yet lower than that obtained for cowpeas (7). According to a previous study by La Favre and Focht (12), however, pigeon peas fixed 91-94% of the nitrogen in the plant. The difference in the amount of nitrogen derived from fixation, was most probably due to the higher organic content of the soil used in this study (5.0%) in comparison to the other [0.5%; (12)]. Nevertheless, the total amount of biologically fixed nitrogen in our study (150 kg N/ha) is comparable to that calculated by La Favre and Focht (12).

P 132 increased seed yield significantly in comparison to uninoculated plants. Strains 401 and IHP 147 also effected higher yields in terms of increases in pods and seeds. Yield performance by strain P 132 had been demonstrated in earlier experiments performed under axenic conditions in Leonard jars (13) and in the greenhouse (12). Seed yields obtained with this inoculum strain, however, are considerably higher (2350 kg/ha) than those reported by La Favre and Focht (12) (825 kg/ha), Chowd-hury and Bhatia (6) (700-800 kg/ha), and Akinola et al. (1) (500-1500 kg/ha). Stem and leaf yields were, on the other hand, not increased by inoculation. The latter observation sheds doubt on the use of shoot biomass for assessment of strain effectiveness in biological nitrogen fixation.

Field inoculations of selected superior strains can successfully compete with less desirable indigenous strains. Superior strains may

also influence the partitioning of biologically fixed N, giving higher N contents in the more valuable parts of the crop plant. The selection of strains with both of these qualities would be valuable for biotechnological applications.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from USDA/SEA/AID (#83-CRSR-2-2320).

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Table 1. Dry matter yields (g) of *Cajanus cajan* (per single plant) as affected by inoculum strain.

Strain	Leaves	Pods	Seeds	Stems
P 132	23.1a[1]	70.0ab	121 a	90.5a
IHP 147	16.9a	59.7ab	91.8ab	77.0a
22A1	21.4a	52.7b	77.7b	71.5a
401	26.4a	71.4a	100 ab	106 a
Control	24.7a	46.9b	43.9c	90.5a

[1]Numbers followed by the same letter in each column are not significantly different by Duncan's Multiple Range Test ($P < 0.05$).

Table 2. Nitrogen content (g) of above-ground portion of plants after harvest.

Strain	Total N	BNF[2]
P 132	5.97a[1]	2.94a
IHP 147	4.59b	1.12b
22A1	4.37bc	1.03b
401	5.50ab	2.11ab
Control	3.14c	1.03b

[1]Numbers followed by the same letter in each column are not significantly different by Duncan's Multiple Range Test ($P < 0.05$).

[2]Biological Nitrogen Fixation as determined by ^{15}N isotope dilution: 1 g per surface area of lysimeter is equivalent to 51.0 kg/ha.

Table 3. Effects of inocula strains on partitioning of available soil nitrogen (0.431 ± 0.021) among plant parts of *Cajanus cajan*. Values are expressed in atom % ^{15}N.

Strain	Leaves	Pods	Seeds	Stems	F[1]
P 132	0.395a	0.414b	0.393a	0.421b	9.08**
IHP 147	0.402	0.426	0.417	0.427	2.17*
22A1	0.406	0.423	0.415	0.421	2.16*
401	0.392a	0.414b	0.403ab	0.411b	5.37**
Control	0.396	0.417	0.411	0.414	0.85NS

[1]*, ** significant at the 0.05 and 0.01 levels, respectively, and NS = not significant for analysis of variance among rows.

All values followed by the same letter in each row are not different ($P > 0.05$) from each other by Duncan's Multiple Range Test. ANOV comparison among columns of plant parts showed no significant difference at $P < 0.05$.

Table 4. Fractional nodule occupancy in *Cajanus cajan* based on intrinsic antibiotic resistance.

Inoculant Strain	Number of Nodules Sampled	Fraction of Antibioti Resistant Nodules
P 132	32	1.00
IHP 147	15	0.87
22A1	12	0.75
401	15	0.67
None (Control)	11	0.09