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ISOLATION, SELECTION AND EVALUATION OF RHIZOBIUM UNDER CONTROLLED CONDITIONS

KEY WORDS: Bean, Cowpea, Lentil, Nodulation, Peanut, Rhizobium, Total nitrogen

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ABSTRACT

Rhizobia were screened in growth chamber and greenhouse tests with the host plants: bean (*Phaseolus vulgaris* L.), lentil (*Lens esculenta* Moench.), cowpea (*Vigna unguiculata* (L.) Walp) and peanut (*Arachis hypogaea* L.). Rhizobial isolates varied in effectiveness, time to nodulation, and host plant specifities. Initial screening procedures in plastic growth pouches allowed selection of infective strains of *Rhizobium*. These tests enabled the selection of a small number of highly effective isolates for more critical evaluation. Highly significant correlations (p=0.01) were obtained between shoot dry weight and total nitrogen, suggesting that the technique was applying sufficient selection pressure to differentiate variation in N_2 fixation among strains of *Rhizobium*.

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Results indicate that highly effective rhizobia can be efficiently selected under controlled conditions following a step-wise procedure.

INTRODUCTION

The use of various legumes as grain and oilseed crops has increased in the southeastern United States and developing countries of the world. As the production of these crops expand into areas where legumes have not been previously planted and as the cost of nitrogen fertilizers rises, the potential for increasing legume production through biological dinitrogen fixation should be explored. The success of increasing legume production may depend on successful inoculation with superior N_2-fixing strains of *Rhizobium* that will effectively nodulate each legume grown.

Inoculation of grain legumes with effective strains of *Rhizobium* significantly increased seed yields of *Glycine max* (L.) Merr.[2,27] and *Cicer arietinum* L.[23] planted on soils free of native rhizobia. The use of effective *Rhizobium* strains on legumes planted on soils with indigenous populations of rhizobia can also increase plant yields. Seed yield of *Phaseolus vulgaris* L. increased from 20 to 145% when a locally selected strain of *R. phaseoli* was used as an inoculant[14]. *G. max* grain yields in Nigeria increased 5 to 8 times when selected strains of *R. japonicum* were used as inocula[6].

Under optimum conditions the full growth potential of leguminous plants occurs through effective symbiosis with superior N_2-

fixing rhizobia. Evaluation of Rhizobium for effectiveness through screening procedures allows for selection of superior rhizobia. Initially, many isolates of rhizobia must be tested under bacteriologically and environmentally controlled conditions[5,7,11,24]. Selection procedures may also identify differences among effective rhizobia due to plant genotype[22,28] and host of isolation[8,17,19]. Superior strains can then be evaluated for effectiveness under field conditions.

The objectives of this study were to develop procedures for evaluating isolates of Rhizobium with specific host legumes under controlled and greenhouse conditions. Superior N_2-fixing strains of Rhizobium identified by these screening procedures will then be used in inoculant development and field studies.

METHODS AND MATERIALS

Collecting and Sampling Soils and Plants

The study examined R. phaseoli, R. leguminosarum and cowpea Rhizobium isolated from a variety of soils and host plants in Mississippi. Soil samples were collected from areas under crop or garden production. Soils collected included Tippah silt loam, Lexington silt loam, Cahaba fine sandy loam, and Houston clay.

The soils were mixed with washed river sand in a ratio of 1:4 by volume (soil:sand) and placed in 3.8-liter (1 gal) cans lined with plastic bags. Each contained 3.5 kg of soil:sand mixture which was equivalent to 875 g of soil. Bean (Phaseolus vulgaris L.) cv. 'Commodore' and peanut (Arachis hypogaea L.) cv. 'Flo-

runner' seeds were surface-sterilized by immersion for 10 min. in 10% hydrogen peroxide (H_2O_2) followed by 5 rinses in sterile distilled water. Lentil (Lens esculenta Moench.) cv. 'Chilean' seeds were surface-sterilized in a similar manner using 1.0% sodium hypochlorite (Clorox) as the sterilant. Surface-sterilized seeds were planted 1 to 3 cm deep (depending on the legume) in duplicate soil:sand mixtures and placed in the greenhouse. Each pot received 200 ml of filter-sterilized Hoagland's nitrogen-free nutrient solution[16], taking care to avoid cross contamination. This was equivalent to 80% of water-holding capacity of the potting mixture. Sterile distilled water was alternated with nutrient solution as needed throughout the growth period.

Nodules were also collected at soil sampling sites in October 1977 for subsequent rhizobial isolations. Additional nodules were collected at other sites in Mississippi, Alabama, Florida and Missouri in 1977 and 1978.

Isolation and Collection of Rhizobium from Soil

All plants in the greenhouse were harvested after 8 weeks of growth. Prominent nodules on the tap or upper root system were collected from each plant. Nodules excised from the root systems were washed thoroughly in distilled water to remove gross surface contamination. The washed nodules were then immersed for 10 min in 5% H_2O_2 followed by 10 min exposure to 70% ethanol. The nodules were rinsed thoroughly 5-6 times in sterile distilled water. Isolation of rhizobia from nodules was accomplished by crushing the nodule aseptically and streaking the contents on yeast

extract-mannitol (YEM) agar plates[13]. The plates were incubated at 24°C. After colonies appeared, a typical colony was selected and cloned by transferring to 10 ml of 0.01% Tween 40 in 0.1% peptone and vortexed[18]. Serial tenfold dilutions were made in 0.1% peptone and spread-plated on YEM agar. Again, a single colony was selected and transferred to YEM slants. Isolates were stored at 8°C and transferred bimonthly. Gram stained slides and cultural characterizations were helpful in determining if the isolates appeared to be rhizobia-like. The above procedures were also followed for nodules from field-collected plant samples.

Rhizobium strains obtained from various culture collections were included in the studies as control strains. Sources of strains included J. C. Burton, Nitragin Corp.; F. Davidson, Research Seeds, Inc.; V. G. Reyes, NifTAL Project; D. F. Weber, Cell Culture and Nitrogen-Fixation Laboratory, USDA; J. M. Vincent, Univ. of Sydney.

Infectivity Determinations

Isolates tentatively classified as rhizobia by Gram staining and cultural characterization were examined for infectiveness on host legumes planted in plastic growth pouches[26] containing N-free nutrient solution. Nodule formation by an isolate indicated infectiveness. Surface-sterilized seeds were planted in growth pouches supported by record racks. Seedlings in the pouches were inspected after 3 days growth for the presence of healthy seedlings and adjusted as necessary to contain two seedlings. Five-day old cultures of R. phaseoli and R. leguminosarum or 8-day old cultures of cowpea Rhizobium on slants were suspended in 5 ml of 0.1% peptone.

Plate counts of random slant suspensions detected an average concentration of 10^9 cells ml^{-1}. One-milliliter of this suspension was used to inoculate seedlings in a pouch. Three pouches were inoculated for each isolate. Racks containing the inoculated pouches were placed in a growth chamber maintained at 28°C during a 16-hour light period and 20°C during an 8-hour dark period. Each pouch received 75 ml of N-free nutrient solution initially and this level was maintained throughout the experiment by periodic additions of nutrient solution. Leguminous hosts included Commodore beans, Chilean lentil, Mississippi Silver cowpeas and Florunner peanuts. Uninoculated controls without supplemental nitrogen and those receiving 70 ppm N (.05% KNO_3) in the nutrient solution were included for each host legume.

Plants were removed after four weeks and infectiveness determined subjectively according to a ranking scale of 1 to 5, with 1 = no nodules, poor plant vigor and 5 = abundant and large nodules on upper root system, healthy plants.

Preliminary Effectiveness Screening

Isolates rated 2.5 and above in the infectiveness test were selected for preliminary effectiveness screening. An attempt was made to include in these selections representative isolates from each soil and different plant species. Test seedlings were prepared as previously described. The growth pouches containing the seedlings were inoculated with slant suspensions of each isolate according to a completely random design with three replications. Pouches supported in record racks were placed in the growth cham-

ber. Nodulation was examined periodically throughout the experiment. Four weeks after inoculation the plant shoots were harvested, oven-dried and weighed. Uninoculated controls, nitrogen controls (0.05% KNO_3) and plants inoculated with control strains were included as reference treatments.

Additional Tests for Effectiveness of N_2 Fixation

Plants inoculated with isolates that yielded shoot dry weights $\geq$ their respective nitrogen controls were subjected to a final effectiveness test. Plants of each legume were grown in modified Leonard jar assemblies constructed of autoclavable plastic bottles [25]. Each assembly received 500 g of a 2:1:1 sand:vermiculite:perlite mixture (pH 6.70), covered with autoclavable plastic (Reynolds Oven Film) and autoclaved for 90 min before use. Surface-sterilized seeds were placed in the sterilized mixtures in the assemblies and each seed was inoculated with a 1-ml suspension of the appropriate culture from slants prepared in 100-cc prescription bottles. Cell density of the suspensions ranged from 1 to 5 x 10^9 cells ml^{-1}. Uninoculated controls were included. The inoculated and uninoculated seeds were covered with sterile sand at a depth appropriate for the particular legume. The assemblies received filter-sterilized N-free nutrient solution except for half of the uninoculated controls which received nutrient solution supplemented with 0.05% KNO_3. Assemblies were placed in the greenhouse according to a completely random design replicated four times. Plants were harvested after 8 weeks of growth. Nodules were removed from

the root systems and weighed. Plant shoots were dried at 60°C for 48 hours and weighed. The dried shoots were ground in a Wiley mill and approximately 250 mg of the ground material analyzed for total nitrogen by the tube digestion[21] and ammonia electrode[3] methods.

RESULTS

Infection and Nodulation

Nodulation of beans, lentils and peanuts grown in soil:sand mixtures varied among soils. Nodulation of bean and lentil occurred only in soils with histories of previous cultivation of legumes in the bean or pea cross-inoculation groups. The average number of nodules per plant in these soils was only 4.5 and 3.0 for lentil and bean, respectively. Nodulation of peanut plants occurred in all soils. Peanut plants averaged 15 nodules per plant for all soils, with plants grown in some soils having 40 or more nodules.

Gram stains and cultural observations aided in reducing the number of isolates prior to infectiveness screening. The number of *R. phaseoli* isolates was reduced from 75 to 62 and the number of *R. leguminosarum* isolates was reduced from 90 to 56. All 87 isolates of cowpea *Rhizobium* appeared as typical rhizobia using these methods. The cloning procedure[18] was essential in obtaining isolated colonies from a single rhizobia. This procedure eliminated problems of mixed cultures that are often encountered in selection of rhizobia[11].

Variable ratings were obtained for isolates in the infectivity tests. The ratings were not associated with type of soil from

which isolations were made. Of the isolates and control strains of R. phaseoli tested on bean, 44/67 (65%) were found to be infective. High ratings (>2.5) were obtained for 24 isolates (35%).

Isolates of Rhizobium leguminosarum and control strains were infective on lentil in 32/63 cases (51%). Only 18/63 isolates attained high ratings (28%). Cowpea rhizobia were infective on cowpea in 55/92 cases (60%), with 29% isolates rated ≥ the positive control (32%). Cowpea was infected by only 36% of the isolates from peanut, but was nodulated by 71% of the isolates from cowpea nodules and 82% of the isolates from other plants of the cowpea miscellany. Infectivity of cowpea Rhizobium isolates on peanut followed a similar pattern. A total of 33/92 (36%) isolates were infective on peanut in growth pouches, with 27 isolates (29%) ≥ N control. Isolates from peanut, cowpea and miscellaneous plants comprised 75, 0 and 27% of the infective cases on peanut.

When peanuts were grown in the growth pouches, problems with inconsistent nodulation were encountered. When germinated peanut seedlings were inoculated with 1-ml aliquots of serial ten-fold dilutions of a broth culture of peanut Rhizobium strain CA22 nodulation only occurred in pouches inoculated with 3.2×10^4 to 3.2×10^8 rhizobia per seedling. Nodulation did not occur with less than 3×10^4 rhizobia per seedling. Based on these results, infectivity determinations on peanut were repeated in the modified Leonard jar assemblies. The results from this test showed that 86% of peanut rhizobia, 44% of cowpea rhizobia and 32% of rhizobia from miscellaneous legumes formed nodules on Florunner peanuts. Only

strains CV020 and CV030 (cowpea rhizobia) and isolates from *Cassia* and *Crotalaria* formed effective nodules while 20/36 (55%) of the peanut rhizobia formed effective nodules.

Preliminary Screening

Preliminary screening for N_2 fixation revealed a wide range of effectiveness based on shoot dry weight of *P. vulgaris* cv. 'Commodore' inoculated with strains of *R. phaseoli* (Table 1). Isolates

TABLE 1

Preliminary Effectiveness Screening of *R. phaseoli* Isolates and Controls on *P. vulgaris* cv. 'Commodore'.

Isolates †	Shoot Dry Weight ‡ (mg/plant	
Nitrogen Control	649.30	a
931C	630.83	a
971A	622.08	a
970B	581.37	b
931A	568.82	b
B84	536.64	c
962C	504.78	d
B	464.11	e
970A	462.11	e
P009	453.62	e
P921	438.93	ef
920B	433.61	ef
960D	425.11	efg
946D	419.90	efg
PK17	396.13	fg
P020	388.69	fg
P004	369.65	gh
971C	358.44	gh
951C	341.30	hi
Uninoc. Control	186.58	j

† All isolates obtained from *P. vulgaris* plants.

‡ Means not followed by a common letter differ significantly (p= 0.05) according to Duncan's Multiple Range Test (DMRT) ($S_{\bar{x}}$ = 15.06).

931C and 971A were as effective in dry matter production as was the nitrogen control. All isolates were significantly higher in effectiveness than the uninoculated control. Similar results were obtained when L. esculenta was inoculated with strains of R. leguminosarum (Table 2). Strain P28 and isolates 771B and 762A equalled or exceeded the nitrogen control in effectiveness. Only isolate LL010 failed to give significantly higher dry matter yields than the uninoculated control.

TABLE 2

Preliminary Effectiveness Screening of R. leguminosarum Isolates and Controls on L. esculenta cv. 'Chilean'.

Strain	Host of Isolation	Shoot Dry Weight † (mg/plant)
P28	Pisum sativum L.	314.00 a
71B	Lens esculenta	272.83 b
Nitrogen Control		270.93 b
762B	L. esculenta	270.43 b
730H	L. esculenta	255.66 c
711M	L. esculenta	249.55 c
760 'O'	L. esculenta	234.42 d
LC53	P. sativum	222.81 d
762K	L. esculenta	220.53 d
700E	L. esculenta	220.34 d
LV011	Vicia dasycarpa Ten.	194.22 e
711R	L. esculenta	193.30 e
LP004	P. sativum	180.72 e
760A	L. esculenta	180.42 e
740B	L. esculenta	152.35 f
731K	L. esculenta	148.40 f
LP008	P. sativum	143.32 f
Uninoc. Control		131.97 g
LL010	Lathyrus sp.	128.78 g

† Means not followed by a common letter differ significantly (p= 0.05) according to DMRT ($S_{\bar{x}}$ = 8.72).

Results summarized in Table 3 indicate that 16 isolates of cowpea rhizobia were equivalent to the nitrogen control in promoting dry matter accumulation by V. unguiculata cv. 'Mississippi Silver'. These isolates included several from host plants other

TABLE 3

Preliminary Effectiveness Screening of Rhizobial Isolates and Controls on V. unguiculata cv. 'Mississippi Silver'.

Strain	Host of Isolation	Shoot Dry Weight † (g/plant)
CV030	V. unguiculata	2.00 a
CV020	V. unguiculata	1.88 ab
CV041	V. unguiculata	1.82 abc
Nitrogen Control		1.74 abcd
CV031	V. unguiculata	1.62 abcde
C510D	Crotalaria spectabilis Roth	1.59 abcde
C346A	Cyamopsis tetragonolobus	1.58 abcde
C510C	C. spectabilis	1.51 abcdef
CV017	V. unguiculata	1.48 abcdefg
CV061	V. unguiculata	1.48 abcdefg
C236	A. hypogaea	1.42 bcdefg
C861C	A. hypogaea	1.40 bcdefg
C346B	C. tetragonolobus	1.38 bcdefg
CAA1	Astragalus sp.	1.35 bcdefg
C651C	V. radiata	1.34 bcdefg
CV012	V. unguiculata	1.32 cdefg
CB756	V. unguiculata	1.21 defgh
CV060	V. unguiculata	1.17 efgh
CV072	V. unguiculata	1.16 efgh
CC011	Cassia fasciculata Michx.	1.14 efgh
CL001	Lespedeza stipulacea Maxim	1.09 efghi
CA22	V. unguiculata	0.98 fghi
CL005	L. stipulacea	0.97 fghi
C8B4	A. hypgaea	0.96 fghi
CV026	V. unguiculata	0.94 ghi
CV018	V. unguiculata	0.67 hi
CV027	V. unguiculata	0.57 i
Uninoc. Control		0.56 i

† Means not followed by a common letter differ significantly (p=0.05) according to DMRT ($S_{\bar{x}}$ = 0.23).

than Vigna spp. Fifteen isolates ranked higher than control strain CB756. In contrast, cowpea rhizobia exhibited a wider range of effectiveness when used to inoculate A. hypogaea cv. 'Florunner' (Table 4). Strain CA22 (positive control) ranked best in promoting dry matter production, isolate C870B was superior to the N ferti-

TABLE 4

Preliminary Effectiveness Screening of Rhizobial Isolates and Controls on A. hypogaea cv. 'Florunner' (in Modified Leonard Jars).

Strain	Host of Isolation	Shoot Dry Weight † (g/plant)
CA22	V. unguiculata	13.70 a
C870B	A. hypogaea	12.08 b
Nitrogen control		10.25 c
C831B	A. hypogaea	10.01 cd
C811K	A. hypogaea	9.85 cd
C820F	A. hypogaea	9.80 d
C810I	A. hypogaea	9.75 d
CC012	C. fasciculata	9.06 e
CA001	A. hypogaea	8.86 e
C820K	A. hypogaea	8.72 e
C830E	A. hypogaea	8.14 f
C860J	A. hypogaea	8.10 f
C810E	A. hypogaea	8.00 fg
C841L	A. hypogaea	7.94 fg
C810J	A. hypogaea	7.58 gh
C870F	A. hypogaea	7.32 hi
C510A	C. spectabilis	7.20 hij
PN1	A. hypogaea	6.95 ijk
C870P	A. hypogaea	6.84 jk
C861C	A. hypogaea	6.66 k
C850J	A. hypogaea	6.23 l
CV031	V. unguiculata	6.17 l
C862C	A. hypogaea	6.02 l
C834	A. hypogaea	5.98 l
C811M	A. hypogaea	3.62 m
Uninoc. Control		3.31 m

† Means not followed by a common letter differ significantly (p= 0.05) according to DMRT ($S_{\bar{x}}$ = 0.21).

lized control, and isolates C831B and C811K were statistically equivalent to the nitrogen control in promoting dry matter accumulation. Most isolates highly effective in fixing N_2 for peanut were originally isolated from peanut nodules.

An advantage of performing effectiveness screening in growth pouches was the ability to observe time required for initial nodulation of the host legume by rhizobia. Early nodulation of bean was recorded at 8 days for R. phaseoli isolates 931A, 962C, 970B and strain B84 while 10-14 days were required for the remaining isolates. Early nodulation of lentil was recorded at 11 days for R. leguminosarum isolates 711M and 762K and strains P28 and LC53 while 12-16 days were required for the remaining isolates. No differences in time to initial nodulation for cowpea rhizobia on cowpea were observed. The most dramatic observations occurred for peanut where time to nodulation varied among strains from 18 to 35 days.

The influence of nodulation time on plant growth was examined on peanut. Peanut seedlings in growth pouches were inoculated with ca. 10^9 organisms/ml from slant cultures of selected cowpea rhizobia. Growth pouches were placed in the growth chamber in a completely random design replicated four times. Roots were observed for appearance and distribution of nodules every second day beginning 14 days after inoculation. Plant shoots were harvested at 5 weeks, dried at 60°C for 48 hours and weighed. Isolates that promoted early nodulation also increased shoot dry weight (Table 5). The correlation between nodulation time and shoot dry weight was

TABLE 5

Influence of Rhizobium on Time to Nodulation of A. hypogaea cv. 'Florunner'.

Strain	Time to Nodulation (days)	Shoot Dry Weight † (g/plant)
CA22	18.6	1.45 a
C870B	20.0	1.30 ab
C8315	25.0	1.28 ab
C860J	29.0	1.20 ab
C811K	20.0	1.17 ab
C830E	21.0	1.16 ab
CB756	24.6	1.12 abc
C810I	21.6	1.10 abc
C820F	21.6	1.09 abc
C841L	25.0	0.96 bcd
C850J	29.0	0.76 cd
PN1	20.0	0.68 d
CA006	27.6	0.66 d
Uninoc. Control	ND	0.59 d

† Means not followed by a common letter differ significantly (p= 0.05) according to DMRT ($S_{\bar{x}}$ = 0.78).

-.60 and was significant at the 10% level of probability. Strains promoting early nodulation formed extensive nodules on the upper tap root and, conversely, strains that were slow to nodulate formed nodules on lateral roots.

Final Effectiveness Tests

Generally, results from final effectiveness tests indicated a positive relationship between shoot dry weight and total nitrogen content (Tables 6-9). High dry matter production was usually associated with high total nitrogen content, although this relationship varied more for bean (Table 6) and peanut (Table 9) than for lentil (Table 7) and cowpea (Table 8). The relationship be-

TABLE 6

Effect of Inoculation with Selected Strains of *R. phaseoli* on Nodulation, Shoot Dry Weight, and Total Nitrogen Content of *P. vulgaris* cv. 'Commodore'.

Strain	Nodule Fresh Weight (g/plant)	Shoot Dry Weight (g/plant)	Total Nitrogen (mg/plant)
971A	2.20 ab†	4.32 ab	100.52 a
931A	1.81 b	4.81 a	88.40 ab
Nitrogen Control	0.00	4.44 ab	79.44 bc
931C	1.65 b	4.05 ab	78.24 bc
962C	1.41 b	4.25 ab	74.14 bc
970 B	1.78 b	3.85 ab	65.98 cd
B84	2.78 a	3.72 b	57.46 d
Uninoc. Control	0.00	2.08 c	27.24 e
$S_{\bar{x}}$	0.36	0.42	7.58

† Means within a column not followed by a common letter differ significantly (p=0.05) according to DMRT.

TABLE 7

Effect of Inoculation with Selected Strains of *R. leguminosarum* on Shoot Dry Weight and Total Nitrogen Content of *L. esculenta* cv. 'Chilean'.

Strain	Shoot Dry Weight (g/plant)	Total Nitrogen (mg/plant)
P28	1.50 b†	25.14 a
Nitrogen Control	2.09 a	23.20 ab
C53	1.42 b	19.94 b
L760A	1.22 bc	18.80 b
L771B	1.15 bc	19.76 b
L762B	0.89 cd	9.89 c
Uninoc. Control	0.70 d	9.70 c
$S_{\bar{x}}$	0.21	2.18

† Means within a column not followed by a common letter differ significantly (p=0.05) according to DMRT.

16

TABLE 8

Effect of Inoculation with Selected Strains of Cowpea Rhizobium on Nodulation, Shoot Dry Weight and Total Nitrogen Content of V. unguiculata cv. 'Mississippi Silver'.

Strain	Nodule Weight (g/plant)	Shoot Dry Weight (g/plant)	Total Nitrogen (mg/plant)
Nitrogen Control	0.00	3.86 a	88.68 a
CV041	0.93 a †	2.68 b	74.40 b
CV030	0.78 ab	2.20 bcd	64.70 bc
CV031	0.58 cd	2.44 bc	54.78 cd
CB756	0.82 ab	1.85 de	54.48 cd
C346A	0.68 bc	1.82 de	49.46 de
CV018	0.84 ab	1.98 cd	36.64 ef
CV012	0.50 d	1.45 e	27.84 f
Uninoc. Control	0.00	0.82 f	4.48 g
$S_{\bar{x}}$	0.25	0.24	6.60

† Means within a column not followed by a common letter differ significantly (p=0.05) according to DMRT.

TABLE 9

Effect of Inoculation with Selected Strains of Peanut Rhizobium on Nodulation, Shoot Dry Weight and Total Nitrogen Content of A. hypogaea cv. 'Florunner'.

Strain	Nodule Fresh Weight (g/plant)	Shoot Dry Weight (g/plant)	Total Nitrogen (mg/plant)
Nitrogen Control	0.00	3.94 a	110.28 a
CA22	1.60 a †	4.30 a	92.47 b
870B	0.86 b	4.25 a	79.82 bc
811K	0.75 b	3.80 a	69.36 cd
850J	0.61 b	4.10 a	61.09 d
831B	1.30 a	4.35 a	56.30 de
860J	0.72 b	3.38 ab	42.53 ef
841L	0.83 b	2.78 b	36.56 f
Uninoc. Control	0.00	1.38 c	9.71 g
$S_{\bar{x}}$	0.46	0.45	8.12

† Means within a column not followed by a common letter differ significantly (p=0.05) according to DMRT.

tween dry matter and total nitrogen with nodule mass was more variable, however. For example, low shoot dry weight and total nitrogen content obtained for bean inoculated with R. phaseoli strain B84 was not due to lack of nodulation but apparently to lower efficiency in N_2-fixation by the nodular tissue (Table 6). Simple correlation coefficients computed for shoot dry weight and total nitrogen were highly significant for all tests (Table 10). Nodule mass was not significantly correlated with either shoot dry weight or total nitrogen with any of the rhizobia-host legume systems that were evaluated.

Local isolates of R. phaseoli and cowpea Rhizobium were found to be more effective in N_2-fixation than control strains. However, control strains of R. leguminosarum and peanut Rhizobium

TABLE 10

Correlation Coefficients for N_2-Fixing Traits.

Trait	Rhizobium Test	Trait: Shoot Dry Weight	Trait: Total Nitrogen
Nodule Mass			
	R. phaseoli	-.40	-.65
	Cowpea Rhizobium	.51	.60
	Peanut Rhizobium	.47	.52
Shoot Dry Weight			
	R. leguminosarum		.84**
	R. phaseoli		.98**
	Cowpea Rhizobium		.92**
	Peanut Rhizobium		.79**

** Significant at p=0.01.

were more effective than isolates. The final isolates selected as most effective on bean (Table 6) and lentil (Table 7) originated from soils previously cropped to beans and peas, respectively. The final effective isolates of the cowpea and peanut rhizobia were of a diverse origin.

DISCUSSION

Nodulation results of plants grown in soil:sand mixtures indicated that R. phaseoli and R. leguminosarum were not abundant in any of the Mississippi soils tested. Burton et al.[9] found that ≤ 18 nodules per plant formed on any variety of bean grown in 10 Midwestern soils. Sparse nodulation on bean plants collected from several areas in Mississippi also indicated the limited distribution of R. phaseoli in these soils. Additional strains of R. leguminosarum and R. phaseoli may reside in these soils but were not detected in this study due to possible host specificity. One cultivar of each legume was used as a host, which may not have been nodulated by all representative strains of rhizobia in the soil. The effectiveness of R. phaseoli and R. leguminosarum isolates obtained from soils and host plants varied over a 2-fold range in shoot dry weight production (Tables 1 and 2).

Abundant nodulation of cowpea and peanut plants in soil:sand mixtures or on field-collected plants indicated the wide distribution of cowpea rhizobia capable of nodulating diverse legume species of the cowpea cross-inoculation group. Previous reports verify the wide distribution and diversity of the cowpea rhizobia[8,19,20].

The effectiveness (shoot dry matter production) of the cowpea rhizobia, however, in the present study varied up to 4-fold among the isolates (Tables 3 and 4). These results agree with those obtained in several studies[1,8,17,19,20] which report the broad range of effectiveness among different isolates of the cowpea rhizobia when inoculated on various species within the cowpea cross-inoculation group. Inoculation of legumes in the cowpea group with highly effective rhizobia may improve plant growth and nodulation on soils containing poorly effective native rhizobia and low availability of soil nitrogen. This would require additional screening of the selected strains to determine the ability to compete against native rhizobia for nodule sites[5,7,11,17,24,28].

Cowpea and peanut are members of the cowpea cross-inoculation group and may be nodulated by rhizobia isolated from a wide range of legumes within that group. Peanut exhibits a greater specificity for rhizobia than cowpea[8]. Peanut nodulates with most rhizobial isolates from other peanuts, but nodulates infrequently with rhizobial isolates from other legume species (Table 4). Cowpea, however, was nodulated by rhizobia from a diverse array of legumes (Table 3). Cowpea was effectively nodulated by isolates from *Cyamopsis tetragonolobus* DC. and *Lespedeza stipulacea* Maxim, but peanut was not nodulated by the same isolates. Cowpea was nodulated by 12 isolates from peanut but peanut was nodulated effectively by only two cowpea isolates. This semi-selective or one-way promiscuity by cowpea has been reported previously[1,8,17,19]. The increased selectivity of peanut for specific rhizobia compared to cowpea em-

phasizes the need for development of effective inocula containing rhizobia selected for a specific host-Rhizobium association.

Highly effective rhizobia were selected and non-infective isolates eliminated from a large culture collection. This selection provides the first level of authentication required for allocating isolates to a species level[24]. However, the possibility exists that the non-infective bacteria could have lost the genetic ability to infect a compatible host legume. Infectiveness screening also permitted preliminary tests for effectiveness of N_2-fixation on known infective isolates. Thus, a small number of infective and effective rhizobia were obtained for more critical evaluation of N_2-fixation traits.

The use of dry matter measurements as an estimate of effectiveness of N_2-fixation in preliminary effectiveness tests was advantageous for screening a large number of rhizobial isolates. The high correlation coefficients between dry weight and nitrogen content obtained in the final effectiveness tests (Table 10) indicate that selection of isolates initially on the basis of shoot dry weight was an adequate selection criterion. Erdman and Means[12] found highly significant correlation coefficients between dry weights and nitrogen contents for 12 different leguminous plants grown under controlled conditions. They suggested that dry weights could be used as indicators of N_2-fixing efficiency of rhizobia. Dry weight of peanut can be used as an estimate of peanut-Rhizobium strain efficiency[28]. A recent report contends that the direct relationship between dry matter yield and nitrogen content is valid

for only fully effective strains of Rhizobium[15]. Our procedure inherently eliminates ineffective and partially effective isolates in the infectiveness and preliminary effectiveness screening stages. Final effectiveness screening is performed on the elite strains of the collection. The validity of this approach is supported by the high correlation between dry matter and nitrogen content obtained in the final stage of screening.

Plastic growth pouches[26] were suitable for screening Rhizobium isolates for infectivity and preliminary effectiveness in the growth chamber. Bacteriological control was achieved as no nodules were observed on any of the uninoculated host plants. Legume seeds to be inoculated can be positioned and supported directly on the germination paper insert of the pouch. This eliminates the preparation and distribution of sterile seedling agar required as a support matrix for seeds when the tube method is used for screening[24]. Large-seeded legumes can be accomodated and grown in pouches while tubes can only handle forage legume species with a maximum seed size of about 10 mg[24], Thus, cowpea rhizobia can be tested directly on Vigna spp. rather than only on Macroptilium atropurpureum (DC) Urb. cv. 'Siratro' which has been suggested when cowpea rhizobia are evaluated in tube culture[24].

Precaution must be taken when testing peanut for nodulation in growth pouches. Inconsistent nodulation of peanut in pouches encountered in this study is apparently related to the mode of rhizobial infection. Most legumes are infected by rhizobia through numerous root hairs present over the developing root system. Peanuts are infected at the base of root hairs located only at root

junctures[10]. Such localization of nodule sites may require rhizobia to survive in the rooting medium for an extended time period before infection occurs. If low numbers of peanut rhizobia were added to the nutrient solution, it is conceivable that rhizobia may die off before locating a nodule site. Also peanuts, unlike clovers, may not secrete sizeable amounts of root exudates which can cause multiplication of added rhizobia in nutrient solution and insure subsequent nodulation[4]. These are all plausible explanations for the inconsistent nodulation and discrepancies between plate count and most-probable number (MPN) analyses of peanut rhizobia. Use of growth pouches is not recommended for MPN analyses involving peanut.

An important characteristic observed for the Rhizobium isolates in this study was the time required for initial nodulation. Results summarized in Table 5 indicate that most of the peanut Rhizobium capable of early nodulation also contributed significantly to plant growth. Most isolates selected for final effectiveness tests were capable of early nodulation. Other reports also indicate that early nodulation has a positive influence on growth and N_2-fixation of clovers[5,22]. Nutman[22] speculated that early nodulation may be a genetic trait of the host legume that is inherited in a complex manner.

The results of this study indicate that highly effective rhizobia can be selected under controlled conditions from a large collection of isolates by following a step-wise screening procedure. Selections can be made based on differences among isolates in infectiveness, dry matter production and total nitrogen production of

their host legumes. Additional characteristics such as time to first nodule formation may be observed. Other selection tests that could be easily performed in growth pouches include tolerance of rhizobia to different pH and nitrate levels and competitiveness with other rhizobial and/or non-rhizobial species of bacteria.

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MEMORANDUM

Date 2 Sep 88

TO: PPC/CDIE/DI, Tina Wilson/Acquisitions - 209 SA-18

FROM: S&T/AGR/RNR, Lloyd R. Frederick

SUBJECT: Reprints of Articles with Partial Support of A.I.D. Contracts/Grants

Attached is one copy of the following article with the supporting contract/grant number:

Kremer, R.J. and H.L. Peterson. 1982. Isolation, selection and evaluation of Rhizobium under controlled conditions. Commun. in Soil Sci. Plant Anal 13: 749-774

~~Contract/Grant~~ PASA Number: AG/TAB 610-9-76

Project: 931-0610

Attachments: a/s

cc: T.Gill
D. Bathrick

27