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Nodulation, N_2 Fixation, and Seed Yield of Navy Beans as Influenced by Inoculant Rate and Inoculant Carrier[1]

S.D. Sparrow, Jr., and G.E. Ham[2]

ABSTRACT

Peat is the most commonly used carrier of *Rhizobium* sp. (rhizobia) in inoculants. The lack of suitable local peat in many areas of the world leads to interest in other materials as carriers of rhizobia. The purposes of these studies were 1) to compare potential carriers of *Rhizobium phaseoli* for their ability, under field conditions, to supply viable rhizobia to promote nodulation, N_2 fixation, and increased seed yield of navy beans (*Phaseolus vulgaris* L.), and 2) to determine the optimum rate of inoculation with *Rhizobium phaseoli* to promote nodulation, N_2 fixation, and increased seed yield of navy beans in two soils in Minnesota, a loamy sand (sandy, mixed, Udorthentic Happloboralf) and a silt loam (fine-silty over sandy, mixed, mesic Typic Hapludoll).

In 1978, five inoculation rates (ranging from 1 to 10^8 cells/cm of row) of liquid suspensions of *R. phaseoli* inoculants were applied at planting. In 1979, *R. phaseoli* in six inoculant carriers, peat, charcoal, vermiculite, corn (*Zea mays* L.) cobs, peanut (*Arachis hypogaea* L.) hulls, and liquid medium, was used to inoculate navy beans at rates of 2.5×10^4 and 5×10^6 cells/cm of row. Nodule numbers and weight, acetylene reduction rates, seed yield, and seed N percentage were determined.

In 1978, in most parameters measured, inoculation rates of 10^5–10^6 cells/cm were required for a statistically significant increase over the lowest inoculation rate. In 1979 there was no effect of inoculation on a soil with a relatively high population of soil *R. phaseoli* (10^4 cells/g soil) and relatively high mineral N content (30 ppm). On a soil with an indigenous population of <10 cells/g soil and mineral N content of 8 ppm, peat and charcoal inoculants resulted in the highest seed yield, nodule numbers and weight, and acetylene reduction rates. Peanut hulls and corn cobs gave the poorest responses. These results indicate that peat, charcoal, and vermiculite can be successfully used as carriers of *R. phaseoli* but peanut hulls and corn cobs are unsatisfactory.

Additional index words: Rhizobium phaseoli, Phaseolus vulgaris L., Inoculation, Acetylene reduction.

THE common bean (*Phaseolus vulgaris* L.) is a frequently cultivated food legume in most agricultural areas of the world and is the primary food staple for people in many developing countries, especially in Latin America (Bazan, 1975; Pinchinat, 1977). Bean yields in these areas are often lower than the potential yield (FAO, 1979; Hernandez-Bravo, 1973; Graham, 1978). Low available soil N often limits bean yields (Bazan, 1975). Often, farmers in developing countries cannot afford expensive technological inputs such as N fertilizers (Aguirre and Miranda, 1973). One alternative solution is to utilize N_2 fixation through the legume-*Rhizobium* symbiosis (National Research Council, 1979). Obtaining nodulation that will supply the N needs of the plant is a problem. Sometimes inoculation of beans results in increased seed yield, but frequently plants are poorly nodulated and yield is not increased (Bazan, 1975; Franco and Day, 1980; Graham, 1978; Stephens, 1967). This has been the case in areas with developed agricultural systems, such as in Minnesota (Robinson et al., 1974) as well as in developing countries.

Failure of beans to respond to inoculation has been attributed to high soil N or to the presence of a large indigenous population of *R. phaseoli*. Sometimes however, this has not been the case and the poor response to inoculation may have been due to inadequate numbers of rhizobia cells being added or to poor survival of the bacteria in the soil during the time between inoculation and the onset of nodulation. Thus, one aspect of the inoculation problem that needs to be studied is methods of insuring adequate numbers of viable nodule bacteria for good nodulation. Bean inoculants must contain large numbers of an effective strain of *R. phaseoli* and the inoculant should provide protection for the rhizobia between the time of inoculation and the formation of nodules (Bonnier, 1960).

Peat is used widely as a carrier of rhizobia in commercial inoculants; however, suitable sources of peat are not available in many areas of the world (Halliday and Graham, 1978; Tilak and Subba Rao, 1978; Corby, 1976; Wu and

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[2] Former graduate research assistant (now assistant professor, Agric. Exp. Stn., Univ. of Alaska, Fairbanks) and professor of soil science (now head, Dep. of Agronomy, Kansas State Univ., Manhattan), respectively.

Kuo, 1969). Thus materials which can serve as alternative carriers of rhizobia need to be evaluated.

The objectives of this research were to determine optimal inoculation rates needed under field conditions in Minnesota to provide for high rates of nodulation, N_2 fixation, and high seed yield of navy beans; and to compare the effectiveness of different carrier materials for their ability to supply adequate numbers of rhizobia to produce good nodulation of navy beans. Peanut (*Arachis hypogaea* L.) hulls, corn (*Zea mays* L.) cobs, and charcoal were selected for evaluation as inoculant carriers because these materials are likely to be readily available in many developing countries; vermiculite was selected because this material has been reported to have a protective effect on rhizobia (Bonnier, 1960); and peat was used because it is the most commonly used carrier for commercial rhizobia inoculants. Most of these materials have been evaluated as carriers of rhizobia (Bonnier, 1960; Corby, 1976; Strijdom and Deschodt, 1976; Tilak and Subba Rao, 1978), but with the exception of peat, these materials have not been tested adequately as carriers of *R. phaseoli*.

METHODS AND MATERIALS

Field studies were conducted in 1978 to determine the optimum rate of inoculation of navy beans in Minnesota and in 1979 to evaluate the effectiveness of different carriers of *R. phaseoli* to promote nodulation, N_2 fixation, and increased seed yield of navy beans under field conditions. In both years, experimental plots were at two locations in east-central Minnesota, one at the Sand Plain Research Farm at Becker on a Hubbard loamy sand (sandy, mixed, Udorthentic Haploborall), and at the Rosemount Agricultural Exp. Stn. at Rosemount on a Waukegan silt loam (fine-silty over sandy, mixed, mesic, Typic Hapludoll).

In 1978, 'Seafarer' and 'Upland' navy bean cultivars and strains 3644 (from Rothamsted, Herts, England), CIAT 75 (from Centro Internacional de Agricultura, Cali, Colombia), and 650R (from Rhodesia, now Zimbabwe) of *R. phaseoli* were used. Inoculants were prepared by growing *R. phaseoli* in yeast extract mannitol (YEM) broth (Vincent, 1970); cells were harvested by centrifugation and then resuspended in dilute yeast extract salts medium (0.1 g yeast extract/liter in YEM salts solution). This was done to remove bacterial toxins or nutrients from the suspensions in order to maintain numbers of viable cells as constant as possible. Plate counts were done on the suspensions prior to making dilutions. Dilutions of 10^0, 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} were made and 16 ml aliquots added to 500 ml whirl-pak bags (Nasco[3] and stored overnight under refrigeration. Inoculants were diluted to 200 ml at the time of planting to facilitate even distribution of the cell suspension in the seed furrow. Inoculation rates were 1.3, 1.3×10^2, 1.3×10^4, 1.3×10^6, and 1.3×10^8 cells/cm row on the Waukegan silt loam and 1.5×10^{-1}, 15, 1.5×10^3, 1.5×10^5, and 1.5×10^7 cells/cm row on the Hubbard loamy sand. For each site, inoculants were made the day prior to planting.

Each plot consisted of four rows, 6.1 m long and 36 cm apart resulting in a population of approximately 187,000 viable seeds/ha. Inoculation was done by distributing the cell suspensions into the furrow at the time of planting.

A split plot experimental design was used, with cultivars as the main plots and *R. phaseoli* strain and inoculation rate factorial combinations as subplots. This resulted in a $2 \times 3 \times 5$ factorial experiment plus a control treatment for each cultivar. Each treatment was replicated five times. Plots on the Hubbard loamy sand were irrigated as needed; plots on the Waukegan silt loam were not irrigated.

Plate counts were done within 24 hours after planting on a few samples to confirm that numbers of viable rhizobia in the inoculants had not changed appreciably since making the suspensions. Soil samples were collected from the plot areas, composited, and sent to the Univ. of Minnesota Soil Testing Laboratory where chemical analyses were done using published methods (Dahnke, 1980). Numbers of *R. phaseoli* in the soil were estimated by the "plant dilution technique" (Weaver and Frederick, 1972). Serial dilutions of soil were made and used to inoculate pre-germinated 'Upland' navy bean seeds planted in growth pouches (Scientific Products)[3] and grown in a growth chamber. After 5 weeks, each plant was observed for the presence or absence of nodules and numbers of *R. phaseoli* estimated by use of a "most probable number" table (Alexander, 1965).

Acetylene reduction assays were used to estimate relative nitrogenase activity before flowering (about 35 days after planting), at full-flowering (about 45 days), and at midpod-fill (about 60 days). Samples were taken on sunny days in late morning or early afternoon. A plant was randomly selected from each plot, clipped at ground level, and a 10×15 cm soil core containing the plant root was collected. The core was placed in a 3.8 liter plastic bucket and partially disrupted to facilitate gas exchange. Within 5 min of taking the core, 60 cc of air was removed through a rubber septum fitted in the bucket lid, and replaced with 60 cc commercial grade acetylene. This resulted in 2.2% (calculated) acetylene by volume. This concentration of acetylene was found in earlier experimentation in this laboratory to be sufficient to saturate the nitrogenase enzyme in our system. The assembly was vigorously shaken and allowed to incubate exactly 1 hour, at which time the buckets were again shaken, and a 10 cc gas sample removed, placed in a vacutainer, and stored until determination of ethylene concentration by gas chromatography. At the full-flowering sampling date, root nodules were collected and frozen. They were later counted and weighed. Seed yield was measured at the end of the growing season and seed N content was determined by the semi-micro Kjeldahl procedure (Bremner, 1965).

In 1979, 'Upland' navy beans were inoculated with the same strains of *R. phaseoli* used in the 1978 study. The 1979 studies were on sites near those of the 1978 studies. The soil types and most of the soil characteristics were similar in both years. Two rates of inoculation (2.5×10^4 and 5.6×10^6 cells/cm row) and six rhizobia carriers (milled peat, powdered charcoal, ground corn cobs, peanut hulls, vermiculite, and liquid) were used. This resulted in a $3 \times 6 \times 2$ factorial experiment plus controls. The experimental design was a randomized complete block with five replications. *Rhizobium phaseoli* was grown in YEM broth, centrifuged, and resuspended in dilute mannitol salts medium (0.1 g mannitol per liter in YEM salts). Cell numbers were maintained at approximately the same concentration as those in the initial culture for the low inoculation rate and at 200 times the initial concentration for the high inoculation rate. Cell suspensions were added to autoclaved carrier materials, mixed, and dispersed into 170 ml whirl-pak bags. Plate counts were made on the cell suspensions to determine the number of viable cells added to the carrier materials. At the time of planting the inoculants (except for the liquid inoculants) were diluted with sand to facilitate even distribution in the furrow. Planting was done within 48 hours after the inoculants were made. Planting, sampling, and sample analysis procedures were the same as in 1978 except that acetylene reduction was done at only two sampling dates (full-flowering and midpod-fill) in 1979.

Statistical analysis consisted of conventional analysis of variance techniques. Two separate analysis of variances were calculated. The first included all treatments; this determined the overall response to inoculation, but main effects and interactions could not be assessed. Therefore, a second analysis, with control treatments omitted, was made to determine factor effects and interactions. The Bayes LSD multiple comparison test (Smith,

[3] Mention of a trademark, proprietary product, or company name does not constitute a guarantee or warranty of the product and does not imply its approval to the exclusion of other products that may also be suitable.

1978) was used for mean separation. In the BLSD procedure, a k-ratio rather than an α value is used. The k-ratio is the Type I to Type II error seriousness ratio. A k-ratio of 100 corresponds approximately to an α value of 0.05.

RESULTS AND DISCUSSION

Soil characteristics at both field locations are shown in Table 1. The 1979 Hubbard sandy loam site was on an isolated area of the research farm where beans had not been previously grown; this, plus the very acid soil conditions, probably accounts for the low numbers of *R. phaseoli*. We have no explanation for the very high numbers of soil *R. phaseoli* on the Waukegan silt loam in 1979 since the site had similar soil characteristics and a similar cropping history to that of the 1978 site.

1978 Experiments

Inoculation at both locations resulted in significant ($P \leq 0.05$) increases over the controls in nodulation, acetylene reduction rates at all sampling dates, and seed yield (Table 2). Although statistically significant differences in seed N percentage were noted on the Waukegan silt loam, these data appear to have little biological significance.

Acetylene reduction rates at both sites for both cultivars peaked at full-flowering (Fig. 1). Other workers (Graham and Rosas, 1977; Waters et al., 1980) have reported maximum nitrogenase activity of *Phaseolus vulgaris* at or near flowering. Except at midpod-fill, when rates were higher for 'Upland' than for 'Seafarer', no significant differences were noted between cultivars for acetylene reduction rates.

Seed yields for all treatments were much higher on the Waukegan than on the Hubbard soil (Table 2), probably due to the higher inorganic N content of the Waukegan soil (Table 1).

About 10^5 cells/cm of row were required for significant ($P \leq 0.05$) increases in acetylene reduction rates, nodule numbers and weight, and seed yield on the Hubbard loamy sand. On the Waukegan silt loam, 10^6 cells/cm of row resulted in significant increases in acetylene reduction rates and nodule numbers and weights; about 10^8 cells/cm of row were required for a significant increase in seed yield (Table 2). Although the highest inoculation rate resulted in a higher percentage increase in yield over the control on the Hubbard soil than on the Waukegan soil (22% vs. 10%), the absolute yield increase was greater on the Waukegan soil (Table 2). This is surprising since the Waukegan soil had a higher inorganic N content. Positive responses in most parameters measured were noted on both soils up through the highest inoculation rate, although the differences were not statistically significant. Thus the rate needed for maximum response may be greater than the highest rate used in this study.

There were no significant effects of strain of *R. phaseoli* on seed yield. For other parameters in which there was a significant strain effect, the greatest response was always due to CIAT 75 (Table 3). There were no significant main effects for bean cultivar and the only significant interactions were inoculation rate × strain and inoculation rate × strain × cultivar for nodule numbers.

1979 Experiments

Inoculation significantly ($P \leq 0.05$) increased seed yield, acetylene reduction rates at both sampling dates, and nodule numbers and weight on the Hubbard loamy

Table 1. Soil characteristics at the field experimental sites.

Characteristic	Site location: Rosemount	Site location: Becker
Soil series	Waukegan	Hubbard
Classification	Typic Hapludoll	Udorthentic Haploboroll
Textural class	Silt loam	Loamy sand
Organic matter (%)	3.5	2.0
Inorganic N (ppm)	30	8
pH (1978)	6.5	6.2
pH (1979)	6.5	5.5
Indigenous *Rhizobium phaseoli* (cells g^{-1} oven-dry soil)		
1978	2.4×10^2	7.1×10^2
1979	6.2×10^4	<10

Table 2. Navy bean seed yield acetylene reduction rates, and nodule numbers and weight as influenced by *Rhizobium phaseoli* strain and inoculation rate in 1978.

	Treatment	Seed yield: Hubbard soil	Seed yield: Waukegan soil	C_2H_2 reduction rates at full-flowering: Hubbard soil	C_2H_2 reduction rates at full-flowering: Waukegan soil	Nodule numbers: Hubbard soil	Nodule numbers: Waukegan soil	Nodule weight: Hubbard soil	Nodule weight: Waukegan soil
	Dilution rate	kg ha^{-1}		μmoles plant^{-1} hr^{-1}		nodules plant^{-1}		grams plant^{-1}	
	Control†	911	2,821	1.76	0.59	27	24	0.74	0.33
	10^{-8}	932	2,900	2.17	0.47	19	16	0.43	0.14
	10^{-6}	866	2,901	2.60	0.59	20	18	0.49	0.23
	10^{-4}	1,004	2,956	2.65	1.01	24	25	0.63	0.33
	10^{-2}	1,053	3,055	4.45	3.32	34	48	0.79	0.64
	10^{0}	1,141	3,217	5.72	3.50	43	60	0.95	0.85
Significance:		**	**	**	**	**	**	**	**
$BLSD_{(k=100)}$		100	168	1.34	1.07	10	19	0.20	0.24
Strain									
	3644	974	2,976	3.15	1.41	22	24	0.59	0.36
	CIAT 75	1,010	3,036	3.94	2.60	35	53	0.71	0.58
	650R	1,013	3,005	3.47	1.33	26	23	0.61	0.37
Significance:		NS	NS	NS	*	*	**	NS	NS
$BLSD_{(k=100)}$		--	--	--	0.34	8	13	--	--

*,** Significant at $P \leq 0.05$, and 0.01.
† Control not used in main effects comparisons.
NS = Not significant.

sand (Table 3). On the Waukegan soil no inoculation responses were noted. As in 1978, average seed yield was much higher on the Waukegan soil than on the Hubbard soil (1947 kg/ha vs. 870 kg/ha).

Acetylene reduction rates at the Waukegan site in 1979 were comparable to those at the Hubbard site, suggesting a similar level of nitrogenase activity. Numbers of *R. phaseoli* were quite high in the Waukegan soil in 1979; the introduced rhizobia were probably not able to successfully compete with the indigenous population enough to affect N_2 fixation rates. Acetylene reduction rates at both locations had not decreased at midpod-fill relative to those at full-flowering, indicating that N_2 fixation was still occurring at this late sampling date.

A significant inoculant rate, strain, and carrier effect was found for seed yield, nodule numbers, and nodule weight on the Hubbard soil; the only main effect for acetylene reduction rate was for inoculant carriers (Table 3). For parameters with a significant main effect for inoculant rate, the high rate always gave the higher values. For parameters which showed a significant strain effect, CIAT 75 gave the greatest response, as was true in 1978. For all parameters except seed N percentage, charcoal- and peat-based inoculants produced the highest values, peanut hulls and corn cobs produced the lowest, and vermiculite and liquid inoculants were intermediate.

For seed yield, acetylene reduction rates, and nodule numbers and weight there was no inoculation rate response for peanut hulls, corn cobs, charcoal, or peat, but there was a rate response for vermiculite and liquid inoculants (Fig. 2, only results of acetylene reduction shown) with the high inoculation rate giving the higher response.

The results of the 1979 field experiments indicate that the kind of rhizobia carrier used can have an effect on the survival of *R. phaseoli* cells in soil between the time of inoculation until they are able to infect bean plants. It appears that peat and charcoal have a protective effect against adverse soil conditions since even at the low inoculation rate inoculants made from these carriers generally resulted in better nodulation, higher N_2 fixation activity, and higher yields than did inoculants made from other carriers. Liquid and vermiculite were intermediate in their effects on most parameters measured, with peanut hulls and corn cobs having little or no effect, even at the high inoculation rate. Peanut hulls and corn cobs apparently failed to support viable *R. phaseoli* in the soil long enough for nodule initiation to occur.

CONCLUSIONS

Inoculation with effective strains of *R. phaseoli* increased nodulation, N_2 fixation, and seed yield of navy beans in east-central Minnesota, provided the numbers

Table 3. Navy bean seed yield, acetylene reduction rates, and nodule numbers and weights as influenced by inoculation rate, *Rhizobium phaseoli* strain, and inoculant carrier on a Hubbard loamy sand in 1979.

	Seed Seed yield	C_2H_2 reduction rates at full-flowering	Nodule numbers	Nodule weight
	kg ha^{-1}	μmoles plant^{-1} hr^{-1}	nodules plant^{-1}	grams plant^{-1}
Inoculation rate				
Control†	644	0.02	3	0.04
Low	875	1.65	17	0.25
High	943	2.05	25	0.41
Significance:	*	NS	*	**
Strain				
3644	911	1.72	13	0.27
CIAT 75	1,007	2.39	38	0.47
650R	803	1.45	12	0.26
Significance:	**	NS	**	**
$BLSD_{(k=100)}$	62	--	7	0.08
Carrier				
Liquid	882	1.33	17	0.30
Peat	1,112	3.10	30	0.53
Peanut hulls	756	0.86	9	0.27
Corn cobs	687	0.44	7	0.07
Vermiculite	971	2.13	27	0.36
Charcoal	1,035	3.16	36	0.46
Significance:	**	**	**	**
$BLSD_{(k=100)}$	86	0.83	11	0.12

*,** Significant at $P \leq 0.05$ and 0.01.
† Control not used in comparison for main effects and interactions.
NS = Not significant.

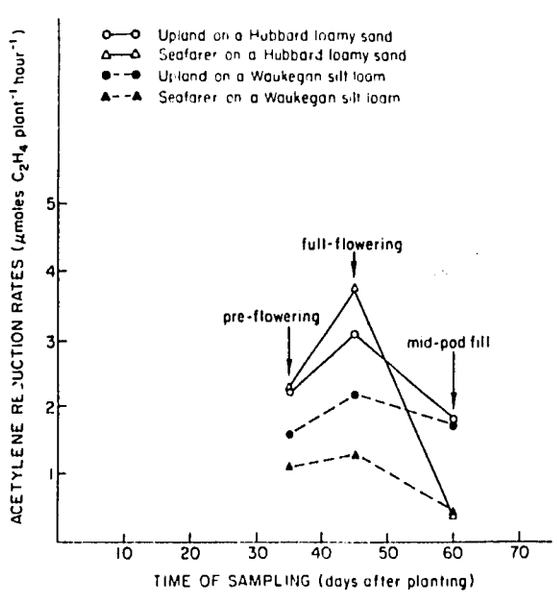

Fig. 1. Acetylene reduction rates of two cultivars of navy beans on three sampling dates on a Hubbard loamy sand and a Waukegan silt loam.

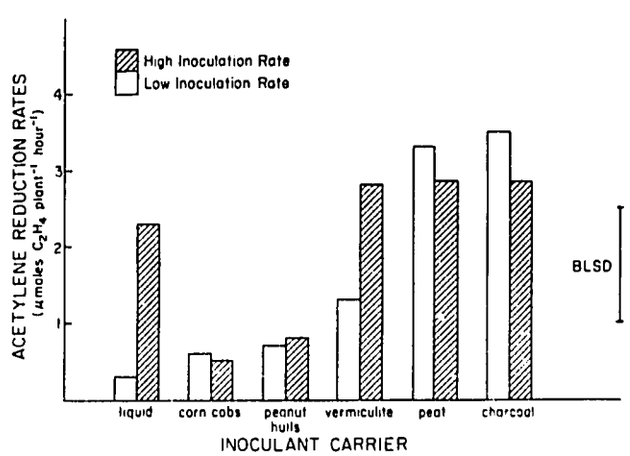

Fig. 2. Influence of inoculation rate with rhizobia inoculants made from various carriers on acetylene reduction rates of navy beans at full-flowering on a Hubbard loamy sand.

of indigenous *R. phaseoli* in the soil were not excessively high. In soils containing a few hundred cells of indigenous *R. phaseoli* per gram of soil, an inoculation rate of 10^5 to 10^6 cells/cm of row were required to increase nodulation and N_2 fixation when liquid inoculants were used and further responses were noted at rates of 10^7 to 10^8 cells/cm of row. In a soil containing a relatively large population of indigenous *R. phaseoli* (10^4 cells/g soil) no inoculation response was noted with inoculation rates of up to 5×10^6 cells/cm of row.

For positive responses in most parameters measured, lower inoculation rates were required for *R. phaseoli* inoculants made from peat and charcoal than from liquid or vermiculite. Results of this study and a companion study in which survival of *R. phaseoli* in peat, charcoal, and vermiculite was shown to be good for more than 6 months, indicate that these materials, especially peat and charcoal can be used successfully as carriers of *R. phaseoli*. On the other hand, peanut hulls and corn cobs are unsatisfactory as carriers of *R. phaseoli*. Charcoal is likely available in many countries where good sources of peat are scarce and may be useful in the production of rhizobia inoculants in these countries.

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