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Semienclosed Tube Cultures of Bean Plants (*Phaseolus vulgaris* L.) for Enumeration of *Rhizobium phaseoli* by the Most-Probable-Number Technique†

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The semienclosed tube culture technique of Gibson was modified to permit growth of common bean (*Phaseolus vulgaris* L.) roots in humid air, enabling enumeration of the homologous (nodule forming) symbiont, *Rhizobium phaseoli*, by the most-probable-number plant infection method. A bean genotype with improved nodulation characteristics was used as the plant host. This method of enumeration was accurate when tubes were scored 3 weeks after inoculation with several *R. phaseoli* strains diluted from aqueous suspensions, peat-based inoculants, or soil. A comparison of population sizes obtained by most-probable-number tube cultures and plate counts indicated that 1 to 3 viable cells of *R. phaseoli* were a sufficient inoculant to induce nodule formation.

An understanding of the ecology of rhizobia frequently requires their enumeration in natural habitats. For instance, it is useful to know the size of the native soil population capable of nodulating a legume plant during an evaluation of different rhizobial strains as potential inoculants. The only method currently available to enumerate the total population of viable, infective rhizobia for a particular legume host in natural environments is the plant infection technique (2, 3, 6–8), which uses most-probable-number (MPN) tables. This method is based on the crucial assumption that the addition of an infective bacterium to a host root system will ultimately lead to nodule formation. To verify this assumption, it is necessary to grow a plant so that the root system is maintained under microbiologically controlled conditions. This is accomplished easily with small-seeded legumes (e.g., clover) grown in enclosed tube cultures (2). However, this criterion has not been verified adequately for rhizobia nodulating large-seeded legumes (e.g., the common bean [*Phaseolus vulgaris* L.]). MPN enumerations of bean rhizobia (*Rhizobium phaseoli*) are traditionally performed in Leonard jars (3, 6, 7) and perhaps could be performed in cellophane growth pouches (5, 8). The main problem with these culture methods is that they are open to cross-contamination with other rhizobia and deleterious microbes (particularly fungi), thus affecting the accuracy of the results. Futhermore, the Leonard jar requires considerable time and space to assemble, thus restricting the number of samples which can be analyzed by the MPN procedure simultaneously. For our purposes, the Leonard jar is precluded owing to limited space for growing plants.

Some large-seeded legumes can be grown as seedlings in large test tubes which enclose the entire plant (D. H. Hubbell, M.S. thesis, North Carolina State University, Raleigh, 1962) or, alternatively, in a semienclosed culture tube (4) in which only the root system is enclosed and grown on an agar slope. However, common beans do not grow well in either of these culture systems, owing to the crowding effects and poor growth of its roots through agar. Thus, the development of a convenient, space-saving culture technique to grow beans under microbiologically controlled conditions is needed. In this paper, we (i) modify the semienclosed tube culture technique for beans, (ii) verify the accuracy of the MPN plant infection technique to enumerate *R. phaseoli* with this modified tube culture, (iii) establish the minimum inoculant of *R. phaseoli* cells required for nodule formation, and (iv) enumerate the native populations of bean-nodulating rhizobia in Brazilian and Mexican soils, with which we are currently conducting field inoculation trials.

(Preliminary reports of this work were presented at the 10th North American Rhizobium Conference, Maui, Hawaii, 11 to 17 August 1985.)

R. phaseoli (*R. leguminosarum* biovar *phaseoli*) CNPAF 150, CNPAF 238, and CNPAF 312 were from the culture collection at Empresa Brasileira de Pesquisa Agropecuaria-Centro Nacional de Pesquisa de Arroz e Feijão, Goiânia, Goiás, Brazil. Strain MSU45 was isolated from root nodules of beans (*P. vulgaris* L. cv. Domino) grown on the Michigan State University campus. Strain Kim5R3 was isolated as a spontaneous rifampin-resistant mutant of Kim5 provided by Dave Bezdicek, Washington State University, Pullman.

The modified tube culture (Fig. 1A and B) consisted of a large outer tube (25 by 200 mm or larger) containing an inverted smaller tube (16 by 150 mm) or a stack of four 13-mm plastic caps. A strip of Whatman no. 1 filter paper (25 by 160 mm) to serve as a wick was inserted between the larger and smaller tubes and covered one half of the inside circumference of the larger tube. A piece (1 cm) of the paper could be bent over the top of the inverted smaller tube to help support the seedling. Each assembly received 5 ml of a nitrogen-free plant nutrient solution, which is ideal for dry, edible beans (B. D. Knezek and A. J. M. Smucker, personal communication) and which fully wetted the paper wick. Preliminary experiments demonstrated that this medium supports the growth of beans better than does the commonly

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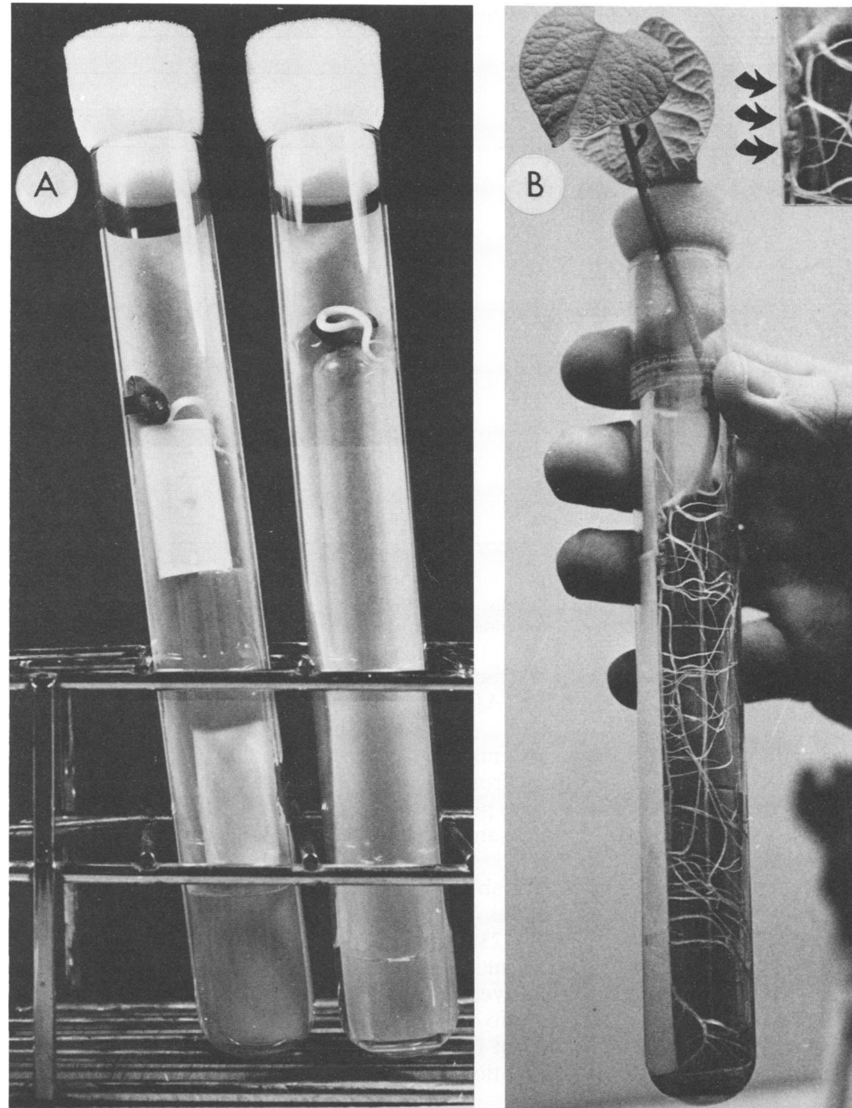

FIG. 1. Tube cultures of bean seedlings for axenic studies of the bean-*R. phaseoli* symbiosis. (A) Seedling supported by plastic caps on the left and an inverted smaller glass tube on the right; (B) nodulated bean plant grown with *R. phaseoli*. The insert is an enlargement of the same root nodules.

used nitrogen-free Fahraeus medium (3; unpublished observation). This growth medium contained 5 ml of 0.5 M K_2SO_4; 2.5 ml of 1 M $MgSO_4$; 0.36 g of $CaHPO_4 \cdot 2H_2O$; 200 ml of 10 mM $CaSO_4$; 1 ml of micronutrient stock; 1 ml of 20 mM ferric citrate; and deionized, distilled water to 1 liter. Micronutrient stock contained 2.86 g of H_3BO_3, 1.81 g of $MnCl_2 \cdot 4H_2O$, 0.22 g of $ZnSO_4 \cdot H_2O$, 80 mg of $CuSO_4 \cdot 5H_2O$, 20 mg of $H_2MoO_4 \cdot H_2O$, and 4 mg of $CoCl_2$ in one liter of deionized, distilled water. The tubes were capped with foam plugs and sterilized by autoclaving. Bean line 21-58, selected for its improved nodulation and nitrogen fixation characteristics (1), was used as the trap host. Seeds were surface sterilized for 1 min in 70% ethanol followed by 2 min in a 0.1% $HgCl_2$ solution acidified with 5 ml of HCl per liter; they were then washed 10 times with sterile water. Seeds were germinated on moistened filter paper in sterile 15-cm petri dishes in the dark for 2 days at 30°C. One seedling was transferred aseptically to each tube and positioned so that the primary root would grow between the inner and outer tubes. The root was then inoculated with 1 ml of a diluted, aqueous suspension of *R. phaseoli*. Either four- or five-tube replicates of each decimal dilution were used for MPN determinations. The tubes were covered individually with aluminum foil and put into test tube racks or in plastic baskets covered with aluminum foil so that the root systems remained dark. After 3 to 4 days of incubation (ideally in a growth chamber, but window light was adequate), the shoot was grasped with sterile forceps and removed gently from the tube without damaging the stem. After the foam plug aseptically reinserted, the tubes were incubated further with the addition of 5 ml of sterile water or sterile nitrogen-free medium twice weekly or as needed. The root systems were examined for nodules at 7, 14, 21, and 28 days after inoculation, and the number of positive tubes was recorded for MPN calculations. When pure cultures were used (suspensions of *R. phaseoli* from plate cultures or an inoculant prepared from γ-irradiated peat), the MPN calculation was compared with the count of viable *R. phaseoli* of

TABLE 1. Enumeration of *R. phaseoli* populations by plate counts of viable cells and MPN plant infection tube culture technique

Expt no.	Culture vessel	*R. phaseoli* strain or soil sample	Log$_{10}$ no. of cells[a]				
			Plate count	MPN at incubation day:			
				7	14	21	28
1	Tube	MSU45	8.0	0	7.5	7.5	7.5
		CNPAF 150	7.9	0	7.1	7.9	7.9
		CNPAF 238	8.8	0	8.0	9.0	9.0
		CNPAF 312	8.7	0	8.0	8.3	8.3
2	Leonard jar	CNPAF 238	8.8	—[b]	—	—	8.5
		CNPAF 312	8.7	—	—	—	8.1
3	Tube	Kim5R3 (peat)	5.1	—	—	5.2	—
		Kim5R3 (soil)	4.8	—	—	4.8	—
4	Tube	Goiânia oxisol[c]	—	—	—	3.5	—
		Salvatierra vertisol[d]	—	—	—	4.2	—
		Irapuato vertisol[d]	—	—	—	4.5	—
		Apaseo vertisol[d]	—	—	—	4.8	—
		CIAB vertisol[d]	—	—	—	5.0	—

[a] Per milliliter of cell suspension or gram of soil.
[b] —, Not applicable.
[c] Collected at Centro Nacional de Pesquisa de Arroz e Feijão, Goiânia, Goiás, Brazil.
[d] Collected near Instituto Technologico Regional de Celaya, Celaya, Mexico.

the same sample plated on the surface of yeast extract-mannitol agar (7). Sterile peat was purchased from NifTAL, Paia, Hawaii.

Leonard jars were filled with a 2:1 sand-vermiculite mixture and autoclaved for 2 h. Germinated seedlings from surface-sterilized seeds were planted in the mixture (one per jar), placed in a greenhouse, inoculated with diluted samples, and irrigated as described above.

In experiment 1, we compared the plate counts of viable cells and MPN enumerations of standardized suspensions of four *R. phaseoli* strains (MSU45, CNPAF 150, CNPAF 238, and CNPAF 312). There was good agreement between these two methods of enumeration when the MPN counts were made after at least 21 days of incubation (Table 1). A ratio of MPN to plate counts indicated that 1 to 3 viable cells of *R. phaseoli* were a sufficient inoculant to induce root nodulation of beans.

In experiment 2, the MPN counts obtained with Leonard jars were compared with those obtained with tube cultures of strains CNPAF 238 and CNPAF 312. Because the roots had to be removed from the Leonard jars for the inspection of any nodules, we extended the incubation to the recommended period of 28 days. The Leonard jar method gave acceptable MPN enumerations, with a ratio of plate count to MPN count of 2 to 4.

In experiment 3, the accuracy of MPN counts with tube cultures was measured with strain Kim5R3 in pure culture in a peat inoculant carrier and in a nonsterile sandy soil previously shown to contain no native *R. phaseoli*. In this case, plate counts were obtained on yeast extract-mannitol plus rifampin (20 μg/ml). Again, the MPN enumerations were accurate. To illustrate the application of this tube culture method, we determined the MPN counts of the native population of *R. phaseoli* in soils from Goiânia, Brazil (oxisol), and near Celaya, Mexico (vertisols) (Table 1). The native population was higher in the Mexican soil samples.

In summary, our modification of the semienclosed tube culture method of Gibson now makes it possible to grow bean roots under microbiologically controlled conditions and obtain accurate MPN counts of *R. phaseoli* populations in the presence of other soil microorganisms. This method minimizes the problems of space requirements and cross-contamination inherent in the Leonard jar culture. Since nodulation can be observed without disturbing the root system, it should be possible to determine the time required for nodule formation and to gain access to the preferential sites for bean nodulation. Furthermore, this modified tube culture technique could be adapted for acetylene reduction assays of nitrogen fixation and used to screen for symbiotically defective mutant strains of *R. phaseoli* in a small space.

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