

Dilution of Liquid *Rhizobium* Cultures To Increase Production Capacity of Inoculant Plants†

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Experiments were undertaken to test whether peat-based legume seed inoculants, which are prepared with liquid cultures that have been deliberately diluted, can attain and sustain acceptable numbers of viable rhizobia. Liquid cultures of *Rhizobium japonicum* and *Rhizobium phaseoli* were diluted to give 10^8 , 10^7 , or 10^6 cells per ml, using either deionized water, quarter-strength yeast-mannitol broth, yeast-sucrose broth, or yeast-water. The variously diluted cultures were incorporated into gamma-irradiated peat, and the numbers of viable rhizobia were determined at intervals. In all of the inoculant formulations, the numbers of rhizobia reached similarly high ceiling values by 1 week after incorporation, irrespective not only of the number of cells added initially but also of the nature of the diluent. During week 1 of growth, similar multiplication patterns of the diluted liquid cultures were observed in two different peats. Numbers of rhizobia surviving in the various inoculant formulations were not markedly different after 6 months of storage at 28°C. The method of inoculant preparation did not affect the nitrogen fixation effectiveness of the *Rhizobium* strains.

Leguminous crops can be self-sufficient for all or part of their nitrogen requirements when their roots are nodulated with an effective nitrogen-fixing strain of *Rhizobium*. Some legumes nodulate only with specific strains of *Rhizobium* or may form fully effective nitrogen-fixing associations with a few of the array of strains that can nodulate them. Therefore, seed or soil inoculation with preselected rhizobia is practiced to ensure nodulation and nitrogen fixation in grain and forage legumes. Production of inoculants involves culture of *Rhizobium* on a large scale and incorporation into a carrier material, usually finely milled peat. In addition to their nutritional requirements, rhizobia need stirring and aeration to reach a high population density in liquid culture. A basal nutrient medium with mannitol as the carbon source and including yeast extract is the standard formulation for culturing rhizobia (11). Stirring and aeration are readily achieved in Erlenmeyer flasks plugged with cotton wool and mounted on a rotary shaker. Rhizobia are microaerophilic (12), and oxygen requirements are satisfied at the minimum speed necessary to prevent sedimentation of cells in the flasks. Because of technical difficulties in the development of large-scale, continuously operating shakers and the impracticality of autoclaving

very large flasks, the shaker-flask system has been considered suitable only for production of inoculants on a laboratory scale. Large-scale production has involved the use of fermentors, commonly of 1,000 to 2,000 liters, with special provisions for stirring and aeration (2).

Such fermentors are expensive to acquire and maintain and costly to install and operate. They are prone to contamination because of the difficulty in sterilizing all access lines and ensuring contaminant-free aeration throughout culture growth. The need for skilled operators contributes to the high operating cost of such production systems. These factors have not prevented establishment of viable inoculant production enterprises in industrialized nations. In developing countries, however, they are major disincentives for public and private entities contemplating production of legume seed inoculants.

It is known that *Rhizobium meliloti*, *Rhizobium trifolii*, and a cowpea *Rhizobium* reach similar ceiling populations in peat after 3 weeks of incubation, when liquid cultures of different ages (which, therefore, contain different numbers of viable cells) are used (8). So far, this phenomenon has not been recognized as a way of greatly increasing the production output of shaker-flask-based inoculant plants.

This research was undertaken to test whether peat-based legume seed inoculants prepared with liquid cultures that have been deliberately

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TABLE 1. Influence of initial inoculum levels on subsequent growth and survival of *R. japonicum* USDA 110 and *R. phaseoli* TAL 182 in Badenoch peat incubated at 28°C

Approx level of diluted liquid culture (log ₁₀ viable cells per ml) ^a	Log ₁₀ viable cells per g of moist peat after indicated time (days):							
	<i>R. japonicum</i>				<i>R. phaseoli</i>			
	0	7	28	70	0	7	28	70
6	5.42	9.96 ^b	9.98	9.83	4.95	9.72 ^b	9.97	10.07
7	6.31	10.06 ^b	9.98	9.99	6.25	9.87 ^b	9.91	9.63
8	7.45	10.18 ^b	10.03	9.77	7.43	10.00 ^b	9.85	9.69

^a YM broth (25%) was used as diluent.

^b The difference in population density between time 0 and 1 week for each inoculum level was significant ($P \leq 0.01$). Other differences were not significant.

diluted can attain and sustain acceptable numbers of viable rhizobia.

MATERIALS AND METHODS

Bacteria. *Rhizobium japonicum* USDA 110 and *Rhizobium phaseoli* TAL 182 were obtained as desiccated ceramic-bead cultures (7) from the NifTAL *Rhizobium* germ plasm resource.

Culture medium. Yeast-mannitol (YM) medium was prepared as described by Vincent (11), with 0.5 g of yeast extract (Difco Laboratories, Detroit, Mich.) per liter substituted for yeast-water.

Diluents. Deionized water, quarter-strength YM medium, quarter-strength yeast-sucrose medium (prepared by substituting sucrose for mannitol in the YM medium), and quarter-strength yeast-water medium (mannitol was omitted from YM medium) were used for diluting liquid cultures of *Rhizobium* before incorporation in peat. Diluents were autoclaved at 121°C at 15 lb/in² for 30 min.

Peat. Finely milled Badenoch peat (pH 6.8, without neutralizing with calcium carbonate) was obtained prepackaged and sterilized by gamma irradiation in 50-g polyethylene bags from Agricultural Laboratories, Regents Park, New South Wales, Australia. Milled Wisconsin peat (pH 4.5 to 5.0) was obtained in bulk from Nitragin Co., Milwaukee, Wis. The Wisconsin peat was adjusted to pH 6.5 to 6.8 by mixing with 5% (wt/wt) food grade-precipitated calcium carbonate (J. T. Baker Chemical Co., Phillipsburg, N.J.). It was then packaged in 50-g lots and gamma irradiated (5 Mrad). The polyethylene bags had a thickness of 38 µm for both peats. The moisture content of the peats was 8 to 10%.

Preparation of inoculants. Rhizobia were cultured in batches of 100 ml of YM medium in 250-ml Erlenmeyer flasks on a shaker platform rotating at 100 rpm. Late log-phase cultures with approximately 1×10^9 to 2×10^9 viable rhizobia per ml were serially diluted with each of the four different diluents to give 10^8 , 10^7 , or 10^6 rhizobia per ml. A sample of approximately 40 ml of diluted culture was injected aseptically into each bag of peat, the exact volume being that which gave a moisture content of 50% in each case. Each inoculant formulation was replicated three times. All bags were massaged thoroughly to mix their contents and incubated at 28°C throughout the investigation.

Counting rhizobia. A 1.0-g sample from the inoculant in each package was transferred aseptically into 99 ml of quarter-strength YM medium in a milk dilution

bottle. After 15 min of agitation on a wrist-action shaker, serial dilutions were performed. Using the drop-plate method of Miles and Misra (6), 30-µl drops from each dilution in the series were spotted on one set of YM agar plates containing 25 mg of Congo red per liter and on a second set of plates containing 1.25 mg of brilliant green per liter. (Both Congo red and brilliant green were incorporated in the YM agar to recognize rhizobia should contamination occur. Brilliant green suppresses non-rhizobia, and therefore it is a useful check on Congo red, especially with bacteria not absorbing Congo red.) Plates were incubated at 28°C and counted after 3 days for strain TAL 182 and after 7 days for strain USDA 110.

Nitrogen-fixing effectiveness of inoculants. Inoculants prepared in the various formulations were tested for effectiveness on their respective host plants in Leonard jars (5) containing nitrogen-free nutrient solution (1). Surface-sterilized and pregerminated seeds were inoculated at sowing with 2 ml of a peat inoculum prepared by suspending 1 g of peat inoculant in 99 ml of quarter-strength YM medium. Test samples were taken from inoculants aged for 3 and 6 months.

Immunofluorescence. Immunofluorescent examination was done only on cultures from the short-term experiment, which was designed to confirm that the rapid population increases observed during week 1 of growth were caused by the inoculant strains. Fluorescein isothiocyanate-conjugated sera of strains TAL 182 and USDA 110 were obtained from the NifTAL serum bank. Smears were prepared from colonies picked from plates (from 0-, 4-, and 7-day counts) and stained by the method of Schmidt et al. (10). At least 50% of the colonies resulting from a single drop (20 to 30 colonies per drop) were examined for contaminants by epifluorescence alone or in combination with the phase-contrast system. When colonies were too numerous to pick, a composite of the growth was made. A sample smear from the composite was then stained and examined.

RESULTS AND DISCUSSION

R. japonicum USDA 110 and *R. phaseoli* TAL 182 attained population densities of close to 10^{10} viable cells per g of moist peat by 1 week after incorporation, even though the initial numbers in the diluted liquid culture varied in the range 10^6 to 10^8 rhizobia per ml (Table 1). Numbers of viable rhizobia were sustained well above 10^9 for

TABLE 2. Effect of different diluents on growth and survival of *R. japonicum* USDA 110 in Badenoch peat during long-term storage at 28°C

Diluent (concn)	Log ₁₀ viable cells per g of moist peat after incorporation for (days):								LSD (<i>P</i> ≤ 0.01) ^a
	0	14	30	60	90	120	150	180	
Sterile, deionized water	4.32	10.01	9.83	9.73	9.43	8.65	9.08	7.89	0.31
YM medium (25%)	4.41	10.06	9.96	9.93	9.60	8.74	9.23	8.03	0.45
Yeast-water medium (25%)	4.37	9.81	9.88	9.77	9.63	8.81	9.17	7.72	0.32
Yeast-sucrose medium (25%)	4.53	9.94	9.98	9.91	9.46	9.08	9.31	7.94	0.23

^a Zero-time viable counts were not included in the analysis. LSD, Least significant difference.

10 weeks. All multiplications were more rapid than those in the experiments described by Roughley (8). The results indicated that a 1,000-fold dilution (from 10⁹ to 10⁶) of the liquid culture is acceptable and suggest that even greater dilutions might be possible without affecting the final population density attained in the inoculants.

The tests with a range of diluents revealed that inoculants with high populations of rhizobia could be prepared with each diluent (Tables 2 and 3). Analysis of the data showed that the four diluents did not differ significantly in their abilities to support growth and survival of both strains. Although no measurements were made to confirm the moisture contents of the inoculants after long-term storage, significant moisture loss was indicated by the dry appearance of the peat. A gradual and significant decline in population increasing with storage time was observed which may be attributed to the effects of desiccation of the inoculants. All three strains of rhizobia were adversely affected during storage by a low moisture content in the peat (9). Numbers of viable rhizobia surviving in each formulation after 6 months of storage were equal or very close to the accepted standard of 10⁸ cells per g of moist peat (3). The high populations of rhizobia sustained in the inoculants in which water was used as a diluent indicated that the carry-over of nutrients in the diluted YM medium and in the peat itself could support high populations of rhizobia during the 6-month shelf

life. The chemical composition of the peat (3) may have an intrinsic nutritional value for rhizobia.

The nitrogen-fixing effectiveness of the rhizobia in the various inoculant formulations was confirmed to have been maintained. The appropriate host plants inoculated with samples from 3- and 6-month-old inoculants produced healthy, green plants. Roots were well nodulated, and nodule sections showed pink-to-red interiors.

Based on the typical mean generation times for fast- and slow-growing rhizobia given by Vincent (12), the rapid increases in population observed in all inoculants after incubation for only 1 week are theoretically possible. Nevertheless, a follow-up experiment was warranted to monitor cell multiplication at daily intervals after incorporation of cultures in the peat carrier. In this short-term experiment, inoculants of each strain were prepared as described above. Both Badenoch and Wisconsin peats were investigated.

Cells of each of the two strains multiplied rapidly (Fig. 1) in both Badenoch and Wisconsin peats. Maximum populations were attained after 4 to 5 and 7 days by undiluted and 1,000-fold-diluted cultures, respectively. The inoculants were confirmed to be free of contaminants by plating and typing colonies by immunofluorescence with the specific antisera. The mean generation times for *R. phaseoli* were 2.7 and 2.8 h in Badenoch and Wisconsin peats, respectively. *R. japonicum* had a mean generation time (7.0 h)

TABLE 3. Effect of different diluents on growth and survival of *R. phaseoli* TAL 182 in Badenoch peat during long-term storage at 28°C

Diluent (concn)	Log ₁₀ viable cells per g of moist peat after incorporation for (days):								LSD (<i>P</i> ≤ 0.01) ^a
	0	14	30	60	90	120	150	180	
Sterilized, deionized water	4.46	9.75	9.69	9.36	8.89	8.67	8.28	8.29	0.43
YM medium (25%)	4.39	9.98	9.75	9.58	8.93	8.47	8.42	8.04	0.46
Yeast-water medium (25%)	4.43	9.69	9.53	9.44	8.81	8.60	8.24	8.05	0.39
Yeast-sucrose medium (25%)	4.31	9.98	9.79	9.60	9.02	8.67	8.50	8.22	0.38

^a Zero-time viable counts were not included in the analysis. LSD, Least significant difference.

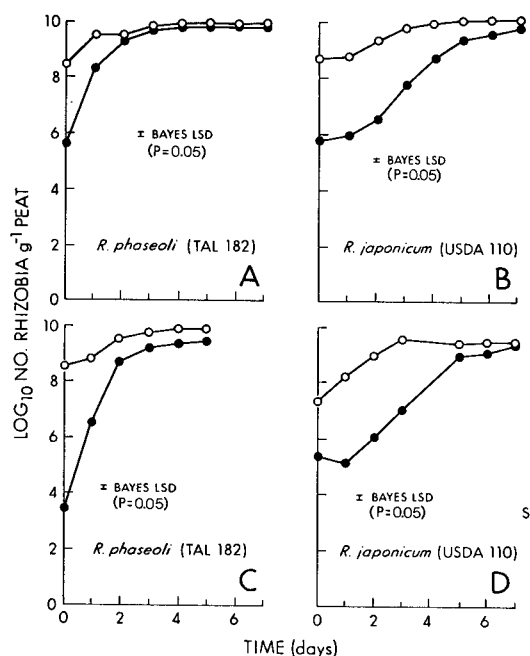


FIG. 1. Multiplication of *Rhizobium* in sterilized Badenoch (A and B) and Wisconsin (C and D) peats after incorporation of full-strength (○) and diluted (●) liquid culture. Diluent was 25% YM broth. The full-strength culture was diluted 1,000-fold for the diluted liquid culture treatment. Each treatment was set up in triplicate. BAYES LSD (least significant difference): $k = 100$.

in the Badenoch peat that was slightly shorter than the 7.7-h value found for Wisconsin peat. These values compare well with the expected mean generation times for fast- and slow-growing rhizobia in liquid culture (12).

The results showed that, for these representative fast- and slow-growing strains of *Rhizobium*, the continued and rapid multiplication of rhizobia after incorporation in the peats tested can be relied upon to produce acceptable inoculants from diluted cultures. The method may also be extended to strains of rhizobia which have specific requirements (4) of a fairly expensive carbon source, especially some strains of soybean and lupine rhizobia which prefer arabi-

nose and other pentoses. Such organisms may be grown in small volumes before dilution. These advantages offer the opportunity to devise large-output inoculant production systems in which rhizobia are cultured simply and inexpensively in flasks or shakers.

Although the method described above was performed with irradiated peat, initial studies have shown that this method can be applied to steam-sterilized peat. The data from these experiments will be presented elsewhere.

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