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Inoculant Production with Diluted Liquid Cultures of *Rhizobium* spp. and Autoclaved Peat: Evaluation of Diluents, *Rhizobium* spp., Peats, Sterility Requirements, Storage, and Plant Effectiveness

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Fully grown broth cultures of various fast- and slow-growing rhizobia were deliberately diluted with various diluents before their aseptic incorporation into autoclaved peat in polypropylene bags (aseptic method) or mixed with the peat autoclaved in trays (tray method). In a factorial experiment with the aseptic method, autoclaved and irradiated peat samples from five countries were used to prepare inoculants with water-diluted cultures of three *Rhizobium* spp. When distilled water was used as the diluent, the multiplication and survival of rhizobia in the peat was similar to that with diluents having a high nutrient status when the aseptic method was used. In the factorial experiment, the mean viable counts per gram of inoculant were log 9.23 (strain TAL 102) > log 8.92 (strain TAL 82) > log 7.89 (strain TAL 182) after 24 weeks of storage at 28°C. The peat from Argentina was the most superior for the three *Rhizobium* spp., with a mean viable count of log 9.0 per g at the end of the storage period. The quality of inoculants produced with diluted cultures was significantly ($P = 0.05$) better with irradiated than with autoclaved peat, as shown from the factorial experiment. With the tray method, rhizobia in cultures diluted 1,000-fold or less multiplied and stored satisfactorily in the presence of postinoculation contaminants, as determined by plate counts, membrane filter immunofluorescence, and plant infection procedures. All strains of rhizobia used in both the methods showed various degrees of population decline in the inoculants when stored at 28°C. Fast- and slow-growing rhizobia in matured inoculants produced by the two methods showed significant ($P < 0.01$) decline in viability when stored at 4°C, whereas the viability of some strains increased significantly ($P < 0.01$) at the same temperature. The plant effectiveness of inoculants produced with diluted cultures and autoclaved peat did not differ significantly from that of inoculants produced with undiluted cultures and gamma-irradiated peat.

In the usual method of peat-based inoculant production, rhizobia are multiplied in fermenters to reach maximal populations before being added to the peat. In the pure-culture or aseptic technique, a definite volume (30 to 50% of the total final weight) of a fully grown culture (10^9 cells per ml) is added aseptically to a relatively dry peat sample (8 to 10% moisture) which has been presterilized in polyethylene bags by gamma irradiation. The added rhizobia multiply further to reach maximal populations since the peat was presterilized, but minimal to no multiplication may take place if the peat was not sterilized. General experience has shown that, with the exception of a few strains that can reach a population of 10^{10} cells per g of peat inoculant, most strains rarely exceed 5×10^9 cells per g in irradiated peat (12). In heat-treated sedge peat used in the United States, the number of added rhizobia reaches 2.5×10^9 to 3.0×10^9 cells per g of inoculant (4). The choice of technique used depends very much on the scale of production, with the nonsterile peat method being the choice of most commerical producers. However, in Australia, all peat used in commerical production is gamma irradiated in polyethylene packages (13, 20). The production capacity of both techniques is directly proportional to the volume of liquid cultures that can be produced, and this in turn is limited by the size of the fermenter. The problem is further magnified when liquid cultures for several species of legumes are needed.

In developing countries, where there is a great need for legume inoculants, numerous problems are associated with inoculant production, e.g., in Guyana a frequent problem is the availability of a suitably sized and low-cost fermenter (11).

The high costs and operational problems associated with large-scale fermenters led to an investigation of the potential of using diluted liquid cultures of *Rhizobium* spp. for inoculant production with irradiated peat (16). In developing countries, costly gamma-irradiation facilities are not always feasible for peat sterilization, but steam sterilization is a realistic alternative. Steam sterilization has been used in the sterilization of peat for inoculant production with undiluted cultures (9, 19, 22). The use of autoclaved peat and diluted liquid cultures of *Rhizobium* spp. has not been investigated as a new and potential method for inoculant production. This report characterizes inoculants produced by the dilution method.

MATERIALS AND METHODS

Rhizobia. The various *Rhizobium* spp. (Table 1) used in this investigation were obtained from the NifTAL *Rhizobium* germ plasm resource. Cultures were maintained on yeast mannitol agar (YMA) as described by Vincent (23) and checked for purity by the fluorescent-antibody technique (15).

Diluents. Various formulations of diluents were used to dilute late-log-phase liquid cultures to investigate the influence of the nutrient status of the diluent on the multiplication and survival of the rhizobia in the peat. The preparation of these diluents was described previously (16), and they are listed in Table 2. Only *R. phaseoli* TAL 182 and *R. japonicum* TAL 102 were used to evaluate the influence of diluents.

In subsequent experiments with other strains of rhizobia, only sterile, deionized water was used to dilute the cultures.

TABLE 1. Sources of *Rhizobium* cultures

Designations[a] of *Rhizobium* spp.		Cross-inoculation group	Parent host
TAL	Original		
82	TAL 82	*Rhizobium* sp.	*Leucaena leucocephala*
102	USDA 110	*R. japonicum*	*Glycine max*
169	Nitragin 176A22	*Rhizobium* sp.	*Vigna unguiculata*
182	TAL 182	*R. phaseoli*	*Phaseolus vulgaris*
379	USDA 136b; CB1809	*R. japonicum*	*Glycine max*
380	SU 47	*R. meliloti*	*Medicago sativa*
620	ICRISAT 3889	*Rhizobium* sp.	*Cicer arietinum*
651	UMKL 44	*Rhizobium* sp.	*Calopogonium mucunoides*
1376	C-34	*R. phaseoli*	*Phaseolus vulgaris*

[a] Labratory sources were as follows: TAL, NifTAL Project, University of Hawaii, Honolulu; USDA, U.S. Department of Agriculture, Beltsville, Md.; Nitragin, Nitragin Co., Milwaukee, Wis.; SU, University of Sydney, Sydney, Australia; ICRISAT, International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India; UMKL, University of Malaya, Kuala Lumpur, W. Malaysia; C, Centro de Energia Nuclear na Agriculture, Piracicaba, Brazil.

Sterilization of the deionized water was achieved by autoclaving or by filtration through a 0.2-μm Ultipor disposable self-contained membrane filter unit (Pall Corporation, Cortland, N.Y.). This filter was reused several times after sterilization by autoclaving. Pressure for filtration was provided by a liquid circulation pump (Flotec Inc., Norwalk, Calif.).

Presterilization of peat in plastic bags. Finely milled Demilco peat (pH 4.5 to 5.0; moisture content, 8 to 10%) was purchased from the Nitragin Co., Milwaukee, Wis. The peat was mixed with food-grade precipitated calcium carbonate (J. T. Baker Chemical Co., Phillipsburg, N.J.) at a ratio of 50 g of peat to 2.5 g of calcium carbonate to give a final pH of 6.5 to 6.8. The peat was then packaged in 50-g lots in thin (38-μm) polyethylene bags, heat sealed, and gamma irradiated (5 Mrad).

Similar quantities of peat were placed in high-density (38 μm) autoclavable polypropylene bags (Sealcraft Packaging Corp., Milwaukee, Wis.). The open end of each bag was folded back to produce a narrow flap, 4 cm in width, which was held in place by two no. 1 paper clips. The bags were arranged in rectangular wire baskets with sufficient space for steam circulation between bags. Before placement of the bags, the wire baskets were lined with paper towels on the sides and bottom to prevent metal-plastic contact during autoclaving. The peat was sterilized for 60 min at 121°C and at 1.05 kg/cm^2. The bags were removed for sealing after overnight cooling in the autoclave. They were sealed in a laminar flow hood. The two paper clips were removed, and a seal was made along the crease of the flap.

Peat from Mexico (FERTIMEX, Anaxagoras 25, Mexico City) Argentina (Nitrasoil Argentina S.A., Buenos Aires), Spain (Instituto Nacional de Investigaciones Agrarias, San Jose de la Rinconada), and Peru (Universidad Nacional de Cajamarca, Cajamarca) was packaged and sterilized by irradiation and autoclaving as with the Demilco peat from the United States. These imported peat samples did not require the addition of the calcium carbonate, as their pHs were 6 to 7.0.

Presterilization of peat in trays. A 1-kg portion of pH-amended Demilco peat was placed in a heavy-duty autoclavable polypropylene tray (18 by 18 by 6 in) and spread out to give a layer of even thickness. An aluminum foil cover was placed over the tray. The peat was autoclaved at 121°C and 1.05 kg/cm^2 for 60 min and left to cool overnight in the autoclave. The aluminum foil cover was left in place until removal just before inoculant preparation.

Preparation of inoculants and storage. Late-log-phase cultures were obtained in YM medium and diluted to the required levels with the various diluents.

To examine the effect of sterility of the peat on the multiplication and survival of the rhizobia, the peat samples were inoculated with the diluted cultures under aseptic or septic conditions. All inoculants were prepared with single strains.

For the aseptic method, presterilized peat samples in polyethylene or polypropylene bags were aseptically inoculated with 40 ml of the diluted culture (50- to 100-fold dilution) with a sterile, plastic syringe fitted with a hypodermic needle (8). The bags were thoroughly kneaded to ensure absorption of the liquid culture into the peat. Inoculants were stored (incubated) at 28°C. To study survival under low-temperature storage conditions, inoculants were sampled for enumeration and immediately transferred to a refrigerator (4°C). The same inoculants were reenumerated after a fixed period of storage at 4°C.

For the septic method (referred to as the tray method henceforth), peat inoculation was done in the open laboratory environment. The diluted culture (approximately 50- to

TABLE 2. Shelf life of inoculants prepared from autoclaved peat and diluted liquid cultures of *R. phaseoli* (TAL 182) and *R. japonicum* (TAL 102)

Strain and diluent	Log_{10} (no. of rhizobia/g of moist peat)[a] after storage for (weeks):				
	0	2 (22)[b]	8	16	24
R. phaseoli					
Sterile, deoinized water	4.41	9.18 (6.47)	8.08	7.53	7.50
25% yeast-mannitol broth	4.85	9.40 (5.68)	8.14	7.87	7.60
25% yeast-water	4.92	9.09 (3.79)	8.08	7.64	7.37
25% yeast sucrose broth	4.81	9.54 (5.75)	7.99	7.73	7.47
R. japonicum					
Sterile, deoinized water	4.68	8.11 (9.03)	9.32	9.29	9.18
25% yeast mannitol broth	4.76	7.93 (9.20)	9.26	9.16	9.10
25% yeast-water	4.75	8.02 (9.19)	9.35	9.13	9.26
25% yeast sucrose broth	4.67	8.54 (9.22)	9.36	9.40	9.27

[a] Mean of three replications.
[b] Inoculant was stored at 28°C for 2 weeks and then at 4°C for 22 weeks. Values in parentheses are plate counts after storage at 4°C.

100-fold dilution) was mixed at a rate of 35 ml per 50 g of peat. Mixing was done by hand; sanitized, disposable polyethylene gloves (VWR Scientific, San Francisco, Calif.) were worn throughout the mixing process. Immediately after being mixed, 100-g quantities of the inoculant were packaged in clean, previously unused polyethylene bags and heat sealed. The survival of rhizobia in these inoculants was compared with that of inoculants of the same strains prepared with undiluted culture inoculated aseptically into bags of irradiated peat as described for the aseptic method. The inoculants prepared by this tray method were also tested for storage characteristics at 4°C.

Enumeration of rhizobia by plate counts and plant infection (MPN) technique. Serially diluted samples of the inoculants were plated on Congo red YMA (CRYMA) and brilliant green YMA (BGYMA) media (16) by the drop and spread plate methods as described previously (8). The drop plate method was routinely used for enumerating pure cultures of rhizobia in inoculants prepared from presterilized peat. Rhizobia in inoculants prepared by the tray method were enumerated by the spread plate (CRYMA and BGYMA) method and the standard plant infection (most probable number [MPN]) technique (23) with plastic growth pouches (24) or in enclosed seedling agar tubes (23). Tenfold serial dilutions and four replications per dilution were used for MPN counts. The following host plants were used for the MPN counts: *Glycine max* cv. Davis for *R. japonicum* TAL 379, a small, white-seeded commercial variety of *Phaseolus vulgaris* for *R. phaseoli* TAL 182, *Leucaena leucocephala* K8 for *Rhizobium* sp. strain TAL 82, *Medicago sativa* for *R. meliloti* TAL 380, and *Macroptilium atropurpureum* var. Siratro for *Rhizobium* spp. strain TAL 169 and TAL 651. Surface-sterilized seeds were pregerminated on water agar plates. Seedlings of *Medicago sativa* and *Macrophilium atropurpureum* var. Siratro were established in seedling agar tubes, and the other hosts were in growth pouches. Uninoculated controls were included in all treatments, and plants were scored for the presence or absence of nodules at 3 weeks after inoculation.

Enumeration of rhizobia by the MFIF technique. Direct counting of the rhizobia by membrane filter immunofluorescence (MFIF) was done with polycarbonate Nuclepore filters (Nuclepore Corp., Pleasanton, Calif.) stained with irgalan black (7), since the inoculants prepared by the tray method would contain contaminants. A 5-ml sample from the 10^{-4} dilution was filtered, and the filter was treated with gelatin-rhodamine isothiocyanate conjugate (2). The direct fluorescent-antibody technique was used for staining the rhizobia (15), and 20 randomly chosen microscope fields were counted per filter at ×100 magnification. MFIF observations were made with a Zeiss standard microscope 14 with an incident light fluorescence illuminator equipped with an HBO 50-W mercury vapor light source.

TABLE 3. Shelf life of inoculants prepared from autoclaved peat and diluted liquid cultures of *Rhizobium* spp.[a]

Rhizobium spp.	Log_{10} (no. of rhizobia/g of moist peat) after storage for (weeks):				
	2 (22)[b]	3	8	16	24
Rhizobium sp. TAL 82	9.45 (ND)[c]	8.92	8.71	8.23	7.89
Rhizobium sp. TAL 169	6.26 (7.30)	8.29	9.14	8.88	8.77
R. japonicum TAL 379	9.77 (9.53)	9.72	9.51	9.31	9.16
R. meliloti TAL 380	9.62 (9.23)	9.77	9.11	8.70	8.65
Rhizobium sp. TAL 620	9.41 (9.87)	9.61	9.30	8.94	8.72
Rhizobium sp. TAL 651	9.41 (8.00)	9.45	9.22	9.01	8.89
R. phaseoli TAL 1376	9.35 (8.47)	9.08	8.10	7.67	7.67

[a] Late-log-phase cultures were diluted to 10^6 rhizobia per ml in deionized water that had been sterilized by membrane filtration.
[b] Inoculant was stored at 28°C for 2 weeks and then at 4°C for 22 weeks. Values in parentheses are plate counts after storage at 4°C.
[c] ND, Not done.

Immunofluorescence examination of colonies on plates. To test the accuracy of recognition of rhizobial colonies from contaminants during plate counts, smears were made from colonies picked from CRYMA and BGYMA plates. All inoculants produced by the tray method were examined. Five colonies recognizable as those of the inoculant *Rhizobium* sp. were picked for observation from every replication at each sampling period.

Nitrogen-fixing effectiveness of inoculants. Inoculants of *R. japonicum* TAL 102, *R. phaseoli* TAL 182, and *Rhizobium* sp. strain TAL 82 prepared with diluted cultures and presterilized peat (autoclaved and irradiated) from various countries were tested for their effectiveness on their appropriate host plants. The performance of each of these inoculants was compared with that of the appropriate multistrain NifTAL inoculant which was prepared with an undiluted culture containing three strains and irradiated peat. The inoculants were tested after aging for 6 months at 28°C. Inoculation procedures and plant culture techniques were as previously described (16). Host-*Rhizobium* combinations and harvest time were as follows: *Phaseolus vulgaris* cv. Bountiful-*R. phaseoli* TAL 182 harvested at 30 days, *G. max* cv. Davies-*R. japonicum* TAL 102 harvested at 31 days, and *Leucaena leucocephala* cv. K8-*Rhizobium* sp. strain TAL 82 harvested at 48 days. At harvest, plant shoots were excised and dried at 70°C for 48 h for dry weight determination. Roots were examined for nodulation. Sample nodules were cut open, and the color of the nodule interior was noted.

RESULTS

Evaluation of diluents. The influence of various diluents on the subsequent multiplication and long-term survival of *R. phaseoli* TAL 182 and *R. japonicum* TAL 102 in inoculants prepared from autoclaved peat and 1,000-fold-diluted liquid cultures is shown in Table 2. The sterile, deionized water was not significantly different from the other three nutrient-enriched diluents in supporting multiplication and survival of both species. The rates of multiplication of the two species differed: maximum populations of *R. phaseoli* were counted at 2 weeks, and maximum populations of *R. japonicum* were counted at 8 weeks. Long-term storage (24 weeks at 28°C) data for *R. phaseoli* TAL 182 indicated a significant ($P < 0.001$) decline in viability, with similar trends for all four diluent treatments. Also, long-term storage of *R. phaseoli* TAL 182 at 4°C for 22 weeks significantly (paired t test, $P < 0.01$) affected survival in the peat, by a decrease of 3.88 logs. With *R. japonicum* TAL 102, long-term storage at 4°C for the same period resulted in a significant (paired t test, $P < 0.01$) increase in the population, by approximately 1.0 log.

Performance of water-diluted liquid cultures of different strains in autoclaved peat. Since sterilized, deionized water was not different from any of the nutrient-enriched diluents tested with *R. phaseoli* TAL 182 and *R. japonicum* TAL 102, seven other *Rhizobium* spp. were selected for further investigation. Inoculants were prepared with diluted liquid cultures and autoclaved peat, and the inoculant shelf life was evaluated (Table 3). An analysis of variance indicated that the growth and multiplication of the seven species differed significantly ($P < 0.01$). Except for *Rhizobium* sp. strain

TABLE 4. Compatibility of different peat samples with diluted cultures of *Rhizobium* spp. for inoculant production

Peat source	Peat presterilization	Log_{10} (no. of rhizobia/g of moist peat)[a] after storage for (weeks):								
		R. phaseoli TAL 182			*R. japonicum* TAL 102			*Rhizobium* sp. TAL 82		
		2[b]	4[b]	24[b]	2	4	24	2	4	24
Mexico	A[c]	8.61	8.58	7.69	9.00	9.20	9.13	9.02	9.02	9.01
	G[c]	9.30	9.39	7.65	9.47	9.55	9.20	9.56	9.47	9.18
Argentina	A	9.06	9.07	8.32	9.43	9.58	9.42	9.25	9.26	9.20
	G	9.51	9.70	8.56	9.67	9.86	9.62	9.88	9.70	9.25
Spain	A	8.99	9.19	7.73	9.31	9.19	9.09	9.38	9.18	9.04
	G	9.17	9.50	7.92	9.39	9.48	9.14	9.58	9.32	9.05
United States	A	8.90	8.97	8.00	9.37	9.52	9.47	9.28	9.05	8.37
	G	8.91	8.59	8.00	9.39	9.48	9.20	8.96	9.05	8.93
Peru	A	ND[d]	ND	ND	9.23	9.20	9.18	ND	ND	ND
	G				9.64	9.65	9.22			

[a] Mean of three replications.
[b] Sampling time in weeks.
[c] A, Autoclaved; G = gamma irradiated.
[d] ND, Not done.

TAL 169, which multiplied very poorly and reached maximum populations at 8 weeks, all the other rhizobia reached populations in excess of 10^9 rhizobia per g in 2 weeks. The shelf life showed similar trends, in that all various species had significant ($P < 0.01$) loss in viability when stored at 28°C for 24 weeks. However, the shelf life of the rhizobia was highly variable; e.g., *R. japonicum* TAL 379 still maintained high populations (10^9 per g) at 24 weeks, whereas *R. phaseoli* TAL 1376 and *Rhizobium* sp. strain TAL 82 populations decreased to 10^8 per g of inoculant.

Low-temperature (4°C), long-term (22 weeks) storage qualities were also evaluated with these different species (Table 3). The survival data for each strain at 4 and 28°C were analyzed for differences by a one-way analysis of variance. The differences were highly significant ($P < 0.01$) for each strain, indicating that storage at 4°C affected growth and survival in the peat. Increases in populations at 4°C were obvious with the cowpea *Rhizobium* sp. strain TAL 169 (+1.25 logs) and the chickpea *Rhizobium* sp. strain TAL 620 (+0.5 log). However, storage at 4°C affected the viability of cowpea *Rhizobium* sp. strain TAL 651 (−1.50 logs), and *R. phaseoli* TAL 1376 (−0.87 log). Also affected, but less seriously, were *R. japonicum* TAL 379 (−0.24 log) and *R. meliloti* TAL 380 (−0.49 log).

TABLE 5. Analysis of variance on the compatability of diluted cultures of three *Rhizobium* spp. for inoculant production with diverse peat samples[a]

Source of variation	df	Mean square variation in viable counts after storage for (weeks)[b]:		
		2	4	24
Rhizobium (*R*)	2	0.440**	0.845**	11.953**
Peat (*P*)	3	0.321**	0.618**	0.847**
R × *P*	6	0.060	0.128**	0.224**
Carrier sterilization (*S*)	1	0.929**	1.511**	0.176*
R × *S*	2	0.017	0.030	0.102*
P × *S*	3	0.487**	0.344**	0.051
R × *P* × *S*	6	0.055**	0.045*	0.133**
Error	48	0.034	0.013	0.020

[a] Peat from Peru was not included in the analysis.
[b] *,**, Significance at the 0.01 and 0.001 levels of probability, respectively.

Compatibility of diverse peat samples with diluted liquid cultures of *Rhizobium* spp. The compatibility of diluted liquid cultures of *Rhizobium* spp. for inoculant production with peat from five diverse sources was evaluated in a factorial experiment (Table 4). The three *Rhizobium* spp. were evaluated at each storage period, and the peat from Peru was excluded from the statistical analysis (Table 5). Viable counts differed significantly ($P < 0.01$) for the three *Rhizobium* spp., peat samples, and the method of peat sterilization at each storage period. These differences were consistent throughout the shelf life of the inoculants. Significant interactions among the *Rhizobium* spp. (*R*), peat (*P*), and the method of peat sterilization (*S*) were evident at different storage periods. Of these interactions, only $R \times P \times S$ persisted throughout the shelf life of the inoculants. The $R \times S$ interaction was significant only at 24 weeks, and the $R \times P$ interaction was evident in the 4- and 24-week-old inoculants. An analysis of variance ($\alpha = 0.05$ and Duncan's multiple range test) to compare the differences between inoculants produced with irradiated and autoclaved peat samples indicated that the quality of inoculants (viable number of rhizobia per gram of inoculant) in irradiated peat

TABLE 6. Quality of inoculants produced with presterilized peat and liquid cultures of rhizobia[a]

Method of inoculant preparation	Log_{10} (no. of rhizobia/g of inoculant) after storage for (weeks):		
	2	4	24
Diluted culture and irradiated peat	9.36 (a)[b]	9.43 (a)	8.73 (a)
Diluted culture and autoclaved peat	9.31 (a)	9.14 (b)	8.63 (b)

[a] Information is a combined analysis of the same raw data obtained for compatibility experiment (Table 4).
[b] Means in the same column with the same letter are not significantly different by Duncan's multiple range test ($\alpha = 0.05$).

TABLE 7. Quality and shelf life of peat inoculants prepared by the tray method compared with the aseptic method

Rhizobium sp.	Log_{10} (no. of rhizobia/ml of culture):		Log_{10} (no. of rhizobia/g of moist peat)[a] after storage for (weeks):									
			2				12			24		
			Autoclaved[c]			Irradiated[d]	Autoclaved		Irradiated	Autoclaved		Irradiated
	Undiluted[b]	Diluted[b]	CR, BG[e]	MPN[f]	MFIF[g]	CR, BG	CR, BG	MPN	CR, BG	CR, BG	MPN	CR, BG
R. phaseoli TAL 182	8.84	6.24	9.38, 9.56	9.26[h]	ND[i]	9.26, ND	9.11, 8.68	8.92	7.67, 8.03	8.55, 8.55	8.08	8.24, 8.26
R. meliloti TAL 380	8.91	6.67	9.65, 9.73	9.76[h]	9.41	9.75, ND	9.41, 9.22	8.84	8.48, 8.35	9.29, 9.17	9.39	9.15, 9.08
Rhizobium sp. TAL 169	9.13	6.81	9.33, 9.26	9.36[h]	9.23	9.80, 9.76	9.41, 8.93	9.69	9.41, 9.33	9.23, 9.08	7.42	9.08, 9.01
Rhizobium sp. TAL 651	9.10	6.95	9.25, 9.56	9.66[h]	9.00	9.75, 9.63	9.20, 9.26	9.45	9.39, 9.48	9.22, 9.16	9.05	9.02, 9.02
Rhizobium sp. TAL 82	9.18	6.70	9.68, 9.69	9.25[j]	9.08	9.33, 9.30	9.51, 9.39	8.52	7.79, 7.87	9.58, 9.12	9.00	7.88, 7.76
R. japonicum TAL 379	9.22	7.12	9.62, 7.21	9.25[j]	9.38	9.72, 8.76	9.64, 9.44	9.18	9.60, 9.42	9.38, ND	9.05	9.00, ND

[a] Counts are means of three replications.
[b] Number of viable rhizobia in the liquid culture before incorporation into irradiated and autoclaved peat, respectively.
[c] Counts of inoculants prepared by the tray method with autoclaved peat.
[d] Counts of inoculants prepared by injecting undiluted culture into gamma-irradiated peat.
[e] CR, BG, Plate counts on CRYMA and BGYMA media, respectively.
[f] MPN counts by plant infection.
[g] MFIF counts.
[h] MPN counts in plastic growth pouches.
[i] ND, Not done.
[j] MPN counts in enclosed seedling-agar tubes.

was significantly higher than in the autoclaved peat for all the storage periods (Table 6).

In the combined analysis of the raw data from the factorial experiment (Table 4), the order of strain performance was *R. japonicum* TAL 102 > *Rhizobium* sp. strain TAL 82 > *R. phaseoli* TAL 182, regardless of peat source or method of peat sterilization. The mean viable counts per gram of inoculant, in order of strain performance, were log 9.23, log 8.92, and log 7.89 after 24 weeks of storage at 28°C.

The same analysis revealed that the peat from Argentina was the most superior for the three *Rhizobium* spp., with a mean viable count of log 9.0 per g of inoculant at the end of the storage period. The mean counts in the other peat samples were log 8.63 (United States), log 8.56 (Mexico), and log 8.54 (Spain).

Inoculant preparation by the tray method. The quality and shelf life of the inoculants produced by the tray method with six *Rhizobium* spp. are summarized in Table 7. Enumeration of the rhizobia in these inoculants was done by the spread plate, MPN, and MFIF (done only on the 2-week-old inoculants) methods. The counts at 2 weeks indicated that the diluted cultures multiplied in the autoclaved peat in the presence of contaminants which were introduced during the septic mixing operation. The inoculants were still of acceptable quality at 12 and 24 weeks, although higher counts were observed at 12 weeks.

Even though the inoculants prepared by the tray method were packaged immediately after mixing, the contaminant increase was considerable, averaging from 35% (at 2 and 12 weeks) to 49% (at 24 weeks) of the CFU. The CFU clearly distinguishable as those of the *Rhizobium* inoculant strain on the basis of colony appearance on the plating medium were further examined by immunofluorescence. Results of the immunofluorescence examination were generally positive with few negative reactions, but the number of examinations (total of 15 CFU) for each replicated treatment was insufficient for quantitative analysis. An earlier experiment with the tray method was abandoned because of very serious contamination which resulted from not wearing sanitized polyethylene disposable gloves during mixing. Scrupulous cleaning of the hands before mixing in of the inocula was inferior to sanitization provided by the polyethylene gloves.

Since plate and MPN counting techniques were routinely used for the tray method to evaluate multiplication of the rhizobia from diluted cultures, the reliability of these techniques was examined. The data in Table 7 were analyzed for correlation between plate counts (i.e., CRYMA versus BGYMA) and between plate counts and MPN (i.e., CRYMA versus MPN and BGYMA versus MPN). Results of both the tray and aseptic methods were analyzed. The results were (i) tray method, CRYMA versus BGYMA, $r = 0.73$ ($P < 0.001$); CRYMA versus MPN, $r = 0.44$ (not significant); BGYMA versus MPN, $r = 0.20$ (not significant); and (ii) aseptic method, CRYMA versus BGYMA, $r = 0.98$ ($P < 0.001$). These determinations were especially important in accepting the data from plate counts which involved selective recognition of colonies of *Rhizobium* spp. in the presence of contaminants, particularly for the tray method. The spot checks on single colonies by immunofluorescence further supported the acceptability of the plate counts. The MPN counts were not significantly correlated with the plate counts on CRYMA or BGYMA. In the analysis, results of *R. japonicum* TAL 379 were not included because brilliant green inhibited the growth of this strain, especially in the young peat inoculants. The counts by MFIF were somewhat lower than the counts obtained by other methods and showed no significant relationship with those from the other methods.

Low-temperature (4°C) storage of inoculants produced by the tray method. Inoculants prepared by the tray method were aged to 3 weeks and transferred to storage at 4°C for 23 weeks. A parallel experiment with the same *Rhizobium* spp. in irradiated peat was set up. The data (Table 8) were analyzed by a paired *t* test, and the results indicated a

TABLE 8. Influence of low storage temperature (4°C) and time (23 weeks) on the survival of rhizobia in inoculants prepared by the tray and aseptic methods

Rhizobium spp.	Log_{10} (no. of rhizobia/g of peat)[a]				
	Autoclaved (tray method)			Irradiated (aseptic method)	
	MPN[b]	Initial[c]	Final[d]	Initial	Final
R. phaseoli TAL 182	9.01	9.49, 9.31[e]	9.19, 9.20	8.27, 8.24	6.94, 6.59
R. meliloti TAL 380	9.85	9.97, 9.89	9.76, 9.71	9.42, 9.36	9.35, 9.32
Rhizobium sp. TAL 169	9.58	9.44, 8.94	9.35, 8.96	10.00, 9.97	9.07, 9.05
Rhizobium sp. TAL 651	9.61	9.38, 9.41	8.59, 8.47	9.49, 9.61	9.33, 9.40
Rhizobium sp. TAL 82	9.25	9.73, 9.64	9.47, 9.46	9.42, 9.43	6.72, 6.82
R. japonicum TAL 379	9.03	9.88, 8.68	9.63, ND	9.98, 9.51	9.62, ND
Means		9.55	9.25	9.38	8.38

[a] Counts are mean of three replicatons.
[b] Viable counts of the 3-week-old inoculants (tray method only) by the plant infection (MPN) technique before storage at 4°C.
[c] Initial counts were taken after 3 weeks of storage at 28°C after which inoculants were immediately transferred to storage at 4°C for 23 weeks. Liquid cultures were diluted for the tray method but not for the aseptic method with the irradiated peat.
[d] Plate counts after 23 weeks of storage at 4°C.
[e] Plate counts on CRYMA and BGYMA, respectively.

significant ($P < 0.001$) overall decrease in the viability of the rhizobia in the inoculants prepared by both the methods. The data were also analyzed for correlation between plate counts used in both methods. The results for the tray method were as follows: initial counts, CRYMA versus BGYMA, $r = 0.84$ ($P < 0.05$); final counts, CRYMA versus BGYMA, $r = 0.89$ ($P < 0.05$). With the aseptic method the results were as follows: initial counts, CRYMA versus BGYMA, $r = 0.94$ ($P < 0.05$); final counts, CRYMA versus BGYMA, $r = 0.99$ ($P < 0.01$).

Inoculant effectiveness. Inoculants of *R. japonicum* TAL 102, *R. phaseoli* TAL 182, and *Rhizobium* sp. strain TAL 82 prepared with diluted cultures incorporated in autoclaved and irradiated peat from the various countries were plant tested. The results (Table 9) showed that the effectiveness of the inoculants prepared with diluted cultures were not significantly different from those of the multistrain inoculants from NifTAL as measured by plant shoot dry weight. Examination of nodule sections showed pink to red pigmentation, indicative of effective symbiosis.

TABLE 9. Plant inoculation tests for effectiveness of inoculants prepared with diluted cultures of *Rhizobium* spp. and peat from different countries

Inoculation treatments and controls	Peat presteril-ization[b]	Dry wt of shoot (g)[a] with:		
		R. japonicum (TAL 102)	*R. phaseoli* (TAL 182)	*Rhizobium* sp. (TAL 82)
Mexico	A	3.37	2.75	0.73
	G	3.48	2.42	0.81
Argentina	A	3.41	2.90	0.80
	G	3.34	3.40	0.91
Spain	A	3.44	3.00	0.85
	G	3.53	2.37	0.79
United States (Demilco)	A	2.42	3.23	0.67
	G	2.73	3.68	0.78
Peru	A	2.44	ND[c]	ND
	G	2.30	ND	ND
NifTAL inoculant[d]	G	2.74	2.81	0.88
Control (uninoculated)		0.83	0.98	0.16
Control (70 ppm of N)		4.18	6.00	1.79

[a] Mean of three replications with two plants per replication; controls were not included in the statistical analysis.
[b] A, Autoclaving; G, gamma irradiation.
[c] ND, Not done.
[d] NifTAL multistrain inoculant prepared with undiluted cultures of *Rhizobium* spp.

DISCUSSION

The potential for using diluted cultures of *Rhizobium* spp. with autoclaved peat for inoculant production is demonstrated in this investigation. The significance of using diluted cultures lies in the fact that the *Rhizobium* spp. must multiply and survive in high numbers in the presterilized peat on introduction. On reaching maximum numbers, the rhizobia should reflect characteristics parallel or similar to those of strains in peat inoculants prepared in the normal procedure whereby undiluted cultures and presterilized peat are used.

The use of water as a suitable diluent without detrimental effects, as evidenced in all the experiments described in this work, has two interesting implications.

First, water brings out the intrinsic attributes of peat, indicating that the peat samples used for inoculant production are naturally self sufficient in the nutrients required for multiplication and long-term survival of *Rhizobium* spp. in the inoculants. However, the carry-over of unmetabolized YM nutrients may not be ignored during initial stages of multiplication, but long-term influence of nutrient carry-over is probably negligible. The exact mechanisms of nutrient extraction by the rhizobia from the peat during growth and survival (especially during long-term storage) have not been established, despite widespread speculations about nutrient release from the peat during sterilization. The chemical nature, array, and concentration of the constituent nutrients and the influence of sterilization on their release and availability for growth and survival of *Rhizobium* spp. warrants further research. It is conceivable that the peat environment is more complex than is reflected by its basic chemical composition (6). It was shown that undiluted and 1,000-fold-diluted fully grown cultures of *R. phaseoli* TAL 182 and *R. japonicum* TAL 102 showed no differences in the survival or shelf life of the inoculants in irradiated peat (16).

Second, the suitability of water as the diluent may have

economic implications for inoculant production systems with presterilized peat, in that sterilized water can be used for diluting smaller volumes of fully grown cultures of *Rhizobium* spp. before incorporation into autoclaved or irradiated peat. Furthermore, diluting liquid cultures significantly reduces the size and capacity of expensive fermenters and accessories needed for growing rhizobia without loss in the production capacity of inoculant production plants. A prototype system for producing inoculants with diluted cultures and presterilized peat has been validated and will be published elsewhere. The use of diluted cultures and presterilized peat (autoclaved in polypropylene bags) may be a cheaper option for small- to medium-scale inoculant production compared with the use of undiluted cultures and gamma-irradiated peat.

The wider applicability of the dilution technique is further supported by its performance with peat from Mexico, Argentina, Spain, the United States, and Peru (Tables 4 and 6). The slight differences in quality between inoculants produced with autoclaved and irradiated peat, although statistically significant, should have no practical drawbacks, as these inoculants had 10^9 rhizobia per g at manufacture and 10^8 rhizobia per g after 6 months of storage, thus meeting quality requirements set for sterilized peat (13). This slightly lower quality in inoculants produced with diluted cultures is not related to the dilution process, as the difference has been shown to occur with autoclaved peat and undiluted cultures (14). Inoculants produced with water-diluted cultures and presterilized peat (autoclaved or irradiated) did not suffer from any lack of effectiveness of the rhizobia or indicate the restriction of the method to any one peat or *Rhizobium* strain (Table 9). Three *R. japonicum* strains and two slow-growing cowpea-type *Rhizobium* strains were found to remain viable and able to rapidly nodulate their respective hosts after being stored in purified water at ambient temperature for a period of at least 1 year (5).

Differences in multiplication and survival in autoclaved peat (Table 2) were noted between *R. japonicum* TAL 102 and *R. phaseoli* TAL 182. Maximum populations of *R. japonicum* TAL 102 were not attained until 2 weeks longer than those of *R. phaseoli* TAL 182. A similiar lag phase in the multiplication was shown by *Rhizobium* sp. strain TAL 169 (Table 3). Such lag phases in multiplication in autoclaved peat may be considered undesirable traits for potential inoculant strains from the viewpoint of inoculant readiness for distribution and incompatibility with autoclaved peat. The reasons for the slower multiplication in autoclaved peat by some rhizobia have not been established. These examples of sensitivity of rhizobia to multiplication in autoclaved peat may not be widespread, as high-quality inoculants of several *Rhizobium* spp. were produced with autoclaved peat (19, 21).

Viability of the rhizobia during storage at 28 or 4°C after maturation gave mixed results, indicating that there was a strain preference for storage temperatures. Long-term storage at 28°C usually results in a decline in the population, and this was observed with all *Rhizobium* spp. in the peat samples from the various countries. The decline in population at 28°C was not related to use of diluted cultures, as similar observations were made on rhizobia in peat inoculants prepared with irradiated peat and undiluted cultures (17). With peat after maturation at 28 to 30°C, low-temperature (4°C) storage is generally more favorable for survival than storage at higher temperatures, including those at which growth would normally occur, e.g., 26°C (20).

Also, Thompson (20) reported that storage at 4°C was favorable to fast-growing *Rhizobium* strains, and survival of slow-growing strains was superior at 26°C. Survival of slow-growing strains such as CB 82 (for fine stem stylo), CB 627 (for *Desmodium* spp.), and CB 1024 (for *Lablab purpureus*) was better at 28°C than at 4°C (12).

In this investigation, fast-growing strains of *R. phaseoli* TAL 182 and TAL 1376 and *Rhizobium* sp. strain TAL 82 from *Leucaena leucocephala* indicated significant loss in viability when stored at 4°C. Similarly, inoculants of slow-growing *Rhizobium* sp. strain TAL 651 did not indicate good storage characteristics at 4°C. Low-temperature storage was not always detrimental, as two other strains *Rhizobium* sp. strains TAL 169 and TAL 620 showed significant increases in the population.

The experiment on the compatibility of the dilution approach for inoculant production with various peats and strains indicated significant survival differences among *Rhizobium* strains which could be related to the peat, method of sterilization of the peat, and various interactions (Table 5).

Strain survival differences with various peats has been well established (14). Marked *Rhizobium*-carrier interactions were demonstrated with *R. meliloti*, *R. japonicum*, and a cowpea *Rhizobium* sp. (18). Sterilization of peat by autoclaving was inferior to gamma irradiation with three *Rhizobium* spp. (14). Strains of *R. japonicum* and *R. meliloti* had significantly higher population in peat presterilized by gamma irradiation at 50 than at 25 kGr (19).

These data clearly demonstrate that the survival of *Rhizobium* spp. in peat is influenced by various factors which are common in inoculant production. As long as strain differences and interactions do not severely limit multiplication and survival, leading to low-quality inoculants, any new method of inoculant production should be considered feasible. Such a method is described in this investigation.

The general inference of the tray method (Table 7) of inoculant production was that rhizobia in a water-diluted culture (e.g., 10^6 to 10^7 cells ml^{-1}) were able to multiply to high populations in autoclaved peat in the presence of low numbers of aerial microorganisms introduced at the time of mixing with the peat. Once the high populations were attained, these populations survived like rhizobia in inoculants produced by the normal procedure. The critical factors thay may have influenced initial multiplication of *Rhizobium* spp. were probably the brief period of peat sterility, low numbers of introduced aerial microorganisms relative to the high numbers of *Rhizobium* spp. present in the diluted culture, the use of sanitized polyethylene gloves during mixing, and the immediate packaging of the inoculants. Posttreatment contaminants in gamma-irradiated peat can occasionally reach levels of 10^7 per g, and even at this level the survival of rhizobia is not seriously affected (10). Survival of *R. japonicum* WB1 in irradiated peat was high (log 9.254 per g of inoculant) after 6 months of storage at 27°C in the presence of contaminants (19). High-quality inoculants can be produced with partially sterilized peat (4). Keeping contamination to the lowest possible levels is the best option for producing good-quality inoculants by the tray method, and this is emphasized as a important prerequisite. Specially constructed mixing cabinets which can be easily fumigated or sterilized may be better than the regular laboratory environment for the tray method of inoculant production.

The close relationship (as measured by the correlation coefficient, *r*) of the plate count data suggested that recognition of *Rhizobium* colonies in the presence of contaminants was not a severe problem in the enumeration of inoculants

produced by the tray method, even though inclusion of contaminants in the counts could not be entirely eliminated. The lower r values for the tray method in comparison with that for the aseptic method indicated that the enumeration may have been subject to errors introduced by inclusion of contaminants. The lack of correlation between the standard MPN (plant infection) counts as described by Vincent (23) gave significantly lower estimates of *Rhizobium* numbers in soil (3), and a modification of the standard MPN produced higher and more definitive population estimates (1). However, despite its inaccuracy, the standard MPN method was useful in estimating the *Rhizobium* population relative to other methods used in the validation of the tray method for inoculant production in this investigation. The MFIF technique used in this investigation gave comparable results in relation to the other techniques, but it must be developed further for its application in inoculant quality control.

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