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Effect of Steam Sterilization and Gamma Irradiation of Peat on Quality of *Rhizobium* Inoculants

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Data obtained by independent tests on each of 483 batches of *Rhizobium* inoculants for *Glycine max*, *Medicago sativa*, and *Arachis hypogaea*, manufactured commercially in South Africa, are reported and discussed. Whereas the average cell count per gram per batch was well in excess of 10^9, inoculants for *G. max* and *M. sativa* manufactured with peat treated with gamma irradiation at a dose of 50 kGr contained significantly higher numbers of *Rhizobium* cells than inoculants from peat which received 25 kGr. Inoculants for *M. sativa* manufactured with steam-sterilized peat were similar in quality to those prepared with peat irradiated at a dose of 50 kGr. Contrary to the inoculants for *G. max* and *M. sativa*, the *Rhizobium* strain used in inoculants for *A. hypogaea* was apparently insensitive to the effect on peat of the higher gamma irradiation dosage.

The relatively slow progress made with the exploitation of the legume-*Rhizobium* symbiosis as a supplier of nitrogen to agriculture in many countries can be attributed partially to the inferior quality of *Rhizobium* inoculants in general. Until a decade ago, inoculants were prepared with unsterilized peat carriers by most manufacturers. Some of these were of acceptable quality (9), but it became apparent that the adverse effects of unfavorable environmental conditions were frequently accentuated in inoculants prepared with nonsterile peat (5, 6, 9).

Although it is now generally accepted that a sterilized carrier is superior to a nonsterilized one, there is some disagreement on the most suitable method of sterilization (6). Whereas steam sterilization has been used in many countries to produce inoculants of high quality (6-8), it is known that excessive heat renders peat unfavorable for subsequent growth and survival of rhizobia. Roughley and Vincent (6) showed, for example, that gamma irradiation of peat at a dose of 50 kGr was more favorable for subsequent survival of rhizobia than steam sterilization for 4 h at 121°C. Tests conducted regularly in our laboratory on inoculants produced commercially with either gamma-irradiated or steam-sterilized peat indicated that numbers of rhizobia in irradiated peat were not necessarily higher than those in steam-sterilized peat, although variation in quality among batches appeared to be less with peat treated with gamma rays (unpublished data).

That gamma irradiation at a dose of 50 kGr does not sterilize peat completely (9), in conjunction with claims that peat partially sterilized by flash drying at high temperatures supports satisfactory survival of rhizobia (2), indicates that complete sterility might not be imperative for success. The reported inferior quality of peat partially sterilized by treatment with ethylene oxide or methyl bromide (3, 6) apparently resulted from toxic side effects of the gaseous treatments and not from the presence of surviving contaminants.

In South Africa two inoculant manufacturers use peat from the same source. Their methods of production are essentially the same, differing mainly in the sterilization procedure used. Manufacturer X uses gamma irradiation at a dose of 50 kGr and, occasionally, steam sterilization; manufacturer Y uses gamma irradiation at a dose of 25 kGr. By monitoring the numbers of *Rhizobium* cells in packets of inoculant sampled randomly from each of 483 batches manufactured over a period of 2 years, a reliable comparison could be made of the effects of the various sterilization procedures on the quality of the inoculants. These results are reported.

MATERIALS AND METHODS

Bacteria. *Rhizobium* strain CB756, *Rhizobium meliloti* U45, and *Rhizobium japonicum* WB1 were supplied by the Plant Protection Research Institute to each of two inoculant manufacturers for production of inoculants for *Arachis hypogaea* (groundnuts), *Medicago sativa* (alfalfa), and *Glycine max* (soybeans), respectively. Strains CB756 and U45 were of Australian origin; strain WB1 was obtained from the South African Rhizobium Collection.

Broth cultures. Both manufacturers X and Y obtained inoculum for peat inoculation by cultivating a *Rhizobium* strain in well-aerated broth in small fer-

mentors of 20- or 40-liter capacity. Manufacturer X used yeast extract-mannitol broth (1); manufacturer Y used the same medium but with sucrose instead of mannitol as the carbon source. The fast-growing *R. meliloti* U45 was cultivated at 27°C for 3 to 5 days; the slow-growing strains WB1 and CB756 were cultivated for ca. 7 days. At this stage the broth cultures of either manufacturer had reached an optical density representing a cell concentration of ca. 10^9 cells ml^{-1}.

Preparation of peat and its inoculation. Portions, 230 g, of finely ground (150 to 200 mesh, B.S.) Putfontein peat (6), with the pH adjusted to between 6.5 and 7.5 with $CaCO_3$ and with a moisture content of ca. 45%, were sealed off in low-density polythene bags for gamma irradiation. Both manufacturers used the same facilities for gamma irradiation; a cardboard container with sealed bags of peat not exceeding a total mass of 22 kg was exposed to gamma irradiation on two sides from a ^{60}Co source. The irradiation dose was determined by red poly(methyl methacrylate) dosimetry and verified by serum sulfate chemical dosimetry (2). Peat from manufacturer X received a 50-kGr dose, and that from manufacturer Y received a 25-kGr dose. When steam sterilization was used, 230-g quantities in high-density polythene bags, with open ends folded back, were autoclaved for 3.5 h at 124°C. The peat was allowed to cool down in the autoclave before the bags were sealed off.

The sterilized peat in each bag was inoculated aseptically by puncturing the surface with a sterilized automatic syringe connected to a fermentor containing the broth culture. A 15-ml amount of culture was injected into the peat before the hole was sealed off, and the contents were mixed by hand. The moisture content of the inoculated peat was 50 to 55%. Packets of inoculant of each batch were incubated at 27°C for approximately 10 days before three randomly selected bags were collected for independent quality tests by the Plant Protection Research Institute.

Quality tests. The number of *Rhizobium* cells in a packet of inoculant was determined by placing 20 g of well-mixed peat aseptically into 180 ml of sterile water. The suspension was left on a shaker for 30 min before a 10-fold dilution series was prepared. Portions, 0.1 ml, of 10^{-5}, 10^{-6}, and 10^{-7} dilutions were streaked onto yeast mannitol agar plates containing Congo red (1). Plates were incubated for 5 to 7 days at 27°C. Strain identity was confirmed serologically by means of spot tests on single colonies, using the agglutination method (10). Inoculants were rejected for marketing on any one of the following grounds: number of *Rhizobium* cells $< 5 \times 10^8$ g of moist $peat^{-1}$; contaminants present on plates streaked with 10^{-5} dilutions; pH of inoculant below 6.5 or above 7.5; *Rhizobium* strain identity doubtful.

RESULTS

Before manufacturer X went into production in 1977, batches of soybean inoculant prepared on an experimental basis from steam-sterilized peat and peat subjected to gamma irradiation at doses of either 25 or 50 kGr were submitted for evaluation to the Plant Protection Research Institute. The results in Table 1 show that, over a 6-month period, numbers of rhizobia in peat irradiated at a dose of 25 kGr were significantly lower than those in either steam-sterilized peat or peat subjected to gamma irradiation at 50 kGr (Table 1).

The quality of 483 batches of inoculants for soybeans, alfalfa, and groundnuts subsequently marketed by manufacturers X and Y during 1978 and 1979 is reflected by the results in Table 2.

The most important deductions to be made are as follows. (i) Average numbers of *Rhizobium* strain CB756 in 106 batches of inoculants for groundnuts were apparently unaffected by the method of peat sterilization. (ii) Average cell numbers of 100 batches of inoculants for soybeans and 32 batches for alfalfa manufactured by X with peat irradiated at a dose of 50 kGr were significantly higher than those of 229 batches produced for the same legumes by Y with peat treated at a dose of 25 kGr. Similarly, inoculants for alfalfa manufactured by X from steam-sterilized peat had higher cell numbers than inoculants from peat irradiated at 25 kGr. (iii) Whereas the average cell numbers of inoculants manufactured by X and approved for marketing were generally higher than those manufactured by Y, larger percentages of X's inoculants for groundnuts and soybeans were rejected because of excessive contamination. Although the reason for this is unknown, contamination of rejected batches appeared to have occurred during inoculation of the peat.

TABLE 1. *Survival of R. japonicum WB1 in peat carriers subjected to steam sterilization or gamma irradiation*

Treatment of peat	Log no. of *Rhizobium* cells in peat after:						
	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	Mean[a]
Irradiation							
25 kGr	8.555[b]	9.015[b]	8.629[b]	8.741[b]	8.315[b]	8.417[b]	8.612
50 kGr	8.968	9.228	9.516[b]	9.061[b]	9.320[b]	9.254[b]	9.224
Steam[c]	9.445	9.471	9.713	9.306	9.578	9.260	9.462

[a] Least significant difference: P (0.01) = 0.292; P (0.05) = 0.258. Coefficient of variation = 3.06%.
[b] Contaminants present.
[c] 124°C for 3.5 h.

TABLE 2. *Effect of method of sterilization of peat carrier on quality of three types of inoculants manufactured and approved in South Africa during 1978 and 1979*

Inoculant for:	Manufacturer	Treatment of peat	No. of batches approved (%)[a]	% of approved batches containing given no. of cells ($\times 10^9$) g^{-1}				Log no. of cells g^{-1} $batch^{-1}$ (mean)	LSD between means[b]	CV (%)[c]
				0.5-0.99	>0.99	>2.99	>4.99			
A. hypogaea CB756	X	50 kGr	22 (91.3)	13	87	50	9	9.3484	NS[d]	3.4
	Y	25 kGr	84 (100)	18	82	35	7	9.3184		
G. max WB1	X	50 kGr	100 (89.3)	6	94	57	31	9.4935	0.1139	3.9
	Y	25 kGr	85 (96.7)	25	75	32	13	9.2597		
M. sativa U45	X	50 kGr	32 (100)	0	100	47	13	9.5440		
	X	Steam (124°C)	16 (89.9)	6	94	69	13	9.4998	0.0599	3.6
	Y	25 kGr	144 (100)	17	83	29	6	9.2896		

[a] Numbers in parentheses indicate the percentage of batches manufactured that were approved for marketing.
[b] LSD, Least significant difference.
[c] CV, Coefficient of variation.
[d] NS, Not significant.

DISCUSSION

Analysis of data obtained with 483 batches of inoculants manufactured for soybeans, alfalfa, and groundnuts in South Africa showed that excellent quality can be maintained by manufacturers whose products are subjected to independent quality control. Whereas the minimum requirement had been 5×10^8 cells g (wet weight) of inoculant^{-1}, average numbers ranged from 1.819×10^9 cells g^{-1} (log value, 9.2597) for soybean inoculants prepared with peat irradiated at 25 kGr to 3.499×10^9 cells g^{-1} (log value, 9.5440) for alfalfa inoculants prepared with peat irradiated at 50 kGr (Table 2). These results indicated that 10^9 rhizobial cells g^{-1} would be a realistic and fair minimum requirement for peat-based inoculants in South Africa, as is the case in Australia (J. A. Thompson, personal communication).

Interpretation of the findings with the commercially manufactured inoculants in terms of sterilization practices for peat before inoculation is somewhat complicated by the fact that two manufacturers were involved. However, it should be emphasized that essentially the same manufacturing procedures were followed by both except for sterilization procedures. Similarly treated peat from the same source was inoculated with cell suspensions of the same *Rhizobium* strains containing approximately 10^9 cells ml^{-1}. With such a high cell concentration, a possible small variation in numbers from time to time would not have influenced the cell numbers in the peat inoculant (4). The small percentage of inoculants from either manufacturer being rejected because of inferior quality (Table 2) also testifies to the technical competence of both. Furthermore, the results obtained by manufacturer X with soybean inoculants prepared from steam-sterilized peat and peat submitted to gamma irradiation at either 25 or 50 kGr (Table 1) showed the same pattern as the data obtained over 2 years with the commercially manufactured inoculants. We are therefore confident that sterilization practice, and not manufacturer, was primarily responsible for differences in quality.

The results showed differences in the responses of the *Rhizobium* strains used to sterilization treatment of the peat carrier. Whereas cell numbers of *R. japonicum* WB1 and *R. meliloti* U45 were significantly higher when gamma irradiation of the peat was increased from 25 to 50 kGr, *Rhizobium* strain CB756 appeared to be unaffected. This indicates the relevancy of determining, as one of the criteria for strain selection for inoculant production, the capacity of a *Rhizobium* strain to benefit from an expensive sterilization practice (9).

Considering the acceptable quality of even those inoculants produced with peat irradiated at 25 kGr, manufacturers might argue that the additional cost does not justify a 50-kGr radiation treatment. We are of the opinion that, with legume inoculants, the aim should always be to obtain the highest numbers practically possible.

Subsequent attempts by manufacturer X to quantify differences in sterility of peat irradiated at 25 and 50 kGr failed to show marked differences in the numbers and types of microorganisms surviving (P. L. Steyn, personal communication). The numbers of bacterial colonies on agar plates inoculated with 10^{-1} dilutions of the irradiated peat were apparently too small, and varied too much between replicates, to allow

statistical analysis of the data. It therefore seems unlikely that the superiority of peat exposed to gamma irradiation at a dose of 50 kGr solely resulted from a higher degree of sterility at the time of inoculation. A higher release of nutrients from peat that was bombarded twice as long by gamma rays could also have contributed to the 50-kGr treatment being superior for survival of rhizobia.

Whereas the results indicated that gamma irradiation at a dose of 50 kGr and steam sterilization for 3.5 h at 124°C were equally effective for the manufacturing of high-quality inoculants with the local peat, both manufacturers preferred gamma irradiation as the more convenient and reliable method. Apparently, the high risk of contaminating sterilized peat when bags are removed from the autoclave before being sealed off makes steam sterilization a less attractive alternative.

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