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Growth and survival of cowpea bradyrhizobia in various carrier materials

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Summary. Pakistan does not yet have the technology for commercial production of *Rhizobium* and *Bradyrhizobium* inoculum. Therefore, investigations were undertaken to evaluate the suitability of different materials like compost, sawdust, rice husks, sugar cane, filter mud, and peat as *Bradyrhizobium* carriers. The growth and survival of bradyrhizobia (strain TAL 441 of the cowpea type) was studied in sterilized and unsterilized carriers mixed with loam and enriched with lucerne meal and sucrose. Three different sterilization methods (autoclaving, gamma irradiation, and dry heating of the carriers) were used. The growth and survival of bradyrhizobia in the inoculants were studied at two different storage temperatures, 4° and 20 °C. After $2\frac{1}{2}$ months of inoculation, maximum survival of rhizobia (7.6×10^9 cells g^{-1}) was observed in autoclaved filter mud containing loam-lucerne meal and sucrose. The survival of rhizobia in autoclaved peat was 3.4×10^9 cells g^{-1}. The maximum viable number of rhizobia per seed of mungbean (*Vigna radiata*) was 7.7×10^8 in gamma-irradiated compost and least (1×10^7 cells $seed^{-1}$) in rice husks.

Key words: Carrier materials – *Bradyrhizobium* inoculum – Cowpea – *Vigna radiata* – Survival at 4° and 20 °C – Mungbean

N_2 fixation by legumes is an essential input for high-protein foods produced in both crop and pasture systems. In many situations the establishment of effectively nodulated legumes cannot be left to chance but requires the introduction of effective strains of rhizobia into the soil at sowing. In principle the establishment of effectively nodulated legumes can be achieved by using legume inoculant containing effective and competitive strains of rhizobia (Roughley 1976). The supply of quality legume inoculants is limited mostly to developed countries, however, and developing countries generally lack a regular supply.

The application of artificially grown *Rhizobium* and *Bradyrhizobium* spp. cultures to legume seeds has been known since the beginning of this century (Fred et al. 1932). Initially, agar- or soil-based cultures were used, but recently, inoculant technology has advanced considerably. Peat has been most commonly used for inoculum production, and is considered to be the most dependable (Strijdom and Deschodt 1976; Halliday and Graham 1978; Burton 1981, 1982; Roughley 1982; Aaron and Ahmad 1986). Peat, in general, is not abundantly available in the tropical, subtropical, arid and semiarid regions of the world and, if available, may not always be of a quality suitable for use as a carrier (Roughley 1982). The ability of rhizobia or bradyrhizobia to survive, proliferate, and maintain growth and effectiveness in a particular carrier must be examined before the carrier is selected for inoculum production (Wilson and Trang 1980; Chao and Alexander 1984; Somasegaran 1985). In Pakistan peat is not abundantly available, except in Swat valley in the north-western part of Pakistan where small quantities may be found. However, its value and quantity as a suitable *Rhizobium* or *Bradyrhizobium* spp. carrier has not been evaluated. The present study was, therefore, undertaken to examine the survival of cowpea (*Vigna radiata*) bradyrhizobia (strain TAL 441) in various carrier materials locally available in Pakistan.

Materials and methods

Sawdust, compost, rice husks and filter mud were selected for study as carrier materials for *Bradyrhizobium* sp. inoculum in comparison with peat received from NifTAL (Nitrogen fixation in Tropical

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Table 1. Survival of *Bradyrhizobium* sp. strain TAL 441 in gamma-irradiated carrier materials at 4 °C

Carriers	pH	Mean number of viable cells $\times 10^8$ g^{-1} carrier						
		Week 1	Week 2	Week 3	Week 4	Week 6	Week 8	Week 10
Compost	6.5	48	90	5	1	18	14	0.06
Compost M		16	63	3	4	30	8	0.02
Compost M·S		21	2	6	4	2	1	0.07
Sawdust	7.5	53	4	33	15	21	15	0.06
Sawdust M		82	66	6	16	14	35	0.05
Sawdust M·S		24	41	7	4	4	11	0.03
Rice husks	7.4	29	16.6	22	7	16	20	0.02
Rice husks M		23	7	23	24	13.2	12.6	15.5
Rice husks M·S		56	31	3	4	7	57	0.03
Filter mud	7.6	17	18	34	23	25	66	9.37
Filter mud M		5	53	32	37	25	24	54.2
Filter mud M·S		14	13	12	16	12	22	46.6
Peat		46	12.5	52	16	10	70	12.1
LSD ($P \leqslant 0.05$)		22.7	29.8	16.1	9.6	19.4	NS	3.27

M, lucerne meal + loam; S, sucrose; LSD, least significant difference; NS, not significant. Initial population of *Bradyrhizobium* sp. was 3×10^9 cells g^{-1}

Agricultural Legumes Project), Hawaii, U.S.A. Sawdust, rice husks and filter mud were obtained as byproducts of wood industries, rice growing and sugar-cane milling, respectively, while the compost was prepared from Kallar grass (*Leptochloa fusca*). Ten-month-old compost was used in this study. The dry carriers were milled to 18–20 mesh. The water-holding capacity and pH of each carrier material were determined. For some treatments, the carriers were amended with 42% loam and/or 0.5% sucrose and then packed in 0.05-mm gauge polyethylene bags.

The growth and survival of *Bradyrhizobium* sp. in the carrier materials were studied under sterilized and unsterilized conditions. Three different methods of sterilization were used, autoclaving at 121 °C for 3 h (interval of 24 h after 1 h of autoclaving), gamma irradiation at a dose of 5 Mrad performed in a gamma cell (Model 220, Atomic Energy Canada Ltd.) and dry heating at 100 °C for 48 h.

Rhizobium culture. Strain TAL 441 of *Bradyrhizobium* sp. (cowpea group) received from NifTAL, Hawaii, U.S.A., was used in this investigation. The *Bradyrhizobium* sp. strain was grown on yeast extract mannitol broth (Vincent 1970) in a fermentor vessel at 28 ± 2 °C for 7 days. In 40-g packages of the presterilized carriers, 45% – 50% (on a wet weight basis) of the broth culture was injected aseptically with a 50-ml syringe. Three replicates for each carrier were used. The mean number of viable cells present in the fresh culture at the time of injection was 3×10^9 cells g^{-1}, as determined by the drop-plate method (Vincent 1970). The inoculants were matured for 1 week at room temperature (28 ± 2 °C) and stored at two different temperatures, 4 °C and 20 °C.

In unsterilized carriers, broth culture was added and mixed mechanically. The mixture was sieved coarsely to remove lumps and then matured for 14 days at 28° – 30 °C in trays in triplicate covered with polyethylene sheets. Finally, the unsterilized carrier mixtures were packed in polyethylene bags and stored at 4 °C. The mean number of viable *Bradyrhizobium* sp. present in fresh culture was 1×10^9 cells g^{-1}.

Plate counts. Numbers of bacteria on yeast extract mannitol agar with 25 ppm congo red (Vincent 1970) were obtained by plate counts. Three plates were used for each dilution. The viability of cells was determined weekly.

Pelleting of seeds. Gum arabica (3.5 ml, 40% solution) was added to 100 g mungbean (*Vigna radiata*) seeds. The seeds were inoculated with 9 g of inoculum. Then they were quickly mixed and rolled in 60 g pulverized rock phosphate until they were thoroughly coated with the rock phosphate.

Viability of Rhizobium on seed. The number of viable rhizobia per seed (*Vigna radiata*) was determined for various carrier cultures after the inoculant had been stored at 4 °C for 2 weeks. Five pelleted seeds from each treatment were aseptically placed in 5 ml sterilized water. The seeds were shaken vigorously for 5 min with glass beads to dissolve all *Bradyrhizobium* sp. cells into the solution. Viable *Bradyrhizobium* sp. cells per seed were counted by a spread-plate method (Vincent 1970), in triplicate, after incubation at 28° ± 2 °C for 7 days.

Results and discussion

The results (Table 1) revealed that all the carriers tested were close to neutral pH and a water-holding capacity of 60%. The sawdust and rice husks had higher water-holding capacities than the peat. None of these media was proved to be the best rhizobia carrier (Tables 1 and 2). Irradiated rice husks proved less favourable (1.5×10^9 cells g^{-1} carrier) than the filter mud-lucerne combination (5.4×10^9 cells g^{-1} carrier), while compost and sawdust proved the least satisfactory. The growth and survival of *Bradyrhizobium* sp. in the autoclaved filter mud-lucerne-sucrose combination was higher (7.5×10^9 cells g^{-1} carrier) than in peat (3.3×10^9 cells g^{-1} carrier). Philpotts (1976) reported filter mud as an alternative carrier for *Rhizobium* sp. inoculum production. A lucerne and sucrose combination with peat also had a beneficial effect on the growth and survival of rhizobia (Schreven 1970). In the present investigations, filter mud showed a better

Table 2. Survival of *Bradyrhizobium* sp. strain TAL 441 in autoclaved carrier materials at 4 °C

Carriers	Mean number of viable cells $\times 10^8$ g^{-1} carrier						
	Week 1	Week 2	Week 3	Week 4	Week 6	Week 8	Week 10
Compost M	48	75	27	21	45	10	31.4
Compost M·S	27	5	7	2	10	56	0.05
Sawdust	4	4	5	4	4	9	0.01
Sawdust M	34	60	3	1	35	87	0.04
Sawdust M·S	7	43	24	7	5	70	0.01
Rice husks	13	63	14	10	25	66	0.09
Rice husks M	10	2	21	4	1	6	0.08
Rice husks M·S	11	5	3	22	18	38	0.04
Filter mud	24	60	45	97	10	74	22.8
Filter mud M	19	24	13	97	11	96	41.6
Filter mud M·S	48	10	24	15	10	47	75.9
Peat	59	16	14	24	17	40	33.7
LSD ($P \leqslant 0.05$)	NS	93.0	94.0	32.1	36.5	28.0	15.92

M, lucerne meal + loam; S, sucrose; LSD, least significant difference; NS, not significant. Initial population of *Bradyrhizobium* sp. was 3×10^9 cells g^{-1}

result when mixed with lucerne meal and/or sucrose (Tables 1 and 2). Similar results have been observed in a coal bentonite-lucerne combination (Strijdom and Deschodt 1976). The use of loam, lucerne meal and carriers for the rhizobia ensures the presence of sufficient quantities of micro- and macroelements in the medium.

Table 3 shows that storage of various inoculants at low temperatures was beneficial for the survival of rhizobia. The favourable effect of low-temperature (4 °C) storage on the survival of *Bradyrhizobium* sp. may be due to maintenance of a suitable long-term moisture content. Further, at low temperatures bacterial cell division and activity slows down, resulting in a slower use of nutrients. *Rhizobium meliloti* and *R. trifolii* cultures stored at 2 °C showed better survival than those stored at 28 °C (Schreven 1970). Roughley (1982) noted that storage temperatures between 4 °C and 26 °C affected the rate of growth but not survival in pure peat culture for up to 26 weeks, provided moisture was not limited. In contrast, *Bradyrhizobium* sp. survived better in the saw dust-lucerne-sucrose and compost-lucerne-sucrose combinations when stored at 20 °C than at 4 °C. Similar results have been reported by the Australian Inoculant Research and Control Service with three *Rhizobium* spp. strains (Roughley 1982).

As shown in Table 4, higher numbers of *Bradyrhizobium* sp. were found in sterilized than in unsterilized carriers. It has been reported that as an inoculant base, sterile peat is superior to non-sterile peat (Schreven 1970; Thompson 1980; Burton 1982). In the present study three sterilization methods were used. A harmful effect on the number of rhizobia was noted with the dry- heat-treated carrier. Autoclaved carriers were superior to irradiated carriers while irradiated filter mud and rice husks enriched with lucerne showed much better survival of bradyrhizobia than the corresponding autoclaved carriers (Tables 1 and 2). The growth and survival of bradyrhizobia during storage was much better in the sterilized carriers than in non-sterile carrier cultures. The advantage of a sterilized base for the preparation of inoculant is that it excludes the antagonistic effect of harmful microorganisms and ensures a more precise determination of the total number of viable bradyrhizobia cells by means of plate counts than is possible with an unsterilized base.

Table 3. Survival of *Bradyrhizobium* sp. strain TAL 441 in various carrier materials at different temperatures after 6 weeks

Carriers	Mean number of viable cells g^{-1} carrier ($\times 10^7$)	
	4 °C	20 °C
Compost M	30.3	1.8
Compost M·S	2.0	15.0
Sawdust	21.7	23.3
Sawdust M	14.3	2.1
Sawdust M·S	3.5	112.7
Rice husks	15.7	3.1
Rice husks M	132.0	3.3
Rice husks M·S	6.9	4.3
Filter mud	24.7	7.4
Filter mud M	246.7	61.7
Filter mud M·S	119.3	50.7
LSD ($P \leqslant 0.05$)	19.42	

M, lucerne meal + loam; S, sucrose; LSD, least significant difference; NS, not significant. Initial population of *Bradyrhizobium* sp. was 3×10^9 cells g^{-1}

Table 4. Survival of *Bradyrhizobium* sp. strain TAL 441 in sterilized and unsterilized carrier materials after 15 days

Carriers	Mean number of viable cells g^{-1} carrier ($\times 10^7$)	
	Sterilized	Unsterilized
Compost	90.0	95.0
Compost M	63.3	6.6
Compost M·S	1.8	19.0
Sawdust	3.8	6.5
Sawdust M	65.7	9.0
Sawdust M·S	41.2	26.3
Rice husks	106.0	20.7
Rice husks M	6.8	4.5
Rice husks M·S	31.0	12.3
Filter mud	18.0	1.1
Filter mud M	533.3	2.5
Filter mud M·S	130.0	14.0
LSD ($P \leqslant 0.05$)	29.83	

M, lucerne meal + loam; S, sucrose; LSD, least significant difference; NS, not significant. Initial population of *Bradyrhizobium* sp. was 3×10^9 cells g^{-1}

Table 5. Viability of *Bradyrhizobium* sp. strain TAL 441, in various carrier materials, on pelleted seeds of *Vigna radiata*

Carriers	Mean number of viable cells $seed^{-1}$ ($\times 10^7$)	
	Autoclaved	Gamma-irradiated
Compost	–	77 ± 7.0
Compost M	14 ± 3.5	11 ± 0.6
Compost M·S	2 ± 0.5	–
Sawdust	19 ± 6.1	3 ± 0.3
Sawdust M	–	8 ± 0.6
Sawdust M·S	4 ± 2.5	12 ± 0.6
Rice husks	–	1 ± 0.1
Rice husks M	6 ± 0.6	8 ± 1.7
Rice husks M·S	10 ± 0.1	12 ± 1.5
Filter mud M·S	2 ± 1.0	2 ± 1.0
Peat	48 ± 15.9	40 ± 12.3

M, lucerne meal + loam; S, sucrose; LSD, least significant difference; NS, not significant. Initial population of *Bradyrhizobium* sp. was 3×10^9 cells g^{-1}

The mean number of viable bradyrhizobia per seed (*Vigna radiata*) as shown in Table 5 was very high in comparison with those described for most of the inoculants. According to NifTAL, 1×10^5 viable rhizobia cells per seed are required for *Vigna radiata*.

Further studies are underway on *Vigna radiata* and *Glycine max* under field conditions to check the suitability of filter mud as a carrier for inoculant. If the results are promising, large-scale production of commercial inoculum will be carried out.

References

Aarons S, Ahmad MH (1986) Examining growth and survival of cowpea rhizobia in Jamaica peat. Lett Appl Microbiol 2:115–118

Burton JC (1981) *Rhizobium* inoculation for developing countries. Trop Agric (Trinidad) 58:291–355

Burton JC (1982) Modern concepts in legume inoculation. In: Graham PH, Harris SC (eds) Biological nitrogen fixation technology for tropical agriculture. CIAT, Cali Colombia, pp 105–114

Chao WL, Alexander A (1984) Mineral soils as carrier for *Rhizobium* inoculants. Appl Environ Microbiol 47:94–97

Fred EB, Baldwin IL, McCoy E (1932) Root nodule bacteria and leguminous plant. University of Wisconsin Studies in Science No. 5. University of Wisconsin Press, Madison, Wisc

Halliday J, Graham P (1978) Coal compared to peat as a carrier of rhizobia. Turrialba 28:348–349

Philpotts H (1976) Filter mud as a carrier for *Rhizobium* inoculants. J Appl Bacteriol 41:277–281

Roughley RJ (1976) The production of light quality inoculants and their contribution to legume yield. In: Nutman PS (ed) Symbiotic nitrogen fixation in plants. Cambridge University Press, pp 125–136

Roughley RJ (1982) The storage, quality control and use of legume seed inoculants. In: Graham PH, Harris SC (eds) Biological nitrogen fixation technology for tropical agriculture. CIAT, Cali Colombia, pp 115–126

Schreven DA van (1970) Some factors affecting growth and survival of *Rhizobium* spp. in soil peat cultures. Plant and Soil 32:113–130

Somasegaran P (1985) Inoculant production with diluted liquid cultures of *Rhizobium* spp. and autoclaved peat: Evaluation of diluents, *Rhizobium* spp. peats, sterility requirements, storage and plant effectiveness. App Environ Microbiol 50:398–405

Strijdom BW, Deschodt CC (1976) Carriers of rhizobia and the effects of prior treatment on the survival of rhizobia. In: Nutman PS (ed) Symbiotic nitrogen fixation in plants. Cambridge University Press, London, pp 151–168

Thompson JA (1980) Production and quality control of legume inoculants. In: Bergersen FJ (ed) Methods for evaluating biological nitrogen fixation. Wiley, New York, pp 489–534

Vincent JM (1970) A manual for the practical study of root nodule-bacteria. IBP Handbook No. 15, Blackwell, Oxford

Wilson DO, Trang KM (1980) Effects of storage temperature and enumeration method on *Rhizobium* spp. numbers in peat inoculants. Trop Agric (Trinidad) 57:233–238

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