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Effects of storage temperature and enumeration method on Rhizobium *spp. numbers in peat inoculants*

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Commercial peat inoculants containing rhizobia of the cowpea miscellany (about 10^8 cell/g) were stored at 25, 35, 45 and 55°C to simulate extreme tropical storage conditions. At intervals of three weeks for 21 weeks, numbers of rhizobia were determined by the plant infection most probable number (*MPN*), plate *MPN* and plate colony enumeration techniques. Survival after 21 weeks at 25°C was good as measured by all counting methods. At 35°C, plate counts remained unchanged throughout the period, but plant infection counts declined slowly. Plate counts declined slowly at 45°C, whereas plant infection counts decreased rapidly. After six weeks of storage at 55°C, there were no detectable viable rhizobia as determined by plant infection counts, but plate counts showed approximately 10^4 rhizobia per gramme even after 15 weeks when sampling was discontinued at this temperature. At all sampling times for all temperatures, counts from the two plating techniques were very similar, but values obtained with the plant infection method were generally lower. This difference increased with storage time and was accentuated at the higher temperatures. This finding was attributed to a decline in cell vigour during storage such that the surviving cells, while able to grow when transferred to agar media, were incapable of surviving in plant nutrient solution and causing nodule development.

INTRODUCTION

Food grain legumes constitute an important part of the diet of many people in the world. It is generally recognized that legume inoculation is a beneficial and sometimes indispensable management practice to include in any efficient legume production programme. The availability of high quality rhizobial inoculants to farmers in the tropics depends on many factors. Successful local production of good inoculants requires a readily available source of good carrier material, the use of effective strains of rhizobia, and careful attention to detail during the production process. However, even superior inoculants can rapidly deteriorate in quality if stored under adverse conditions. The

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possibility that inoculants will be exposed to high temperatures during storage is particularly likely in tropical areas.

The effect of high temperatures on survival of rhizobia has been reported in soils (WILLATT, 1966; CHATEL and PARKER, 1973), in laboratory growth media (GRAHAM, *et al.*, 1963; GILLBERG, 1968; OKAFOR and ALEXANDER, 1975) and on seed (HERRIDGE and ROUGHLEY, 1974; PHILPOTTS, 1977). Little attention has been given to the effects of prolonged exposure of peat inoculants to the extreme temperatures possible in enclosed storage areas in the tropics. It has also been reported that the calculated number of viable rhizobia in inoculants is influenced by the enumeration method that is used (BROCKWELL, 1963; VINCENT, 1970). It is important to know the relative differences among enumeration methods and how these differences may be affected by adverse inoculant storage conditions. The purpose of this research was to determine the effects of storage temperature and enumeration method on calculated numbers of viable cells of *Rhizobium* spp. in commercial peat inoculants.

MATERIALS AND METHODS

Commercial peat inoculants (The Nitragin Co., Milwaukee, Wisconsin, U.S.A.) containing effective strains of *Rhizobium* spp. for use with the cowpea group of legumes were obtained in both powder and granular forms. Both forms of inoculant were dispensed in lots of 5 g into polyethylene film (0·1 mm) packages and heat sealed. Packages of the inoculants were stored in environmental chambers controlled at 60 per cent relative humidity and 25, 35, 45 or 55°C. At intervals of three weeks for 21 weeks, a sealed package of each inoculant was removed from each of the chambers for counting. Duplicate samples of each inoculant were initially diluted by adding 1 g of inoculant to 9 4 ml of sterile 0·1 per cent (w/v) peptone solution. Glass beads were used to aid dispersion of the inoculants during the mechanical mixing of the first two dilutions. Further tenfold dilutions were made with sterile 0·1 per cent peptone solution to give a dilution series ranging from 10^{-2} to 10^{-10}.

For plate colony and plate most probable number (*MPN*) counting, 0·1 ml of the appropriate dilutions was spread on the surface of a yeast extract–mannitol (*YEM*) agar medium containing 0·025 mg/ml rose Bengal and 0·03 mg/ml cycloheximide. At this concentration, cycloheximide had no detectable influence on growth of the rhizobia and was used to retard the growth of fungal contaminants. The plates were inverted and incubated at 28°C and plate colony and plate *MPN* counts were made seven to ten days after plating. Plant infection *MPN* counts were made using the plastic pouch method (WEAVER and FREDERICK, 1972). A 1 ml aliquot from each dilution in a dilution series was added to quadruplicate pouches, each containing two five-day-old cowpea (*Vigna unguiculata* (L.) Walp. cv. 'Mississippi Silver') seedlings growing in sterilized N-free nutrient solution. The nutrient solution had the following composition: K_2SO_4, 0·4 mM; $CaCl_2$, 0·4 mM; K_2HPO_4, 0·2 mM; $MgSO_4$, 0·2 mM; H_3BO_3, 5 μM; Fe-EDDHA, 4 μM; $MnSO_4$, 2 μM; $ZnSO_4$, 1 μM; $CuSO_4$, 0·2 μM; Na_2MoO_4, 0·2 μM; $CoCl_2$, 0·02 μM. Uninocu-

lated pouches were included to detect possible contamination. After inoculation, the pouches were placed in wooden racks and grown for 28 days in a growth chamber at 60 per cent relative humidity and 29°C during a 16 h light period and 22°C during an 8 h dark period. Photon flux in the chamber was 420 Einstein m^{-2} sec^{-1} at the top of the plants. Plant nutrient solution in the pouches was maintained by periodic additions of sterilized nutrient solution. At harvest, the number of pouches with nodulated plants at each dilution level was determined and the number of rhizobia was estimated using the *MPN* tables of VINCENT (1970).

RESULTS AND DISCUSSION

The numbers of viable rhizobia in the inoculants determined by three enumeration methods over 21 weeks of storage at 25, 35, 45 and 55°C are shown in *Tables 1, 2, 3* and *4*, respectively. A comparison of rhizobial numbers within each enumeration method showed no significant difference between the powder and granular forms at any storage temperature. Consequently, the mean of these two forms is also presented and is used to compare the effects of time and enumeration method on rhizobial numbers. At 25°C (*Table 1*), the number of viable rhizobia in the inoculants showed no significant decrease over the 21 week storage period. At 35°C (*Table 2*), rhizobial numbers obtained with the plate *MPN* and plate colony methods showed no decline over the entire period, but counts from the plant infection method showed a significant decrease at the end of 12 weeks. By the end of 21 weeks, plant infection counts were only two per cent of initial values. Counts at 45°C (*Table 3*) declined rapidly when determined by the plant infection method but much less so with the other two methods. The values at the end of the storage period at this temperature were 0·00004, 0·6 and 0·7 per cent of initial values for the plant infection, plate *MPN* and plate colony methods, respectively. Because no rhizobia were detected with the plant infection method for three successive sampling periods, the experiment at

Table 1. Log number of viable rhizobia per gramme in granular and powder peat cowpea inoculants stored at 25°C

Storage time (week)	Enumeration method								
	Plant infection			*Plate* MPN			*Plate colony*		
	Granular	*Powder*	*Mean*	*Granular*	*Powder*	*Mean*	*Granular*	*Powder*	*Mean*
0	7 62	8·00	7·85	8 62	8 88	8·77	8·66	9 12	8·95
3	7 62	7 49	7·56	8·12	9·12	8·86	8·22	9 27	9·01
6	7·24	6·86	7·09	8·86	8 49	8·71	8·54	9·01	8·84
9	7·36	7·24	7·30	8·12	8·12	8·12	8·59	8·38	8·59
12	7·24	7·00	7·14	8·62	8·62	8·62	8·62	8 63	8·63
15	7·12	6·86	7·01	8·76	8·86	8·81	8·81	9·11	8·99
18	6·36	7·00	6·79	8 36	8·49	8 43	8·42	8·82	8·66
21	6·76	7·76	7·50	8·54	8·86	8·73	8 57	8 92	8·78

95 per cent confidence interval for all values = ± 0 58.

55°C was terminated after 15 weeks of storage. Counts with the plant infection method declined to non-detectable levels within six weeks, whereas values for the other methods were still measurable after 15 weeks (*Table 4*), being 0·0005 and 0·002 per cent of initial values for the plate *MPN* and plate colony methods, respectively.

Table 2. Log number of viable rhizobia per gramme in granular and powder peat cowpea inoculants stored at 35°C

Storage time (week)	Enumeration method								
	Plant infection			*Plate* MPN			*Plate colony*		
	Granular	*Powder*	*Mean*	*Granular*	*Powder*	*Mean*	*Granular*	*Powder*	*Mean*
0	7·62	8·00	7·85	8·62	8·88	8·77	8·66	9·12	8·95
3	7·00	6·76	6·90	8·36	8·12	8·26	8·47	8·52	8·50
6	6·86	6·49	6·71	8·49	8·00	8·31	8·55	8·03	8·36
9	6·36	7·00	6·79	8·00	7·62	7·86	8·36	7·46	8·11
12	6·49	6·62	6·56	8·24	7·76	8·06	8·24	7·78	8·07
15	6·62	6·00	6·41	8·62	8·62	8·62	8·13	7·36	7·90
18	6·24	6·49	6·38	8·36	7·49	8·11	8·19	7·76	8·03
21	6·00	6·36	6·22	8·36	8·62	8·51	8·41	8·44	8·43

95 per cent confidence interval = ±0·58.

Table 3. Log number of viable rhizobia per gramme in granular and powder peat cowpea inoculants stored at 45°C

Storage time (week)	Enumeration method								
	Plant infection			*Plate* MPN			*Plate colony*		
	Granular	*Powder*	*Mean*	*Granular*	*Powder*	*Mean*	*Granular*	*Powder*	*Mean*
0	7·62	8·00	7·85	8·62	8·88	8·77	8·66	9·12	8·95
3	6·76	5·36	6·48	8·26	8·23	8·25	8·47	8·42	8·45
6	2·12	2·49	2·34	7·99	7·24	7·76	7·48	7·09	7·33
9	1·76	0·78	1·50	6·76	6·23	6·57	7·55	6·82	7·32
12	1·49	1·00	1·31	6·23	6·49	6·38	6·96	6·90	6·93
15	2·00	1·36	1·79	6·65	6·23	6·49	6·65	6·45	6·56
18	1·62	0·78	1·38	5·24	6·00	5·77	5·58	4·78	5·34
21	0·62	1·76	1·49	6·56	6·49	6·53	6·97	6·41	6·77

95 per cent confidence interval = ±0·58.

Table 4. Log number of viable rhizobia per gramme in granular and powder peat cowpea inoculants stored at 55°C

Storage time (week)	Enumeration method								
	Plant infection			*Plate* MPN			*Plate colony*		
	Granular	*Powder*	*Mean*	*Granular*	*Powder*	*Mean*	*Granular*	*Powder*	*Mean*
0	7·62	8·00	7·85	8·62	8·88	8·77	8·66	9·12	8·95
3	4·62	4·49	4·56	5·76	4·88	5·51	6·08	5·35	5·85
6	1·36	0·49	1·11	4·49	4·23	4·38	4·98	4·72	4·87
9	0·00	0·00	0·00	4·23	3·23	3·97	4·74	4·31	4·58
12	0·00	0·00	0·00	4·36	3·49	4·11	4·64	4·39	4·53
15	0·00	0·00	0·00	3·62	3·23	3·47	4·36	3·79	4·16

95 per cent confidence interval for all values = ±0·58.

It is apparent from the data that calculated values for numbers of viable rhizobia in these inoculants are strongly influenced by enumeration method. At all sampling times and at all temperatures, plate *MPN* and plate colony counts were very similar but values obtained with the plant infection method were generally lower. The difference between the plant infection method and the other methods increased as storage time increased at all storage temperatures, with the difference being accentuated at the higher temperatures. The numbers of surviving rhizobia after 21 weeks' storage calculated from the plant infection method were 5·5, 0·6, and 0·0007 per cent of the mean of the plate *MPN* and plate colony values at 25, 35, and 45°C, respectively.

There are several possible explanations for the observed discrepancies among enumeration methods. There is the possibility that many of the colonies counted in the plating methods arose from contaminant organisms in the inoculants and thus erroneously high estimates were calculated. This explanation was rejected since numerous colonies plated from various dilutions were examined microscopically and subsequently tested for infectivity on cowpeas and always proved to be rhizobia. Since the colonies appearing on the plates were known to be rhizobia, it was thought that long storage times and high temperatures might possibly have decreased the infectiveness of the rhizobia, thus giving rise to the low values in the plant infection method. To test this, several individual colonies from plates that had received dilutions of inoculants stored for 21 weeks at 45 and 55°C were transferred to liquid *YEM* medium, allowed to grow, and a dilution series was counted using the plant infection method. Since these cells proved to be as infective as those in cool-stored inoculant, it was concluded that no permanent or genetically transmissible loss of infectivity was involved. Another possibility, which is still being investigated, is that many of the rhizobia were not surviving when transferred to the plant nutrient solution in the plant infection method. This appears to be the most likely possibility and if confirmed, means of improving survival and growth of the rhizobia in the plant nutrient solution will be sought.

Most studies dealing with inoculant storage temperatures have been restricted to the 0 to 26°C range (ROUGHLEY, 1968; DATE, 1977). Storage and transportation problems are particularly serious in developing tropical countries and inoculants may be subjected to ambient temperatures of 20 to 45°C for varying lengths of time (AYANABA, 1977). The higher storage temperatures used in the present study give results applicable to tropical storage conditions. It should be emphasized that the relative humidity during these experiments was maintained at 60 per cent. Moisture loss through polyethylene packages will occur at this relative humidity and is accelerated at the higher temperatures. Although 60 per cent relative humidity may be somewhat low for a representative tropical value, the results include the effects of both temperature and drying as would occur under actual storage conditions.

In most areas of the tropics, temperatures in well-ventilated storage areas should not exceed 35 to 45°C for any extended period of time. Average daily temperatures will be substantially lower. The data from this study indicate that if a storage temperature of 25°C or less can be maintained, cowpea inoculant quality can be maintained for a period of at least 21 weeks. If the

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storage temperature consistently remains near 35°C, plate counts may remain unchanged, but a slow decline in cell numbers as measured by plant infection will occur. Prolonged storage temperatures of 45°C and above result in a serious decline in inoculant quality and should clearly be avoided.

As noted previously, plant infection counts were generally lower than plate counts but the difference between the methods was much greater after lengthy storage at the higher temperatures. This enhanced difference is interpreted as resulting from a progressive loss of cell vigour during storage at the higher temperatures. The surviving cells retain the ability to multiply and grow normally when transferred to the optimum growth environment of *YEM* agar plates, but their ability to survive and multiply in the plant nutrient solution and cause subsequent nodule development is apparently impaired. The plate count data indicate that rhizobial numbers are not severely reduced by up to 12 weeks storage at 45°C. However, inoculants stored under these conditions have very low plant infection counts and may perform quite poorly in the field because the apparent reduced vigour of the cells would probably reduce their ability to survive under the suboptimal conditions characteristic of most soils. If this is indeed the case, counts obtained by the plant infection technique may be more closely correlated with inoculant performance than counts obtained by plating methods.

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