Loss of Desiccation Resistance Following the Artificial Cultivation of Cowpea Bradyhizobia Isolated from Previously – Desiccated Soil

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Abstract

Soil kept in a desiccated condition (6% moisture content, equivalent to 38.6 Mpa tension) for 2 years, was found to contain 300 viable cells of cowpea bradyrhizobia per gram of soil, contrary to the general consensus that rhizobia are highly sensitive to desiccation. Two strains of the native bradyrhizobial population were isolated via host legume infection. From each of the strains, doubly-labelled, antibiotic resistant mutants were developed in order to follow-up their survival when re-introduced into natural (non-sterile) soil. The double labeling was achieved by selection for sequential resistance to high concentrations (500 mg/l) of the antibiotics streptomycin and spectinomycin. The antibiotic-resistant strains were then stored in yeast-extract mannitol agar for 3 months at 4°C. The survival ability of the antibiotic-resistant strains was then studied over a 6-week period in the same soil of their origin under conditions of favourable moisture (field capacity) or under slow desiccation to the moisture level at which the same strains had previously survived for 2 years. This evaluation was undertaken both in previously sterilized soil and in natural (non-sterile) soil. Results of this study indicated that both strains generally survived poorly under conditions of soil desiccation. In fact, the population of one of the strains declined to extinction within 6 weeks even in previously sterilized soil in which all probable antagonistic biotic factors were precluded. It was concluded from the findings of this study that the common practice of artificial cultivation and storage of rhizobia in agar culture media and other forms of laboratory manipulations of the micro-organisms was partially implicated for their loss in survival ability under desiccation. It is therefore suggested that superior strains of rhizobia or bradyrhizobia intended for longer term storage for commercial inoculant production, be maintained in sterile carriers other than in agar media.

Keywords: Antibiotic-resistant mutants, bradyrhizobial survival, cowpea, soil desiccation

Introduction

The root-nodule bacteria (rhizobia and bradyrhizobia) subsequently referred to simply as rhizobia, are an important group of soil micro-organisms due to their ability to form symbiotic relationships with legumes and fix nitrogen. Biological nitrogen fixation (BNF) has the potential of meeting most if not all of the N needs of legumes and increase the legume grain yields in many cropping systems, depending on the symbiotic effectiveness of the association (Giller and Wilson, 1993). For the case of the non-grain legumes, most of the fixed N in the plant biomass can be returned into the soil, resulting in substantial improvements in soil fertility and crop yields (Mugwe and Mugendi, 1999). Due to the variability in the efficiency of legume nodule bacteria in fixing N_2 with their associated legumes, it is necessary to select the most effec-

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Tanzania J.Agric.Sc.(2002) Vol. 5 No. 2, 71-79

Manuscript accepted on the 26th of July, 2004

tive strains and store them for large scale inoculant production (Singleton *et al.*, 1990). Symbiotic effectiveness is not the only desirable attribute of rhizobia. The effective strains must also be more competitive than the native, inferior strains in nodulating the host legumes (Msumali and Kipe-Nolt, 1998). Another desirable ecological attribute of rhizobia is that which is referred to, as *saprophytic competence*. This is the ability of rhizobia to survive in large numbers, in soils with complex antagonistic factors both edaphic and biotic (Parker *et al.*, 1977; Alexander, 1991). For the effective utilization of the BNF technology, rhizobial ecological studies including saprophytic competence and population dyanamics are important in providing information on the frequency of inoculation (Chowdhury *et al.*, 1983) or on predicting the likelihood of inoculation response when desirable strains are introduced into soils already containing native but inferior strains (Woomer *et al.*, 1997).

It is however important to note that in most ecological or agronomic studies with rhizobia, cultures which have been selected and maintained on artificial media are more often used than freshly-isolated cultures. Yeast-extract mannitol is the regular medium used for the growth and maintenance of rhizobia (Vincent, 1970; Dye, 1979). As a source of nitrogen and/or growth factors, yeast extract may contain high proportions of some amino acids such as glycine, alanine or valine, or other polyamines like, for example spermine, which are known to be inhibitory or capable of causing mutations on rhizobia (Schwinghamer, 1977; Chakrabarti *et al.*, 1981; Ozawa and Tsuji, 1992).

Apart from or in addition to the possibility of causing genetic alterations, rich laboratory media may also cause rhizobial maintained on agar slopes to develop nutritional dependence on some of the ingredients contained therein. This postulate may appear to be logical on the grounds that the ingredients used in laboratory media are not likely to be present in natural soil in any substantial amounts. As pointed out above, rhizobial screening programmes involve the selection and retention of few, symbiotically-superior strains of rhizobia. This means some strains have been maintained in culture media for many years since they were first isolated from soil. Prior to their isolation from soil, rhizobia in their natural habitats are probably capable of utilizing the nitrogen, carbon or growth factor sources, often present at much lower concentrations than those provided in artificial culture media.

The extent to which strains of rhizobia, with naturally low nutrient requirements, lose their nutritional or ecological independence due to artificial cultivation in laboratory media, has not been investigated. Indeed, the nutritional requirements of rhizobia as a factor affecting their adaptation to a particular soil, have received little attention.

There is abundant literature (Chao and Alexander, 1982; Singleton *et al.*, 1990; Friedricks, 1996) which indicates that rhizobia are generally susceptible to desiccation and therefore, easily lose their viability under such conditions of stress. This is to be expected given that rhizobia lack features such as endospores which enable other bacterial species to persist during unfavourable conditions. The susceptibility of rhizobia to desiccation is exemplified by the general recommendation that seeds inoculated with rhizobial culture should be planted soon enough in order to avoid loss of viability of the inoculum (Vincent, 1970; Salema *et al.*, 1982). Msumali and Harris (1985) reported that strains of cowpea bradyrhizobia, obtained from the Rothamsted culture collection, failed to tolerate slow desiccation in an 8-week period of study in an Oxisol from Morogoro, Tanzania. However, some of the Rothamsted cultures were isolated from tropical soils as early as 1964 (Dye, 1979). In such tropical soils, periodic or prolonged droughts are a common feature. The observation that these strains failed to tolerate slow desiccation when re-introduced into another tropical soil could have been caused by the long periods of storage in artificial media. On the basis of previous reports (Chakrabarti *et al.*, 1981; Ozawa and Tsuji, 1992), it is plausible to advance the hypothesis that laboratory cultivation and maintenance of rhizobia under refrigerator conditions could impair the ability of rhizobia to subsequently survive in nutrient-poor and edaphically – inhospitable soil. But such are the conditions which the bacteria do encounter in nature as free-living saprophytes, if not isolated in artificial culture media. The objective of this study was therefore to determine if artificial culture and cold storage (4°C) of cowpea bradyrhizobia

isolated from previously dedicated soil, could impair the ability of the rhizobia to tolerate drought when re-introduced into the soil undergoing desiccation.

Materials and methods

The soil used in the study

The soil used in this study was a sandy clay loam Oxisol (Typic Acrorthox – Kesseba et al., 1972). This soil was later, further classified as a Kandiustalfic Eutrustox (Kaaya *et al.*, 1994). Surface (0-20 cm) soil samples were collected during the dry season (August, 1996) at a site adjacent to the SUA Botanical Garden, as part of the Rhizobium Ecology Research Network for Eastern and Southern Africa (RENEASA) Project. The soil was air dried to about 6% moisture content, ground and sieved (2mm) and then stored in water proof polythene bags until 1998.

The population of indigenous cowpea bradyrhizobia in the dried, stored soil

The cowpea bradyrhizobial population in the dried, stored soil was determined essentially by the plant-infection test (Vincent, 1970), employing the modification proposed by Woomer (1993). In this procedure, five-fold soil dilution series were aseptically prepared in the range $5^{-1} - 5^{-6}$ i.e. 6 dilution steps. One ml of soil suspensions of each of the dilution steps was inoculated onto previously-sterlized cowpea seeds (*V. unguiculata* (L) cv VITA 4) in quadruplicated modified Leonard jars (Vincent, 1970). Following the nodulation patterns of the inoculated plants across the 6 dilution steps, the Most Probable Number (MPN) concept was used to estimate the population of the cowpea bradyrhizobial population in the soil, using MPN tables (Woomer, 1993) which revealed a population estimate of 300 viable cowpea bradyrhizobial cells per gram of soil (Figure 1).

Isolation of bradyrhizobial strains and selection of their antibiotic resistant mutants

Surface sterilized seeds (Vincent, 1970) of cowpea were sown in disinfected plastic pots, each containing 1 kg of the desiccated soil. The plants were raised under normal glass-house management procedures up to 5 weeks at which maximum nodulation was expected to have occurred.

Pure culture isolates of cowpea bradyrhizobia were obtained from nodules of the uninoculated plants. They were designated as MG-CP$_1$ and MG-CP$_2$ and were presumed to represent two separate strains as judged by their differential absorption of the Congo red dye, incorporated into a yeast-extract mannitol agar (YMA) medium. Each strain was selected for sequential resistance to streptomycin (str) and spectinomycin (spc) each at a concentration of 500 mg/l. The antibiotic-resistant mutant isolation procedure described by Park (1978) was used.

When sufficient growth had occurred following successive transfers onto YMA containing 500 mg/l each of str and spc, at least 3 well isolated colonies of the doubly-labeled antibiotic – resistant mutants (MG-CP1/str^r-spc^r and MG-CP2/str^r-spc^r) were picked off. Individual colonies from each mutant were combined on slopes of antibiotic – free YMA, incubated at 28^{0}C to obtain confluent growth and then stored for 3 months at 4^{0}C. Antibiotic – resistant mutants were isolated from the wild type cultures in order to permit the study of the survival of the cowpea bradyrhizobia in the native (non-sterile) as well as the sterilized soil, an approach used by earlier workers with similar objectives (Danso and Alexander, 1974; Rosas *et al.*, 1998).

Testing the desiccation response of indigenous cowpea bradyrhizobia when re-introduced into the soil

Agar slant cultures of strains MG-CP1/str^r-spc^r and MG-CP2/str^r-spc^r previously stored for 3 months at 4^{0}C (as stated above), were used to inoculate sterile yeast extract mannitol broth (Vincent, 1970). When the culture suspension had achieved turbidity corresponding to about 10^9 cells/ml, it was centrifuged at 1500 g to harvest the cells. The washed cell suspensions (1 ml) were inoculated into 10 g air dried, sterile or non-sterile soil in 125 – ml medical flat bottles. The viable cell numbers in the inocula were determined by plate counts. The inoculated soil was divided into two moisture treatments one of which was slow desiccation while the other was maintained at field capacity. The moisture treatments were prepared as follows.

Desiccation

After inoculating the air-dried soil (c. 6% moisture content), the bottles, with their caps removed, were placed horizontally in glass desiccators, containing CaO, for 14 days at room temperature (25°C). In a previous study (Msumali and Harris, 1985), it was shown that 14 days were required to remove 1 ml of moisture from 10 g of the same soil initially at 6% moisture content and under similar conditions of incubation in a desiccator cabinet. After the 14-day desiccation period, the inoculated soil was therefore brought to its original moisture content of c. 6%, corresponding to a final moisture tension of slightly > 38.6 MPa, as determined from the soil's moisture characteristic curve. Since the survival study was to be monitored over a 6-week period, the soil that was under slow desiccation for 14 days already represented the second sampling period. Bottles intended for further incubation than the 14 day period were transferred to an incubator at 28°C where no further moisture loss occurred.

Field capacity (FC) moisture condition

After inoculation, soil in this treatment received distilled water up to a moisture content of 28% corresponding to a moisture tension of 0.03 MPa and was transferred to the incubator (28°C). The field capacity conditions were maintained by the addition of sterile distilled water at regular intervals to off-set slight evaporation losses, rates of which were previously determined in a separate study (Msumali and Harris, 1985).

Experimental design and treatment

The survival of each of the antibiotic-resistant bradyrhizobial strains was evaluated in soil of two moisture regimes i.e. soil maintained under field capacity or that which was desiccated. In each case, previously sterilized or non-sterile (natural) soil was used. The survival was monitored on bi-weekly intervals for a period of 6 weeks. The treatment structure was therefore, a 2x2x4 factorial in a completely randomized design (Gomez and Gomez, 1984), the factors being the two moisture regimes, two soil sterility statuses and four sampling periods namely 0, 2, 4 and 6 weeks. Each of the above treatments was replicated 3 times. Sufficient incubation bottles were prepared for individual sampling at the bi-weekly intervals.

Recovery of antibiotic-resistant cowpea bradyrhizobia inoculated in the soil

At each sampling time, ten-fold soil dilutions were prepared. Numbers of surviving bradyrhizobia were determined by drop-plating (for sterile soil) or spread-plating (for natural soil) using appropriate dilutions of the soil suspensions on YMA supplemented with streptomycin, spectinomycin and cycloheximide each present at a final concentration of 200, 200 and 400 mg/l respectively. A medium incorporating these antimicrobial substances was previously found (Msumali and Harris, 1985) to be totally selective against native bacteria and the majority of fungi in the same soil, thus, permitting a reliable recovery of the doubly-labeled, antibiotic-resistant bradyrhizobial mutants from the native (non-sterile) soil. For statistical analysis, the cell counts per gram of soil were transformed to $\log_{10}$ cell number or $\log_{10}$ (cell number + 1) (Crozat *et al.*, 1982), the latter transformation being necessary when populations of survivors fell to 'zero' i.e. numbers below detection by the soil dilution method employed.

Results and discussion

Except in the sterile soil held at field capacity (FC) moisture, populations of both strains (MG-CP1 and MG-CP2) declined with time in the desiccated soil (Fig. 1). Surprisingly, however, populations of both strains were higher in desiccated non-sterile than in sterile soil. These differences (i.e. populations in desiccated sterile vs non-sterile soil) were significant (P=0.05) for strain MG-CP2 (Table 6) but non-significant for strain MG-CP1 (Table 3). In the sterile soil, desiccation caused the decline of strain MG-CP2 to undetectable numbers in 6 weeks (Fig. 1).

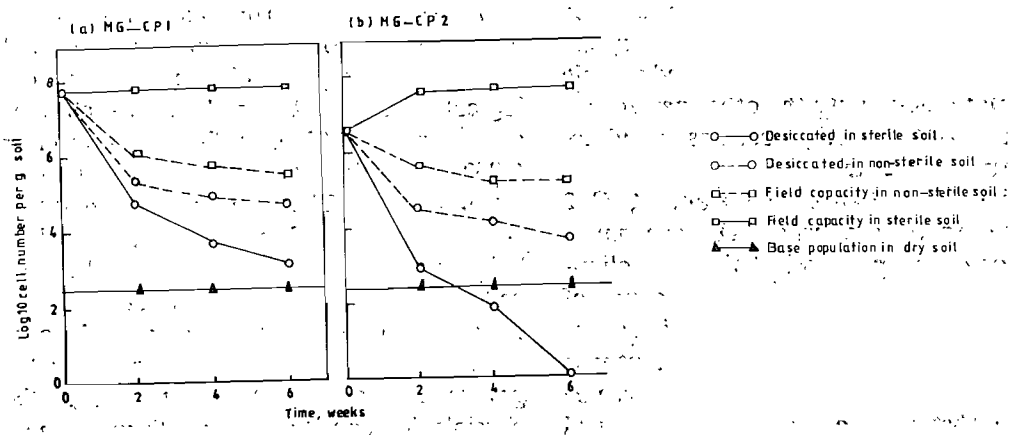

Figure 1: Effect of artificial cultivation on native strains of cowpea bradyrhizobra on their ability to withstand desiccation after reintroduction into the soil.

When averaged over incubation time for moist and desiccated soil. survival of both strains was not significantly different in sterile and in non-sterile soil (c.f. Tables 2 and 5). However. the mean numbers of survivors for either strain (averaged over time for sterile and non-sterile soil) was significantly higher (P = 0.05) in the moist soil at FC than in desiccated soil (c.f. Tables 1 and 4). Thesc results imply that moisture stress per se rather than biological factors affected survival of the native bradyrhizobial isolates. This is proba-

Table 1. Effect of moisture stress on survival of strain MG-CP1 (averaged over 3 replications for sterile and non-sterile soil. [Data in all Tables 1-2 are log 10 cell numbers per gram of soil]

Descriptive moisture Content (DMC)	Time. weeks				Moisture effect mean (MEM)	L S D ± (P=0.05)
	0	2	4	6		
Air-dried (AD)	7.82	5.03	4.33	3.94	5.28	0.37
Field Capacity (FC)	7.82	6.94	6.74	6.64	7.04	
Time effect mean (TEM)	7.82	5.98	5.54	5.29		
LSD= (P=0.05)	0.51					

Table 2: Survival of strain MG-CP1 with time, in sterile or non-sterile soil (averaged over 3 replications for desiccated and moist soil)

	Time. weeks				Moisture effect mean (MEM)	LSD= (P=0.05)
Soil sterility status	0	2	4	6		
Sterile	7.82	6.28	5.76	5.49	6.34	0.37
Non-sterile	7.82	5.69	5.32	5.09	5.98	
TEM	7.82	5.98	5.54	5.29		

bly to be expected since the strains of cowpea bradyrhizobia, being indigenous to the experimental soil, are expected to have established a stable biotic relationship with the other native soil microflora. Antagonistic reactions would not therefore be expected to occur.

It is interesting to note that strain MG-CP2 which had survived desiccation in the experimental soil for 2 years prior to isolation, was eliminated within only 6 weeks by the same degree of desiccation in sterile soil. The desiccation of the inoculated soil to 6% moisture content was achieved slowly in 14 days, a situation considered similar to what would occur under tropical field conditions at the cessation of rainfall. Abrupt desiccation stress causing cellular disorganization (Bushby and Marshall, 1977) would not therefore explain the elimination of strain MG-CP2.

Artificial cultivation or storage in agar at low temperatures prior to re-introduction into soil could partly be implicated for this change in ecological behaviour. Reports by Pena-Cabriales and Alexander (1979). or Chao and Alexander (1982) and Friedricks (1996) appear to support this hypothesis. These researchers found that rhizobia which proliferated in soil prior to their being subject to desiccation, survived drying better than rhizobia grown in broth and then introduced into soil and subjected to similar conditions of desiccation. It would thus appear that growth of rhizobia in artificial media alters the physiological condition of the cells in a manner that has not yet been adequately explained. However, one of the effects could be to make the cells more susceptible to environmental stress. This suggestion however, fails to explain

Table 3: Effect of moisture stress in sterile or non-sterile soil, on survival of strain MG-CP1 (averaged over 3 replications for all sampling times).

	DMC		
Soil sterility status	AD	FC	SEM
Sterile	4.88	7.80	6.34
Non-sterile	5.68	6.28	5.98
MEM	5.28	7.04	

Table 4: Effect of moisture stress on survival of strain MG-CP2 (averaged over 3 replications for sterile and non-sterile soil)

(DMC)	Time, weeks				(MEM)	LSD =(P=0.05)
	0	2	4	6		
AD	6.74	3.76	3.13	1.89	3.88	0.39
Field Capacity (FC)	6.74	6.69	6.51	6.49	6.61	
TEM	6.74	5.23	4.82	4.19		
LSD=(P=0.05)	0.53					

why strain MG-CP2 was not eliminated under identical conditions of desiccation in non-sterile soil.

In previous studies (Msumali and Harris, 1985) other strains of cowpea bradyrhizobia were found to be able to tolerate similar conditions of desiccation better in non-sterile than in sterile soil thus suggesting the behaviour may not be uncommon among cowpea bradyrhizobia. The factors giving cowpea bradyrhizobia these apparent survival advantages cannot easily be explained and are yet to be identified. A suggestion that the native heterotrophic flora probably produced stimulatory substances that enhanced survival of the bradyrhizobia in non-sterile soil during desiccation would be inconsistent with the fact that in a soil undergoing desiccation, the physiological activities of most microorganisms (including the potential stimulators) are slowed down. If there were soil microflora capable of producing substances stimulatory to cowpea bradyrhizobia, their effect would have been greater in moist than in dry soil. On the contrary, survival of the two strains tested was better in sterile than in non-sterile soil under the field capacity moisture conditions (Fig. 1).

The strains used in this study were not just subject to artificial cultivation and storage but were also mutants selected for resistance to antibiotics, unlike the indigenous population of the same organisms. In similar ecological studies (Bushby, 1981; Friedricks, 1996; Rosas *et al.*, 1998), it was normally assumed that, apart from resistance to antibiotics and infrequent symbiotic modifications, the mutants did not otherwise differ in survival ability from the wild type parent cultures. Information on rhizobial ecology derived from the use of mutants is thus considered to be adequate in answering questions of survival problems of wild type rhizobia in natural soils. Such assumptions may be challenged by some evidence (unpublished) indicating that antibiotic-resistant mutants of rhizobia tend to differ from their corresponding wild type cultures in growth rates both in broth and in sterile soils, factors which may impair the survival of the mutants in natural soils. Species of bacteria have their definite patterns of carbon-substrate metabolism (Collins *et al.*, 1998). It is interesting to note that such patterns were altered in three species, each of Bacillus and Pseudomonas when they were stored for 32 weeks at 4°or −10°C (Shishindo and Chanway, 1998). The above named species are also known to have growth promoting properties on spruce seedlings. Shishindo and Chanway (1998) also observed that spruce root colonization and growth promotion were greater with fresh soil isolates than with the bacteria that had been stored at the low temperatures although this was not due to loss of viability. The above changes were however observed only in some but not all soils included in the study of Shishindo and Chanway (1998).

Table 5: Survival of strain MG-CP2 with time in sterile or non-sterile soil (averaged over 3 relications for desiccated and moist soil)

Soil sterility status	Time, weeks				Sterility effect mean (SEM)	LSD ± (p=0.05)
	0	2	4	6		
Sterile	6.74	5.35	4.88	3.83	5.19	0.39
Non-sterile	6.74	5.13	4.77	4.55	5.29	
TEM	6.74	5.23	4.82	4.19		

Table 6: Effect of moisture stress in sterile or non-sterile soil on survival of strain MG-CP2 (averaged over 3 replications for all sampling times)

Soil sterility status	DMC		SEM
	AD	FC	
Sterile	2.92	7.47	5.19
Non-sterile	4.84	5.75	5.29
MEM	3.88	6.61	

Regarding the artificial (laboratory) cultivation of micro-organisms, there appears to be convincing evidence of changes in microbial behaviour being caused by some chemical ingredients used in the preparation of the culture media. As stated earlier, yeast-extract mannitol agar is the regular medium for culturing and storage of rhizobia (Vincent, 1970). While yeast-extract is a recommended source of nitrogen and growth factors, it is also known to contain substantial amounts of the polyamines spermine and spermidine (Ozawa and Tsuji, 1992). Spermine

(Spm) and other polyamines e.g. spermidine (Spd) and putrescine (Put) have long been know to have inhibitory or mutagenic effects on rhizobia (Schwinghamer, 1977; Chakrabarti *et al.*, 1981). In a study involving the Bradyrhizobium-Glycine max symbiosis, the polyamines adversely affected the growth and viability of bacteroids in the G. max nodules (Ozawa and Tsuji, 1992). Wolff *et al.*, (1995) have found that the nodulation of *Phaseolus vulgaris* was negatively correlated with concentrations of Put, Spd and Spm. Vassileva and Ignator (1999) have reported concentration-dependent, polyamine-induced changes in symbiotic parameters of the *Galega orientalis – Rhizobium galegae* N_2 fixing system. In that study, it was observed that while lower concentrations (10 – 50 µM) of Put, Spd and Spm improved nodulating ability of *Galega orientalis*, higher concentrations (100 µM) of these polyamines, not only reduced nodulating ability but also depressed the nitrogenase activity. The study further revealed that the growth of R. *galegae* was not only depressed by poyamines but that these chemical substances also reduced the ability of the microsymbiont to attach to the host (*Galega orientalis*) roots (Vassileva and Ignator, 1999). There is thus, ample evidence from the above sources of the literature that cold storage of bacteria (rhizobia included) and some of the ingredients commonly used in artificial culture and storage media, have the potential of modifying the behaviour of bacteria. The commonly-reported problems of rhizobial survival in laboratory or field studies are probably artifacts caused by the isolation, cultivation or storage in rich media and other manipulations in the laboratory. It can thus be concuded from the findings of this study, that where facilities for lyophilization of cultures are not available, sterilized natural substrates such as high organic matter soils or peat, could be the preferred bases for long-term storage of superior strains of rhizobia.

Acknowledgements

This study was undertaken with the support of the Rockefeller Foundation, under the Rhizobium Ecology Research Network for Eastern and Southern Africa (RENEASA) Project. This financial support is gratefully acknowledged.

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