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SHELF-LIFE OF LEGUME INOCULANTS IN DIFFERENT CARRIER MATERIALS AVAILABLE IN EAST AFRICA

I.K. BALUME, O. KEYA, N.K. KARANJA and P.L. WOOMER¹

Department of Land Resource Management and Agricultural Technology, University of Nairobi,
P. O. Box 29053, Nairobi, Kenya

¹N2 Africa Program, CIAT-Kenya, P. O. Box 79, The Village Market, Nairobi 00621, Kenya

Corresponding author: isaacbalumem@yahoo.fr, isaacbalume@gmail.com

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ABSTRACT

Adoption of legume inoculation with rhizobia by small-scale farmers in East Africa, and the resultant increase in biological nitrogen fixation requires that quality inoculants meet minimum standards. BIOFIX is one of the commercially available rhizobia/legume inoculants in East Africa, whose standard is at least 10^9 rhizobia g^{-1} . We examined the effect of carrier material and storage conditions on the populations of two industry standard rhizobia, *Bradyrhizobium japonicum* USDA 110 for soybean (*Glycine max*) and *Rhizobium tropici* CIAT 899 for common bean (*Phaseolus vulgaris*) over 165 days, using the drop plate method on Congo Red Yeast Mannitol Agar. Viable populations of rhizobia differed significantly between carriers and rhizobia strains ($P < 0.05$). *Rhizobium tropici* CIAT899, prepared with filter mud carrier, achieved a shelf-life of 135 days and *B. japonicum* USDA110 contained over 10^9 cells g^{-1} for 105 days. Both of these results fall below the stated six months expiry period of BIOFIX. Replacing filter mud carrier with vermiculite, resulted in an inferior product; although, both more thorough sterilisation and refrigerated storage, after a 14 day curing stage, improved the shelf-life rhizobia in the inoculant packet.

Key Words: BIOFIX, CIAT 899, Kenya, USDA 110

RÉSUMÉ

L'adoption d'inoculation de légumineuses avec des rhizobiums par les petits producteurs en Afrique de l'Est, et l'augmentation de la pratique de fixation biologique d'azote qui en résulte nécessite que de bonnes qualités d'inoculum soient fournies aux producteurs. BIOFIX est l'une des maisons de commercialisation d'inoculum en Afrique de l'Est, dont la norme minimale est de 10^9 cellules g^{-1} . Nous avons examiné l'effet du support et les conditions de stockage sur les populations de deux inocula de norme industrielle, *Bradyrhizobium japonicum* USDA 110 utilisé pour le soja (*Glycine max*) et *Rhizobium tropici* CIAT 899 utilisé pour le haricot commun (*Phaseolus vulgaris*) pendant 165 jours, la méthode de goutte étalée sur extrait de Congo Red Yeast Mannitol Agar a été utilisée. Les populations de rhizobiums viables différaient significativement d'un support à un autre et d'une souche de rhizobium à une autre ($P < 0.05$). *Rhizobium tropici* CIAT899, préparé avec un support en boue filtrée, assuraient la plus longue durée de vie (135 jours) et *B. japonicum* USDA110 contenait plus 10^9 cellules g^{-1} pour une durée de 105 jours. Tous ces deux résultats sont en dessous des six mois de délai d'expiration mentionné sur les produits BIOFIX. En remplaçant le support en boue filtrée par des vermiculites, on obtient un produit de qualité inférieure, malgré que la stérilisation et réfrigération minutieuse, après 14 jours solidification, améliorent la durée de vie des rhizobiums dans l'inoculum.

Mots Clés: BIOFIX, CIAT 899, Kenya, USDA 110

INTRODUCTION

Wider use of legume inoculants by African small-scale farmers, offers potential for a sustainable source of nitrogen and increase nutrient-use efficiency (Dieker *et al.*, 2011). Nitrogen is still the most demanded nutrient in agricultural production that will play role in overcoming low productivity in Sub-Saharan African (Batiano *et al.*, 2011). Legume crops face low yields in East Africa due to declining soil fertility and reduced symbiotic biological nitrogen fixation (BNF) (Giller, 2001). Greater availability of legume inoculants offers the potential to better manage BNF (Herridge *et al.*, 2008) and substitute for inorganic nitrogen fertiliser requirements (Sofi and Wani, 2007; Sanginga and Woomer, 2009; Woyessa and Assefa, 2011). But the inoculants must be of the desired quality to effectively nodulate legume hosts and offer strong economic returns (FAO, 1984).

Nodulation is improved when the number of viable rhizobia cells inoculated per seed increases. This is accomplished by having more viable rhizobia cells in the inoculant or delivering larger doses (Catroux *et al.*, 2001). The key to ensuring high quality legume inoculants is through promotion of an effective quality control system (Thompson, 1984), through internal monitoring or functional regulations (Beck *et al.*, 1993). Thus, it is important to determine the duration of the bacteria survivability in different carrier materials, to ensure that the expected level of bacterial population remains viable for the inoculants to be effective. Besides, the solid carrier materials should bear properties that protect rhizobia and permit easy application to seeds (FAO, 1984).

Thus, the objective of this study was to assess the shelf-life of industry standard rhizobia in East Africa within a commercially available BIOFIX inoculant, and evaluate the means to improving its quality through better carriers and preservation practices.

MATERIALS AND METHODS

BIOFIX legume inoculant recovered off the curing shelf from its production facility in Nakuru, Kenya, one of the commercially available inoculant in

East Africa made of filtermud carrier and rhizobium strain, was compared to alternative production approaches in the laboratory. Its carrier is filter mud recovered as sludge from crushing of sugarcane. Dried filter mud and commercially-available horticultural vermiculite, were ground using a hammer mill, sieved through 2.12 μm , and wetted to 35% field capacity. The chemical and physical characteristics of filter mud and vermiculite carriers were evaluated (Table 1). Then, 10 g of non-sterile carrier was placed in polythene bags, sealed and autoclaved thrice for three hours each at 121 °C.

Rhizobium tropici CIAT899 and *Bradyrhizobium japonicum* USDA110 strains were cultured in yeast extract mannitol broth, for seven days, on a rotating shaker, resulting in a log-phase broth culture of $>10^8$ cells ml^{-1} . The volume of inoculants added from the broth culture was injected using a sterile syringe at 50% of the water holding capacity of the respective carrier materials; and then mixed with the carrier. The syringe-punctured area was wiped with 70% alcohol, and an adhesive seal was applied. These packets were then incubated for curing, under room temperature, for 14 days as is the curing practice at BIOFIX factory. They were then stored under refrigeration at 4°C, and room condition (24 °C) for shelf-life evaluation after 164 days.

A ten-fold dilution series of up to 10^{-7} was prepared from the inoculants, using the Miles & Misra drop plate technique (1938). Three drops of 20 μl , from the last three dilutions, were plated onto Congo Red Yeast Extract Mannitol Agar, with three replicates for each dilution. All agar plates were incubated at 28 °C for 3-7 days. Only colonies in the range from 5-55, were counted and colony forming units per gramme back-calculated. Occasional presence of fungal contaminants was also recorded. The number of rhizobia and occasional contaminants were counted, six times (14, 45, 75, 105, 135 and 165 days after injection of broth cultures), and compared with commercial BIOFIX. Each treatment (refrigeration and room temperature) was replicated five times, in a completely randomised design (Herridge *et al.*, 2002).

Effects of rhizobial strain, carrier, storage temperature and time of storage, and their

interactions were determined using the General Linear Model procedure of SAS version 9.2 (SAS Institute, 2008).

RESULTS

The chemical and physical characteristics of the two test carriers and a North American peat are presented in Table 1. Filter mud closely resembled peat, but had a lower water holding capacity and pH. Vermiculite is an inorganic material with low carbon and nitrogen concentrations, and greater bulk density, but with high porosity.

The population dynamics of two rhizobia in different carriers, over time, are shown in Table 2. There were significant effects among carrier materials ($P < 0.001$), storage conditions and time, and strains ($P < 0.05$). There were also many interactions, including carrier \times strain and carrier \times storage ($P = 0.05$) and carrier \times time, but not strain \times storage or strain \times day (data not presented). Contamination of inoculants only occurred after 14 days of curing at $0.58 \times 10^6 \text{ g}^{-1}$ in the commercial product, among 35% of the samples (data not presented).

The BIOFIX carrier represents the commercial product itself, while filter mud is essentially a more thoroughly sterilised and carefully prepared alternative within the laboratory. BIOFIX displayed a large initial increase in rhizobia population, followed by a steady decline (Fig. 2). More careful preparation of filter mud led to larger populations and greater survival than 1.15×10^9 per g^{-1} rhizobia, after curing; and less than 10^6 per g^{-1} fungi contaminants. Vermiculite did not support rhizobia, neither in terms of initial colonisation, nor longer-term survival. Note that the agreed industry threshold for inoculants in Kenya lies at a minimum of 1×10^9 rhizobia g^{-1} .

The population dynamics of the two strains across all carriers and storage conditions are presented in Figure 1. CIAT 899 colonised carriers better than USDA 110. After a 14 day curing interval, both populations increased up to 45 days and then declined. After 105 days, both strains exceeded industry standards.

The effect of refrigeration on storage of BIOFIX inoculant containing USDA 110 is presented in Figure 2. Lower temperatures resulted in higher populations with time and

TABLE 1. Physical and chemical properties of the carrier used in the experiment

Carrier	Total C (%)	Total N (%)	Bulk density (1 kg^{-1})	Porosity (%)	Water holding capacity (1 kg^{-1})	pH
Filtermud	18.4	2.0	0.79	56	155	6.8
Vermiculite	2.1	0.1	0.98	63	152	6.6

TABLE 2. Survival of two rhizobia in inoculants of different carriers over time ($\times 10^9 \text{ cell g}^{-1}$)

Carrier	Strain	14	45	75	105	135	165
		Time in days ^a					
BIOFIX	<i>R. tropici</i> CIAT 899	1.15	6.17	4.06	2.70	0.04	0.03
	<i>B. japonicum</i> USDA 110	1.13	5.60	5.62	1.86	0.21	0.04
Filter mud	<i>R. tropici</i> CIAT 899	8.79	6.20	5.00	3.14	2.30	0.44
	<i>B. japonicum</i> USDA 110	6.36	3.93	4.13	2.77	0.44	0.25
Vermiculite	<i>R. tropici</i> CIAT 899	0.78	1.77	0.53	0.27	0.06	0.04
	<i>B. japonicum</i> USDA 110	0.54	0.63	0.54	0.08	0.05	0.02

^a $\text{LSD}_{0.05}$ carrier = 2.78, strain = 4.06 and time = 4.98

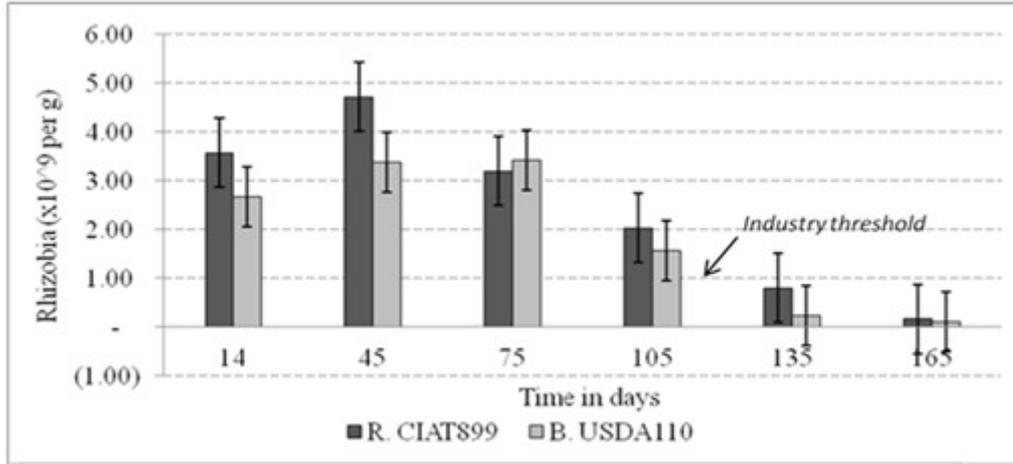


Figure 1. Populations of CIAT 899 and USDA 110 across the three carriers over time.

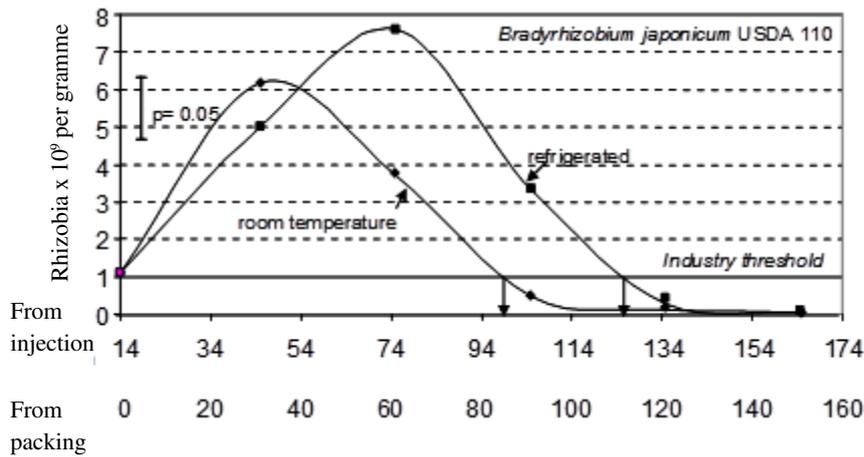


Figure 2. The effect of storage temperature on BIOFIX inoculant containing USDA 110 includes extended shelf life.

extended shelf-life; by 30 days. A similar trend was observed with CIAT 899 and filter mud prepared at MIRCEN laboratory, but not for vermiculite carrier as it lacked a pronounced population increase following injection (Table 1).

Shelf-lives of different inoculants are presented in Table 3. Note that these values were calculated by interpolating the two values falling above and below 10⁹ rhizobia g⁻¹. In the case of some vermiculite carriers this could not be performed (Table 1).

TABLE 3. Shelf lives of different inoculant formulations including the 14 day curing period

Carrier	Storage	days since injection	
		CIAT 899	USDA 110
BIOFIX	Room	131	100
BIOFIX	Refrigerator	132	130
MIRCEN	Room	131	127
MIRCEN	Refrigerator	134	129
Vermiculite	Room	n.a.	41
Vermiculite	Refrigerator	n.a.	43

DISCUSSION

Among the three sources of inoculant examined at Mircen Laboratory, filter mud held large numbers of rhizobia population for both strains *R. tropici* CIAT 899 used bean and *B. japonicum* USDA 110 for soybean (Table 2). However, an inoculant should meet the quality standard of at least one billion (1×10^9) live rhizobia g^{-1} inoculant, as reported in Australia (Herridge *et al.*, 2002). Three approaches were considered to improve the inoculant formulation to meet the quality standard: altering its carrier material, manner of sterilisation and lowering its storage temperature. Filter mud was found the best and was able to colonise carriers beyond 131 days (Table 2). Maintaining the standard is due to the carrier properties of high water holding capacity, carbon and nitrogen which affect positively the increasing of rhizobia population. A contrast observation appeared for vermiculite carrier, which was not able to increase rhizobia population for the time given; meanwhile the process for inoculant preparation was the same compared to filter mud the rhizobia survivability did not extend more than 45 days due to poor physical properties specially porosity which is high making inoculant not to maintain moisture for a long period and lower nutrient content, carbon and nitrogen (Table 1).

Rhizobium tropici CIAT 899 seems to slightly cope with various carriers, compared with *B. japonicum* USDA 110, which originated from the United State of America (Table 2). This might be due to its adaptability, since the strain was isolated from the tropics by the International Center of Tropical Agriculture. After production cures the product for 14 days, increased rhizobia population above the threshold; What do you mean? packets of inoculants were kept in a cooler place to allow exponential multiplication of rhizobia cells, even though vermiculite carrier was still unable to meet the industry standard. BIOFIX®, the commercial product which was used in this experiment, is also produced from filter mud and has a shelf life of six months (about 180 days) from the time of injection to allow the use of inoculant by farmers during the cropping period. This study disagrees and indicates that the six-month shelf life may be too long, as both soybean and bean inoculants

fell below the expiry interval by 80 and 49 days, respectively (Table 3). Nonetheless, from the results in (Table 2) filter mud was the best to store rhizobia for long. This carrier was selected many years ago after careful comparison to other available sources such as coconut fiber, local peat and animal manure (Anyango *et al.*, 1985; Kibunja 1985).

One problem with the carrier materials, is heavy contamination of the bulk material by other microbes. This study found some fungal contamination in BIOFIX® (35% of samples (data not presented)). This is much less than reported in the past and below the target standard of $1 \times 10^6 g^{-1}$ (Herridge *et al.*, 2002). Meanwhile, when BIOFIX® was replicated at the MIRCEN laboratory, this contamination was eliminated with careful and repeated autoclaving. We observed significantly higher populations in the inoculant, especially for CIAT 899 (Table 2), and extended shelf life for USDA 110 (Table 3). Attempts to substitute horticultural-grade vermiculite as a carrier were not successful as both rhizobia strains were less able to colonise and persist in this material (Tables 2 and 3). Some contamination was also noted among other carriers late in the investigation (day 105 and 135), likely due to repeated sampling of packets.

The last approach to consider was refrigeration of BIOFIX® from the production line immediately after curing resulted in slightly delayed colonisation of the carrier material (Fig. 2). However, this colonisation continued for a longer period and larger populations were later achieved. What was not observed, however, was a greatly extended shelf life (Table 3) in large part because both room temperature and refrigerated inoculants declined in a linear fashion following colonisation of the carrier, but later attenuated with extend of 20 days for USDA 110 in refrigeration. This is due to low microbial activity affected by low temperature effect (Fig. 2). Both CIAT 899 and USDA 110 were not able to maintain the industry threshold after 130 days, probably because the substrate in which they were growing declined in nutrient content, most likely carbon (Fig. 1). During this steady decline, the die-off of rhizobia was roughly between 90 and 170 million cells $g^{-1}d^{-1}$ (Table 2); although too few observations between the peak and

attenuation diminished the strength of this observation.

It was hoped that attenuation of refrigerated inoculant would fall above the industry threshold, but it did not. These findings disagree with those of Khavazi *et al.* (2007); who reported that the number of rhizobia cells in carrier was not significantly different after six months of storage. Lupwayi *et al.* (2000) expressed concern that inoculant quality declines quickly if contaminated. Swelim *et al.* (2010) also emphasized the importance of complete sterilisation of carrier. However, even our numerous contamination-free samples displayed linear decline to levels below the industry standard.

One disadvantage of solid over liquid formulation inoculants is that contaminants may persist in carriers (NifTAL Project, 1998; Woome *et al.*, 1999). Proper sterilisation is important to meet quality standard. Our findings disagree with those of Boonkerd (1991) who reported differences in inoculant quality due to storage temperatures. However, room temperature fluctuation for storage during experimentation period did not vary significantly. The excessive die-off observed in this experiment may be attributable to the experimental conditions themselves and the need for curing. Dieker *et al.* (2007) reported that rhizobia survive best when changes in moisture status of cells are minimised. Yet at the same time, it is important to cure solid inoculants in a manner that slowly dries them, so that the cells harden and the solid formulation becomes friable rather than caked for ease of application on seeds. Curing itself is a two phase process where rhizobia, first colonise the carrier, increasing several fold, but then populations decline as surviving rhizobia harden. For BIOFIX®, the inoculant is cured in sealed, semi-permeable plastic bags and later packaged into a labeled, air tight outer bag after 14 to 20 days for marketing. In this study, inoculants remained in their inner bags throughout the time series, and were opened at sampling intervals. Between times, they were stored together in a sealed plastic container with different water vapour exchange properties than sealed commercial inoculant. The expediency of repeated sampling, rather than preparing samples for individual time points may have altered the results. As no humidity

measurements were made, this methodological consideration cannot be tested.

CONCLUSION

BIOFIX® legume inoculants for soybean and bean exceed their target industry standard of 1×10^9 rhizobia g^{-1} up to 100 and 131 days, respectively; somewhat less than its stated expiry date of six months. This suggests that the inoculant must be used during the growing season for which it is produced, and not carried over to the next, even when stored under refrigeration. Careful preparation of inoculants in the laboratory suggest that there is an opportunity of slight improvement along its production line, particularly in better sterilisation of the carrier material and achieving higher populations several days following injection. Altering the carrier material from organic (filter mud) to mineral (vermiculite) material results in an inferior product, but search for a better carrier material and production approach, able to support greater than $1 \times 10^9 g^{-1}$ over an extended shelf life should continue.

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