ASSESSMENT OF QUALITY CONTROL OF INOCULANTS USED ON BEAN AND SOYBEAN IN EASTERN AND CENTRAL AFRICA

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DECLARATION

This thesis is my original work and has not been presented for award of a degree/research in any other university.

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DEDICATION

I dedicate this work to my parents; NGABOYEKA KAYANI Albert and NABAKAZI Jeannette for their support, inspiration and encouragement during pursuit of this degree.

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GENERAL ABSTRACT

There is increased adoption of rhizobia based legume inoculation by small-scale farmers in East and Central Africa. This technology supports the exploitation of biological nitrogen fixation on the assumption that quality inoculants in the market meet minimum standards. In East Africa BIOFIX® developed at the MIRCEN laboratory has been used for over 30 years and when applied to soybean or common been seeds the product is able to furnish at least 10^9 rhizobia g^{-1}. This study examined the effect of carrier materials and storage conditions for inoculants. The inoculants were prepared using two industry standard rhizobia, *Bradyrhizobium japonicum* USDA 110 for soybean (*Glycine max*) and *Rhizobium tropici* CIAT 899 for common bean (*Phaseolus vulgaris*). The storage experiments were conducted for 165 days and the number of viable rhizobia was determined using the drop plate method on Congo Red Yeast Extract Mannitol Agar. The study established that viable populations of *rhizobia* differed significantly between carriers ($P<0.001$) *Rhizobia* strain *R. tropici* CIAT 899 prepared with filter mud as carrier achieved a shelf life of 135 days while *B. japonicum* USDA110 maintained over 10^9 cells g^{-1} for 105 days. The results fall below the stated six month expiry period of BIOFIX®. Studies on potential alternative carriers such as vermiculite were carried out but it turned out that these materials were inferior to filter mud. While BIOFIX® meets reasonable standards in terms of rhizobia counts; it must not be used beyond the specified expiry period. The study also found out that even under refrigeration, the expiry period does not change significantly. Contaminants found in carriers were also evaluated in BIOFIX® legume inoculants. Contaminants in BIOFIX® inoculants determined after 105 days of incubation were found to be mainly fungi. The number of contaminants was in the magnitude of 10^6 cells g^{-1}.

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ACRONYMES

BNF	Biological Nitrogen Fixation
CFU	Colonies Forming Unit
CIAT	International Center for Tropical Agricultural
CR	Congo Red
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization
MIRCEN	Microbial Resources Center
MPN	Most Probable Number
PGPB	Plant Growth Promoting Bacteria
TSBF	Tropical Soil Biology and Fertility
UK	United Kingdom
USA	United States of America
USD	United States Dollar
USDA	United state Department of Agriculture
YEMA	Yeast Extract Mannitol Agar
YEMB	Yeast Extract Mannitol Broth
SSA	Statistical System of Analysis

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Importance of legume inoculants in African smallholder farming systems.

Low crop productivity is a problem facing most farming systems in sub-Saharan Africa (SSA). These low yields are pronounced in grain legumes and are often associated with declining soil fertility and reduced N2-fixation due to biological and environmental factors (Murage *et al*., 2010). Legumes are an important component of all agricultural systems because of the nitrogen fixation provided through symbiotic nitrogen fixation entailing rhizobia (Maria et al 2000). In addition, legume inoculation is an established agricultural practice used since the end of the last century. It has contributed to increased N2 fixation and yield of legume crops in situations where the natural N2fixation is deficient, thereby increasing yield and adding nitrogen to the soil. Biological nitrogen fixation is economical and environmentally suitable hence improves soil condition (Dieker et al., 2011). Microbial inoculants are becoming more available as sustainable alternatives to nitrogen fertilizers however, little is known about the shelf life and survival of the inoculants under different conditions.

1 .2 Regulation and quality of legume inoculants

Generally inoculants should contain a magnitude of rhizobia bacteria sufficient to nodulate plants well and fix nitrogen at an economic benefit. The required level of bacteria cannot be established as a general standard because it varies from one species to another. Rhizobial inoculants have legally established standards in some countries, but not in East and Central Africa. Since this is a new research field, standards for minimum numbers of nitrogen fixing bacteria in inoculants do not yet exist and every manufacturer can claim whatever he deems appropriate for his product. In fact, some commercial preparations do not even contain any viable cells (Anonymous, 1995; Bashan, 1992 unpublished data). Quality control methods to

determine the numbers of bacteria in inoculants are not standardized either. In order to determine the number of viable rhizobia, standard methods of microbiology are used. For this study the traditional Plate Count method, Most Probable Number (Woomer *et al*., 1990), ELISA, and Immunoblot (Olsen and Rice, 1989; Olsen *et al*. 1983; Rice and Olsen 1988) were employed.

The need for improved quality standards implies that minimum number of rhizobia delivered per g of carrier, at any one point must be free from contaminants (Cartoux *et al.,* 2001). Many developed countries (but not the USA) have regulations for inoculant, quality but monitoring is not widely enforced. The level of rhizobia required in the inoculant varies worldwide (between 10^7 and 4 x 10^9 cfu/g carrier) and no set of common international standards exists (Olsen *et al*., 1994). Since the introduction of government regulations, there has been an improvement in the quality of commercial inoculants in several countries, including Australia, Canada, and the UK (Brockwell *et al*., 1987). In the USA, intense competition among inoculant procedures serves to enhance quality through internal assessment. The minimum standard in Australia is 1 X 10^9 viable rhizobia up to the expiration date. An exception is made for *Lotononis* rhizobia where only 3 X 10^7 is acceptable (Date and Roughley, 1977). Canada has a minimum standard of 10^6 viable rhizobia per gram, but the inoculum must provide a minimum of 10^3 viable rhizobia for small seed (200,000 or more seed/kg), 10^4 viable rhizobia for intermediate size seed (30,000 to 200,000 seed/kg) and 10^5 for larger seeds such as soybeans or faba beans (less than 30,000 seed/kg), again up to the expiration date. This new concept of expressing a standard number of viable rhizobia per carrier has considerable merit.

France has the highest standards for inoculant quality and mandated field testing of new formulations and a strict requirement that prohibits contaminants within rhizobia inoculants (Cartoux, 1991). Australia permits low levels of contaminants (0.1 % of the total bacterial

population), but at the same time requires high population levels of rhizobia (Thompson, 1991). Even some developing countries have very high standards for inoculants. In Rwanda, high rhizobia counts and no more than 0.001% contaminants are allowed (Scaglia, 1991), but it is doubtful whether these high standards are met or enforced. Surprisingly, the USA and UK have no regulations, perhaps because there have been no reported adverse effects. Actually it is an effort towards the role of government, believing that industries should be permitted to regulate themselves unless the general public is endangered.

Inoculant quality control is left to market forces and at the manufacturers' discretion (Smith, 1992). The self-imposed standards for rhizobia in American industry are also lower than most European standards. This lack of standard regulation makes it particularly risky for small and medium size growers who tend to purchase inoculants from only one source and may end up with non effective inoculants (Anonymous, 1992). Olsen *et al.* (1994) noted that Canadian regulations sometimes allow even low levels of rhizobia to be legally acceptable, perhaps because the cost of regulation is too high, compared to the risk of misuse. Olsen *et al*. (1994) concluded that increased standards not only ensure that the farmer is provided with effective inoculants but are also in the best interest of the inoculation industry. The presence and nature of contaminants encountered in inoculants may represent a risk for humans, plants and the environment, which remains to be assessed. Olsen *et al*. (1996) reported very interesting data on the contaminants found in 60 samples of commercial inoculants processed with a non-sterile carrier. The number of rhizobia per g product was in the range of $5.5x10^5$ to $8.1x10^9$ while the number of contaminants varied from $1.8x10^8$ to $5.5x10^{10}$, in many cases exceeding the number of rhizobia required per g of carrier.

1.3 Factors limiting the quality of inoculants

The aim of legume inoculation is to provide high numbers of viable effective rhizobia in the rhizosphere to allow rapid colonization, nodulation and nitrogen fixation by the selected inoculants strain in order to maximize legume yield potential (Dieker *et al*.,2006)**.** Survival is affected by the initial condition of the cells in the inoculant, particularly the moisture status, age, purity, the initial number, the strain and the type of inoculant. Changes in the physiological and morphological characteristics of cells during the maturation of the inoculant have been shown to affect survival (Lucy *et al*., 2004). Inoculants are usually suspended in polymeric adhesive, often containing other additives such as dyes or pigments, plant nutrients and seed protection agents, before being applied to seed.

1) Temperature

Nearly all inoculants are best stored in a refrigerator. The exceptions are the inoculants for fine stem stylos, centrosema, desmodium and lablab, which should be stored between 20°C and 30°C (Dieker, 2012). Optimum long-term storage conditions for rhizobial survival, has assumed that temperature and moisture status are important for rhizobial viability over time (Dieker, 2012). At farm supply dealerships and on individual farms, legume inoculants may be stored in uninsulated steel sheds in which temperatures can fluctuate widely and can rise far above the constant low temperatures considered optimal for maintenance of rhizobial cultures (Newbould 1951; Gunning and Jordan 1954; Yan Schreven 1970; Sparrow and Ham 1983).

Peat is the most common used carrier for commercial legume inoculants in North America and elsewhere (Date 1972; Sparrow and Ham 1983). Finely ground peat has a high water-holding capacity and provides a nutritive medium for growth of rhizobia. It also offers effective protection against adverse environmental conditions during inoculant distribution and storage

and after application on seed (Kremer and Peterson 1983; Materon and Weaver 1985). Peats can sustain high numbers of rhizobia to the tune of 10^8 g^{-1}; when incubated at temperatures between 30° and 28°C (Van Schreven, 1970). In tropical and subtropical regions of the world, where inoculants are often exposed to extremely hot and dry conditions, survival of rhizobia is generally better in peat than in alternative organic carrier materials because peat provides superior protection against desiccation (Vincent 1965; Van Schreven 1970; Biederbeck *et al*., 1992).

2) Desiccation

Desiccation is one of the major factors causing poor rhizobial survival on legume seeds. Rhizobia are susceptible to drying on surfaces particularly where individual cells are exposed to low ambient relative humidity (Dieker *et al.*, 2006). This has been demonstrated on various surfaces such as glass (Vincent *et al*., 1962), sand (Bushby and Marshall, 1977a, b) and membrane filters (Mary *et al*., 1985; Dieker *et* al., 2006).

Several investigations have focused on effects of suspending medium on the recovery of cells after dehydration. Sugars, sugar alcohols, amino acids, synthetic and natural polymers and clay minerals have improved recovery of dried bacterial cells. Sucrose was superior to sorbitol, lysine, amino acids, and milk and yeast mannitol broth for preserving rhizobia during freeze-drying (Vincent, 1958; Dieker *et al*., 2006). Annear (1962) found peptone mixed with glucose or sorbitol was effective for the preservation of vacuum dried bacteria on cellulose fibers at room temperature. He concluded that a drying medium must be assessed with regard to the protection it affords during drying storage, the simplicity and definability of its composition and the range of organisms with which it is effective. In addition Clays (montmorillonite, bentonite, illite, vermiculite) can also provide protection against desiccation and thus extend survival of

inoculants (Marshall 1964; Biederbeck *et al*., 1992). This is particularly true for the fast-growing rhizobia (e.g., *Rhizobium meliloti, Rhizobium leguminosarum*), which are more susceptible to desiccation than the slow-growing *Rhizobium lupine* and *R. japonicum* (Bushby and Marshall 1977; Beck *et al*., 1993). Consequently, clay-base inoculants have recently gained some popularity in North America.

3) Moisture content

Moisture content plays a crucial role in the growth rate of Rhizobia in inoculant however microbial inoculants; typically bacterial inoculants, have a very short shelf life. The biological activity of the PGPR may decline rapidly if the handling and storage is not done in the correct manner. The use of carrier materials for the microbial inoculants proves to be beneficial to protect the bacteria and has long been practiced (Ardakini *et al*., 2010; Fuentes- Ramirez and Caballero- Mellado, 2005).

Among various types of carrier materials, peats are the most frequently utilized. this is because peats were able to support high number of rhizobia and maintain its survivability due to high moisture holding capacity and large surface area. Peat can be defined as soil-like material that was formed during decomposition of carbonized plant tissues or mosses (Bachan, 1998). The usage of peat however, poses problems typically in the tropics as it is not readily available in many countries (Smith, 1992). The usage of peat however, poses problems typically in the tropics as it is not readily available in many countries (Bachan, 1998).

4) Nature of carrier

Biofertilizers are usually prepared as carrier-based inoculants containing effective microorganisms. Incorporation of microorganisms in carrier material enables easy-handling, long-term storage and high effectiveness of biofertilizers. Various types of material are used as

carrier for seed or soil inoculation. For preparation of seed inoculant, the carrier material is milled to fine powder with particle size of 10-40 μm. According to the "Handbook for Rhizobia" (Somasegaran and Hoben, 1994), the properties of a good carrier material for seed inoculation are: non-toxic to inoculant bacterial strain, high moisture absorption capacity, easy to process and free of lump-forming materials, easy to sterilize by autoclaving or Gamma-irradiation, available in adequate amounts, inexpensive, has good adhesion to seeds, and of good pH buffering capacity. Non-toxicity to the host plant is another important property. However other essential criteria for carrier selection relating to survival of the inoculant bacteria should be considered. Seeds are not always sown immediately after seed coating with the inoculant bacteria. The bacteria have to survive on seed surface against drying condition until placed into soil.

Optimum long-term storage conditions for rhizobial survival on inoculated seed are not well defined. As with inoculants, it is assumed that temperature and moisture states are important for rhizobial viability over time. Polymers also play a role in reducing exposure of cells to environmental stress but protective properties vary with every polymer. In addition, contaminants in inoculant carriers are known to suppress growth of rhizobia during inoculants production (Nápoles *et al*., 2000).

1.4 The importance of bean and soybean .

Use of legumes that fix nitrogen has been shown as one way of improving soil N content. Benefits from Biological Nitrogen Fixation can be accrued in several ways, including breeding legumes for nitrogen fixation, introducing new legumes into cropping systems or using rhizobia inoculant (Giller 2003). The latter two approaches can be achieved within shorter time frames

and may effectively reverse negative N balance. Grain legumes contribute more than 20 million tons of fixed N to agriculture each year (Herridge *et al*., 2008).

Bean seeds contain large amounts of N and P (35-45 mg N/g^{-1} and 4-5mg P/g^{-1}) and these concentrations do not vary largely among different growing condition and bean cultivars (Araujo and Teixeira 2003). This means that with bean production of 2000/kg dry seeds ha^{-1} $year^{-1}$, almost 100/kg N and about 10 kg P will be removed each year from the system in the harvested seeds. Previous survey estimates that over 60% of bean production area in central, southern and Eastern Africa was affected by N deficiency (Wortmann *et al.*, 1998; Thung and Rao 1999). This caused yield losses of up to 40% compared to the N fertilized areas (Singh 1999; Thung and Roa 1999).

Although Rhizobial inoculants will improve common bean yields and production sustainability under field conditions (Vargas *et al*., 2000; Guene *et al*., 2004; Cardoso *et al.*, 2007). Its industrial production may suffer from low economic returns (Ndakidemi *et al.*, 2006). Lack of information (Hungria *et al*., 2000; Martinez- Romero 2005), as well as lacking technology institutions, education, and markets for targeted production, distribution and application of rhizobia, where investments into agricultural innovation are limited because of institutional and market constraints(Sanginga *et al*., 2007; Jama et Pizarro 2008).

The soybean rhizobia symbiosis can fix up to 30 kg N ha^{-1} under optimal conditions. The response is controlled by the level of indigenous, competing rhizobia, the N demand on yield potential of the host, and N availability in the soil (Theis, 1990). The use of high quality inoculants, and education about their benefits and use can still make a significant contribution in many countries (Keyser and Fudili 1992). With grain legumes alone contributing 33% of the dietary protein nitrogen (N) needs of humans (Vance *et al*., 2000). The common bean (*Phaseolus*

vulgaris L.) is the most important food legume crop grown worldwide (Wortmann and Allen, 1994; Wortmann *et al*., 1998; Buruchara, 2006). This legume is considered by many to be the perfect food as they are nutrient dense with high contents of proteins, micronutrients, vitamins, dietary fiber, and also have a low glycemic index (Wortmann and Allen, 1994; Bennink, 2005; Widers, 2006).

Common bean are grown extensively in five major continental areas: Eastern Africa, North and Central America, South America, Eastern Asia, and Western and South-Eastern Europe (Adam, M.W. (1967). Between one- sixth to one-third of the dietary proteins intake by humans in the Eastern and Central Africa highlands comes from common beans, making them the most important pulse in the region. Bean yield can be increased through rhizobium inoculants. Kenya's annual demand for dry beans of 749,000 metric tons is far above the annual production of 380,000 metric tons (Pachico 1993; Broughton et al 2003; Bationo *et al*., 2011).

Common bean provides livestock feed and their crop residues offer benefit to soils through BNF that, in turn reduce the requirement for costly mineral fertilizers. A small-scale farming household that has incorporated legumes into its enterprises is in a better position to raise its wellbeing and to meet expectations in improved living standards (TSBF-CIAT, 2009). It is an important component of the production systems and a major source of protein for the poor in Eastern and southern Africa. Although largely grown for subsistence, mainly by women, approximately 40 percent of production is marketed at a market value of USD 452 million (Wortmann *et al*., 1999; David *et al*., 2000). In recent years, the crop production trend has not kept pace with the annual growth rate (estimated above 2 percent) in population in some countries due to a number of biotic, abiotic and socio-economic constraints (Kambewa 1997; Chirwa *et al*., Forthcoming and Xavery *et al*., 2006).

Soybeans production worldwide is estimated to be in the range of 12 – 20x 10^6 ha (Cartoux *et al*., 2001). The approximate composition of soybean is 40% protein, 21% oil, 34% carbohydrate and 5% ash (Jones, 2003). Soybean supplies about one fourth of the world's fat and oil, about two-third of the world's protein-concentrate animal feeds and about three-fourth of the world trade in high-protein meals. In accounting for utilization of Soybean, 39 products have been identified ranging from livestock feed, salad oil and baby foods to industrial adhesives, putty and use in pharmaceuticals. By weight, the protein of soybean yield is twice that of meat and most of beans and nuts, four times that of eggs and cereals and twelve times that of milk (Keyser *et al*., 1992).

1.5 Statement of the problem and significance of the study

The role of soil fertility is a fundamental biophysical cause for declining food security among small-farm households in SSA (Sachez *et al.,* 1997). Today over 180 million people in SSA live below the poverty line, and the number is expected to exceed 300 milion by 2020 (Johanson and Ives, 2001) while world food production has continued to decrease over recent years. It has been established that low soil Nitrogen is an important constraint to food production in Eastern and Central Africa (Batiano *et al*., 2011). The average annual loss in soil nutrients of 42 kg N, 3 kg P and 29 kg K ha^{-1} in Kenya is among the greatest in Africa (Smaling, 1993). Nutrient depletion at the farm-scale results from an imbalance of nutrient inputs and losses over time and reaches critical proportions when land is continuously cultivated without the addition of adequate external nutrient inputs (Woomer *et al*., 1999). At the same time the average of 660 kg N ha^{-} has been lost during the last 30 years from about 20 million ha of cultivated land (Kimani *et al.,* 2003). Too often, small scale farmers are unable to afford the high cost of mineral

fertilizer. More than 75% of the fertilizers used in Africa are imported, putting pressure on foreign exchange (Murage *et al.,* 2010). The lack of effective rhizobial inoculants for leguminous crops adapted to African conditions is a serious constraint to food production (Burton, 1981).

BIOFIX is an organic fertilizer made at MEA Ltd Nakuru, Kenya to meet specific need of most legumes crops such as beans, soybeans. One of the problems affecting farmers' use of BIOFIX is its quality. Poor quality control in the production process as well as problems associated with its transportation and storage negatively affects the viability of inoculants (Odame, 1997). When farmers see no benefit in the field from inoculation, their confidence in the technology is reduced, and both the manufacturers and the users are ultimate losers. For this reason, it is desirable that the quality of legume inoculant be protected by some sort of institutional regulation (Burton, 1978; FAO, 1991). A series of inoculations has been conducted on farmers' field by N2Africa program to improve legume crop yields and soil fertility through BIOFIX inoculation, problems of high quality, contaminant and storage management has to be established. The benefit of increased knowledge of symbiotic nitrogen fixation is the inoculation of legumes by selected rhizobia, a practice that allows producers to introduce rhizobia into the soil-plant ecosystem (Ben Reban *et al.*, 2006).

The use of inoculants in other countries has increased legumes production. According to Johanson and Ives (2001) about 3 million hectare of beans and 1.1 million hectare of soybeans are established in Sub-Saharan Africa where the soybean is inoculated with rhizobia incoculant. Inoculation helps to increase the number of effective rhizobia (Boahen, 2008).

A potential for inoculants use in Kenya is great since an estimated 800,000 ha are planted to common bean annually (Africa Agriculture, 2008; Mungai and Karubiu, 2009). Inoculation of legumes with rhizobium is an important management practices for sustainable agriculture in

Africa (Ben Rebah *et al.,* 2002). In 2011, MEA limited produced 13,450 packets of bean and soybean inoculants according to Bala (2011). Selected Rhizobium strains fix more nitrogen as compared to applying a recommended 90 kg of mineral nitrogen fertilizer per ha common beans. A good inoculant must be prepared with a strain of rhizobium selected for higher N fixation efficiency and competitive ability for nodulation; the strains must survive in the inoculant formulation, maintain its properties during storage, and tolerate to stress factors such as acidity, desiccation, high temperature and chemical pesticides (Burton 1981, Smith 1992, Bashan 1998, Ben Rebah *et al.*, 2002). Important factors for inoculant quality is a high number of live rhizobia (greater than 10^9 rhizobia per g), and no or minimal contamination by microorganisms detrimental to rhizobia, plants and humans (Lupwayi *et al*., 2000; Ben Reban *et al.*, 2007). The possibility of the disseminating human, animal, or plant pathogens in rhizobial inoculants contained in non-sterile carrier has prompted French regulations to prevent the sale of such inoculants (Catroux *et al*, 1992). Results of independent tests (Schall *et al*., 1975; Skipper *et al*., 1980; Somasegaran, 1991; Vincent and Smith, 1982; Olsen *et al*., 1995) indicate that a substantial proportion of the inoculant produced using non-sterile carrier appears to be unsatisfactory for farmer use, either because of low populations of rhizobia or high numbers of contaminants. The use of N2fixing legumes to address current soil nutrient depletion and increase crop yields is a system that maximizes natural methods of maintaining soil fertility and therefore has more capacity for sustainable crop yields in the long term. Several studies have shown that legumes can be effective in improving soil fertility in many areas (Giller *et al*., 1997; Lathwell, 1990). This study is set to evaluate the quality of inoculants by enumerating the viable rhizobia using different carrier materials, two rhizobia strains in different storage condition over time to identify shelf life and improve inoculants production methods.

1.6 Objectives of the study

1.6.1 Overall objective

The overall objective of the study is to ensure that farmers of Eastern and Central Africa receive quality rhizobia inoculants that are consistent with international standards.

1.6.2 Specific objectives

i. To assess whether commercial inoculants (Biofix) contain at least 10^9 rhizobia per g of carrier in conformity with expiry date 6 moths.

ii. To determine whether inoculants contain fewer than 10-^6 contaminants per gram of carrier material throughout their shelf lives.

iii. To quantify the effect of storage conditions on the quality of inoculants.

1.7 Outline of the Thesis

The thesis is divided into six chapters addressing the quality assessment of legume inoculants on bean and soybean in Eastern and Central Africa. Following general introduction, the chapter 2 presents the description of the procedures used to achieve the result, Chapter 3 documents shelf lives of CIAT 899 and USDA 110 in BIOFIX, filter mud and vermiculite carrier under six months storage condition. The effect of contaminants on the rhizobia population in the inoculants is discussed in Chapter 4 of the thesis. Chapter 5 discusses the proper storage condition of inoculants for it to attain six months expiry date. The last chapter presents the general conclusions of the study and recommendations for improving the quality of legume inoculants in Eastern and Central Africa.

CHAPTER TWO: GENERAL MATERIAL AND METHOD

2.1 Study area

This study was conducted at the Faculty of Agriculture, - University of Nairobi/Mircen Lab and at MEA Ltd factory in Nakuru Town. The MIRCEN Labratory (figure 2.1) is located at Kabete Campus, which is about 1940 m above sea level and lies within latitudes 1° 14’ 20” to 1° 15’ 15’’ S and longitudes 36° 44’ 20’’ to 36° 45’ 20’’ E (Wamburi, 1974; Mwangi, 1998).

Figure 2.1: the author working at the MIRCEN Laboratory

The room temperature during the day and the experimental period, August 2012- February 2013 was within the range of 23 to 24 °C.

Kabete site has a bimodal rainfall distribution with the long rains starting from late March to June and short rains from late October to December. The mean annual rainfall of the station is 925 mm and the evapotranspiration is 1363 mm. MEA factory based in Nakuru Town, Kenya

West of Nairobi produces BIOFIX inoculants for commercial purposes (figure 2.2) under license of University of Nairobi. This public private partnership involving MEA Ltd and the University of Nairobi is helping farmers to increase their yields of legumes trough better BNF.

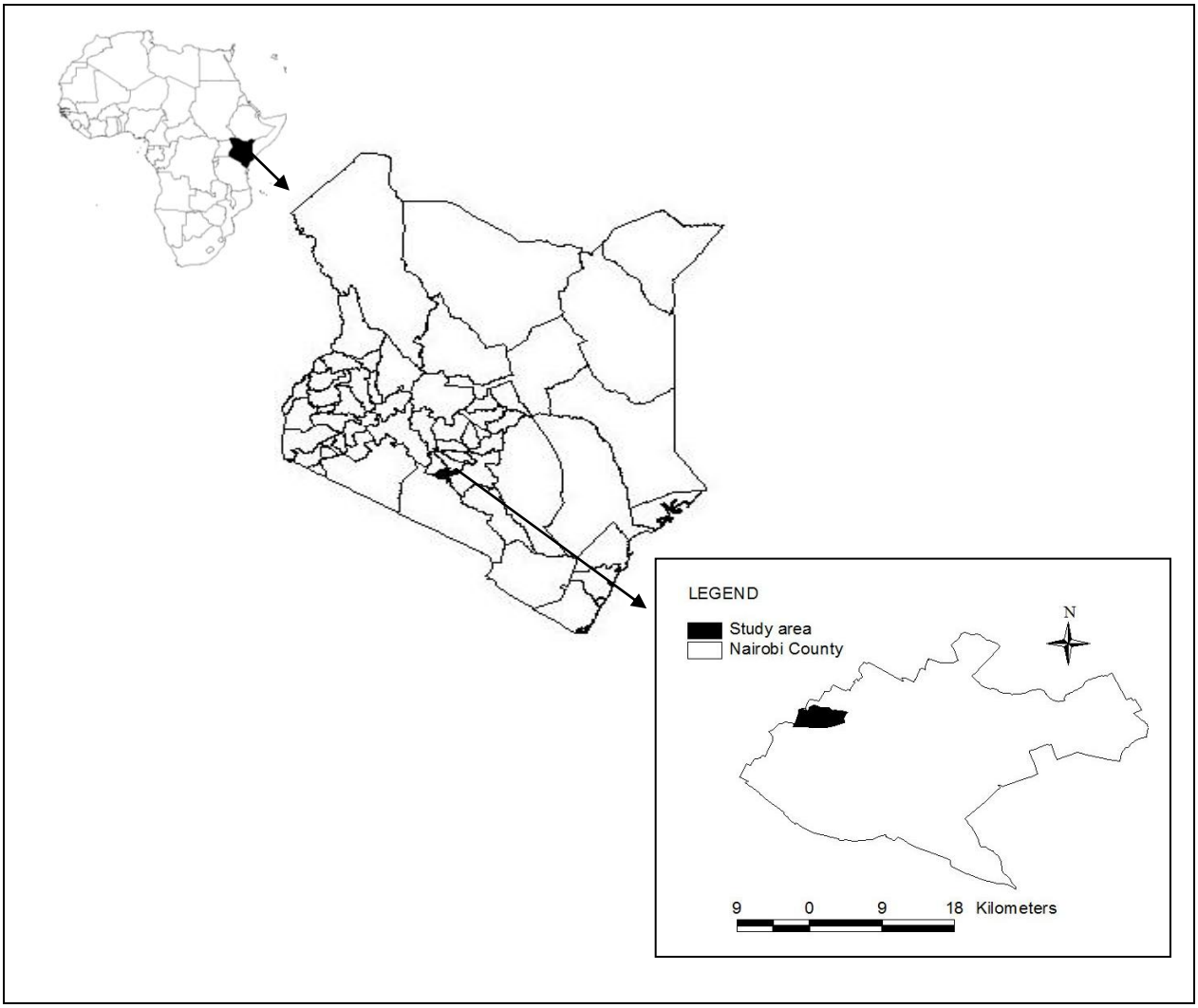


Figure 2.2: Location of the study area, Kabete Campus

2.2 Study approach

The study involved laboratory and green a house experiments.

2.2.1 Laboratory Experiments

A laboratory experiment was conducted to assess rhizobium populations in different sources of carrier preparation using Congo red plate counts. The information gathered from the plate count helped to identify the best carrier with higher population and lower contamination and its proper storage condition that is needed to better maintain the shelf life of legume inoculants used by farmers.

1) Preparation of Carriers

The filter mud was ground using hammer mill then sieved to pass through 2.12 Micron. The filter mud was wetted to field capacity and left to stay for 2-3 days allowing spores to grow then packed in high density polythene bags and sealed. A second autoclaving was performed before testing to ascertain that there were no other micro-organisms in the filter mud. If contaminants were still present a third sterilization was carried out.

2) Preparation of Cultures.

Preparation of media;

Chemicals were weighed and mixed with distilled water to make 1L, then take the pH adjusted about 6.8 -7.0. The media was dispensed into conical flasks of 500 ml and 250 ml wraped with aluminum foil and sterilized for 15-20minutes. The appropriate rhizobia broth was introduced once the media had cooled to below 30°C. Growth and survival of rhizobia in the carrier materials were tested at two different storage temperatures, 4 and 28°C. The carrier materials were packed into small polyethylene bags. It was mixed well in the bag and immediately sealed

to maintain the moisture content. Packaged carrier materials used in this experiment were subjected to triple sterilization (Thompson, 1984) to ensure proper sterility of the pack of carrier.

Each package of sterilized carrier materials was then inoculated with the rhizobia (>10^8 cells ml^{-1}). The volume of inoculants added was 50% of the water holding capacity of the respective carrier materials. The inoculants were injected using sterile syringes into the bags that contained carrier materials aseptically then mixed with the carrier. The punctured area was wiped with 70% alcohol and covered with a small sticker. The packets were stored in incubator at for 14 days before their redistribution in fridge at 4°C and at 23°C room temperature.

Sampling of BIOFIX inoculants

BIOFIX inoculants are commercial inoculants of bean and soybean from MEA Ltd and were used in this study for assessment of viable populations and contaminants at different time in both refrigerated at 4°C and room temperature condition 23°C for a period of 164 days. A random sampling from two batches were picked from seven packets of inoculants, a subset of five were tested first and in case one or two packets failed the other two packets were tested to determined numbers of viable colonies forming unit in packets until the cumulative mean stabilized (Diaker *et al.*, 2001).

Counting of viable cells of rhizobia in carrier materials

A total of 1 g sample from each bag was placed into test tubes containing 9 ml of sterile distilled water. They were then mixed thoroughly to ensure complete separation of the microorganism from the carrier. A tenfold serial dilution (until 10^{-7}) was performed. Three drops of 20 µl from the last three dilutions was plated onto YEMA (Yeast Extract Mannitol Agar) with three replicates for each dilution (Somasegaran and Hoben, 1994). All the plates were incubated at

28°C for at least 24 hours before colony formation was counted and the CFU g^{-1} determined. From this value, the rhizobium population was calculated. Only the number of colonies that grew in the range of 5 to 55 colonies was counted. Viable population of rhizobia/g of inoculants was calculated using the following formula = 1/drop volume X Number of colonies X dilution factor

However, a spread sheet utility developed for the purpose of this study was used to provide mean populations and coefficient of variation (standard deviation/ mean) of rhizobia and contaminants of inoculants package.

Experimental design and treatments

A Completely Randomized Design (CRD) with a factorial arrangement replicated five times (3carriers x 2strains x 2 temperature) was adopted. A total of twelve treatments were counted by drop plate methods and plant infection technique. Two strains of rhizobia, *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA110 were grown in broth culture and introduced into filter mud, and vermiculite. BIOFIX from MEA prepared using filter mud was also evaluated.

Five packets of inoculant of the two crops (soybean ad beans) were selected randomly each month from the two storage conditions. Each sample was plated with the three last dilutions 10^5 to 10^7 replicated three times and the the drop late method according to (Hoben and Semasegaran, 1982) was used to culture viable organisms. Microbial numbers were determined at intervals 14, 45, 75, 105, 135 and 165 days.

2.2.2. Greenhouse experiment

Soybean plants were grown in growth pouches in the greenhouse at the field station, Kabete campus. Nodulation was observed on plants at 45, 105 and 165 days. MPN method was used to determine population of rhizobia.

Seed preparation and planting in growth racks

The growth racks were prepared in accordance to (Somasegaran and Hoben, 1985). Legumes seeds were first rinsed in 95% alcohol for 10 seconds to remove waxy material and trapped air. Sodium hypochlorite solution (2.5%) in sufficient volume was used to immerse the seeds completely for 3-5 minutes. The seeds were rinsed with six changes of sterile water aseptically then poured to submerge the seeds for 1 to 4 hours, allowing them to imbibe the water. Legumes seeds were pre-germinated on sterile (autoclaved) vermiculite for 48 hours in an incubator at 28°C, and regularly inspected to ensure that the radical does not become too long or etiolated. The vermiculite was moistened and sterilized one day in advance. These seeds were then planted in plastic pouches (two seeds per plastic pouch), then inoculated with appropriate rhizobia culture. Six serial dilutions were made with three replicates per dilution. One set of uninoculated pouches served as a control. The plants were then grown in the greenhouse for 28 days, regularly adding N free nutrient solution (Broughton and Dillworth, 1970; Somasegaran and Hoben, 1985) as required, after which they were observed for nodulation. The presence and absence of nodules after 28 days were recorded according to (Woomer *et al*., 1990; Olsen and Rice, 1996).

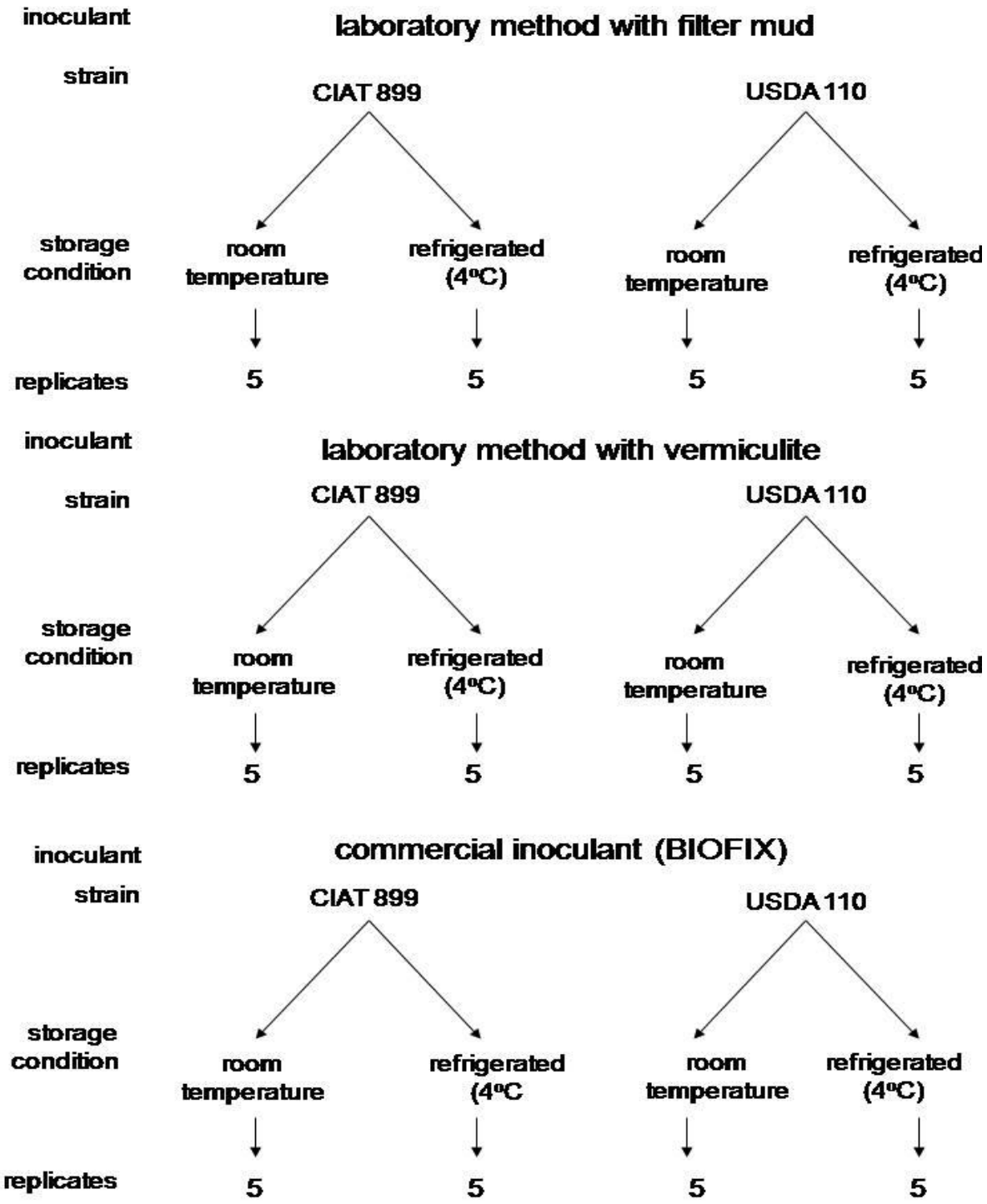
Based on the formula of Somasegaran and Hoben (1985), the number of rhizobia per gram or per ml was estimated by: $\frac{m\ X\ d}{v}$ where:

m = likely number from the MPN table for the lowest dilution series

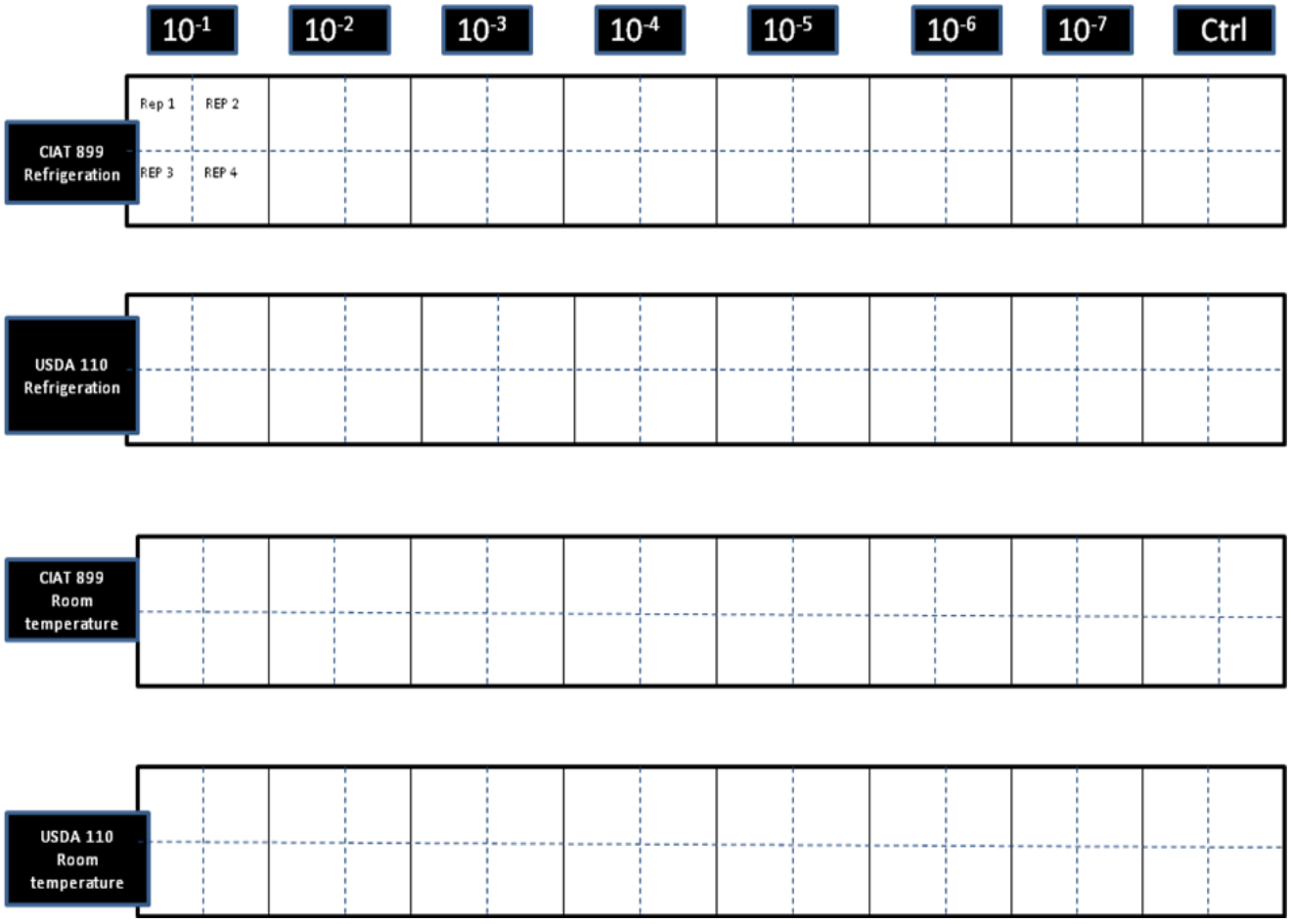
d = lowest dilution (first unit)

v= volume of aliquot, populations were estimated using the MPNS computer program which provides considerable additional flexibility (Woomer *et al*, 1990)

Figure 2.4 Layout of laboratory experiment



Greenhouse experiment layout



The layout for the greenhouse experiment was intended to test the capacity rhizobium in the inoculant to nodulate bean and soybean seed in the pouches. The experiment was repeated three times using growth racks. BIOFIX®, filter mud and vermiculite based inoculants were examined under refrigeration and non-refrigeration conditions when *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110 were diluted to 10^{-6}, each then inoculated to bean and soybean grown in pouches.

Table 2.1: Physical and Chemical properties of the carriers used in the experiment

Carrier	% C	Organic matter	Total N%	Bulk density	Porosity	Water holding capacity	pH
Filtermud	18.44	31.80	1.96	0.79	56	155	7.81
Vermiculite	2.11	3.64	0.08	0.98	63	152	6.63
Coconut coir	35.50	61.20	0.43	0.75	59	171	6.21
Biochar	3.35	5.78	0.15	0.43	73	200	8.53
Nitrogen Peat	29.09	50.15	1.82	0.82	45	200	7.79

2.2.3 Statistical analysis

Analysis of variance (ANOVA) was used to generate the means and least significant differences at 5% significance level. Least significant differences at (LSD) values were used to compare means where significant differences between the mean were detected.

CHAPTER THREE: ASSESSING SHELF LIFE OF LEGUME INOCULANTS USED IN EAST AFRICA

Abstract

Adoption of legume inoculation with rhizobia by small-scale farmers in East Africhas the potential to increase biological nitrogen fixation but it is important that the quality inoculants must meet minimum standards. In the case of BIOFIX®, the only commercially-available inoculant in East Africa fulfills that standard at least 10^9 rhizobia g^{-1}. We examined the effects 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 Extract Mannitol Agar. Viable populations of *rhizobia* differed significantly between carriers ($P<0.001$) and rhizobia strain ($P<0.05$). *R. 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 month expiry period of BIOFIX®. Attempt to replace filter mud carrier with vermiculite proved unsuccessful. While BIOFIX® meets reasonable standards in terms of retaining high rhizobia numbers; it must not be stored or retained over the expiry period. Even under refrigeration conditions, its expiry period should be adhered to because contaminants have the opportunity to affect its quality.

Key words: BIOFIX®, CIAT 899, Kenya, legume inoculant, quality control, USDA 110

Introduction

Wider use of legume inoculants by African small-scale farmers offers potential to supply a sustainable source of nitrogen and increase nutrient-use efficiency (Dieker *et al,*. 2011), two necessary components to overcome low farm productivity in Sub-Saharan Africa (Batiano *et al*. 2011). These low yields are pronounced in grain legumes and are often associated with declining soil fertility and reduced symbiotic biological nitrogen fixation (BNF) due to biotic and environmental stresses (Giller 2001). Greater availability of legume inoculants offers the potential to better manage BNF (Herridge *et al*. 2008) and substitute for inorganic nitrogen fertilizer (Sofi and Wani 2007; Sanginga and Woomer 2009; Woyessa and Assefa 2011) but the inoculants must be of suitable quality to nodulate legume hosts and offer strong economic returns (FAO 1984).

Nodulation is improved when the number of viable rhizobia inoculated per seed increases, as accomplished by having greater numbers of viable rhizobia in the inoculant or delivering larger doses (Catroux *et al*. 2001). The key to ensuring high quality in legume inoculants is an effective quality control system (Thompson, 1984), whether through internal monitoring or government regulation but where producers have little incentive to invest in quality control, some market poor inoculants (Beck *et al*., 1993).Thus, it is important to determine the duration of the bacteria survivability in the respective carrier materials to ensure the desired level of bacterial population remains viable for the inoculants to be effective (Kaljeet *et al*., 2011). Besides that, the carrier materials contained in solid inoculants should have properties that protect rhizobia and permit ease in application to seeds (FAO, 1984). Thus the objective of this experiment was to assess the shelf life of two industry standard rhizobia (*R*.CIAT899 and *B*.USDA110) within a commercially-available BIOFIX® inoculant and to assess the means to improve its quality through better carriers, sterilization and storage temperatures.

Materials and Methods

Dried filter mud and commercially-available horticultural vermiculite were grounded by 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 analyzed by the University of Nairobi Soil Analysis Laboratory (Table 1). Ten g of non-sterile carrier was placed in polythene bags, sealed and autoclaved thrice for three hours 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 broth culture was injected by 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 at room temperature (about 24°C) which is the curing practice at the BIOFIX factory before refrigeration at 4°C and room temperature for 14 days. Half of these packets were then refrigerated at 4°C and one other half was stored at room temperature. After 165 days, both experiments were evaluated.

A ten-fold dilution series to 10^{-7} was prepared from the inoculants after Miles & Misra (1938) and Somasegaran and Hoben (1985). Three drops of 20 μl from the last three dilutions was plated onto Congo Red Yeast Extract Mannitol Agar with three replicates for each dilution. All plates were incubated at 28°C for 3-7 days. Only the number of colonies that grew in the range of 5 to 55 colonies was counted and colony forming unit g^{-1} was back calculated. Occasional presence of fungal contaminants was also recorded. The number of rhizobia and contaminants were counted at different intervals of: 14, 45, 75, 105, 135 and 165 days following with injection

with broth cultures. Each treatment was replicated five times in a completely randomized design (Herridge *et al*., 2002). Effects of rhizobial strain, carrier, storage temperature, length of storage and their interactions were determined using the General Linear Model procedure of SAS version 9.2 (SAS Institute, 2008). This research was conducted at the MRCEN laboratory of the College of Agriculture and veterinary science, University of Nairobi, between August 2012 and March 2013.

3.3 Results

The chemical and physical characteristics of the two test carriers and a North American peat appear in Table 2.1. Filter mud closely resembles peat but it has lower water holding capacity and pH. Vermiculite is an inorganic material with lower carbon and nitrogen, and greater bulk density but with high porosity.

Table 3.1: Survival of two rhizobia strains in different inoculant formulations

Carrier	**Strain**	**Time in days[a]**					
		14	**45**	**75**	**105**	**135**	**165**
		---------- rhizobia (x 10^9) ----------					
BIOFIX	*R. phaseoli* CIAT 899	1.15	6.17	4.06	2.70	0.04	0.03
	B. japonicum USDA 110	1.13	5.60	5.62	1.86	0.21	0.04
Filter mud	*R. phaseoli* CIAT 899	8.79	6.20	5.00	3.14	2.30	0.44
	B. japonicum USDA 110	6.36	3.93	4.13	2.77	0.44	0.25
Vermiculite	*R. phaseoli* CIAT 899	0.78	1.77	0.53	0.27	0.06	0.04
	B. japonicum USDA 110	0.54	0.63	0.54	0.08	0.05	0.02

$LsD_{0.05\%}$ carrier =2.78, strain= 4.06 and time 4.89

The population dynamics of two rhizobia in different carriers over time appears in Table 3.1 Four-way ANOVA of these data resulted in significant effects of carrier material ($p<0.001$), storage condition ($p<0.001$), strain ($p=0.01$) and time ($p<0.001$) and many key interactions including carrier x strain ($p=0.02$) and carrier x storage ($p=0.02$) and carrier x time ($p<0.001$) but not strain x storage or strain x day (data not presented). Contamination of inoculants only occurred in the commercial product among 35% of the samples containing fungi, averaging 0.58 x 10^6 g^{-1} at day 14, presumably through persistent spores (data not presented). Some level of late contamination was also noted among other carriers after day 105 and 135 days. The reason for this late contamination is attributed to repeated sampling of packets.

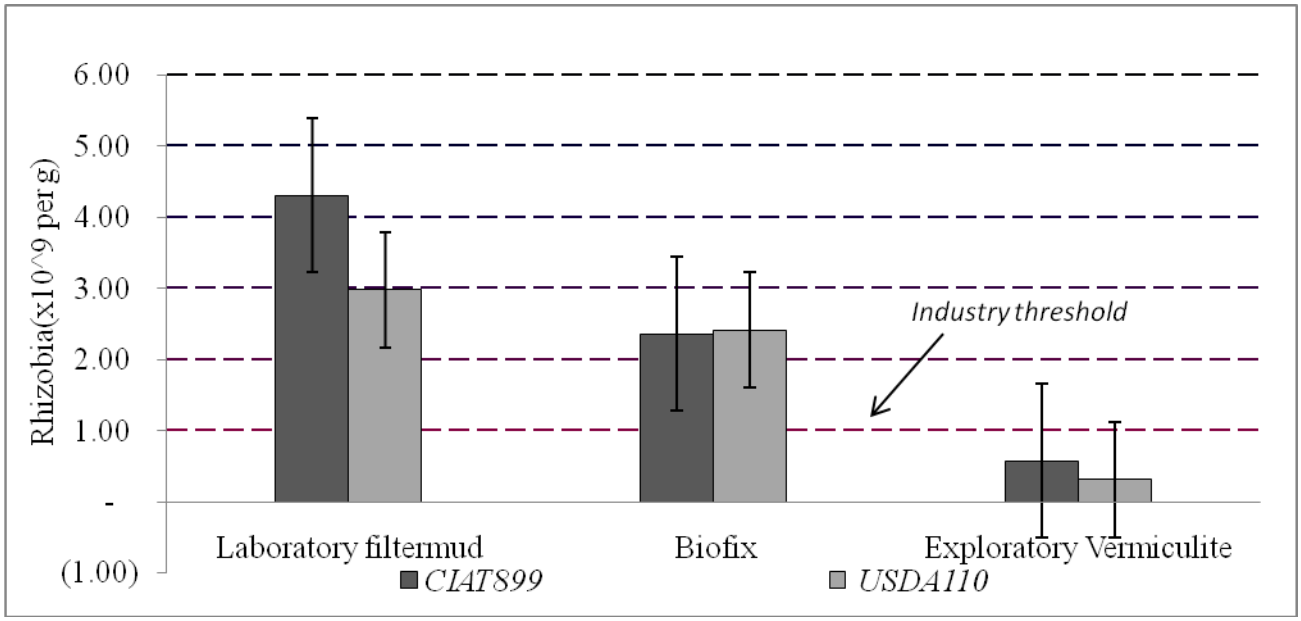


Figure 3.1: *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110 strains across the carrier (laboratory filter mud, BIOFIX and vermiculite).

The BIOFIX® carrier represents the commercial product itself while filter mud is essentially a more thoroughly sterilized and carefully prepared alternative within the MIRCEN laboratory.

BIOFIX® displays a large initial increase followed by steady decline. Thorough and careful sterilization of filter mud leads to larger populations and greater survival of rhizobia. Vermiculite does not support rhizobia, neither in terms of initial colonization nor longer-term survival. Note that the agreed industry threshold for inoculants in Kenya is a minimum of 1 x 10^9 rhizobia g^{-1}. The population dynamics of the two strains across all carriers and storage conditions is presented in Figure 3.2. *Rhizobium tropici* CIAT 899 colonizes carriers better than *Bradyrhizobium japonicum* USDA 110. After a 14 days curing interval, both populations increase to 45 days and then decline. After 105 days both strains exceed industry standards but not thereafter.

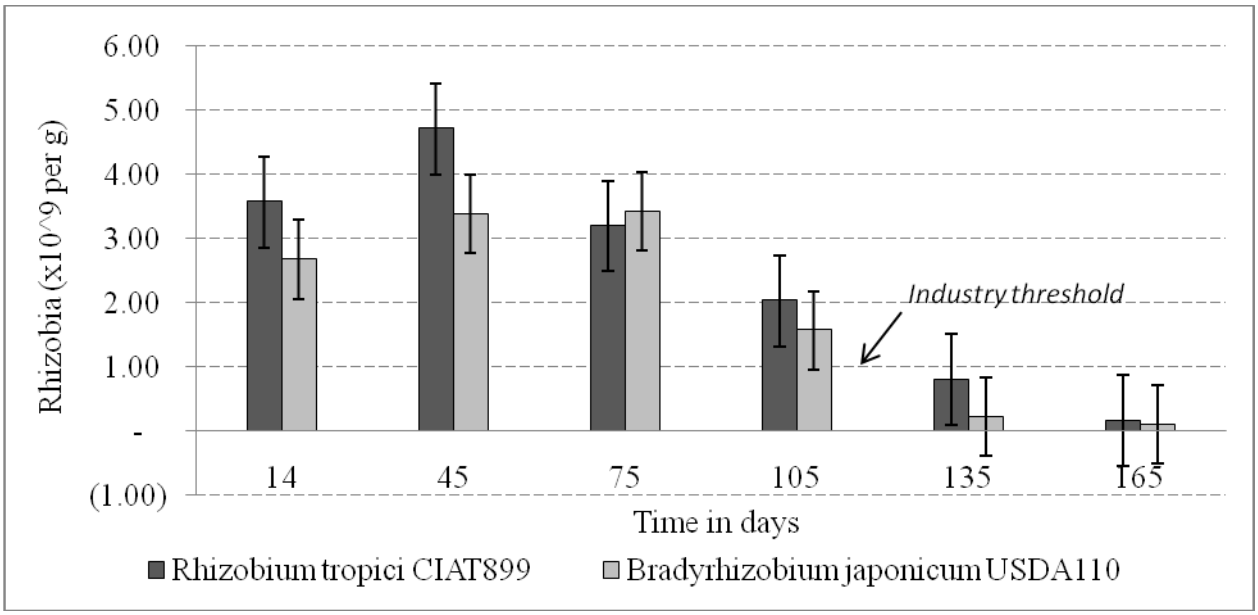


Figure 3.2: Populations of CIAT 899 and USDA 110 across the three carriers over time.

The effect of refrigeration on storage of BIOFIX® inoculant containing USDA 110 is presented in Figure 2. The x-axis is presented as both days after injection and packing (after a 14 day curing period). Note that lower temperature results in higher populations with time and extends

shelf life by 30 days. A similar trend was observed for CIAT 899 and MIRCEN filter mud, but not for vermiculite carrier as it lacked a pronounced population increase following injection (Table 3.1). Shelf lives of different inoculants appear in Table 3.2. Note that these values were calculated by interpolating the two values falling above and below 10^9 rhizobia g-1. In the case of some vermiculite carriers this could not be performed (Table 3.1).

The following figure presents the behavior of *Bradyrhizobium japonicum* USDA 110 strain in BIOFIX® inoculants incubated at two different conditions refrigerated 4°C and room temperature 23°C

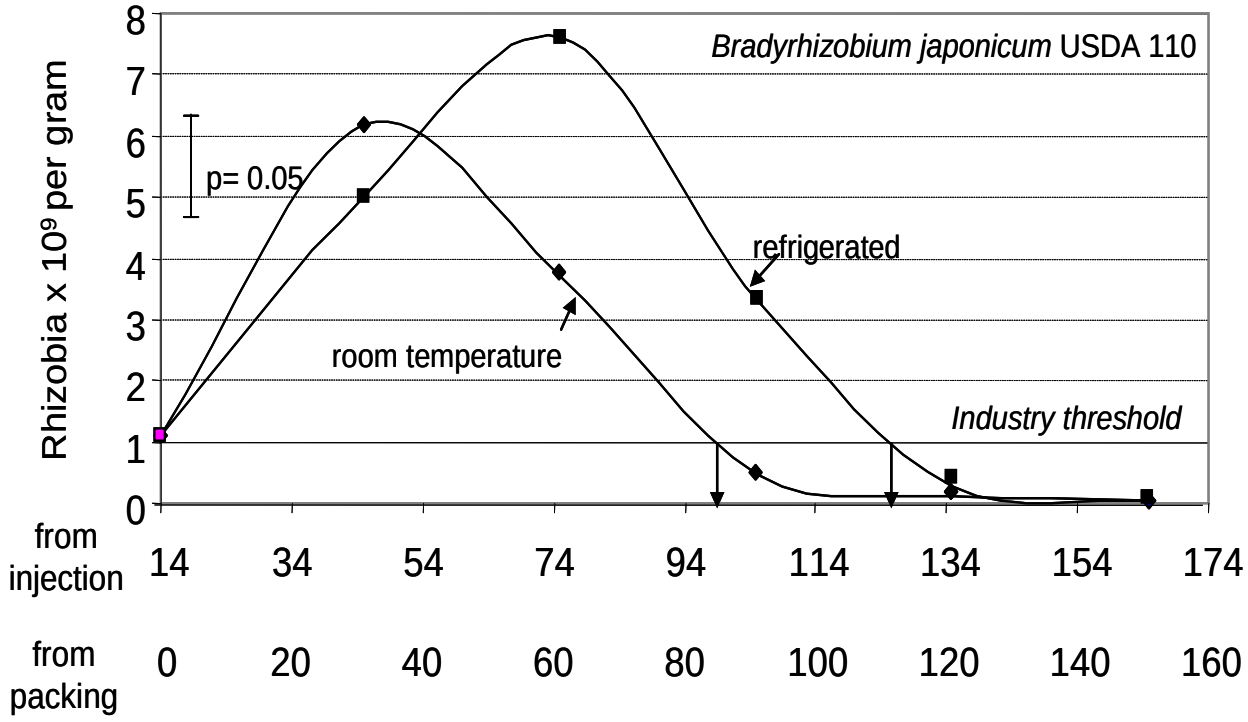


Figure 3.3: The effect of storage temperature on BIOFIX inoculant containing USDA 110 includes extended shelf life.

After long period of storage, shelf life *of rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110 in different inoculants formulation was calculated and presented in the following Table 3.2.

Table 3.2: Shelf lives of different inoculant formulations

Carrier	Storage	CIAT 899	USDA 110
		------- days since injection ------	
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

3.4 Discussion

The persistence of two industry standard rhizobia, *B. japonicum* USDA 110 for soybean and *R. tropicii* CIAT 899 for bean in BIOFIX® legume inoculant was examined over time and possible alternatives to improve the inoculant compared. BIOFIX® is the only commercial legume inoculant produced in East Africa, and its improvement stands to benefit large numbers of African farmers. This inoculant is currently produced in Nakuru, Kenya using a carrier material of sugar cane filter mud from the Muhoroni sugar factory in west Kenya. The factory sterilizes inoculant carrier by autoclave, injects it with rhizobial broth, mixes the broth and carrier, cures the product for 14 days and then packages and ships (Wafulah, 2013). The product has a stated shelf life of six months (about 180 days) from the time of injection. Because much of East Africa receives bimodal rainfall, the factory operates year round, and larger orders must be placed in advance, so the product is usually shipped shortly after curing and packaging, and the factory currently does not have refrigerated storage facilities. MEA Ltd., producers of BIOFIX®

has adopted a quality standard of at least one billion (1×10^9) live rhizobia g^{-1} inoculant, as in Australia (Herridge *et al*. 2002). BIOFIX® meets this standard over much of its expiry period. (Fig.3.2). However, this study also indicates that the six-month shelf life may be too long, as both soybean and bean inoculants fell below that expiry interval by 80 and 49 days, respectively (Table 3.2).

Three approaches were considered towards improving the inoculant formulation: altering its carrier material, manner of sterilization and lowering storage temperature. The filter mud 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 *et al.,* 1985). Its composition is not similar to pre-package sterilized peat from North America used to produce solid inoculants (Table 2.1). Filter mud is collected from the bottom of the crushing vat in sugar production. It is largely organic matter but contains some silt, slightly lowering its moisture holding capacity. One problem with this carrier material, however is, the heavy contamination of the bulk material by other microorganisms. The Nakuru factory currently sterilizes the ground, sealed carrier by autoclaving, and over packing has caused problems with contamination in the past. This study found some fungal contamination in BIOFIX® (35% of samples, overall average of 0.58×10^6 g^{-1}, data not presented. This is much less than reported in the past and below the target standard of 1×10^6 g^{-1}. Nonetheless, when BIOFIX® was replicated at the MIRCEN laboratory, this contamination was eliminated with careful and repeated autoclaving, and we observed significantly higher populations in the inoculant, especially for CIAT 899 (Table 3.1) and extended shelf life for USDA 110 (Table 3.2). Attempts to substitute horticultural-grade vermiculite as a carrier were not successful as both rhizobia strains were less able to colonize and persist in this material (Tables 3.1 and 3.2).

Attempts to improve inoculant quality through refrigeration produce mixed and somewhat unusual results. Refrigeration of BIOFIX® from the production line immediately after curing resulted in slightly delayed colonization of the carrier material (Figure 3.3). However, this colonization continued for a longer period and larger populations were later achieved. What was not observed, however, was a greatly extended shelf live (Table 3.2) in large part because both room temperature and refrigerated inoculants both declined in a linear fashion following colonization of the carrier, but later attenuated (Figure 3.3). During this steady decline, the die-off of rhizobia is roughly between 90 and 170 million cells $g^{-1}d^{-1}$ (estimated from Table 3.1) although too few observations between the peak and attenuation reduces 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 that of Khavazi *et al*. (2007) who reported that the number of rhizobia 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 sterilization of carrier, but even our numerous contamination-free samples displayed linear decline to levels below industry standard. One disadvantage of solid over liquid formulation inoculants is that contaminants may persist in carriers (NifTAL Project 1998; Woomer *et al.* 1999). Our findings also disagree with Boonkerd (1991) who reported differences in inoculant quality due to storage temperatures. However, the temperatures under study were 10°C vs 30°C, the higher temperature somewhat greater than room storage conditions in Nairobi's equatorial highlands.

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 minimized. 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 so it is more easily applied to seed. Curing itself is a two phase process where rhizobia first colonize the carrier, increasing several fold, but then populations decline as surviving rhizobia harden. At the BIOFIX® factory, inoculant is cured in sealed, semi-permeable plastic bags and later packaged into a labeled, airtight outer bag after 14 to 20 days for marketing. In this experiment, inoculants remained in their inner bags throughout the time series, and opened at sampling intervals. The inoculants were stored together in a sealed plastic container with likely different water vapor exchange properties. 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.

3.4 Conclusion

BIOFIX® legume inoculants for soybean and bean were shown to 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. More careful preparation of inoculants in the laboratory suggest that there is opportunity of slight improvement along its production line, particularly by better sterilizing the carrier and achieving higher populations several days following injection. Altering the carrier material from organic (filter mud) to mineral (vermiculite) material resulted in an inferior product, but search for a yet better carrier material and production approach, able to support greater than 1×10^9 g^{-1} over an extended shelf live should continue.

CHAPTER FOUR: CONTAMINANTS OF FUNGI IN LEGUME INOCULANT REDUCES VIABILITY OF RHIZOBIUM.

Abstract

An experiment was designed to evaluate the contaminants in BIOFIX® legume inoculants, inoculants prepared with filter mud by the MIRCEN Laboratory University of Nairobi and vermiculite carrier prepared with *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110, two inoculant industry standards for bean and soybean respectively. Ten grams of each sterilized carrier material was inoculated with *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110 then incubated for 165 days. A follow up of plate count procedure was designed and used Congo red YEMA to determine the number of contaminants and their impact on rhizobia population. Contaminants in BIOFIX® inoculants depicted significant difference at ($p<0.05$). It was established that 55% of BIOFIX® inoculants contained fungal contaminants after 105 days of incubation. The level on fungal contaminants exceeded 10^6 cells $g^{-1,}$ a magnitude not found in inoculant formulation of filter mud prepared at MIRCEN Laboratory. The latter were free from contaminants except vermiculite that showed some low level contamination 165 days of injection with broth.

Key words: inoculants, carrier, contaminants, rhizobium, fungi.

4.1 Introduction

Commercial inoculants of lower quality are being marketed, especially in tropical countries where high quality peat and sophisticated sterilization equipment are not available (Brockwell and Bottomeley, 1995; Herridge, 2008). These inoculants contain contaminants that are known to suppress growth of rhizobia during inoculant production, storage and its application to legumes seed (Roughly *et al*., 1976; Dieker *et al*., 2011). Contaminants on inoculated seed may also reduce cell numbers or effectiveness of rhizobial colonization of the rhizosphere (Ben Rebah *et al*., 2007). However, apart from having levels of contaminant which adversely affect their quality and in turn effectiveness in the field, they contaminate soil with unknown microorganisms (Olsen *et al.,* 1994).

Research in a number of countries has shown that, the most effective inoculants are produced using a sterile carrier and are relatively fresh, for example less than six (6) months old. The carrier (most commonly peat) must contain $<10^6$ contaminants/g (Herridge *et al.,* 2002). In addition, non-sterile inoculants will contain large numbers of contaminants that may depress numbers of rhizobia with time (Date and Roughley, 1977). Even in sterile carriers, numbers of viable rhizobia will decrease over time, although not at the same rate as in non-sterile carriers (Herridge 2002). Conclusion from a survey done by Cartoux *et al*., (2001) confirmed that the quality of legume inoculants remained poor, the technologies to produce high quality inoculants are available and legal requirements and controls can improve the quality of inoculants and thus their efficacy.

Moreover, attempts have been made to reduce the level of contamination in carrier based inoculants. Many countries have overcome the potential risk by placing legislation that prohibits

the use of non-sterile carriers in the manufacture of legume inoculants (Kumar and Jauhri, 2003). India standards institution (1986) also prescribe that there should be no contaminants at 10^{-6} dilution of rhizobia inoculant when enumerated by dilution plate methods. However, it is a paradox that, despite almost 100 years of research and experience, many of the inoculant produced in the world today is of poor quality. Even good quality inoculants are often not used to best advantage of the farmers (Herridge *et al*., 2002).

Mechanisms of sterilization by autoclaving is based on the wet killing of microorganisms under high temperature (121ºC) and pressure (15 pounds/inch) for a period of time, depending on the size and the composition of material. However, the problem of spore forming contaminants remaining in the carrier after autoclaving is a major obstacle to achieving sterility (Panlanda *et al*., 2012). Unfortunately, quality control is not universal in inoculant production and the quality of inoculants sold on the market may be low (Cartoux *et al*., 2001). Shelf-life is an important inoculant characteristic for farmers and manufacturers. It has been shown many years ago that contaminants have detrimental effect on shelf-life of rhizobia (Date and Roughley, 1977). Since contaminant is the main problem that affects the quality and shelf life of rhizobial inoculant, sterilized carrier is necessary to be accomplished prior to injection of the pure culture of rhizobia into the carrier (Panlanda *et al*., 2012), contaminant free inoculants are preferred **(**Brockwell and Bottomley, 1995) offering shelf life longer than one year. The future of the inoculant industry, and its potential benefits for world agriculture, depends on improving inoculant quality, both numerically and in terms of strain effectiveness (Herridge *et al.*, 2002).

4.2 Materials and Methods

4.2.1 Carrier preparation

Filter mud from Muhoroni sugar cane factory was ground and sieved with 2.212 µ. A total of 10g each of non-sterile ground and sieved carrier filter mud and vermiculite were weighed separately into polythene bags. Final moisture content of each carrier was then re-adjusted to 35% of their water holding capacity (Thirukumaran and Pain, 1983) and autoclaved at 121°C for 3 hours three times. Spore forming microorganisms were allowed to germinate during a 24 hours holding period, and then their vegetative cells were eliminated by the second autoclaving. (Tittabutr *et al*., 2012).

4.2.2 Preparation of inoculants.

Rhizobium tropici CIAT899 and *Bradyrhizobium japonicum*USDA110 obtained from MIRCEN Laboratory University of Nairobi were cultured in yeast extract mannitol broth for 7 days to obtain log-phase ($>10^8$ cells ml^-) culture on an orbital shaker. The volume of broth was 50% of the water holding capacity of the respective carrier materials using sterile syringes and then mixed by hand. The punctured area was wiped with 70% alcohol and sealed. The packets were cured for 14 days before their allocation to refrigerated (4°C) and (~23°C) storage.

4.2.3 Quantification and identification of contaminants from inoculants

The contaminant population was assessed 14 days after injection and consecutively after each 30 days to 165 days using the plate count method in Congo Red YMA (Vincent, 1970). A 10- fold dilution series was made from inoculants and a 20 µl aliquot obtained from the last three dilutions, replicated three times, drop plated and then incubated for 3-7days before observation of contaminant colonies (Somasegaran and Hoben ,1985).

4.2.4 Experimental design and statistical analyses

The research work was conducted at the Laboratory of Soil Microbiology Research MIRCEN of the College of Agriculture and Veterinary Science, University of Nairobi Kabete Campus. *R.tropici* CIAT 899 and *B.japonicum* USDA 110 strains were tested in the following treatments: (1) Filter mud inoculated with *R.tropici* CIAT 899 and *B.japonicum* USDA 110; (2) BIOFIX® containing *R.tropici* CIAT 899 and *B.japonicum* USDA 110; (3) Vermiculite inoculated with *R.tropici* CIAT 899 and *B.japonicum* USDA 110. The BIOFIX was supplied by MEA Ltd a private inoculant manufacturer from Nakuru- Kenya.

Ten grams of each sample were weighed; only the three last dilutions 10^{-5}, 10^{-6} and 10^{-7} were plated on Congo Red YMA using the drop plate method (Miles & Misra 1938; Somasegaran & Hoben 1985). The number of contaminants was determined at six time periods: 14, 45, 75, 105, 135 and 165 days after rhizobium broth culture injection. Each treatment was replicated five times in a completely randomized design (Herridge *et al*., 2002). For each experiment, at each time period, treatment effect and interactions were determined using the General Linear Model procedure of SAS version 9.2 (SAS institute, 2008).

4.3. Results.

The population of contaminants observed in different inoculants was mostly fungi occurring in inoculant, with observation after 75 days of storage (Table 4.1). Over time, carriers showed significant difference from 14 days to 165 days ($p< 0.001$), strain ($p=0.03$) and time ($p<0.007$). There was also interaction between carrier x time ($p< 0.0004$), and interaction between storage time ($p=0.002$), but there was no interaction between storage and strain or strain and storage.

Most of the fungal Contaminants appeared in BIOFIX® inoculant and neither in vermiculite, nor filter mud from MIRCEN Laboratory.

Filter mud and vermiculite were carefully prepared and sterilized at the MIRCEN Laboratory and BIOFIX® was sampled from the commercial manufacturer and was prepared using the same filter mud as in MIRCEN Laboratory. BIOFIX® inoculants had greater fungi contaminants than other carriers (>10^6 cells g^{-1}) especially after 75 of incubation data in Table 4.1. Vermiculite carrier did not show any spores of contaminants during early stages while later some contaminants started to appear in the inoculant formulation.

Table 4.1: Fungal populations in different rhizobium inoculants stored for 165 days (x10^6cells g^{-1}).

Rhizobium **Incolant**	**Strains**	**Incubation time of inoculation**					
		14	**45**	**75**	**105**	**135**	**165**
BIOFIX	CIAT 899	1.00	0.50	1.33	1.00	2.17	0.50
	USDA 110	0.67	0.17	1.17	1.17	1.33	0.17
Filtermud	CIAT 899	-	-	-	-	0.17	0.33
	USDA110	-	-	-	-	0.17	0.50
Vermiculite	CIAT 899	-	-	-	0.50	0.33	0.67
	USDA110	-	-	1.67	0.17	0.17	0.17

$LsD_{0.05\%=}$ 1.07

Within strains *Bradyrhizobium japonicum* USDA 110 showed resistance against colonization by fungal contaminants than in *Rhizobium tropici* CIAT 899 where fungi increased significantly.

In the case of BIOFIX® inoculant the situation was highly pronounced than other inoculants formulation. According to data in Figure, 4.2 refrigeration stimulated higher fungal population in BIOFIX® inoculant than at room temperature. It was observed that fungi population increased after 105 days at room temperature and in refrigeration in all carriers of vermiculite and filter mud. Figure 4.1 below present contaminants of fungi in different inoculants formulation.

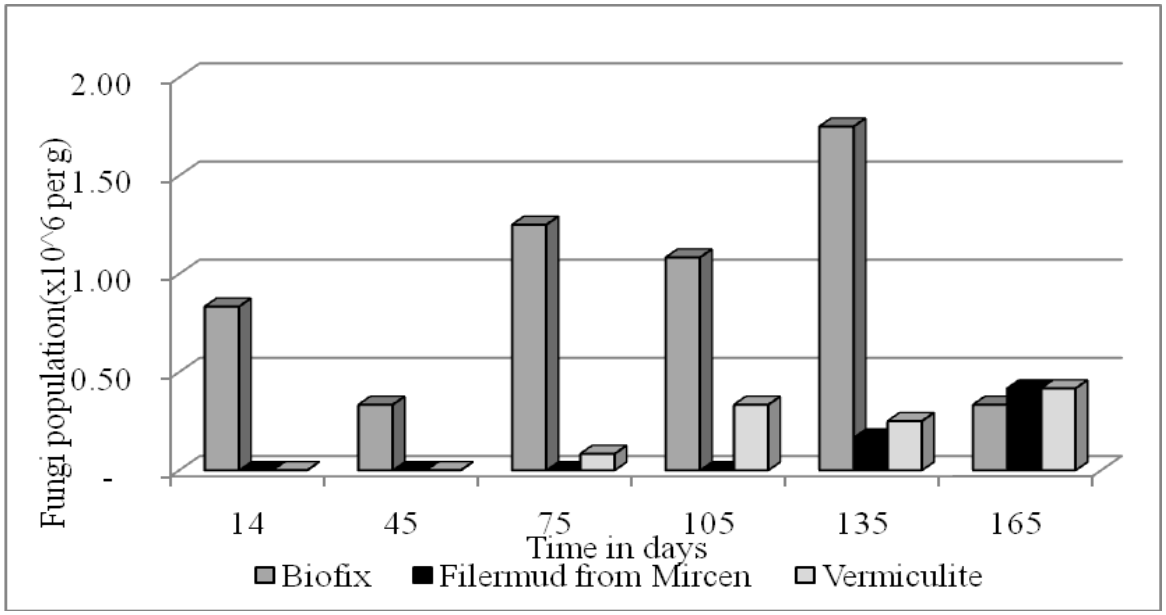


Figure 4.1: Viability of fungi contaminants in different inoculants preparation.

Fungi contaminants were shown to be detrimental for rhizobia growth after 75 and 105 days of incubation depending on inoculant formulation. Data presented in Figure (4.1), confurm the effect of temperature on delayed colonization of this contaminant in different inoculant formulation

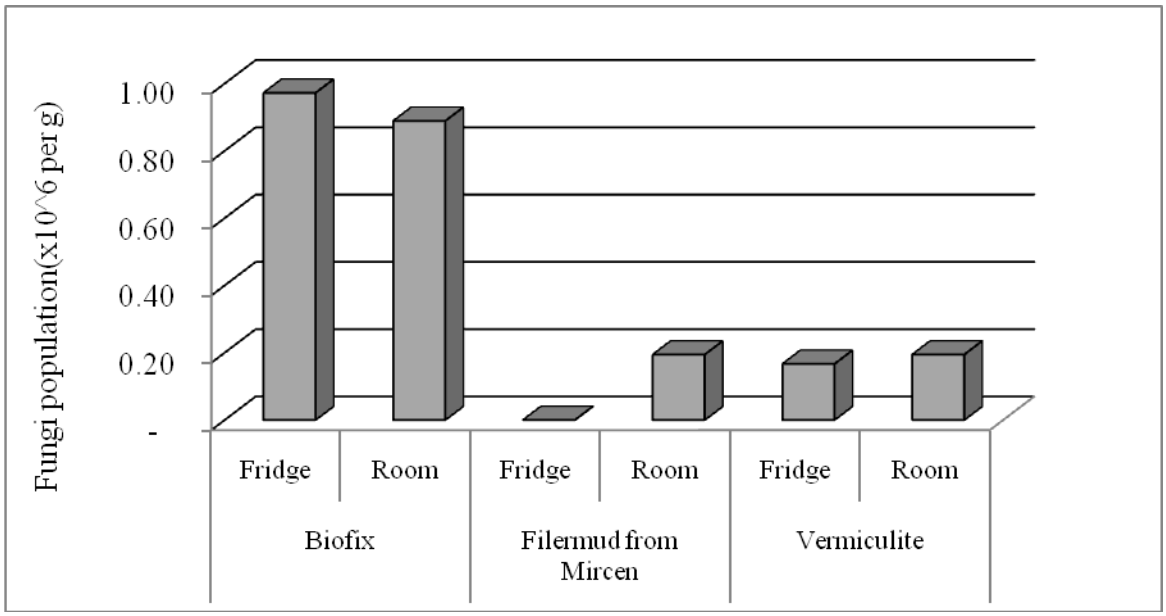


Figure 4.2: Effect of storage temperature on colonization of fungal contaminants in inoculants

4.4 Discussion.

In order to determine whether fungal contaminants had negative impact on survival of *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110 both in BIOFIX®, filter mud and vermiculite, an assessment of these three carriers was carried out. The results in table 4.1 show that BIOFIX® inoculant carrier was different from other carriers. The population of rhizobia declined after 75 and 105 days of incubation when fungal contaminants increased above 10^6 /g of carrier during the incubation. This observation may be due to poor sterilization of the carrier used by BIOFIX®. It is likely that inoculant production for commercial purposes present high demand hence less precision for carrier preparation. Meanwhile sterilization processes of carriers are important whatever quantity is needed for inoculant production. Lateral contamination by fungi was found to be in vermiculite inoculants after 105 days of incubation but not as much as in BIOFIX®. Filter mud which has the same physical and chemical properties

as BIOFIX® showned fewer spores after 135 days of incubation (less than 10^6) when rhizobia were already decreasing bellow to $1x10^9$ cells g^{-1}. This later fact may be explained by first: carrier preparation (sterilization) that was successful. BIOFIX® sampled inoculants were produced using filter mud carrier. Presence of contaminant in BIOFIX indicates problems with factory-level operations. Olsen et al. (1996) also found contaminants were inhibiting growth of rhizobia in culture. It is also well known that contaminants decrease the survival of rhizobia. Later, Yardin et al. (2000) found almost the same results on how contaminant were competitive and would suppress rhizobia growth, then Mohamed and Abdel-Moniem (2010), confirmed declining of rhizobial population can be explained by the fungi contaminants that was a problem with semi decomposed carrier of sugarcane bagasse at the end of the storage period (120 days) an effect that was also observed in our experiment with BIOFIX® inoculants.

It is apparent that presence of fungal contaminants negatively affects the standard rhizobia namely *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110. This increase in fungal contaminants at later stage of incubation and storage in not yet unraveled and deserves future attention. Despite contamination by fungi these two standard strains of rhizobia are still able to cause effective nodulation.

The relationship between storage condition (refrigerated and non- refrigerated) and effect of fungal contaminants was also assessed in this study. Whether BIOFIX® inoculants were stored in refrigerator or not had no effect on the growth of fungi. On the other hand vermiculite and filter mud carriers under refrigerated condition delayed colonization of fungi contaminants. The lower contamination on vermiculite and filter mud may be due to perfect sterilization before injection with broth.

4.5. Conclusion

This study has established the relationships between survival of rhizobia and contaminants. The results from MIRCEN Laboratory indicate that there is a relationship between sterility of the carrier and number of contaminants over time in the inoculants packet. The carrier material had significant influence on rhizobial inoculants quality in this study, Nevertheless the efficiency of sterilization by autoclaving successively three times at 121°C for 3 hours with the holding period of 24 hours during each autoclaving improved the state of the carrier and number of rhizobia remained above $1x10^9$ cells g^{-1} for more than 4 months storage at room temperature. The study reconfirmed that the carrier used by BIOFIX is essentially filtermud based as the one employed at the MIRCEN laboratory and are not different. However, the MEA Ltd carrier is not properly sterilized hence lower numbers of rhizobia in inoculants after storage for six months. The study further showed contamination by fungi is high at MEA LT leading to lower survival of rhizobia in the carrier after 165 days.

CHAPTER FIVE: STORAGE CONDITIONS FOR LEGUME INOCULANTS

Abstract

Storage is an important component in the inoculant delivery systems especially in the tropics where temperatures are higher. The effect of storing inoculants under refrigeration with carriers used by BIOFIX® inoculants containing *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110 legume inoculants was assessed. Standaed Rhizobia strains showed capacity of growth at different refrigerated and room temperature during incubation period of six months. Under both storage condition the counts of rhizobia exceeded the industry standard of 10^9 cells g^{-1} with 2.53 $x10^9$cells g^{-1} in refrigeration against 1.79 $x10^9$cells g^{-1} in room temperature. Response of rhizobia to storage temperature was assessed in Laboratory filter mud, BIOFIX® inoculants from MEA Ltd and vermiculite for a period of 165 days. At the same time, a second experiment assessed the viability of the standard industry rhizobium strains in peat, coconut coir and biochar carriers, under refrigeration and room temperature. The results showed that rhizobia counts were not different was not different ($p= 0.1315$) for a period of 45 days of incubation.

Key words: inoculants, rhizobia, survival, storage, temperature, refrigeration, room temperature.

5.1 Introduction

Microbial inoculants are becoming more available and suitable alternatives to chemical fertilizers. Bacterial inoculants have a very short shelf life. The biological activity may decline rapidly if storage is not done in the correct manner (Kaljeet *et al.,* 2011). Many biotic and abiotic factors affect the growth and survival of rhizobia during its storage process. The type of carrier affects the number of rhizobia that are developed and survival during its incubation process (Dieker *et al*., 2012). However, survival is affected by the initial condition of the cells in the inoculant, particularly the moisture status, age, purity, the initial number, the strain and the type of inoculants (Dieker *et al*., 2004). Inoculant manufacturers prefer products with a shelf-life of 1–1.5 years under warehouse conditions in order to be able to sell them over two cropping seasons. Inoculants must have the potential to increase legume yields. To do so they must meet the standard of an appropriate inoculation rate and thus it must contain the highest possible number of rhizobia after storage (Cartoux *et al.,* 2001). Most inoculants are not used immediately after manufacture and are likely to be 3 to12 months old, or more, when used. Clearly, storage effects are more important as inoculant use increases (Herridge, 2008).

For optimum long-term storage conditions of rhizobial survival in inoculants, it is assumed that, temperature and moisture status remains optimal over time (Roughly *et al.,* 1976; Dieker *et al*., 2011). A suitable rhizobial carrier should have a good water holding capacity, good aeration characteristics, support bacterial growth and survival, and be non-toxic, easily sterilized, manufactured, handled in the field, environmentally friendly, and have good storage quality (Khavasi *et al*., 2007). Moreover, tolerance of the strain to temperature, aeration and storage condition is necessary (Napoles *et al*., 2000; Boiardi *et al*. 1983; Roughley 1970).

NifTAL *et al.,* (1984) reported that shelf life of inoculants will vary with carrier quality and treatment as well as storage temperature. It is important that each country study and formulate an expiration system for inoculants that is realistic and protects the interests of the farmers. However, quality of rhizobial inoculants in many developing countries is limited by the availability of suitable carriers or technological factors (Khavazi *et al*., 2007). The aim of this research was to determine optimum conditions for the storage of inoculated carrier in terms of shelf-life, thus helping smallholder's farmers of East and Central Africa to obtain high quality inoculants during planting season.

5.2 Material and Methods

In order to determine whether refrigeration and room temperature have serious impact on survival of *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110 using different carriers materials for a six months incubation period, shelf life of rhizobia population was monitored using the plate count method as highlighted in section 4.2. In addition to that, peat, coconut coir and biochar were inoculated with *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110 each, and then kept in both refrigeration and room temperature for a period of 30 days after curing.

5.3. Results

Survival of rhizobia strains on inoculants under different conditions were evaluated during this study by the plate counting method, the population dynamic of rhizobia over time under refrigeration are shown in Table 5.1. In additional to carrier type, rhizobium strain and storage condition, as shown in section 4.3, other key interactions related to the storage have significant effect on strain, storage and time interaction (p=0.02). The carrier, strain, storage time, interaction was significant (p< 0.001ed). Similarly the interaction between peat, coconut coir, and biochar were also tested. Results in table 5.2. Report the composition of coconut coir dust from compost of coconut and biochar product obtained from the carbonization of bagasse. The chemical and physical characteristics of these carrier materials found in Table 2.1. Four –way ANOVA of these data resulted in significant effects of carrier material (p<0.001) and time (p<0.001), other interactions were carrier x strain (p=0.002) and carrier x time (p=0.02) but not storage, carrier x storage or strain x storage.

The three carriers (peat, coconut coir and biochar) were sterilized and injected with rhizobia broth culture from the MIRCEN Laboratory, University of Nairobi. Similarly filter mud, vermiculite in the above experiment given similar treatment for comparative purposes and exploring for replacement of filter mud carrier from Muhoroni sugarcane factory. The results demonstrate that peat lead in that group supporting a high population of rhizobia and better survival conditions of rhizobia from day 1 after injection until 45 days when the experiment ended. The inoculants count was more than 1 $x10^9$ rhizobia g^{-1} in both storage at refrigerated and non-refrigerated conditions. Coconut coir came second carrier as it supported the increase of large number of rhizobia after curing. Biochar could not support rhizobia, neither in term of initial colonization nor long- term survival expect on short term after curing with *Rhizobium tropici* CIAT 899. The population dynamics of the two strains across carriers and storage

conditions is presented in Table 5.2. Note that for the holding period of the experiment all the carriers do not show contamination.

Table 5.1: Viability of rhizobium strains in two different storage conditions over six month incubation.

Carrier	Storage condition	Strain	Time in days					
			14	**45**	**75**	**105**	**135**	**165**
Filter mud	Fridge at 4°C	CIAT 899	7.11	6.17	5.37	5.44	4.32	0.84
		USDA110	9.00	3.93	3.57	2.90	0.49	0.46
	Room temperature	CIAT 899	10.47	6.23	4.64	0.83	0.29	0.04
	23°C	USDA110	3.73	3.93	4.70	2.64	0.38	0.04
BIOFIX	Fridge at 4°C	CIAT 899	1.15	8.17	5.00	4.83	0.04	0.03
		USDA110	1.13	4.96	7.57	3.27	0.28	0.05
	Room temperature	CIAT 899	1.15	4.17	3.11	0.56	0.04	0.03
	23°C	USDA110	1.13	6.23	3.67	0.46	0.14	0.03
Vermiculite	Fridge at 4°C	CIAT 899	0.78	1.25	0.70	0.28	0.05	0.04
		USDA110	0.37	0.66	0.28	0.12	0.07	0.07
	Room temperature	CIAT 899	0.78	0.60	0.37	0.26	0.07	0.01
	23°C	USDA110	0.72	2.29	0.80	0.04	0.02	0.01

[a]$LSD_{0.05\%}$ carrier = 2.78, strain= 4.06, storage= 4.05 and time = 4.98

Survival of the two strains in refrigeration and non- refrigeration storage condition is illustrated in Figure 5.1 which shows the extent of *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110 between 105 and 135 days with refrigeration at 4°C. Both methods of inoculants storage demonstrate significant differences over incubation period, although the interaction between strain and storage was not different (p= 0.3981) data in (Figure 5.1).

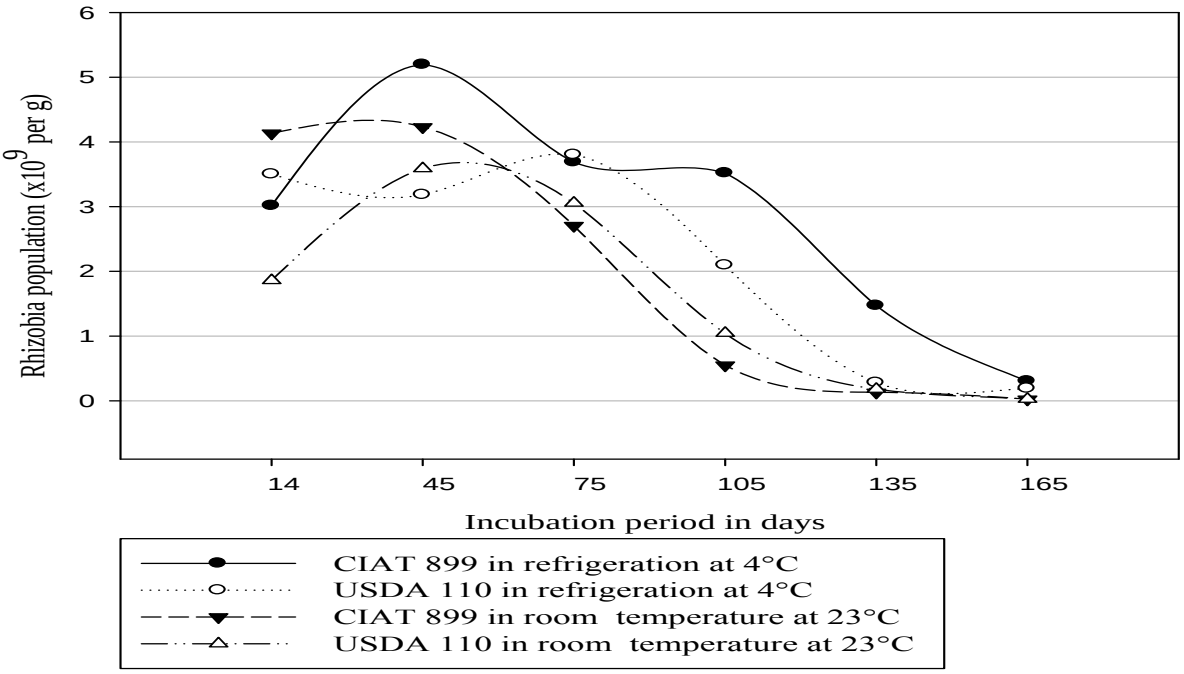


Figure 5.1: Viability of Rhizobia strains incubate in two different conditions

Table 5.2: Variability of two rhizobium strain in Peat, coconut coir and biochar inoculants store in refrigeration and room temperature.

Carrier		Strains	Time in days		
			1	**14**	**45**
Peat	Refrigerated at 4°C	CIAT 899	1.30	3.14	2.63
		USDA110	1.30	3.14	0.46
	Non-refrigerated 23°C	CIAT 899	2.02	4.85	4.33
		USDA110	1.96	3.44	2.63
Coconut coir	Refrigerated at 4°C	CIAT 899	0.73	2.40	0.40
		USDA110	0.73	2.40	0.03
	Non-refrigerated 23°C	CIAT 899	0.09	1.25	0.02
		USDA110	0.09	1.25	0.22
Biochar	Refrigerated at 4°C	CIAT 899	0.54	1.62	0.12
		USDA110	0.54	1.62	0.31
	Non-refrigerated 23°C	CIAT 899	1.79	0.01	0.01
		USDA110	0.80	0.11	0.01

$SE_{0.05\%}$ at (10^8) carrier = 1.97, strain=1.61, storage = 1.51and time=1.97

Survival data (Table 5.2) is compared peat, coconut coir and biochar based incolants in both refrigeration and non-refrigeration conditions in the first experiment. Peat based carrier showed high survival of rhizobia followed by coconut coir and last biochar when standard criteria of $1x10^9$ are considered. Examination of growth of rhizobia over time, revealed an increase of rhizobia population in the all the carriers after 14 days of one magnitude except in biochar based incolant that was stored at 23°C in non-refrigeration. Except for peat the number of rhizobia decreased in all other carriers bellow the standard after 30 days from the curing time. The shelf life of rhizobia is presented in Table 5.3

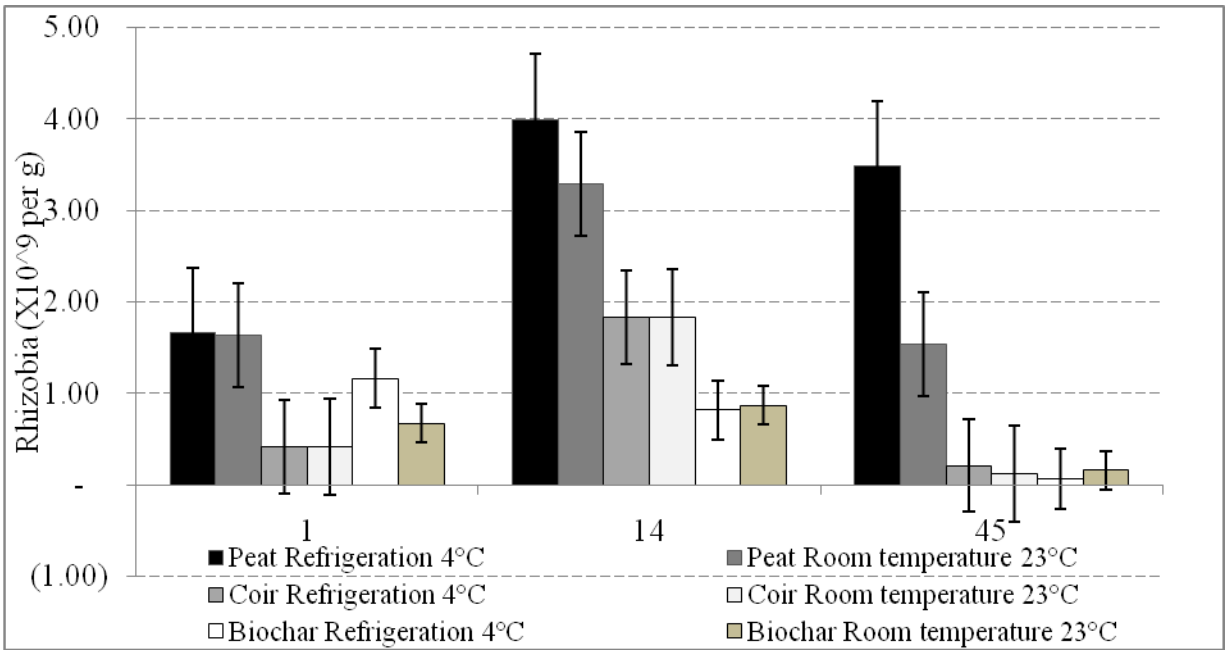


Figure 5.2: Viability of rhizobium in peat, coconut coir and biochar stored in refrigeration and room temperatures

Table 5.3: Shelf lives of peat, coconut coir and biochar based rhizobium inoculant formulations within the 14 days curing period.

Carrier	Storage	CIAT 899	USDA 110
		------- days since injection ------	
Peat	Room	139	107
Peat	Refrigerator	146	244
Coconut coir	Room	35	21
Coconut coir	Refrigerator	36	22
Biochar	Room	27	n.a
Biochar	Refrigerator	29	n.a

5.4. Discussion

The effect of temperature on storage conditions of inoculants was determined in this study using refrigeration set at constant 4°C and non-refrigeration at the room temperature at (about 23°C). The survival of different rhizobia strains in inoculants was assessed. In all cases, survival values of *Rhizobium tropici* CIAT 899 and *Bradyrhizobium japonicum* USDA 110 were higher in refrigerated than non-refrigerated storage condition resulting in $2.52x10^9$ cells g^{-1} compared to $1.79\ x10^9$ cells g^{-1} under room temperature respectively. In BIOFIX® inoculants the initial population of rhizobium was at more than a billion cells g^{-1} and it continued to grow after 105 days of incubation. This population maintained their rhizobial density in refrigeration while in non-refrigeration, rhizobia went below the 10^9 data in (Table 5.1), at the same moment filter mud that was carefully prepared at the MIRCEN Laboratory increased rhizobium numbers to greater than the standard even after 135 days in refrigeration than vermiculite which does not support rhizobia, neither effected by refrigeration. Our results agree with those obtained by NifTAL (1984): shelf life will vary with carrier quality and treatment as well as storage temperature.

From Table (2.1) filter mud carrier from Muhoroni sugarcane factory had pH close to neutral, high water holding capacity, high carbon and nitrogen content compared to vermiculite with high porosity, greater bulk density and lower nutrients contents. This had influence on rhizobia density of greater than 10^9 to be maintained after 105 and 135 days of incubation in filter mud and BIOFIX® inoculants. Anyango *et al*. (1985) reported the physical and chemical characteristics of filter mud approach to the one of peat. Smith (1992) attributed this potential to the high nutrients content to be the key characteristics of a good carrier in peat to support growth of rhizobium in refrigeration. Related to filter mud, Khavazi *et al*. (2007) reported that high organic matter contained in sugarcane bagasse could increase the number of bradyrhizobia after six months of storage. Similar effects upon to our filter mud resulted in an extra 30 days of shelf life. Lower temperature of storage will reduce water loses in the carrier and allow rhizobia to be maintained over time in the inoculant formulation, Yet Dieker *et al*. (2007) indicated that survival is best when changes in moisture status of cells are minimized.

Survival of rhizobia strains in inoculants under storage conditions was different, *Rhizobium tropici* CIAT 899 values were higher in all the carriers and high in refrigeration compared to non-refrigeration and/ or *Bradyrhizobium japonicum* USDA 110 as it appears in Table (5.1 and 5.2). This rhizobial strains will increase or decrease their shelf life depending on nutrients that available in it, the greater population of *rhizobium tropici* CIAT 899 strain in peat may be attributed to genus characteristics which cope and adapt to tropical stress. The same strain can still grow at 40°C. It's thought to be important characteristic in their success as tropical inoculants strain (Hungria *et al*., 2000). Later a study done with liquid inoculants formulation showed survival depended upon the strain of rhizobia as well (Tittubutr *et al*., 2007). However, a study carried out in Australia by Thompson and Roughley (1982) using commercially available

inoculants confirmed the general benefits of cold storage for several rhizobial species. Also, at room temperature large variability in temperature occurs between day and night (17- 23°C), and between months according to Figure 5.1 by using vermiculite carrier, an inorganic material with high moisture retention, the initial. Finally rhizobia density population of the two strains was reduced during the same storage period although it was the same broth culture that was used for filter mud. As we mentioned above the decline may due to lack of nutrients. In the second experiment, biochar inoculant presented the same problem of poor rhizobia colonization by the two strains as in vermiculite inoculant. This could be attributed to lower proportion in carbon content, organic matter and nitrogen sources as well as bulk density as compared to coconut coir that presented higher carbon and organic matter content with lower nitrogen and bulk density (Table 2.1). This allowed the inoculant formulations to increase rhizobia density of one magnitude from 10^8 to 10^9 after curing, before reducing below the industry threshold after 45 days of incubation. Coconut coir carrier results from the composting of coconut and others residues; they may release toxic substance which might be harmful for rhizobia survival. These results agreed with those obtained by Tittabutr (2010) that showed how carrier material had an important influence on inoculant quality; an experiment used composted carrier of reduced bradyrhizobia in the sterile compost after one month of storage. Data in Table 5.2 show how peat maintains the population density to greater than $1x10^9$ rhizobia g^{-1} from day 1 to after 45 days of incubation period for all the strains in both storage conditions. It shows they were more under refrigeration compared to non-refrigerated. After long study Kishore *et al*. (2005) reported the benefit of peat as popular carrier in biofertilizer inoculant production it supports the survival of bacteria in long term storage, it is easy to apply sterilization and facilitating direct colonization

by bacteria. But this solid carrier poses problem of commercial availability especially in the tropic where countries have adopted programs to preserve wetlands.

Long term storage and rapid losses of water on rhizobial inoculants has been reported to be critical to the survival of rhizobia strain, as the case of vermiculite carrier (Table 5.1) and biochar (Table 5.2). Rhizobia cells from these inoculants formulation may be subjected to desiccation and increase in the number of bacteria with compromised membrane. However, previous research indicates that survival is best when changes in moisture status of cells are minimized (Dieker *et al.*, 2007). Boonkerd (1991) used peat carrier in different storage temperatures, and his report showed how temperature was critical with survival of rhizobia substantially greater at 10°C than at 30°C.

5.5. Conclusion

This study was to determine the relationships between storage condition and survival of rhizobia in legume inoculant. Based on the results, refrigeration improved the shelf life of BIOFIX® inoculant and other formulations, but less than expected six months expiry date on the inoculant packet, production and use of rhizobial in Africa (N2Africa, 2011). In some cases (BIOFIX) this may be due to antagonism from contaminants, but in others, where contamination is controlled, there is no easy explanation for this truncated shelf life under improved storage. Refrigeration had a positive impact on exceeding rhizobia shelf life compared to non-refrigeration, although contaminants were not increasing significantly at room temperature. Attempts to improve shelf life by using alternative carriers were only partly successful. Peat works well as a replacement for filter mud, but not coconut coir and biochar which are available in the market and pose problem of poor nutrient content.

CHAPTER SIX: GENERAL CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study conducted at the MIRCEN laboratory of the University of Nairobi established that rhizoium inoculants commercially produced by MEA Ltd for East and Central Africa meet the international standard of 10^{9} cells rhizobium g^{-1}. However when stored at room temperature for 100 days it fall below the standard while if stored in the refrigerator, it attained 140 days which is the guaranteed period of expiry date. The study found out that BIOFIX inoculants are not free from contamination because fungal colonies were identified after 105 days of incubation. Careful preparation of commercial inoculants is suggested as the best way of minimizing contamination. Refrigeration of inoculants before use is recommended in order to maintain the high numbers of rhizobia in the inoculants.

6.2 Recommendations

1. There is a need for an alternative carrier material to organic filter mud that will be able to support greater than $1x10^9$ g^{-1}rhizobia over an extended shelf life, and option for reduction of contaminants in filter mud carrier should continue.

2. Careful preparation of inoculants in the laboratory is needed in order to improve quality of inoculants, particularly through better sterilization and injection of fully grown rhizobia broth culture to the carrier material. Gama irradiation for total sterilization should be applied.

3. Legume inoculants produced in Eastern and Central Africa must be used during the growing season for which they are produced and not carried over to the next season, even when stored under refrigeration

4. MEA Ltd should improve its sterilization process of the filter mud carrier to ensure any or less of contaminants which are harmful to rhizobia inoculant and to revise the 6 months stated expiry date for BIOFIX® inoculant to 4 months when the population is still greater than 10^9 cells rhizobia g^{-1}

APPENDIX 1: YEMA for rhizobial place counting media

Nutrients	g or ml per liter	Micronutrients	mg per liter
K_2HPO_4	0.5	H_3BO_3	1.0
NaCl	0.2	$ZnSO_4$	1.0
$CaSO_4 \cdot 2H_2O$	0.1	$CuSO_4 \cdot 5H_2O$	0.5
$MgSO_4 \cdot 7H_2O$	0.2	$MnCl_2 \cdot 4H_2O$	0.5
Mannitol	10.0	$Na_2MoO_4 \cdot 2H_2O$	0.1
Yeast extract	2.0	Fe-EDTA (Sequestrene)	10.0
Agar	15.0		
Congo red	10		

APPENDIX 2: N- Free Nutrient Solution (Broughton and Dillworth, 1970).

Stock Solution	**Element**	**M**	**Form**	**MW**	**g/L**	**M**
1	Ca	1000	$CaCl_2.2H_2O$	147.03	294.1	2.0
2	P	500	KHP_2 O4	136.09	136.1	1.0
3	Fe	10	Fe-citrate	355.04	6.7	0.02
	Mg	250	$MgSO_4.7H_2O$	246.5	123	0.5
	K	250	K_2SO_4	174.06	87.0	0.5
	Mn	1	$MnSO_4.H_2O$	169.02	0.338	0.002
4	B	2	H_3BO_3	61.84	0.247	0.004
	Zn	0.5	$ZnSO_4.7H_2O$	287.56	0.288	0.001
	Cu	0.2	$CuSO_4.5H_2O$	249.69	0.100	0.0004
	Co	0.1	$CoSO_4.7H_2O$	281.12	0.056	0.0002
	Mo	0.1	$Na_2MoO_2.2H_2O$	241.98	0.048	0.0002

APPENDIX 3: Class level and fixed effect *rhizobia* of Analysis of variance for BIOFIX, FILTERMUD and VERMICULITE inoculants.

Class	**Levels**		**Values**	
carrier	3		Biofix, Filter mud Vermiculite	
strain	2		USDA110 CIAT 899	
storage	2		Refrigeration Room temperature	
time	6		1 2 3 4 5 6	
rep	5		1 2 3 4 5	
day	6		14 45 75 105 135 165	
Total Observations			360	
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
carrier	2	288	80.38	<.0001
strain	1	288	6.10	0.0141
carrier*strain	2	288	4.07	0.0181
storage	1	288	12.22	0.0005
carrier*storage	2	288	4.25	0.0152
strain*storage	1	288	0.72	0.3981
carrie*strain*storag	2	288	0.29	0.7503
day	5	288	39.44	<.0001
carrier*day	10	288	14.43	<.0001
strain*day	5	288	1.22	0.3006
carrier*strain*day	10	288	0.58	0.8274
storage*day	5	288	1.81	0.1116
carrier*storage*day	10	288	1.48	0.1451
strain*storage*day	5	288	2.71	0.0207
carr*strai*stora*day	10	288	3.29	0.0005

APPENDIX 4: Class level and fixed effect of rhizobia of Analyses of variance for BIOCHAR, FILTERMUD and VERMICULITE inoculants.

Class	**Levels**		**Values**	
carrier	3		Biochar , Coconut coir ,Peat	
strain	2		USDA110 CIAT 899	
storage	2		Refrigeration Room temperature	
time	3		1 2 3	
rep	5		1 2 3 4 5	
day	3		0 14 45	
Total Observations			180	
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
carrier	2	144	30.73	<.0001
strain	1	144	0.13	0.7220
carrier*strain	2	144	6.30	0.0024
storage	1	144	2.30	0.1315
carrier*storage	2	144	1.45	0.2389
strain*storage	1	144	0.14	0.7134
carrie*strain*storag	2	144	0.16	0.8556
day	2	144	11.22	<.0001
carrier*day	4	144	2.97	0.0214
carrier*storage*day	4	144	0.91	0.4596
strain*storage*day	2	144	0.25	0.7805
carr*strai*stora*day	4	144	0.24	0.9143
carrier*storage*day	4	144	0.91	0.4596
strain*storage*day	2	144	0.25	0.7805

APPENDIX 5: Class level and fixed effect Fungi contaminants of Analysis of variance for BIOFIX, FILTERMUD and VERMICULITE inoculants.

Class	**Levels**		**Values**	
carrier	3		Biofix, Filter mud Vermiculite	
strain	2		USDA110 CIAT 899	
storage	2		Refrigeration Room temperature	
time	6		1 2 3 4 5 6	
rep	5		1 2 3 4 5	
day	6		14 45 75 105 135 165	
Total Observations			360	
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
carrier	2	284	42. 85	<.0001
strain	1	284	4.57	0.0333
carrier*strain	2	284	2.13	0. 1211
storage	1	284	1.43	0.2328
carrier*storage	2	284	1.02	0.3606
strain*storage	1	284	0.12	0.7266
carrie*strain*storag	2	284	0.19	0.8254
day	5	284	3.25	0.0072
carrier*day	10	284	3. 36	0.0004
strain*day	5	284	0.33	0.8975
carrier*strain*day	10	284	0.56	0.8424
storage*day	5	284	3.77	0.0026
carrier*storage*day	10	284	1.07	0.3843
strain*storage*day	5	284	0.81	0.5459
carr*strai*stora*day	10	284	0.35	0.9646

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