IMPACT OF BIOFERTILIZER INOCULANTS ON YIELDS OF COWPEA VARIETIES GROWN IN OMUSATI REGION OF NAMIBIA

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Abstract

Smallholder farmers in Omusati dependent on subsistence agriculture on poor sandy soils for their livelihood. They rarely apply nitrogen fertilizer during legume production due to their high cost of fertilizer. However, soil microorganisms specifically bacteria called rhizobia can contribute to increasing plant growth and yield. Therefore, the aim of this study was to test the effect of introduced biofertlizer inoculants strain 1-7 and 14-3 on yields and growth of cowpea varieties cultivated in field experiments in Omusati region Namibia, then isolate and identify Rhizobia from nodules collected from field experiments at Mashare Irrigation and Training Center (MITC) (Kavango region). Five cowpea varieties namely Lutembwe, Bira, I2, Nakare and Shindimba were grown in four replicates in Omusati region (Ogongo campus), three treatments were used (Nitrogen fertilizer, Bioinoculant and non-Bioinoculant plots). Rhizobia were obtained from nodules of local varieties of cowpeas and identified by sequence analyses of *rpoB* genes. The average mean of all three treatments showed that Bira had the highest grain yield production with estimated mean of 1769kg/Ha, Nakare 1561 kg/Ha, Lutembwe 1344 kg/Ha, Shindimba 1161 kg/Ha and lastly I2 with lowest grain estimated mean of 841 kg/Ha. Rhizobia Isolated from MITC were identified as *Bradyrhizobium* sp. 1-7 and *Bradyrhizobium elkanii*. The study suggested that application of the bio-inoculants enhanced productivity.

Key words: Cowpea, Nitrogen fixation, grain yield, *Bradyrhizobium*

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List of Abbreviations and/or Acronyms

BNF: Biological nitrogen fixation

DNA: deoxyribonucleic Acid.

FAO: Food and Agriculture Organization

HGT: horizontal gene transfer

***Hsn*:** Host specific nodulation genes

IS: Insertion sequence

MITC: Mashare Irrigation and Training Center

N: Nitrogen

***Nod, Noe, Nol*:** Nodulation genes

***Nif*:** Nitrogen fixing genes

PVP-40: polyvinylpyrrolidone 40

RNA: Ribonucleic acid

SSA: Sub Saharan Africa

Sym: Symbiosis

tRNA: transfer Ribonucleic acid

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Dedication

This paper is dedicated to Family; Namunji Mukelabai, Margret Nyirenda Mukelabai, Thandiwe Mukelabai and Elisha Sinyinza.

DECLARATIONS

I, Mukelabai Muyoba Florence, hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

Mukelabai Muyoba Florence

M. 08/04/2019.

Name of Student **Signature** **Date**

1. Introduction

1.1. Background of the study

Variability of yields, risk for crop failure, and limited financial resources are among the factors that contribute to farmers' reluctance to adopt new technologies in Africa's rainfed agricultural systems (Ogada et al. 2010). Therefore, risks are predicted to increase in many areas in Sub Saharan Africa (SSA) due to climate change, as SSA is projected to suffer a decline in land area suitable for cultivation of crops (Lane & Jarvis, 2007).

The impact of climate change in Namibia is already being felt according to Wilhelm (2012). In 2012 it was observed that the ground water recharge had reduce throughout the country; agricultural dry-land crop productivity was reduced by up to 50% in north-central areas and by about 20% in the north-eastern regions due to climate change (Wilhelm, 2012). Drought is one of the major limiting factors in crop production; it can affect crop yield severely. Furthermore, it has been reported that the availability of water for agriculture purposes had reduced (Abed, 2014).

There are several dimensions to the problem of increased food insecurity; the fundamental cause was declining staple food production in Namibia (Taapopi et al. 2018). The reduced yields of important food crops were attributed to the increase in frequency and intensity of cyclical droughts, the use of rudimentary farming practices and the low input nature of the conventional farming systems in the country (Hase, 2013).

This is also coupled with the lack of incentives to farmers for engaging in optimal land management policies that would speed up the need for technological change, leading to low agricultural productivity and consequently increased food insecurity (Ali et al.,

2017). Furthermore, very few farmers use farming practices that physically conserve the soil, and as a result, farmers are trapped in a vicious circle of poverty and hunger as the soils continue to degrade unabated (Taapopi et al. 2018).

Omusati is the third populated Region in Namibia with a population size of 243 166 out of which 133 621 are females and 109 545 are males, the region has a degraded landscape, rainfall that ranges from 250-300mm and has temperatures that range with a minimum of 14°c and maximum of 32°c according to Lisao et al. (2018). Smallholder farmers in Omusati area depend on subsistence agriculture on poor sandy soils for their livelihood (Reinhold-hurek, 2015: Simon et al. 2014).

Nitrogen (N) is required in large amounts for plant growth, since it is the basic constituent of many chemical compounds, including proteins and nucleic acids. N is applied to the soil mainly in the form of synthetic chemical fertilizers, which in the long run may pollute soil and underground water according to (Farahvash et al. 2010). Besides, it raises the cost of crop production. Therefore, biological means need to be further explored to supply nitrogen for plant growth and reduce these adverse effects (Farahvash et al. 2010).

Fortunately, soil microorganisms specifically bacteria called rhizobia can colonise the rhizosphere, infect legume roots and biologically fix nitrogen in soil, hence making soils fertile for plant growth (Mohammadi &Sohrabi, 2012: Eskin, 2012). Biological nitrogen fixation is a key factor to sustain agricultural productivity. Increasing crop yield cannot be fulfilled unless we carefully make use of biological nitrogen fixation (Farahvash et al. 2010.

Since the nodulation is solely a physiological process, it can be induced by using different plant growth regulators. In this process nitrogen fixing bacteria enter plant through its root

hairs where the nodules will be formed. These nodules can provide a niche for the bacteria to fix atmospheric nitrogen. They not only fix atmospheric nitrogen but also produce certain plant growth promoting hormones (Farahvash et al. 2010). These bacteria belong to several genera including *Azotobacter*, *Azospirillum*, *Bacillus*, *Arthrobacter*, *Enterobacter*, *Pseudomonas*, *Alcaligenes* and *Klebsiella* (Farahvash et al. 2010).

To develop and adopt efficient Biological nitrogen fixation (BNF) and inoculant technologies, SSA still faces several obstacles such as:

- Limited knowledge of inoculation responses of nodulating bacteria to local Cowpea varieties, and studies on other factors inhibiting BNF; hence the basis for decision-making on biotechnology issues is weak in SSA (Chianu et al. 2011).
- Poorly developed marketing strategies and infrastructure, and limited involvement of the private sector in the distribution of inoculants (Chianu et al. 2011).
- Limited farmer awareness about the technology and limited access to inoculants. Furthermore, farmers in the Omusati region mainly use land races of the *Vigna unguiculata* that are not well-defined with respect to disease resistance or resistance to a parasitic weed such as *Alectra vogelii*, which often causes cowpea infections in farmer's fields (Grönemeyer et al. 2012).

No previous studies have been done on the effect of biofertilizers on yields and growth of cowpea variates in Omusati region. This paper therefore explores the effect of introduced biofertlizer inoculants strain 1-7 and 14-3 on yields and growth of cowpea varieties cultivated in field experiments in Omusati region Namibia.

1.2. Statement of the problem

The smallholder farmers in Omusati have faced a very low crop growth and productivity, due to declining soil fertility as a result of low utilization of rhizobial nitrogen fixing strains (Haiyambo et al. 2015; Grönemeyer et al. 2012). Furthermore, no previous studies have been done on the effect of biofertilizer inoculants on yields of cowpea variates in Omusati region.

1.3. Objectives of the study

The aim of this study was to test the effect of introduced biofertilizer inoculants on yields and growth of cowpea varieties cultivated in field experiments in Omusati region Namibia, then isolate and identify Rhizobia from nodules collected from replicated field experiments at Mashare Irrigation and Training Center (MITC) (Kavango region)

a) To test the effect of the introduced biofertilizer inoculants strain 1-7 and 14-3 on yields of cowpea varieties cultivated in field experiments at Ongongo campus (Omusati region Namibia).
b) To isolate and identify Rhizobia from nodules collected from replicated field experiment at Mashare Irrigation and Training Center (MITC) (Kavango region).

1.4. Hypothesis of the study

Null hypothesis: There was no significant difference between the yields of the biofertilizer (inoculant) plots and the non-inoculated plots.

Alternative hypothesis: There was a significant difference between the yields of the biofertilizer (inoculant) plots and the non-inoculated plots.

1.5. Significance of the study

The study will contribute towards improving the soil fertility in Omusati region. Consequently, this will contribute to improving the food security, provide a source of income to the smallholder farmers and improve their nutrition.

1.6. Limitation of the study

This study only used culture dependent methods to isolate Rhizobacteria from nodules, meaning not all bacteria were isolated, hence culture independent bacteria were not isolated.

1.7. Delimitation of the study

The findings of this study can only apply to Omusati region, because this study was done to assess the impact of Rhizobia inoculants on yields of cowpea variates grown in Omusati region Namibia. And study can only be applicable to cowpeas, and not any other crop

2. Literature review

In Namibia cowpea is the third most important staple crop grown after pearl millet (*P. glaucum*) and sorghum (*Sorghum bicolor*) (Grönemeyer et al. 2012: McDonagh & Hillyer, 2003). Poverty reduction in such small farm households could be achieved by improving yields of traditional crops through a well-established cowpea improvement program required to develop farmers-preferred and locally adapted varieties for sustainable production and productivity (Horn, 2016).

2.1. Cowpea plant

2.1.1. Botanical description and nutritional value

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most important food legumes in the tropic and sub-tropic regions where drought is a major production constraint due to low and erratic rainfall (Singh et al. 2003b). The crop is grown mainly as a grain legume, but the leaves and fresh green pods are also eaten as vegetables in many parts of Sub-Saharan Africa. Nutritionally, the grain contains 23.4% protein, 56.8% carbohydrates), while the green leaves eaten as vegetables contain between 27.1% and 34.7% protein (Singh et al. 2003b). Cowpea is a major source of protein, minerals and vitamins in daily human diets and is equally important as nutritious fodder for livestock (Singh et al. 2003b).

Among the popular crops grown in Central and West Africa, cowpeas are drought tolerant plants (Kuykendall et al. 2000; Martins et al. 2003). Nevertheless, cowpea still suffers considerable damage due to frequent drought in the Savanna and Sahel sub region. Early maturing varieties escape terminal drought, but if exposed to intermittent moisture stress during the vegetative growth stage, they perform very poorly (Martins et al. 2003).

Additionally, the early maturing cowpea cultivars tend to be very sensitive to drought that occurs during the early stages of the reproductive phase (Agbicodo et al. 2009). Therefore, genetic enhancement of cowpea for drought tolerance by incorporating drought tolerance into early maturity cowpea lines represents the best and most cost-effective method for insuring sustainable and improved crop yield in variable and changing climates (Agbicodo et al. 2009)

2.2 Production constraints to cowpea

2.2.1. Biotic constraints

2.2.1.1. Parasitic weeds

Striga gesnerioides and *Alectra vogelii* (figure 1) are the two major parasitic weeds affecting cowpea production in SSA (Ayen et al. 2017). The weeds grow and attach themselves on the root surfaces of the host where they absorb nutrients. *Alectra* causes serious yield losses in cowpea production in Namibia (Horn et al. 2015). Noubissietchiagam et al. (2010) documented the negative effects of *Striga* on cowpea production such as severe yield reduction. *S. gesnerioides* has been identified to cause yield losses ranging from 40 to 70 (Ayen et al. 2017). A large part (about 70%) of the damage to the crop occurs even before the parasite emerges from the soil thereby making weeding an ineffective option in reducing yield losses (Ayen et al. 2017). Seeds of the parasitic weed can remain dormant in the soil for over 20 years making it difficult to control using traditional methods (Kabambe et al. 2002; Kabambe et al. 2013).

One of the possible ways in controlling *Striga* and *Alectra* is by reducing its seed bank in the soil, this can be achieved by removing the parasitic weeds after germination and before flowering and seed set (Horn et al. 2015). Timko et al. (2007); Kabambe et al. (2013) reported some of the progress made in breeding cowpea for resistance to Striga and Alectra using conventional breeding methods.

Figure 1: Photo of Alectra sessiliflora which is a parasitic plant. Interestingly it seems the cowpeas it is growing with is actually an Alectra resistant cultivar taken from experimental field at UNAM Ongongo campus.

2.2.2. Socio-economic constraints

Sabo et al. (2014) and Horn et al. (2015) outlined several socio-economic constraints adversely affecting cowpea production in SSA. These includes non-availability of market preferred varieties, low yield potential, high cost of farmland preparation, lack of improved production and harvesting tools, high cost and absence of labour, high cost and adulteration of pesticides, poor harvest prices, and underdeveloped marketing channels. Other major constrains to cowpea production in many SSA countries is lack of defined value chain and poor development of cowpea as a commodity crop according to Horn et al. (2015).

There is no efficient transport systems and cowpea trading is not organized due to limited value addition and lack of cowpea enterprises (Fakayode et al. 2014). In Nigeria and other west African countries farmers solely survive on cowpea farming which is the major

economic mainstay and business (Aboki & Yuguda, 2013). It was also reported in west Africa cowpea trades enables farmers to buy other cereal grains and farm inputs such as fertilizers making it easy to have access to agricultural inputs (Fakayode et al. 2014).

In Namibia farmers earn cash incomes from sales of cowpea grains (Horn et al. 2015) though the monetary values of cowpea products are low. The full economic potential of cowpea will only be realized if other value-added products especially those targeted at the ever-growing urban population, are introduced.

Waddington et al. (2010) suggested that converting cowpea into baby food might bring about a rise in the price of the commodity which will also bring higher returns to the producer. Cowpea is an important weaning food in many communities in Africa and Asia. In SSA its demand is particularly high (Ibro et al. 2014). Raising the average yield per hectare of the crop will therefore increase the annual global production and hence the revenue.

2.3 Drought tolerance mechanisms

Several factors and mechanisms operate independently or jointly to enable plants to cope with drought stress. Therefore, drought tolerance is manifested as a complex trait (Krishnamurthy et al. 1996). Traditionally, drought tolerance is defined as the ability of plants to live, grow, and yield satisfactorily with limited soil water supply or under periodic water deficiencies (Ashley, 1993). According to Mitra (2001), the mechanisms that plants used to cope with drought stress can be grouped into three categories such as drought escape, drought avoidance and drought tolerance. However, crop plants use more than one mechanism at a time to cope with drought (Agbicodo et al. 2009).

Drought escape is defined as the ability of a plant to complete its life cycle before serious soil and plant water deficits occur. This mechanism involves rapid phenological development (early flowering and early maturity), developmental plasticity (variation in duration of growth period depending on the extent of water deficit) and remobilization of parenthesis assimilates (Agbicodo et al. 2009).

Drought avoidance is the ability of plants to maintain relatively high tissue water potential despite a shortage of soil-moisture (Agbicodo et al. 2009). Plants develop strategies for maintaining turgor by increasing root depth or developing an efficient root system to maximize water uptake, and by reducing water loss through reduced epidermal (stomatal and lenticular) conductance, reduced absorption of radiation by leaf rolling or folding and reduced evapotranspiration surface (leaf area) (Mitra, 2001).

Drought tolerance is the ability of plants to withstand water-deficit with low tissue water potential. The mechanisms of drought tolerance are maintenance of turgor through osmotic adjustment (accumulation of solutes in the cell), increased cell elasticity and decreased cell size and desiccation tolerance by protoplasmic resistance (Agbicodo et al. 2009).

However, all these adaptation mechanisms of the plant to cope with drought have some disadvantages with respect to yield potential. For instance, a genotype with a shortened life cycle usually yields less compared to a genotype with a normal life cycle (Simon et al. 2014). The mechanisms that confer drought avoidance by reducing water loss (such as stomatal closure and reduced leaf area) decrease carbon assimilation due to reduction in physical transfer of carbon dioxide molecules and increase leaf temperature thus reducing biochemical processes, which negatively affects yield (Agbicodo et al. 2009).

Plants try to maintain water content by accumulating various solutes that are nontoxic (such as fructans, trehalose, polyols, glycine betaine, proline and polyamines) and do not interfere with plant processes and are called compatible solutes (Yancey et al. 1982). However, many ions concentrated in the cytoplasm due to water loss are toxic to plants at high concentrations leading to what is termed a glassy state. In this condition whatever liquid is left in the cell has a high viscosity, increasing the chances of molecular interactions that can cause proteins to denature and membranes to fuse (Hartung et al. 1998). Consequently, crop adaptation to water stress must reflect a balance among escape, avoidance and tolerance while maintaining adequate productivity.

Drought escape, avoidance, and tolerance mechanisms have been described in cowpea (Agbicodo et al. 2009). However, the drought response pathways associated with these mechanisms are not yet understood, and the degree to which these adaptations operate jointly or separately to allow the crop to cope with drought still needs to be established.

2.4 Nitrogen fixation in legumes and its benefits

Nitrogen fixing organisms.

Organisms that can fix nitrogen, i.e., convert the stable nitrogen gas in the atmosphere into a biologically useful form, all belong to a biological group known as prokaryotes but not all bacteria can fix nitrogen. Nitrogen fixing organisms are generally categorized as (1) symbiotic N_2-fixing bacteria including members of the family rhizobiaceae (*Rhizobium, Sinorhizobium, Bradyrhizobium, Mesorhizobium* and *Azorhizobium*, collectively termed rhizobia) which forms symbiosiswith leguminous plants (Zahran,

2001) and nonleguminous trees (e.g. *Frankia*) and (2) non-symbiotic (free living, associative and endophytes) nitrogen fixing forms such as cyanobacteria (*Anabaena, Nostoc*), *Azospirillum, Azotobacter, Gluconacetobacter diazotrophicus*and *Azocarus,* etc. (Bhattacharyya & Jha, 2012). The signalling processes (Long, 2001), the evolutionary history (Henson, Watson, & Barnum, 2004) and the molecular aspects determining host specificity in the rhizobial–legume symbiosis (Young, Mutch, Ashford, Zézé, & Mutch, 2003), have been reviewed.

All organisms which reduce dinitrogen to ammonia do so with the aid of an enzyme complex, nitrogenase (Zahran, 2001). The nitrogenase enzymes are irreversibly inactivated by oxygen, and the process of nitrogen fixation uses a large amount of energy (Zahran, 2001). Nitrogenase activity is usually measured by the acetylene reduction assay, which is cheap and sensitive (Zahran et al. 2012).

The 15N isotopic method, which is also used to measure N_2 fixation, is accurate but expensive (Zahran, 2001). A wide range of organisms can fix nitrogen. However, only a very small proportion of species can do so; 2 genera of archaea, 38 genera of bacteria, and 20 genera of cyanobacteria have been identified as diazotrophs or organisms that can fix nitrogen (Zahran et al. 2012).

At the genetic level, rhizobial nodulation is controlled by the nodulation genes *nod, noe*, and *nol* (Steenkamp et al. 2008). The nodulation genes determine the formation and structure of Nod factors (NFs), which represent the primary signalling molecules implicated in host infection and nodule organogenesis (Steenkamp et al. 2008). The structural *nodABC* genes are absolutely essential for symbiosis and their products (*NodA*, acyl transferase; *NodB*, deacetylase; *NodC*, N-acetylglucosaminyl transferase) produce a

b-1,4-linked N-acylated-Dglucosamine backbone molecule consisting of three to five sugar units and a fatty-acid group (with varied length and saturation) attached to the non-reducing end (Steenkamp et al. 2008).

This lipo-chitooligosaccharide constitutes the NF core and may be decorated by the products of a class of nodulation genes that are often referred to as host-specific nodulation (*hsn*) genes (Steenkamp et al. 2008). Various studies have shown that such modifications of the NF core, especially at the reducing end, are important determinants of the host range of a specific rhizobial strain (Perret et al. 2000). As rhizobia use different sets of *hsn* genes for decorating their NFs, rhizobial species and lineages differ broadly in their complement of these genes (Moulin et al. 2004).

Numerous studies have demonstrated that horizontal gene transfer (HGT) plays a significant role in the evolution of rhizobia (Flores et al. 2005; Young et al., 2006), as well as their host range (Moulin et al. 2004; Suominen et al. 2001). In some instances, HGT may result in "patchy" compositions of individual genomes where not all individuals have the same complement of genes (Koonin et al. 2001; Welch et al., 2002) as is evident from the erratic distribution of nodulation genes among rhizobial species and lineages (Moulin et al. 2004).

Also, the evolutionary histories of genes acquired through HGT (and usually genes encoded on the accessory genome) are mostly incongruent with those of genes that form part of the core genome (Koonin et al. 2001; Philippe & Douady, 2003). Phylogenetic analyses of nodulation or nif genes typically reveal substructures in rhizobial populations that are remarkably different from those using core genome genes (e.g., Laguerre et al. 2001; Mutch et al. 2003; Qian & Parker, 2002). For example, *Bradyrhizobium nodC* and

nodA sequences from a specific host and/or geographic region, regardless of the *Bradyrhizobium* species harboring the sequence, are more closely related than to those from other legume hosts and/or geographic regions (Ste pkowski et al. 2005; Vinuesa et al. 2005).

Current models for the evolution of rhizobia therefore hold that different rhizobial lineages in various geographic areas, diversify through HGT of sym loci to generate locally adapted genotypes or so-called biovarieties that are associated with a specific group of hosts (Laguerre et al., 2001; Mutch & Young, 2004; Parker et al. 2002; Vinuesa et al. 2005).

A recent study demonstrated that Bradyrhizobium isolates associated with the root nodules of cowpea and peanut (Arachis hypogaea) in Botswana and South Africa are genetically diverse (Law et al. 2007). Genomic fingerprints of these bacteria revealed several clusters of isolates that are less than 60% similar. This diversity was also reflected by the symbiotic properties of these bacteria, as they displayed a range of nodulation and nitrogen fixation abilities on one or both hosts.

Host specificity refers to the ability of rhizobia species to form nodules on specific legumes (Simon et al. 2014). The approach of using effective or superior exotic rhizobia strains as inoculants has failed in various environments due to various reasons including the use of ineffective and non-competitive rhizobia strains as inoculants (Slattery et al. 2004). The host specificity leads to a perfect match between legume and rhizobia resulting into effective nodules (deep red inside) formation and nitrogen fixation (Simon et al. 2014). If cross inoculation with no perfect match has occurred, ineffective nodules (green or white inside) or no nodules may be formed, and nitrogen fixation does not occur (Gwata et al. 2004).

Benefits of Nitrogen fixation in legumes

Nitrogen is the most limiting nutrient for growth of leguminous plants like common beans, soya beans, cowpeas and garden peas (Howieson & Committee, 2007). Nitrogen is crucial in plant cells for synthesis of enzymes, proteins, chlorophyll, Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA), thus vital for plant growth and production of food and feed (Matiru & Dakora, 2004).

Soil microorganisms specifically rhizobia can infect legume roots and biologically fix nitrogen (Mohammadi & Sohrabi, 2012). Especially under unfavorable environmental conditions or under pest pressure, plant-growth-promoting rhizobacteria may lead to increased nutrient uptake or enhanced plant defense responses against pathogens (Reinhold-Hurek et al. 2015). Biological Nitrogen fixation is a process of converting elemental nitrogen into the form ammonia (4 NH^{+}) and nitrate (3 NO^{-}), hence making Nitrogen useable by plants (Simon et al. 2014).

Rhizobia can live on plant residues (saprophytes) or entirely within plants (endophytes) or (rhizobacteria) or in close association with the plant roots (Mohammadi & Sohrabi, 2012). Based on ability to fix nitrogen, rhizobia are classified into slow (*Bradyrhizobium*) and fast-growing *Rhizobium*. The growth of *Rhizobium* visible in Yeast Extract Mannitol Agar (YEMA) is 3 - 5 days while *Bradyrhizobium* takes 6 - 8 days (Simon et al. 2014).

The process in which the rhizobia colonize the rhizosphere, infect the roots and fix nitrogen leads to plant development and grain yield improvement (Matiru & Dakora, 2004). The effectiveness of rhizobial populations in fixing nitrogen is correlated with soil

fertility status where acidic soils have been reported to contain fewer effective rhizobia strains (Doyle & Luckow, 2003).

Legumes are one of the most diverse plants on earth widespread in tropics and temperate zones (Sprent & James, 2007). Legumes belong to the superfamily of angiosperms (*Leguminosae/Fabaceae*) from the order Fabales and the clade eurosid (Doyle & Luckow, 2003: Tran & Nguyen, 2009). Legumes can grow in much degraded soils because they can fix nitrogen in association with rhizobia (Wong, 2003: Freitas et al. 2004). Besides its major role in the traditional diets throughout the world, legumes provide a multiple benefit to both soil and other crops through intercropping (Freitas et al. 2003: Stajković et al. 2011). Despite vast and potential uses of grain legumes like soybean, cowpea, common bean and peas as human food, animal feed and soil fertility enhancer, they can also be grown in different agro-ecological environments (Hendawey & Younes, 2013).

Small-scale farmers who are the major legume producers in Africa rarely apply fertilizers during legume production due to their low income; hence the crop is largely dependent on fixed nitrogen from symbionts (Simon et al. 2014). In most cases, native nitrogen fixers are competitive to inoculants, but they may not be an efficient strain and possibly incompatible to the host plant (Simon et al. 2014). Therefore, relying on native nitrogen fixers without prior information on its efficiency and compatibility with host legume leads to crop production failure (Simon et al. 2014).

The interaction between plants and soil microorganisms occurs much in the rhizosphere (Marschner et al. 2011). The legume-rhizobial symbiosis has a large impact on success of legumes, hence the atmospheric nitrogen the organisms fix can be more than the fertilizer nitrogen an average farmer can afford to buy and apply (Rumjanek, 2003). Therefore,

legume-rhizobia symbiosis can provide easy and inexpensive way to enhance soil fertility and improve crop production (Roychowdhury et al. 2013).

The root nodule rhizobia approximately reduce 20 million tons of atmospheric nitrogen to ammonia which is 50% - 70% of the world biological nitrogen fixation (Rumjanek, 2003). The higher fixed nitrogen in hosts determines the success of symbiotic relationship between legumes and rhizobia (Rumjanek, 2003). However, host range expansion may be limited by the symbiont distribution while hosts can potentially acquire different rhizobia when invading new habitats (Parker, 2006).

Amongst other advantages such as drought tolerance, cowpea may benefit from an efficient N– fixing symbiosis when associated with effective rhizobia (Rumjanek, 2003). The symbiotic specificity and host range of indigenous tropical rhizobia which nodulate cowpea and other tropical legumes has not yet been well characterized, but rhizobia are often described as promiscuous, capable of nodulating a wide range of legumes but with poor effectiveness (Rumjanek, 2003).

Genes involved in nodulation

Host specificity and symbiotic effectiveness

The host specificity and symbiotic interaction is conferred by the particular root exudates (flavonoids) and the Nod factor structure composition (Haru & Ethiopi, 2012). It is enhanced by bacterial recognition of the flavonoids produced by a host species providing opportunity for plant choice as the only correct flavonoid for induction of symbiotic gene expression in a particular rhizobium strain (Spaink, 2000). The particular flavonoid signal perceived by bacteria is mediated in part by the transcriptional regulator *NodD*, varying

functionally among rhizobial strains (Simon et al. 2014). The lipo-chitin oligosaccharide (LCO) ''nod factors,'' is then produced to influence the regulation of plant genes leading to recognition of specific legume host (Spaink, 2000). Bacterial nod factors are composed of four to five beta 1 – 4 linked N-acetyl glucosamine units (a chitin backbone) and a fatty acid (D'Haeze & Holsters, 2002).

The nod factors differ in their fatty acids, lengths of their sugar backbones, saturation of the acyl unit and decorations (glycosylation, sulfation, and methylation) of the reducing and non-reducing ends of the backbone (Simms & Taylor, 2002: Broughton et al. 2003). The nod factors produced by rhizobia are diverse, and plant discrimination of the same contributes the second level of specificity to the interaction and create an opportunity for plant partner choice (Simms & Taylor, 2002).

The *rhizobium* cell walls are composed of varying polysaccharide which provides another opportunity for plant partners choice using the ''lock and key'' cascade that determines the degree of plant and bacterial specificity (Simms & Taylor, 2002). Some researchers have reported that rhizobia of different genera can infect the same plant species, but some plant species, can strictly be infected by rhizobia from only one specific genera (Botha et al. 2004).

Cowpea has been reported to be nodulated by rhizobia isolated from soybean, ground nuts and Bambara ground nuts but these legumes cannot be nodulated by rhizobia isolated from cow pea (Ampomah et al. 2008). This is an essential phenomenon when providing recommendation on efficient inoculants for legume and developing efficient nitrogen fixing rhizobia inoculants because inoculants can be introduced into new environments

that have never been cultivated and lack compatible rhizobia strain according to Bala & Giller (2006).

Therefore, identification of rhizobia strains in African farmers' fields using specific hosts and testing its efficiency in nitrogen fixation and effectiveness in cross inoculation is essential for improvement of legume production (Holtorf et al. 2002).

The process of symbiosis is such that rhizobia supply a constant source of reduced nitrogen to the host plant in exchange of nutrients and energy for its activities (Holtorf et al. 2002), because plants cannot directly utilize nitrogen stored in the soil as organic matter except in NH_4^+ and NO_3^- inorganic forms (Simon et al. 2014). The interaction between root nodules and their symbiotic bacteria has been studied through proteins (proteomics) produced by both partners during their signal exchange and growth of symbiosome (Holtorf et al. 2002). The study has identified hundreds of proteins with development and functioning of rhizobial symbiosis according to Chevalier et al. (2004) and Holtorf et al. (2002).

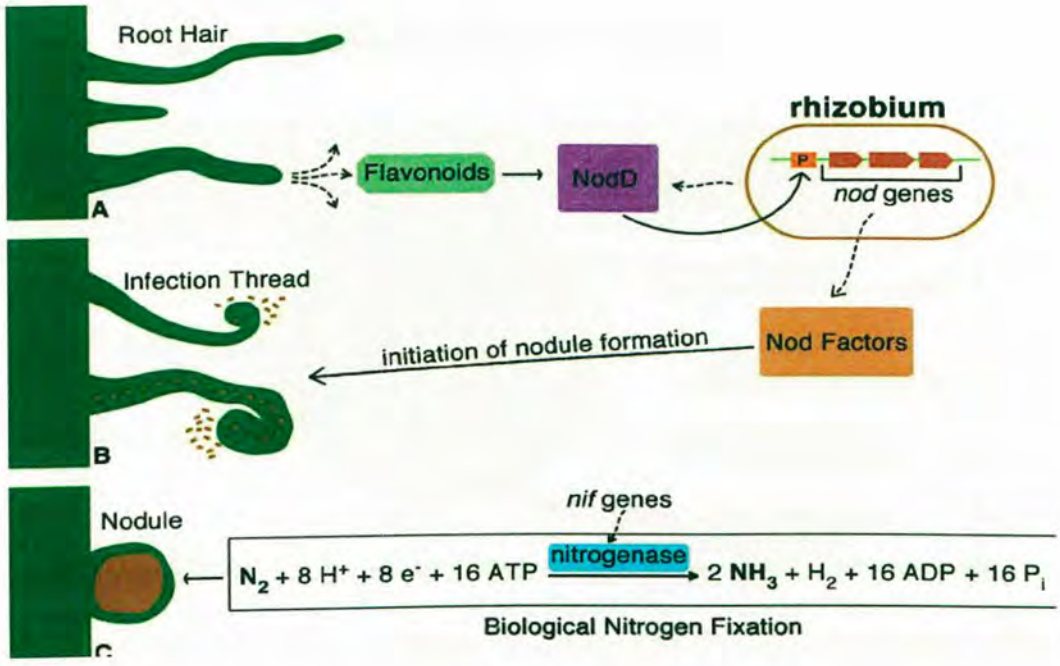

Figure 2: Biological nitrogen fixation (Simon et al. 2014).

When nitrogen in the soil is inadequate, legumes release flavonoids which signal to rhizobia that the plant is seeking symbiotic bacteria (Leidi & Rodríguez-Navarro, 2000: Ndakidemi & Dakora, 2003). In response, the rhizobia releases nodulation factor which stimulates the plant to create deformed root hairs (Ndakidemi & Dakora, 2003). Rhizobia then form an infection thread for allowing them to enter the root cells through root hairs (Gage et al. 1996).

When the rhizobia are inside the root cells, the cells divide rapidly to form nodule (**Figure 3**) (Simon et al. 2014). Then the rhizobia convert atmospheric nitrogen into ammonia, a form that is directly used by the plant for synthesis of amino acids and nucleotides, the plant provides the bacteria with sugars, hence the symbiosis is established (Glick, 2003).

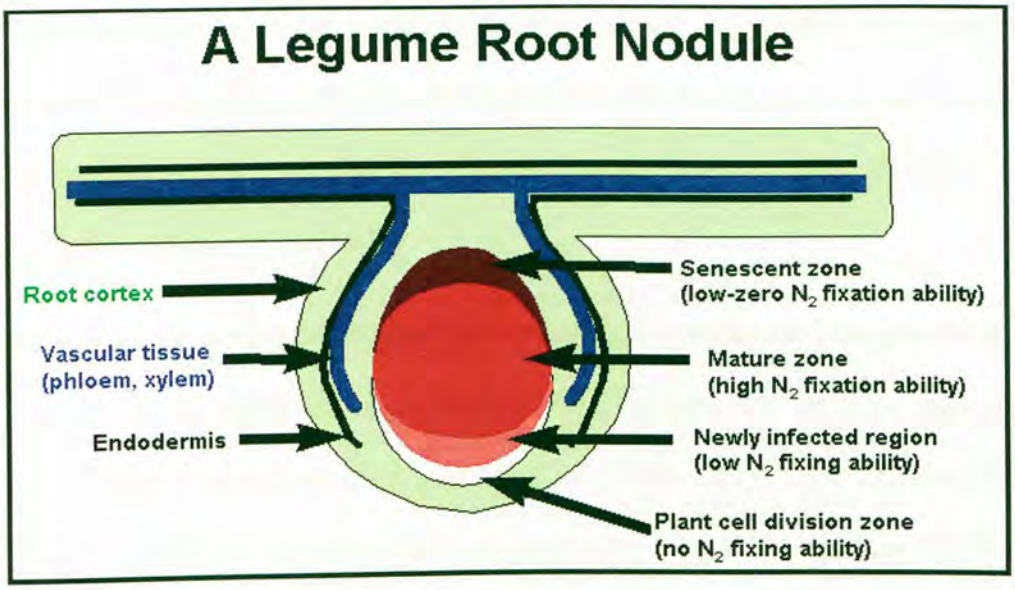

Figure 3: Physiology of a legume nodule and the specific areas of nitrogen fixation (Simon et al. 2014).

Biological nitrogen fixation has gained attention in recent years because it substitutes inorganic fertilizer and its environmentally friendly farm inputs are essential for poor resource farmers (Haru & Ethiopia, 2012). It also limits groundwater's pollution by nitrates (Berrada & Fikri-Benbrahim, 2014).

There are two major groups of bacteria fixing nitrogen within nodules of vascular plants; rhizobia (Alpha-proteobacteria) associating with legumes and Frankia (in Actinobacteria). These are filamentous slow growth rate bacterium forming hyphae colonies without an aerial mycelium and have vesicles and spores, the unique differentiated developmental structures are critical to its survival (Simon et al. 2014).

Grain legume like soybean, cowpea, common bean and peas are used in a wide range of products and are important crops for sustainable agriculture (Abbasi et al. 2010). More studies on interaction between rhizobia and legume are essential in unexploited area.

Therefore, isolation of native nitrogen fixing rhizobia from the soils obtained from farmers' fields and from virgin land using legume trap host crop provides a perfect match between legume and rhizobia for efficient nitrogen fixation leading to improve legume production (Simon et al. 2014).

2.6 The Factors Affecting Rhizobia Population, Legume and Nitrogen Fixation

Nitrogen fixing rhizobia cannot express their full potential in fixing nitrogen if the environment is in a poor state (Simon et al. 2014). The process of nitrogen fixation depends much on the functional state of the legume plant and the optimum environmental conditions supporting the macro and microsymbionts (Simon et al. 2014). The nitrogen fixing rhizobia vary in their tolerance to major environmental factors (Simon et al. 2014).

Environmental stresses can affect the host plant and symbiotic rhizobia according to Hungria &Vargas (2000). The most threatening environments for rhizobia functions are marginal lands with low rainfall, acidic soils with poor water holding capacity, nutrient stress and temperature extremes (Hungria &Vargas, 2000)

2.6.1 Temperature Extremes

High temperatures have effects on the root nodule structure, function and root hair infection (Figueiredo et al. 2008). The best temperature for nodule functioning in common beans (*Phaseolus vulgaris*) is between 25°C - 30°C according to a study done by Alexandre and Oliveira (2013). The optimum temperature range for rhizobia has been reported to be 2°C to 31°C in culture media but rhizobia isolated from hot and dry Sahel Savannah environment were reported to grow well at 40°C (Zahran, 2001).

The favourable recommended temperature for root hair development and large number of infections is between 15 and 20 °C (Simon et al. 2014). The limits of low temperature for crops native to temperate region is 2 °C while for tropical species is 10 °C (Naeem et al. 2008). Legume species can fix nitrogen at different critical levels of temperature such as 35 °C - 40 °C for soybeans and cowpeas, and 30 °C for peas (Naeem et al. 2008).

The rhizobia population in relation to temperature for across different zones in Africa still needs more research. The low temperature experienced at the highlands and high temperature with low moisture content in lowlands has resulted into varied crop yield from one season to another (Simon et al. 2014). Therefore, isolation of native rhizobia species from extreme temperature range as those found in Africa is essential for obtaining temperature tolerant rhizobia species for improvements of legume yield (Simon et al. 2014).

2.6.2 Drought (Soil Moisture Deficiency)

Both rhizobia and legume can exist in soils with low moisture levels with the lowest population densities reported in most desiccated environments (Simon et al. 2014). Drought reduces the rhizobia population in soils, inhibits nodulation and N2 fixation (Hungria &Vargas, 2000). Nitrogen fixation process is highly sensitive to the deficiency of soil moisture (Hungria &Vargas, 2000).

Rhizobia population in relation to drought has received little attention in Tanzania and Africa at large. Most dry lowlands in Africa are characterized with low moisture content and high annual temperature range (Simon et al. 2014). Therefore, successful isolation of rhizobia from such environment will result in obtaining good rhizobia candidates for

establishing successful symbioses in drought environments useful for production of common bean and other legumes (Simon et al. 2014).

2.6.3 Soil Acidity and Related Stress

Soil acidity and related problems of manganese and aluminum toxicity as well as calcium deficiency seriously affect nodulation, N_2 fixation and plant growth (Simon et al. 2014). Nitrogen fixation by rhizobia to most leguminous plants is effective at neutral or slightly acidic soils (Simon et al. 2014). Researchers have reported that most legume species fail to nodulate at pH less than 5.0 because cannot withstand acidic condition (Kellman, 2008).

Rhizobia isolated in Egypt grew at pH ranging from 6 - 8 with some being able to tolerate acidic pH ranging from 3.5 to 4.0 and alkalinity at pH ranging from 9 - 10 (Zahran et al. 2012).

Bother et al. (2004) in South Africa reported that the Bradyrhizobia population serotype 135 for soybeans was observed to be adapted to alkaline soils at Koedoeskop while serotype 122 was adapted to soils of neutral or acid pH thus could not survive well in Koedoeskop soils. Therefore; isolation of rhizobia from varied locations with wide range of soil pH is a pavement to acquire effective native rhizobia tolerant to low and high soil pH (Simon et al. 2014).

2.6.4 Salt and Osmotic Stresses

The response of legume to salinity varies much depending on soil properties, climatic conditions and plant growth stage (Simon et al. 2014). Salt and osmotic stress can affect the initial stage of legume-rhizobial interaction and nodule formation than it does to rhizobia (Sulieman & Tran, 2013). The root hair formation on plants is more sensitive to

salt than rhizobia cells hence rhizobia can tolerate salinity from 4.5 to 5.2 dsm^{-1} (Qureshi et al. 2010).

The *Rhizobium leguminosarum* for common beans (*Phaseolus vulgaris*) can tolerate up to 350 mM NaCl concentration in broth culture while those for *Vigna Unguiculata* can tolerate up to 450 mM NaCl concentration according to a study done by Zahran (2001). Some rhizobia species can tolerate moderate salinity soils and fix nitrogen effectively, to establish a successful rhizobia-legume symbiosis for saline environments, efficient salt tolerant native rhizobia strains should be isolated from saline soils (Bouchmouch et al. 2005).

The study conducted in Meru and Hai Districts northern Tanzania reported that Bradyrhizobia and rhizobia inoculants are efficient supplier of nitrogen to common beans and soy beans respectively and that inoculation is a better option to poor resource farmers who cannot afford to buy expensive inorganic fertilizers (Simon et al. 2014). This process is an environmentally friendly source of nitrogen which is renewable and can replace or complement mineral fertilizer inputs (Simon et al. 2014).

Therefore, isolation and estimation of the population density in relation to salt and osmotic stress for native rhizobia nodulating common beans, cowpeas, soybeans and peas across favorable to unfavorable rhizobia growth conditions across Africa is a means for improvement of legume production (Bouchmouch et al. 2005).

3. Materials and Methods

3.1 Research design

This study will compare five different varieties of cowpeas, including *Alectra*-resistant lines released from other countries, with typical land races. Non-inoculated and inoculant treated (peat carrier) cowpeas will be compared under conditions favorable for nodulation (with low phosphate fertilizer dosage), with and without moderate irrigation at Ogongo campus.

3.2 Study area

The campus is strategically located in Omusati Region (figure 4), the famous Culvelai-Etosha Basin of north-central Namibia (Hiyama et al. 2017). It is approximately 800 km from the city of Windhoek and mid-way between Oshikuku and Outapi townships along C46 highway and roughly 50 kilometres from Oshakati (Hiyama et al. 2017). Omusati Region has seasonal water bodies caused by seasonal flooding, degraded landscape, rainfall that ranges from 250-300mm and has temperatures that range a with minimum of 14°c and maximum of 32°C according to Lisao et al. (2018). The pH of the soil at the study area Ogongo campus was about 6, organic carbon was 0.2242%, the total nitrogen levels where about 0.03323% which is very low for the growth of plants or cowpeas, the phosphorus content was about 6.4769mg/kg (own observations).

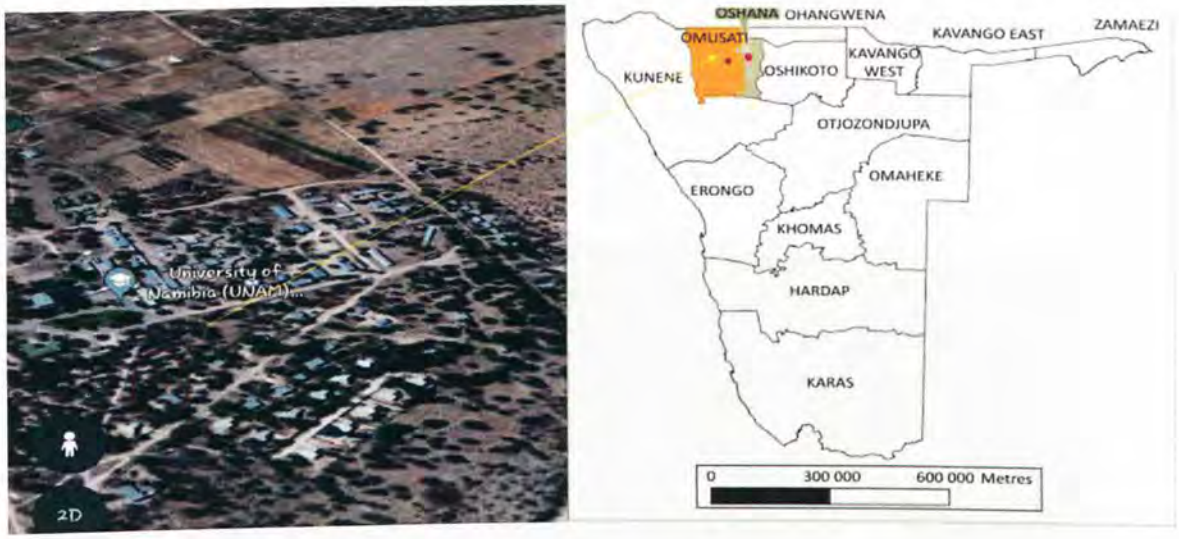

Figure 4: Map of the study area. Ogongo campus is approximately 800 km from the city of Windhoek and mid-way between Oshikuku and Outapi townships along C46 highway and roughly 50 kilometres from Oshakati (Hiyama et al. 2017) and (google maps).

3.3 Procedure

3.3.1 Flied preparation and planting of seeds

Fields were ploughed back and forth to homogenize the test field area. Conservation agriculture was used to make permanent rip lines and Phosphate fertilizer was added to the whole experimental field. For field experiments: No seed surface sterilization was necessary. The integrity of seeds was checked (should not have holes or insects), seeds that were sown in the Non-inoculant plots and fertilizer plots were the first to be planted, followed by seeds that were inoculant treated in their respective plots as.

As seen from the figure below (figure 5) first four columns represent the No inoculant plus nitrogenous fertilizer (positive control), second four columns represent the inoculant columns (test plots or fields), and lastly the last four columns represent the No inoculant plots (negative control). Six different varieties of cowpeas including relatively *Alectra-*

resistant lines released in other countries, with a typical land race, namely Lutembwe, Shindimba, Nakare, Bira and I2 from Nigeria were gown among the three treatments in triplicates. The actual field design can be seen in (figure 5) below.

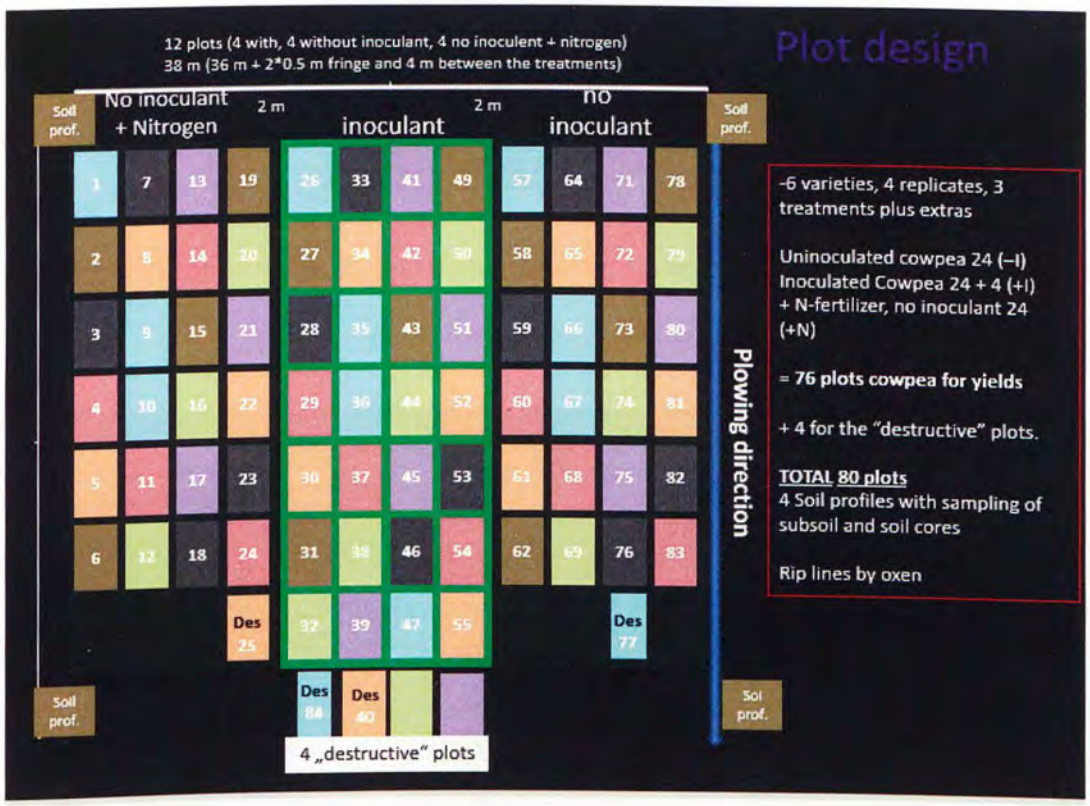

Figure 5: Plot design with the three treatments. first four columns represent the No inoculant plus nitrogenous fertilizer (positive control), second four columns represent the inoculant columns (test plots or fields), and lastly the last four columns represent the No inoculant plots (negative control).

Figure 8: Plots and subplots design on the actual field at Ogongo campus.

3.3.2 Inoculant preparation

To test for the effect of the introduced biofertilizer inoculants on yields of cowpea varieties cultivated in field experiments at Ongongo campus, the strains *Bradyrhizobium* 1-7 and *Bradyrhizobium* 14-3 were used as biofertilizers as shown in the table 1 below.

Table 1: Bioinoculants that were used in fields

strain/inoculant	Genus	Host
1-7	***Bradyrhizobium***	**cowpea (6)**
14-3	***Bradyrhizobium***	**cowpea (6)**

A 2% PVP-40 solution was made and mixed with seeds for the bio inoculum treatment plots, the number of seeds were counted per cultivar and planted with the bio inoculum by using the field plan (figure 9).

Figure 9: Mixture of the PVP- 40 solution with the bio-inoculum and seeds.

3.3.3 Harvesting and data collection of cowpeas grown from plots in Ogongo

About 10 cowpeas plants were randomly selected from each subplot. The border plants were avoided and only plants from each of the 4 middle rows at various positions were harvested (figure 10). The pod number for these 10 cowpeas plant was counted to get the average number of pods per subplot. Forty pods were randomly selected from the 10 cowpeas Plants and then the number of seeds from the forty pods was obtained. Finally, 100 seeds were weighed (g) per subplot to obtain the grain yield in grams (figure 11).

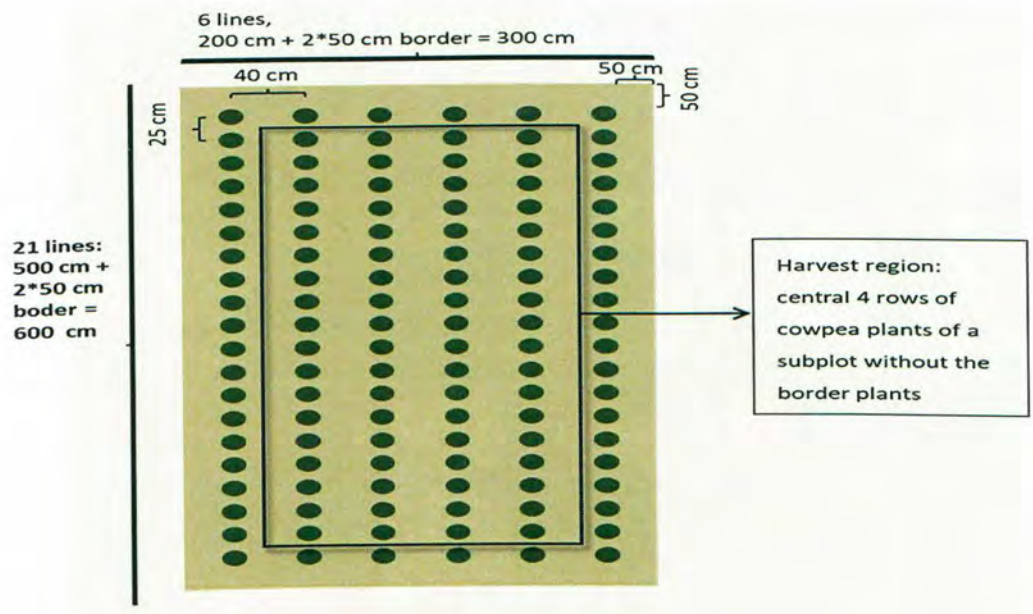

Figure 10: Harvest plan used for all the 76 subplots. From each of the subplot only the four central rows were harvested living the border plants untouched to avoid harvesting cross pollinated seeds or plants.

All the pods were harvested per subplot and sun-dried for 3 days. The grains were weighed on a standard electronic balance and recorded. Finally, the grain yield was calculated using Microsoft excel (kg /hectare) **(figure 11)**.

Figure 11: Ten plants obtained and dried to get the dry weight of the seeds and average pod number.

3.3.4 Isolation and Identification of Rhizobium from the root nodules collected from fields at Mashare Irrigation and Training Center (MITC).

A shovel was used to properly dig out and carefully remove the roots from the soil to obtain the root nodules. The nodules were kept in small valves which contained a red-coloured dry silica (which turns orange-yellow when hydrated), a cotton barrier, and a tightly closing lid.

Figure 12: Nodules were obtained from a replicated field experiment from Mashare Irrigation and Training Centre.

Surface sterilization of Cowpea nodules and isolation of bacteria

The desiccated root nodules stored in the silica valves were rehydrated in sterile water over night at 4°C on petri dishes. The nodules were washed in 70% ethanol for 30 seconds, then rehydrated in sterile water for a minute on petri plates. They were further sterilized in 5% Na-hypochlorite for 2 minutes, then rinsed six times in sterile water to allow the Na-hypochlorite to diffuse out. I used 500 µl (depending on nodule size) of sterile water and crushed the nodules using a mortar and pestle. Then stored the mixture in Eppendorf cups. The mixture was kept at 4 °C for further culturing on MAG Agar.

Table 2: Reagents and stock solutions added to make MAG medium.

Reagents in grams added to the MAG medium	**Percentage of Stock solutions made and added to the MAG medium**
(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1.3g	NH4Cl (16% w/v) 2.0ml

2-(N-morpholino)ethanesulfonic acid (MES) 1.1g	FeCl3 (0.67% w/v) 1.0ml
Yeast Extract 1.0g	CaCl2 (1.5% w/v) 1.0ml
Arabinose 1.0g	MgSO4 (18% w/v) 1.0ml
Gluconic acid 1.0g	
KH2PO4 0.22g	
Na2SO4 0.25g	
Agar 18g	

3.3.4.1 Morphological identification.

Gram staining

The isolated bacterial cells from nodules were placed on a glass slide and heat-fixed and stained with crystal violet for 1 min and rinsed gently under running tap water. The slide was immersed in iodine solution for 1 min and rinsed gently under running tap water. The slide was decolorized, with alcohol/acetone solution and rinsed under running tap water. The slide was flooded with safranin for 30 sec and with rinsed gently under running tap water, then air-dried the slide in an upright position. Finally, a microscope was used to scan the slide to observe any clusters of epithelial cells.

3.3.4.2 Molecular identification

3.3.4.2.1. Bacterial colony lysis from pure cultures

About 50 µl of colony lysis buffer was added to a microcentrifuge tube. A single small colony was picked using a sterile tooth pick and added to 50 µl of colony lysis buffer in a micro centrifuge tube, then the microcentrifuge tube was incubated at 95°C for 15 min on

a thermomixer with gentle shaking. The microcentrifuge tube was taken out and allowed to cool briefly. The microcentrifuge tube as vortexed for 5 min at room temperature to pellet the debris then transfered in to clean tubes. Finally, the supernatant was directly used as template for PCR amplification and stored at 4°C.

PCR amplification of *rpoB* genes Partial *rpoB* genes were amplified using the primers rpoB83F (5′-CCTAATCGAGGTTCACAGAAGGC-3′) and rpoB1061R (5′-AGCGTGTGCGGATATAGGCC-3′) under the cycling conditions described by Laguerre et al. 1996, with increased annealing temperature of 58°C. PCR mixtures were composed of DNA polymerase (Molzym, Bremen, Germany), 5 µl of the respective 10× PCR buffer, 5 µl (each) deoxynucleoside triphosphate (dNTP), 0.5 µl (each) primer, 37.5 µl RNA-H_2O 2 µl template in a total volume of 50 µl. The patterns obtained were visually identified by gel electrophoresis in a 0.5% agarose gel. The PCR products were cleaned up and purified using the Monarch PCR & DNA Cleanup Kit. The purified products were sent to LGC Genomics in Berlin Germany for sequencing.

Bio inoculum isolates from Mashare were obtained from nodules of local varieties of the pulse cowpea, and four of them were identified by sequence analyses of the *rpoB* genes, these sequences were identified using NCBI BlASTn, followed by a phylogenetic tree construction from the information obtained from NCBI using MEGA 10.0.5 software.

3.3.4.3 Biochemical Identification

Catalase test

A small amount of bacterial colony was transferred on to a clean dry glass slide using a sterile loop then added a drop of 15% hydrogen peroxide on to the slide. A positive result for the Catalase test formed bubbles while negative result didn't form any bubbles.

Indole test

I inoculated a test tube of tryptone broth with a small amount of the isolated bacterial culture then incubated the mixture at 35°C for 48 hours. To test for indole production, I added 5 drops of the Kovacs reagent directly to the tube. A positive control was made by growing *E. coli* in tryptone broth which formed a red ring at the interface of the medium when 5 drops of Kovacs reagent, while the negative control was made by growing *Klebsiella pneumoniae* in tryptone broth had no colour development.

3.4 Data analysis

To analyze the impact of Biofertilizer inoculants on yields of cowpea varieties, the grain yield per hector data and pods per hector data were tested for normality using Shapiro-Wilk test, then a two-way ANOVA test (for data normally distributed data) and Friedman's test (for data not normally distributed) were used.

4 Research Ethics

A research permit and ethical clearance was obtained as per UNAM regulations. A collection permit from the Ministry of Environment and tourism. Maximizing benefit and minimizing harm is one of the ethical principles that was used in this research as very few plants were used to extract the *rhizobium*, of which the study will be of great benefit to the people of Omusati region once the project is adopted by any intellectual benefactor e.g. the government for mass production of the bio fertilizer.

5 Results:

5.1 Seeds obtained from the study area

Seed germination was seen after two to three weeks from 13th of February 2017. Most of the plants from the inoculant and nitrogen treatment grew very well. Unfortunately, some cultivars like I2 did not germinate in some plots especially the non-inoculant treatment due to the harsh climatic conditions of the study area. After three months the plants where fully grown, and the pods and seeds were harvested. Figure 14 shows the grains obtained from the fields.

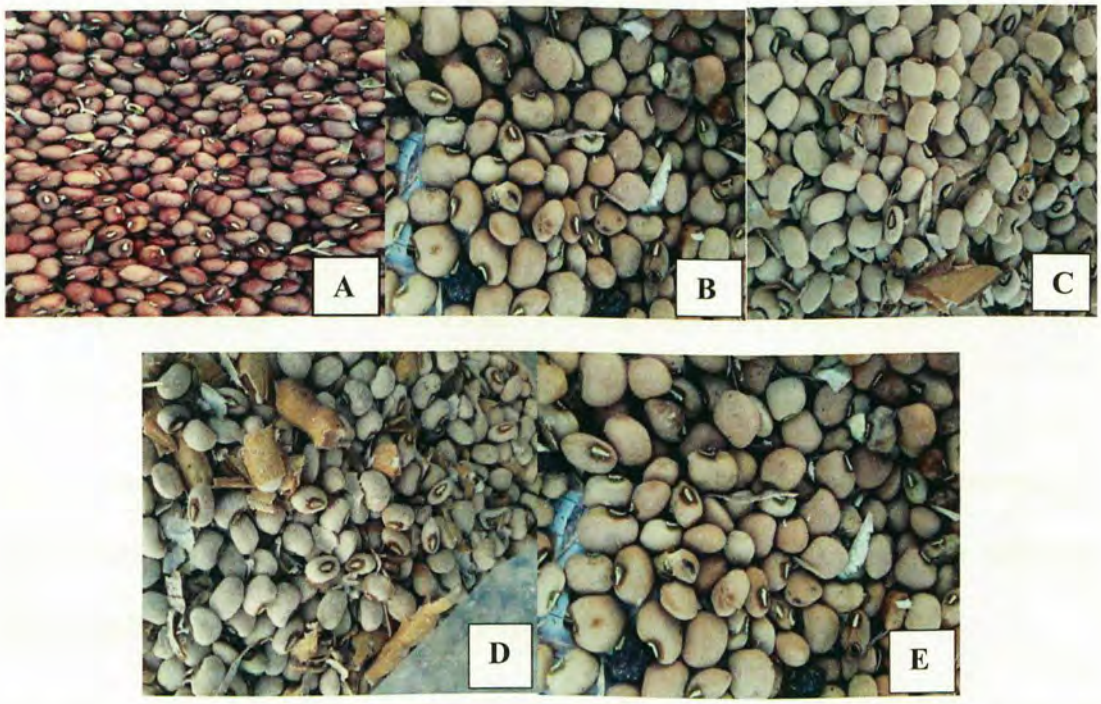

Figure 13: Different cultivars obtained from the Ogongo campus fields after reaping the harvest. A represents Bira, B Nakare, C I2 the Nigerian cultivar, D Shindimba and E Lutembwe.

5.2. Data analysis

Normality test (grain yield)

A Shapiro-Wilk's test showed that the data were normally distributed ($p=0.434>0.05$) (Appendix 1A and Appendix 1B).

Analysis of Variance. (Grain yield)

From the analysis the crop that produced more grain was Bira 1761.007 kg/ha, followed by Nakare with 1562.00 kg/ha, then Lutembwe 1344.57 kg/ha and Shindimba 1161.79 kg/ha and lastly I2 841.34 kg/ha amongst the three treatments (figure 14). Between the three treatments from (figure 14), the cultivar Nakare, indicated that there wasn't much of a difference in grain yield among the three treatments as seen from the error bars.

The graph below (figure 14) showed that Lutembwe had a significant difference between the inoculant treatment and non-inoculant treatment (Appendix 1C and 1D).

The cultivar I2 showed that there was no significant difference between the treatments for (figure 14). The same can be said for Shindimba not much of a significant difference among its three treatments (figure 14) (Appendix 1C and 1D).

For the cultivar Bira there was a significant difference between the fertilizer and the bioinoculant. Thought not much of a difference could be seen between the bioinoculant and the non-bioinoculant (figure 14) (Appendix 1C and 1D).

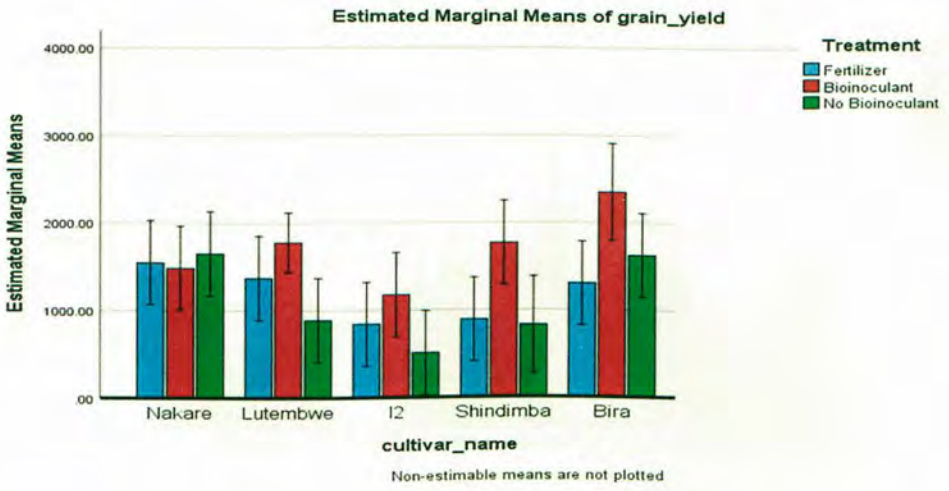

Figure 14: The graph above shows the results of the three treatments on different cultivars.

The two-way Anova analysis of grain yield kg/ha showed that overall the highest input was received from the bioinoculant treatment with 1710.703 kg/ha with an increase in grain yield production of about 61%, followed by the fertilizer treatment with 1445.441 kg/ha with a grain yield production increase of about 53% and lastly no bioinoculant treatment 1100.661 kg/ha which increased the grain yield by only 35% (Appendix 1E). Based on the statistical data (Figure 15) it was concluded that crops that where grown with the bioinoculant actually did better than those grown with nitrogen fertilizer and non-inoculated cowpea crops (Appendix 1E and 1F).

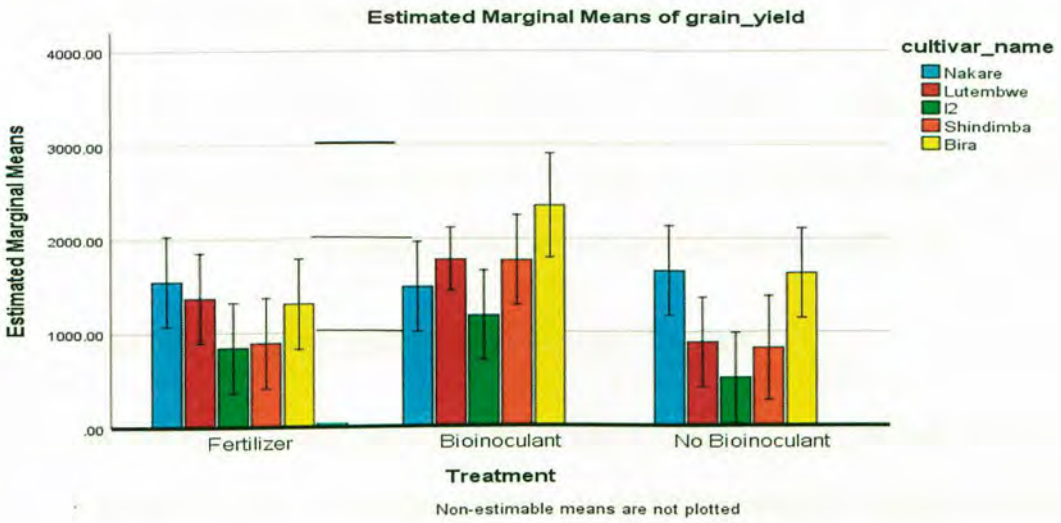

Figure 15: Graph of the two-way Anova analysis of grain yield among the three treatments.

The post Hoc test (grain yield)

The post Hoc test was carried out to establish the statistical differences between the three treatments using Bonferroni correction, which is one of the several methods used to counteract the problem of multiple comparisons, of which in this study was trying to find the differences between the three treatments, specifically which one yielded a better result in terms of grain yield production. There was a statistical difference between fertilizer and bio inoculum P value of $p=0.14<0.05$. And between the inoculum and non-bio inoculum treatments where $p=0.01<0.05$ there by giving a statistical difference. Therefore, it was concluded that the crops grown with bio inoculum treatment had a higher grain yield compared to those grown with fertilizer and non-inoculum (Appendix 1G).

Normality test (average number of pods per hectare)

A normality test was carried out and showed that data were not normally distributed (P=0.008<0.05) therefore we accept the null hypotheses. Hence a Friedman test was used to analyses the data for the average number of pods per hectare (Appendix 2A).

The Friedman's test (average number of pods per hectare)

The Friedman's test gave a P value of 0.001 (p=0.001< 0.05), hence null hypothesis was rejected, because there was statistical difference in the average number of pods per hectare between the three treatments. In order to see these differences a post Hoc test was further carried (Appendix 2B and 2C).

Friedman's post hoc test for the average number of pods per hectare

To assess precisely where the difference was in between the three treatments further analysis was carried out using a Friedman's post hoc test. The test showed that between the bioinoculant and nitrogen fertilizer p value was 0.022 (p=0.022<0.05) meaning there was a significant difference between the bioinoculant and fertilizer treatments on the number of pods per hectare and the statistical difference (Appendix 2C).

And between the no inoculant and Nitrogen fertilizer the p value was 0.156. (p=0.156>0.05) indicating no statistical difference between the two treatments on the number of pods per hectare, and lastly amongst no inoculant and bioinoculant the p value was p=0.003 (P=0.003<0.05) hence there was a significant difference on the number of pods per hectare, hence implying that the bioinoculant plots produced more pods per hectare than the nitrogen fertilizer and the non-inoculated plots according the post Hoc tests (Appendix 2C).

Correlation tests between pod number per hectare and the grain yield per hectare

A correlation test was performed between the pod number per hectare and the grain yield per hectare, results revealed that there was a significant correlation between the number of Pods and grains per hectare ($p=0.00<0.05$) (Appendix 3).

Comparison of the two bioinoculants used on the cowpea cultivars

Normality test

The data were normally distributed ($P=0.937>0.05$) (Appendix 4A), therefore null hypothesis was rejected. Hence a Two-way Anova was used to analyse the data in the next step to get the average grain yield and compare the two strains (Table 1) strains (Appendix 4A).

Strain 1-7 had the lowest estimated mean of 1448.445kg/ha meaning this strain helped increased the grain yield per hectare by 53% and 14-3 had the highest estimated mean of 1509.328kg/ha therefore increasing the grain yield per hectare by 56% (Appendix 4B).

Analysis of Variance for plots treated with strains 1-7 and 14-3 (Grain yield)

(Figure 16) it was concluded that there wasn't a statistical difference in the grain yield per hectare between the two bio inoculums used on the cultivars after conducting a two-way ANOVA. This was further proved by the pair wise comparisons in which showed there was no significant difference in the grain yield between cowpeas are grow with the strain 1-7 and the cowpeas grown with strain14-3 ($p=0.806>0.05$).

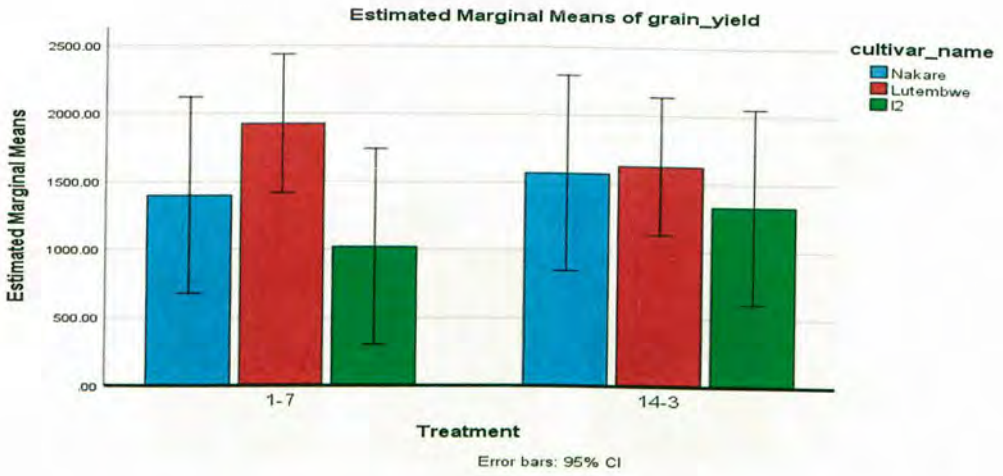

Figure 16: Estimated grain yield mean between the two strains used.

From the crops grown with the introduced *Rhizobium* strains, Lutembwe had the highest mean of about 1775.965kg/ hectare followed by Nakare with a mean of 1484.583kg/hectare and lastly I2 with 1176.112kg/hectare (Appendix 4C).

Pair wise comparisons were done (Figure 17) to see if there were any statistical differences between the three cultivars grown amongst the two bio-inoculum. Between Nakare and Lutembwe there no difference in the yields ($p=0.968>0.05$), between Nakare and I2 there was no statistical difference in the grain yield per hectare ($p=1.000>0.05$). And lastly between I2 and Lutembwe there was no statistical difference ($p=0.174> 0.05$). All in all, this shows and says that both strains 1-7 and 14-3 had the same impact on the grain yield per hectare for the three cultivars. This also shows that both strains where host specific to the cowpea cultivars and lead to a perfect match between legume and rhizobia resulting into effective nodule (deep red inside) formation and nitrogen fixation. As can be noticed all the bars are over lapping hence can be concluded that there was no significant different

in the grain yields of cowpea cultivars grown with 1-7 inoculum and the cultivars grown with the 14-3 inoculum strains (Figure 17).

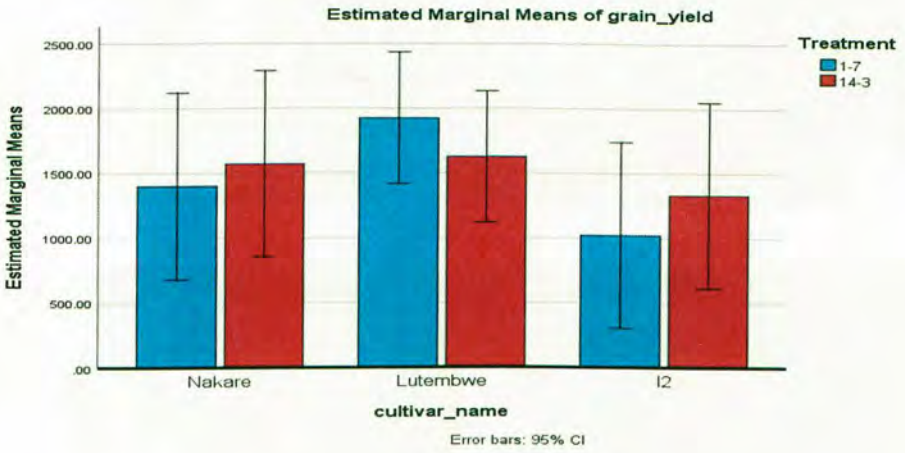

Figure 17: Estimated mean of the different cultivars .

5.3 Identification of the Rhizobium from Nodules collected from Mashare Irrigation and Training Center

5.3.1 Morphology identification of the isolates obtained from Mashare fields

From the results obtained, the first isolates namely A, D, E and F were slow growers and took at least 6 to 8 days to grow on MAG plates and were white in color and seemed to grow in small colonies, their colonies were circular, entire and semitranslucent (figure 18).

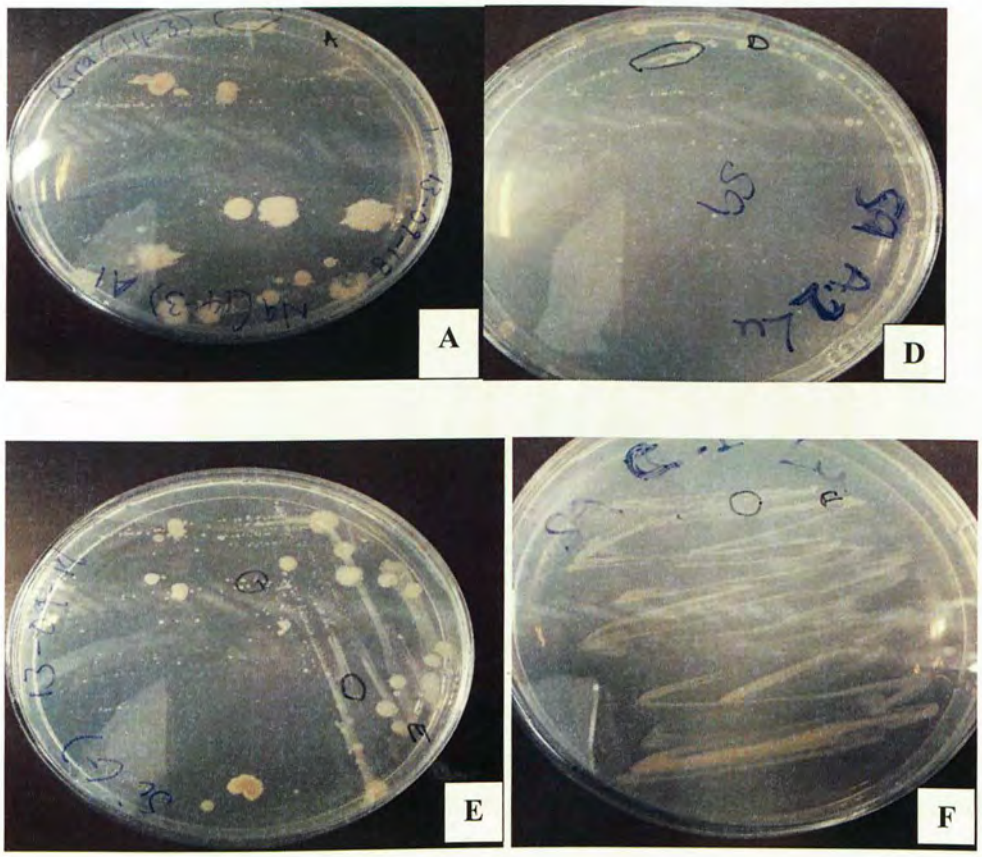

Figure 18: Picture of the bacteria isolates A, D, E and F which were the first isolates grown on MAG plates.

As can be seen (figure 19), all isolates from the microscopic identification where gram negative (pink in colour) and were rod shaped which is very characteristic of the *Bradyrhizobium* genus

Figure 19: Microscopic picture of the isolate A.

5.3.2 Molecular identification of the isolates obtained from Mashare fields

Gel Electrophoresis of the amplified PCR products.

A was a DNA sample from Bira root nodules from the inoculanted treated plots and contained the strain 14-3. D is a DNA sample from the nodules of Lutembwe from the non-inoculant field. E is a product come from root nodules of silwana from the non-inoculated fields. F is another sample from Lutembwe non-inoculated field. G, 8-9, and T13 are positive controls and lastly the negative control. The *rpoB* gene PCR product was at least 1kb as can be seen in the picture below (figure 20).

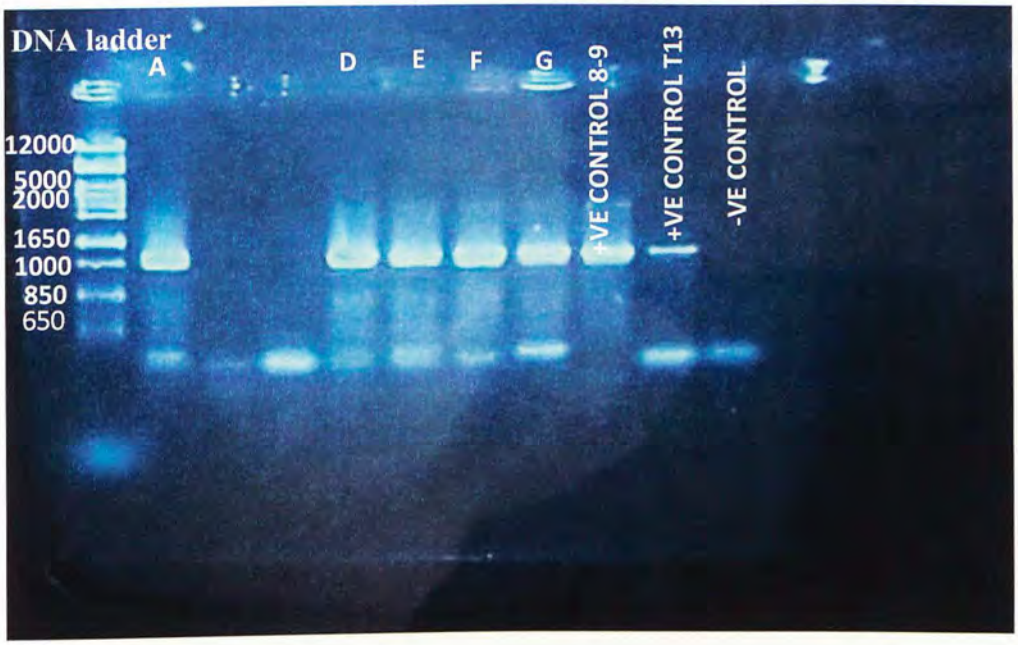

Figure 20: Molecular identification of the isolates obtained from Mashare fields.

Four isolates of Rhizobacteria from three different cultivars namely Bira, Silwana and Lutembwe were isolated and identified. The isolates were selected based on their morphological characteristic of the colonies on the agar plates and microscopic morphology. A total of four isolates were successfully amplified using PCR and sequences were generated (Appendix 5). Analysis by bioinformatics grouped the Rhizobacteria into the following genera *Bradyrhizobium* sp. 1-7 and *Bradyrhizohium elkanii.* All the isolates showed a good homology ranging from 96% to 99% (Table 3).

Table 3: Species isolated and blasted on blastn NCBI

Samples	**Species isolated (accession number)**	**Percentage similarity**
A	*Bradyrhizobium* sp. 1-7 (KM378302)	100%
D	*Bradyrhizobium* sp. 1-7 (KM378302)	100%
E	*Bradyrhizobium* sp. 1-7 (KM378302)	100%
F	*Bradyrhizohium elkanii* (LC167350.1)	99%

The optimal tree with the sum of branch length = 0.97214625 is shown in (Figure 21). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There was a total of 4141 positions in the final dataset.

As seen from the phylogenetic tree (figure 21) the isolates A, D and E were grouped with a 100% similarity with *Bradyrhizobium* sp. 1-7, while the isolate F was closely related to *Bradyrhizohium elkanii* with a similarity of 99%.

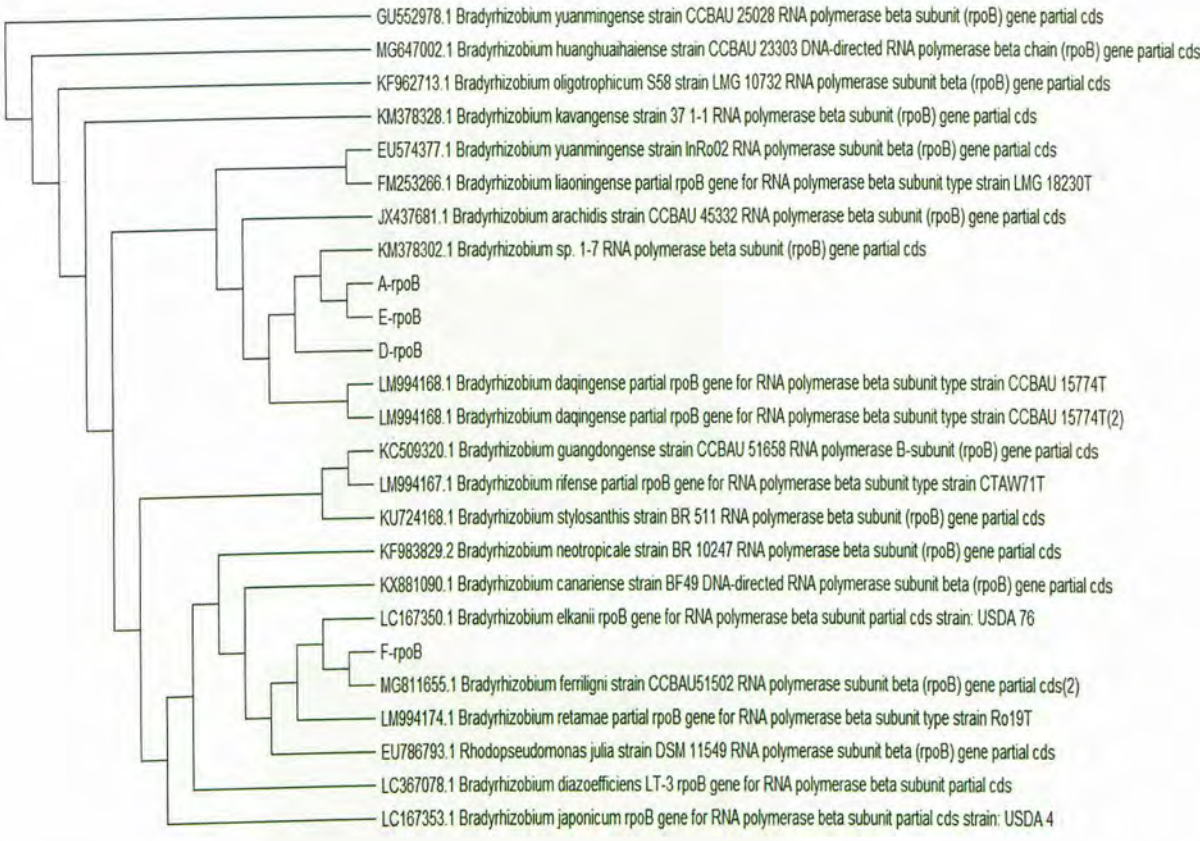

Figure 21: The evolutionary history was inferred using the Neighbor-Joining method. The tree is based on an analysis of *rpoB* sequences of bacterial isolates from cowpea pulses.

5.3.3 Biochemical Identification.

All the isolates showed a positive result for catalase (Table 4 Figure 22) and a negative result for an indole production test (Table 4 and Figure 23).

Table 4: Biochemical tests.

Samples	Catalase test	Indole Production
A	+	-
D	+	-
E	+	-
F	+	-

Figure 22: Catalase test showing a positive result.

Figure 23: Indole test. The positive control had a red ring at the interface of the medium, while the negative control had no colour development.

6. Discussion:

Cowpea is grown by smallholder farmers in Namibia and other areas of Sub-Saharan Africa under low inputs agricultural system with little or no fertilizer application; hence biological nitrogen fixation in the traditional cropping system is of vital importance for system sustainability. The cowpea residue is typically incorporated into the soil and therefore the higher N and P content in the shoots resulting from enhanced plant growth and nitrogen fixation could provide additional residual N and P for subsequent crops (Giller, 2002).

The efficiency of the inoculant strains (Table 1) in fixing nitrogen was demonstrated in the production of higher grain yield per hectare and an increase in the pod number per hectare in inoculated plants when compared to the non-inoculated plants (Figure 14 and 15) (Appendix 2B and 2C). The data is consistent with the report by Martins et al. (2003) which showed that inoculation of cowpea increased nodulation and grain yield. This study showed an increase in the grain yield by 61% when an inoculant is applied. Similarly, in the study by Martins et al. (2003) which involved 10 rhizobial isolates from cowpea nodules, significant increases in grain yield of up to 30% (533 to 693 kg/ha) were observed.

In contrast, De Freita et al. (2012) found no effect of inoculation on cowpea grain yield and nitrogen fixation in Paraiba state in Brazil and attributed the lack of response to the presence of native rhizobia strains that formed efficient symbiosis with the local cowpea varieties. The data in Brazil provide appreciable evidence that increases in grain yield due to inoculation varied considerably depending on location, inoculation history of the sites and the rhizobia strains used (Kyei-Boahen et al. 2017).

In eastern Kenya, Onduru et al. (2008) reported a 6.8% higher grain yield for inoculated cowpea plants compared with non-inoculated plants; and in northern Tanzania, Nyoki and Ndakidemi (2013) observed that cowpea inoculation increased nodulation, number of pods, and seed weight leading to 12% increase in grain yield. The number of pods per plant, seeds per pod, and 100-seed weight for the inoculated plants in the study were higher than those for the non-inoculated control plants.

The study showed that a combination of both phosphate and the bio-inoculum increased the grain weight by 61% while the non-inoculated field or negative control only containing phosphate only increased the grain weight by 35.

Onduru et al. (2008) also reported similar positive interaction between inoculant and P for cowpea grain yield which led to 54% increase in grain yield compared with the yield for the control. Although limited information is available on cowpea inoculation, the response of cowpea to P fertilization in semi-arid areas of Africa is well documented (Bationo et al. 2002; Kolawole et al. 2002; Nyoki & Ndakidemi, 2013; Abaidoo et al. 2016). It has been demonstrated that low soil P availability constrains nitrogen fixation and cowpea productivity.

This has been attributed to the important role P plays in both nodulation, nitrogen fixation and plant growth processes through enhanced root development and root hair formation (Nielsen et al. 2001; Nziguheba et al. 2016), nodule initiation and growth and as energy source for nitrogen fixation process that has direct effect on nitrogenase activity in nodules (Hogh-Jensen et al. 2002) and photosynthesis (Hogh-Jensen et al. 2002). Thus, application of P fertilizer to nitrogen fixing legumes on P-deficient soils further increased nitrogen fixation and crop yields.

Cowpea forms nodules with a group of soil rhizobia classified as *Bradyrhizobium* spp., commonly present in many tropical soils (Martins et al. 2003; Abaidoo et al. 2007). The presence of large indigenous rhizobia population in these soils affects the effectiveness of introduced inoculant strains making it difficult to demonstrate yield response through inoculation (Abaidoo et al. 2007). Consequently, cowpea inoculation is not a common practice according to Kyei-Boahen et al. (2017).

The concept of adequate nodulation of cowpea by indigenous strains limited the interest in identifying effective and competitive strains to overcome the difficult challenge of inoculant strain establishment. The population density, effectiveness in forming nodules and competitive ability are the major factors that determine the degree of inoculation response (Abaidoo et al. 2007). Despite the relatively high indigenous rhizobia population size across the study location, inoculation with the strains 1-7 and 14-3 (table 1) increased the grain weight yields per hectare and the number of pods per hectare in the cultivars used (Figure 14 and 15) (Appendix 2B and 2C).

Similar to this study Gachande and Khansole (2010) identified the Rhizobium *Brodyrhizobium japonicum* based on its morphological, cultural and biochemical characteristics.

The four cowpea isolates collected from MITC were classified in the *Bradyrhizobium* genus. A, D, and E had a 100% homology with *Bradyrhizobium* sp. 1-7 and F with 99% homology as *B. elkanii*. Contrary to this study, another study was carried out in Kavango by Grönemeyer et al. (2014) were the ITS sequence had highest similarity to the strains

of *B. daqingense*, *B. yuanmingense*, or *B. iriomotense* located on the *B. Japonicum* branch according to Grönemeyer et al. (2014).

The reason for such differences in this study and the study done by Grönemeyer et al. (2014), was because this study only used cowpeas to trap the rhizobial strains in the nodules and only collected four rhizobial strains, while the study done by Grönemeyer et al. (2014) used cowpeas, Bambara groundnut, peanut, and hyacinth bean for trapping the *rhizobium* strains.

The host specificity leads to a perfect match between legume and rhizobia resulting into effective nodule (deep red inside) formation and nitrogen fixation. If cross inoculation with no perfect match has occurred, ineffective nodules (green or white inside) or no nodules may be formed, and nitrogen fixation does not occur (Gwata et al. 2004).

Hence host specificity may have played a role and that is why this study only acquired a small amount of rhizobial strains while the other study managed to collect and sequenced a larger number of rhizobial strains of up to 75 isolates due to the different types of crops they used to trap different isolates.

All the samples gave a positive result for the catalase test similar to the results of (Singha et al. 2013). The indole production test was negative for all the isolates, this result was similar to a study by (Monica et al. 2015).

6 Conclusions:

From the results obtained, cowpea cultivars responded very well to inoculant treatment (*Bradyrhizobium* strains 1-7 and 14-3) compared to the native strains, by comparing the grain yield between the non-inoculated fields and the inoculated fields, of which the biofertilizer increased yields by 61% while the native Rhizobia only increased yields by 35%. The study provided evidence that using inoculant can enhance food security through increased grain yield in Omusati region. The study isolated and identified four bacterial strains and identified them *Bradyrhizobium* sp. 1-7 and *B. elkanii* from the *bradyrhizobium* genus from the fields at MITC in Mashare.

7 Recommendations:

Would recommend other researchers to do more research in diversity, and characterization of nodule symbionts should be intensified, particularly the high temperature tolerance of some Namibian *Bradyrhizobium* species, which makes them suitable candidates for application in global climate change scenarios that predict temperature increases. Future research should also address the molecular basis for the unusual temperature tolerance. If domesticated in Namibia, strains that are isolated and paired well with the right leguminous crop will act as a natural fertilizer for cowpeas and other potential crops. Furthermore, this management practice can contribute to the sustainability of the production system by enhancing residual N and P for subsequent crops. Farmers would also benefit economically from using inoculant biofertilizers, because they not expensive and they are easy to use and time saving, because they are applied directly to the seed and sawn.

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9 Appendices

Appendix 1A: Normality test for the grain yield among the three treatments from SPSS software

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
grain_yield	.068	63	.200*	.981	63	.434

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Appendix 1B: Descriptive table for the grain yield from SPSS software

Descriptives

			Statistic	Std. Error
grain_yield	Mean		1376.0825	80.24403
	95% Confidence Interval for Mean	Lower Bound	1215.6770	
		Upper Bound	1536.4881	
	5% Trimmed Mean		1353.7085	
	Median		1342.6700	
	Variance		405663.621	
	Std. Deviation		636.91728	
	Minimum		124.67	
	Maximum		3067.33	
	Range		2942.66	
	Interquartile Range		1026.50	
	Skewness		.423	.302
	Kurtosis		-.139	.595

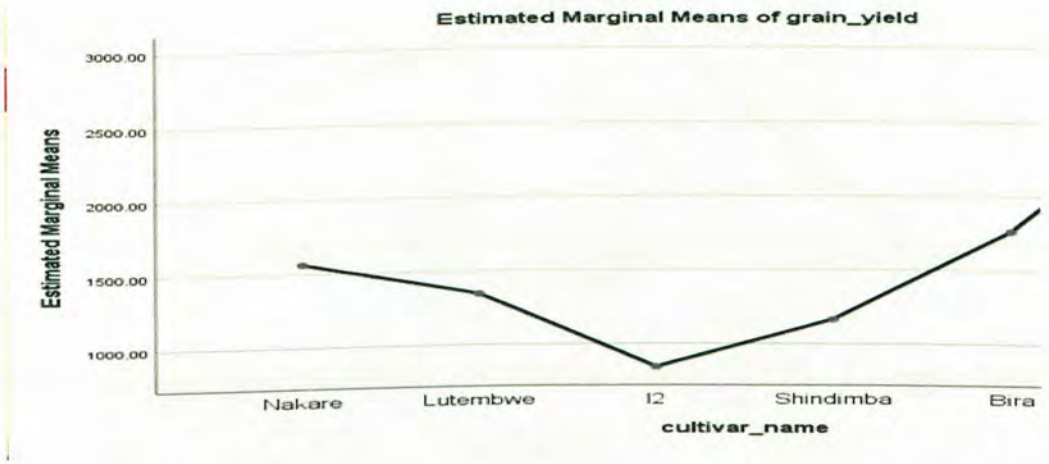
Appendix 1C: Shows the grain yield mean in kg/ha for five different cultivars grown in Omusati region using Two-way anova from an SPSS software.

Estimates

Dependent Variable: grain_yield

cultivar_name	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Nakare	1561.999	138.052	1284.274	1839.724
Lutembwe	1344.572	126.024	1091.045	1598.099
I2	841.343	138.052	563.618	1119.068
Shindimba	1161.791	145.520	869.043	1454.539
Bira	1761.007	145.520	1468.259	2053.755

Appendix 1D



Overall the crop that produced more grain was Bira 1761.007 kg/ha, followed by Nakare with 1561.999 kg/ha, then Lutembwe 1344.572 kg/ha and Shindimba 1161.791 kg/ha and lastly I2 841.343kg/ha. Picture taken from SPSS software.

Appendix 1E: Shows the grain yield estimated mean in kg/ha for the three treatments. (SPSS software)

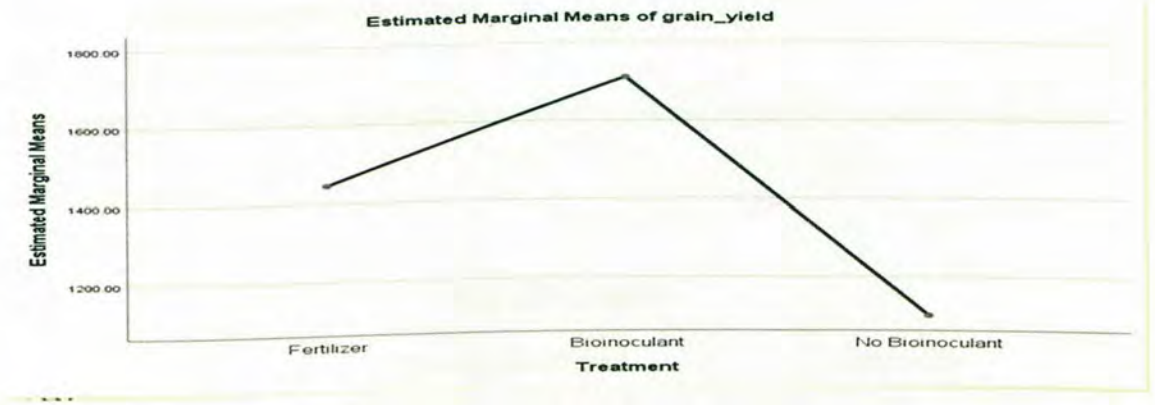
Estimates

Dependent Variable: grain_yield

Treatment	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Fertilizer	1445.441	119.557	1204.924	1685.958
Bioinoculant	1710.703[a]	105.137	1499.194	1922.212
No Bioinoculant	1100.861[a]	110.442	878.681	1323.041

a. Based on modified population marginal mean.

Appendix 1F



Shows the graph of estimated marginal means of grain yield kg/ha among the three treatments.

Appendix 1G: shows a Post Hoc test of grain yield amongst the treatment SPSS software

Multiple Comparisons

Dependent Variable: grain_yield

	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Bonferroni	Fertilizer	Bioinoculant	-430.6800*	144.33992	.014	-789.0319	-72.3281
		No Bioinoculant	148.3811	151.41791	.996	-227.5434	524.3055
	Bioinoculant	Fertilizer	430.6800*	144.33992	.014	72.3281	789.0319
		No Bioinoculant	579.0611*	148.25782	.001	210.9822	947.1399
	No Bioinoculant	Fertilizer	-148.3811	151.41791	.996	-524.3055	227.5434
		Bioinoculant	-579.0611*	148.25782	.001	-947.1399	-210.9822

Based on observed means.
The error term is Mean Square(Error) = 228700.647.

*. The mean difference is significant at the 0.05 level.

Appendix 2A: Shows the normality test for the average number of pods per hectare. SPSS software

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
average_pod_number_per_subplot	.106	63	.074	.946	63	.008

a. Lilliefors Significance Correction

Appendix 2B: Friedman's test for the average number of pods per hectare taken from SPSS software

Test Statistics[a]

N	25
Chi-Square	13.505
df	2
Asymp. Sig.	.001

a. Friedman Test

Appendix 2C: Post Hoc test using Wilcoxon signed rank test used to show the differences between the three treatments for the average number of pods per hectare. Taken from SPSS software

Test Statistics[a]

	bioinoculant - fertilizer
Z	-2.286[b]
Asymp. Sig. (2-tailed)	.022

a. Wilcoxon Signed Ranks Test

b. Based on negative ranks.

Test Statistics[a]

	no_inoculant - fertilizer
Z	-1.419[b]
Asymp. Sig. (2-tailed)	.156

a. Wilcoxon Signed Ranks Test

b. Based on positive ranks.

Test Statistics[a]

	no_inoculant - bioinoculant
Z	-2.929[b]
Asymp. Sig. (2-tailed)	.003

a. Wilcoxon Signed Ranks Test

b. Based on positive ranks.

Appendix 3: Correlation between pod number and the grain yield per hectare. Taken from SPSS software

Correlations

			average_pod_number_per_hector	grain_yield
Spearman's rho	average_pod_number_per_hector	Correlation Coefficient	1.000	.829**
		Sig. (2-tailed)	.	.000
		N	63	63
	grain_yield	Correlation Coefficient	.829**	1.000
		Sig. (2-tailed)	.000	.
		N	63	63

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 4A: shows the normality test for the two bioinoculants used. Taken from SPSS software

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Treatment	Statistic	df	Sig.	Statistic	df	Sig.
grain_yield	1-7	.210	8	.200*	.910	8	.354
	14-3	.179	8	.200*	.975	8	.937

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Appendix 4B: Estimated means of the bio inoculums used in kg/hector. Taken from SPSS software

Estimates

Dependent Variable: grain_yield

			95% Confidence Interval	
Treatment	Mean	Std. Error	Lower Bound	Upper Bound
1-7	1448.445	170.487	1068.575	1828.315
14-3	1509.328	170.487	1129.459	1889.198

Appendix 4C: Mean estimates of cultivates that with grown with the two different bio-inoculums in kg/hectare. Taken from SPSS software

Estimates

Dependent Variable: grain_yield

cultivar_name	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Nakare	1484.583	228.733	974.934	1994.231
Lutembwe	1775.965	161.739	1415.589	2136.341
I2	1176.112	228.733	666.464	1685.761

Appendix 5: Sequences obtained from LGC genomics Berlin Germany

A-rpoB

TTCGCAGCTCGCGTGATCCCGTATCGCGGCTCCTGGCTCGACATCGAGTTCGACGCCAAG
GACATCGTCTATGCGCGTATCGACCGTCGCCGCAAGATTCCGGTGACGTCGCTGATGTTC
GCGCTCGGCCTCGACGGCGAGGCGATCCTGTCCACGTTCTATAAGAGGATCCTCTACAAG
CGGACCAAGGAAGGCTGGCGCGTTCCGTTCGACGCCAACCGCTTCCGCGGCTATTCGACC
GTCAACGATTTGATCGACGCCGATACCGGCAAGGTGGTGCTCGAGGCCGGCAAGAAGCTC
ACCGTCCGTGCCGCCCGCCAGCTCCAGGAGAAGGGGCTGAAGGCGCTGCGCATGGCCGA
GAGGAGCTGGTCGGCAACTACGTCGCCGAGGACCTCGTCAACCCGAAGACGGGCGAGAT
CATGCTGAAGCCGGTGAGGAAATCACCGACAAGCTGATGAAGGCGCTCAACGAGCAGGG
TACAAGGAGCTGCCGCTGCTCGACATCGACCACGTCAATGTCGGTCCCTACATCCGCAAC
ACGCTGTCGGCCGACAAGAACATGACGCGTGAGGACGCGCTGTTCGACATCTATCGCGTC
ATGCGTCCCGGCGAGCCGCCGACGCTGGAATCGGCGCAGGCCATGTTCCAGTCGCTGTTC
TTCGACGCCGAGCGCTACGATCTCTCTGCGGTCGGCCGCGTCAAGATGAACATGCGCCTC
GACCTCGATGCGCCCGATACCCAGCGCACGCTGCGCAAGGAAGACATCCTCTCCGTCATC
AAGACGCTGGTGGACCTGCGCGACGGCAAGGGCGAATCA

D-rpoB

AGGGCAAGACCCATTCCTCGGGCAAGCTGCTGTTCGCCGCTCGCGTGATCCCGTATCGCG
GCTCCTGGCTCGACATCGAGTTCGACGCCAAGGACATCGTCTATGCGCGTATCGACCGTC
GCCGCAAGATTCCGGTGACGTCGCTGATGTTCGCGCTCGGCCTCGACGGCGAGGCGATCC
TGTCCACGTTCTATAAGAGGATCCTCTACAAGCGGACCAAGGAAGGCTGGCGCGTTCCGT
TCGACGCCAACCGCTTCCGCGGCTATTCGACCGTCAACGATTTGATCGACGCCGATACCG
GCAAGGTGGTGCTCGAGGCCGGCAAGAAGCTCACCGTCCGTGCCGCCCGCCAGCTCCAG
AGAAGGGGCTGAAGGCGCTGCGCATGGCCGATGAGGAGCTGGTCGGCAACTACGTCGCC
AGGACCTCGTCAACCCGAAGACGGGCGAGATCCATGCTGAAGCCGGTGAGGAAATCACC
ACAAGCTGATGAAGGCGCTCAACGAGCAGGGCTACAAGGAGCTGCCGCTGCTCGACATC
ACCACGTCAATGTCGGTCCCTACATCCGCAACACGCTGTCGGCCGACAAGAACATGACGC
GTGAGGACGCGCTGTTCGACATCTATCGCGTCATGCGTCCCGGCGAGCCGCCGACGCTGG
AATCGGCGCAGGCCATGTTCCAGTCGCTGTTCTTCGACGCCGAGCGCTACGATCTCTCTG
CGGTCGGCCGCGTCAAGATGAACATGCGCCTCGACCTCGATGCGCCCGATACCCAGCGC
CGCTGCGCAAGGAAGACATCCTCTCCGTCATCAAGACGCTGGTGGACCTGCGCGACGGC
AGGGCGAATCGCC

E-rpoB

GCAACGACCCATTCCTCGGGCAAGCTGCTGTTCGCAGCTCGCGTGATCCCGTATCGCGGC
TCCTGGCTCGACATCGAGTTCGACGCCAAGGACATCGTCTATGCGCGTATCGACCGTCGC
CGCAAGATTCCGGTGACGTCGCTGATGTTCGCGCTCGGCCTCGACGGCGAGGCGATCCTG
TCCACGTTCTATAAGAGGATCCTCTACAAGCGGACCAAGGAAGGCTGGCGCGTTCCGTTC
GACGCCAACCGCTTCCGCGGCTATTCGACCGTCAACGATTTGATCGACGCCGATACCGGC
AAGGTGGTGCTCGAGGCCGGCAAGAAGCTCACCGTCCGTGCCGCCCGCCAGCTCCAGGA
AAGGGGCTGAAGGCGCTGCGCATGGCCGATGAGGAGCTGGTCGGCAACTACGTCGCCGA
GACCTCGTCAACCCGAAGACGGGCGAGATCCATGCTGAAGCCGGTGAGGAAATCACCGA
AAGCTGATGAAGGCGCTCAACGAGCAGGGCTACAAGGAGCTGCCGCTGCTCGACATCGA
CACGTCAATGTCGGTCCCTACATCCGCAACACGCTGTCGGCCGACAAGAACATGACGCGT
GAGGACGCGCTGTTCGACATCTATCGCGTCATGCGTCCCGGCGAGCCGCCGACGCTGGAA
TCGGCGCAGGCCATGTTCCAGTCGCTGTTCTTCGACGCCGAGCGCTACGATCTCTCTGCG
GTCGGCCGCGTCAAGATGAACATGCGCCTCGACCTCGATGCGCCCGATACCCAGCGCACC
CTGCGCAAGGAAGACATCCTCTCCGTCATCAAGACGCTGGTGGACCTGCGCGACGGCAAC
GGCGA

F-rpoB

GACCCATTCGTCCGGCAAGCTGCTGTTTGCCGCGCGCGTGATTCCGTATCGCGGTTCCTG
GCTCGACATCGAGTTCGACGCCAAGGACATCGTGTTCGCGCGCATCGACCGCCGCCGCAA
GCTGCCCGTGACCTCGCTGATGTACGCGCTCGGTCTCGACGGCGAGCAGATCCTGTCGAC
CTTCTACAAGAAGATCACCTACAAGCGGACCAAGGACGGATGGCGCGTGCCGTTCGATGC
CAACCGCTTCCGCGGCTATTCGACCGTCAACGACCTGATCGACGCCGACACCGGCAAGGT
GGTGCTCGAGGCCGGCAAGAAGCTGACCGTGCGCCAGGCGCGCCAGCTTCAGGAGAAGG
CCTGAAGGCGCTGCGCATGTCGGACGAGGAACTCGTCGGCAACTATCTCGCCGAGGATCT
CGTCAACCCGAAGACCGGTGAGATCTACGCCGAAGCCGGCGAGGAGATCACCGAGAAGT
GCTCAAGGTGCTGAACGAGCAGGGCTACAAGGACCTGCCGCTGCTCGACATCGACCACGT
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CGCGCTGTTCGACATCTACCGCGTGATGCGTCCGGGCGAGCCGCCGACGCTGGATTCGGC
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CCGCGTCAAGATGAACATGCGCCTCGAGCTCGATGCGCCCGATACCCATCGCACGCTGCC
CAAGGAAGACATCCTGGCGGTCATCAAGACGCTGGTCGACCTGCGCGACGGCAAGGGCGA
AATCGCCCAACATCGAA