SOYBEAN (*Glycine max*) RESPONSE TO RHIZOBIA INOCULATION AS INFLUENCED BY SOIL NITROGEN LEVELS

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DECLARATION

This thesis is my original work and has not been submitted for a degree in any other University.

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DEDICATION

To: My father, Bernard Mathenge; my mother, Beatrice Mathenge. My parents' vision for a better tomorrow is unrivaled; you have been the best educators in my life. Above all, I extend my sincere gratitude to the Almighty God for making this journey possible.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
BNF	Biological nitrogen fixation
C	Carbon
CRD	Complete randomized design
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ETDA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
ICIPE	International Centre for Insect Physiology and Ecology
MOA	Ministry of Agriculture
MPN	Most probable number
N	Nitrogen
NFW	Nodules fresh weight
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-Restriction fragment length polymerase
RCBD	Randomized complete block design
SAS	Statistical Analysis Software
SDW	Shoot dry weight
SED	Standard error of differences
SOC	Soil organic carbon
SOM	Soil organic matter

UV   Ultraviolet

ABSTRACT

Inoculation of soybean (*Glycine max)* is an efficient and convenient way of introducing rhizobia to soil and subsequently the rhizosphere of the crop. However, its full potential in sub-Saharan Africa is yet to be realized due to effects of varying soil limiting conditions. Critical levels of nitrogen (N) below and above which response is not guaranteed are unknown. The objective of the study was to determine the critical range of N outside which response to inoculation is hindered. Below the lower limit, an adequate starter N would be required, while above the upper limit, inoculation would not be effective. A greenhouse experiment was set up with sixty soils with a varying range of total N (%) and organic carbon $\leq$ 2.1 %. The experiment setup was a Complete Randomized Design (CRD) with and without inoculation replicated three times. A second greenhouse experiment with two soils selected based on the first experiment was used (with 0.06 % N and 0.08 % N). A complete randomized design was used with three replicates and five rates of an organic N source (i.e. 0, 2.5, 5, 7.5 and 10 t Phymyx ha^{-1}) for each soil N level, with and without inoculation to assess whether the organic amendment could improve the response to inoculation within the adequate range of N. A field experiment was set to validate the 2ndgreenhouse experiment results under field conditions. A split plot arrangement in a randomized complete block design (RCBD) design full factorial with three replicates for the two sites was set up. The main plot as Sympal (contains phosphorus, potassium, Sulphur, calcium, magnesium, and zinc) applied at a rate of 30 kg P ha^{-1}and the sub-plots had Phymyx at 5 levels with and without inoculation. Soybean variety TGx1740-2F was inoculated with *Bradyrhizobium japonicum* strain 532c. In the first greenhouse trial, it was demonstrated that soybean response to inoculation was significantly affected by soil fertility based on nodule fresh weight and shoot biomass, but on average the two parameters were improved by inoculation with Legumefix ($p<0.05$). The soil with 0.17 % N gave the highest mean weight of 27.89 g plant^{-1} while soils of 0.029 % N produced the lowest mean weight of 0.17 g plant^{-1} for inoculated soils. In the second greenhouse trial, nodule fresh weight and biomass N uptake were enhanced by application of Phymyx ($p<0.05$. highest shoot dry weight and nodules fresh weight were observed between 0.16 % and 0.17 % nitrogen for both non-amended and after soil amendment. Nitrogen levels above 0.17 % N suppressed the nodulation and resulted in reduced shoot dry weight per plant. A 0.17 % N was the potential threshold level above which soybean response to inoculation would not be achieved in clay soils. In field conditions, soybean grain yields after inoculation were better at 0.08 % N than at 0.06 % N, which confirmed the effect of the soil type found in the first greenhouse conditions and reflected the differences in the initial soil fertility level at the two sites. Yields were also significantly improved by application of Phymyx from 5t of Phymyx ha^{-1}. It was concluded that soybean nodulation in low fertility soils would not be suppressed by starter N in the form of organic amendments like Phymyx up to 7.5 t Phymyx ha^{-1} and that productivity traits including yields of rhizobia-inoculated soybean in such soils could be improved by co-application of organic amendment and a legume-specific fertilizer blend.

CHAPTER 1. INTRODUCTION

1.1 Background information

Soybean (*Glycine max*) is one of the world's most important legumes in terms of production and trade and has been a dominant oilseed since the 1960s (Smith and Huyser, 1987). Soybean is said to have originated from Asia and later introduced into North America, Europe, then into South and Central America (Hymowitz, 2004). Currently, about 50 countries worldwide grow soybean (Boerma and Specht, 2004). The United States of America (USA) accounted for 40 to 45 % of the world's total soybean production in 2003 (Boerma and Specht, 2004). The United States of America and Brazil were the first and second biggest producers of soybean in the world with an output of 73 million metric tons (33 %) and 42 million metric tons (28 %) respectively in 2008. Records in 2008-2009 show Nigeria is the largest Africa's soybean producer (39 %), closely followed by South Africa (35 %) while Uganda is the third African producer (14 %). Africa's soybean production cannot match her demand. According to FAO, Africa spent US$ 1 billion in 2004 to import soybean and soy oil (Kolapo, 2011).

In Kenya, soybean production is very low even within the African context. Earlier FAO records did not recognize Kenyan production in global soybean statistics. Historic data on Kenya's soybean production is poor and scanty, especially in the years before 1990. After 1990, data suggests that production, area and yield have remained almost stagnant, with little annual change (FAOSTAT, 2008). Western province stands out as the leading soybean producing region in Kenya, accounting for nearly 50 % of total national

smallholder planted area and production in 2003. The main soybean production areas are Butere, Mumias, Busia, Bungoma, Teso, Kakamega, Mount Elgon, Lugari, and Vihiga. Butere, Mumias, Busia, and Bungoma districts accounted for approximately 80 % of the total soybean production in Western Kenya in 2003 (Chianu *et al*., 2010). Annual domestic soybean production between 2000 and 2010 was on average 2425 MT (FAO, 2011). The yield ranged between 715 and 1010 kg ha^{-1} with an average harvested area of 2759 ha. The FAO data from 2011 shows a rise in production and yield up to the astonishing value of 2.5 t ha^{-1}. An experiment carried out to assess the performance of rhizobia inoculants in western Kenya found an average yield of up to 2 t ha^{-1} for the variety SB 19 (TGx 1740-2F) (Masso et al., 2016).

Soybean provides a valuable source of protein and thereby sustaining nutritional balances of low-income populations (Appunu, 2009). It contains about 40 % protein (Greenberg and Hartung, 1998). Soybean products are cholesterol free, high in calcium, phosphorous and fiber, and have one of the lowest levels of saturated fat (BIDCO, 2005). About 80 % of soybean produced in Kenya is consumed by the livestock industry with human consumption accounting for about 20-30 %. The demand was expected to rise to about 150,000 T per year by the year 2014 (MOA, 2006; Jagwe and Nyapendi, 2004).

Soybean being one of the legumes has been shown to meet up to 80 % of its nitrogen (N) budget through Biological Nitrogen Fixation (BNF) (Hungria *et al*., 2006). Biological nitrogen fixation is the process whereby atmospheric nitrogen (N_2) is reduced to ammonia in the presence of the enzyme nitrogenase. Nitrogenase is a biological catalyst found naturally in certain prokaryotes such as rhizobia. Biological nitrogen fixation is due to the

association of plants, with both free-living (for example. *Azoarcus, Azospirillum*, and *Azotobacter*) and symbiosis (for example. *Rhizobia* and *Frankia*) with higher plants (Herridge *et al*., 2008). Leguminous plants fix atmospheric nitrogen by working symbiotically with gram-positive bacteria of the genus Rhizobiaceae. Rhizobia infect root hairs of the leguminous plants and form nodules, the *Rhizobium* differentiates to bacteroids- the form in which they are able to fix N. The common soybean nodulating rhizobia that have been identified are *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii, Bradyrhizobium diazoefficiens* and *Sinorhizobium ensifer / fredii* (Delamuta *et al.,* 2012; Jordan, 1982; Young, 2003). However, the amounts of N_2 fixed can vary considerably in time and space (Wagner, 2011). Nitrogen fixation process is influenced by factors such as; presence, population and effectiveness of rhizobia, present nitrogen levels in the soil, plant genotype and age, plant and rhizobia interactions and changes in soil physiochemical conditions.

The amount of nitrogen fixed by a legume in an environment is highly influenced by the proportion of nitrogen derived from the atmosphere as a result of symbiotic nitrogen fixation by rhizobia in the legume's nodules. The N-fixing potential of legumes is expected to be high when soil mineral N is low compared to richer soil conditions (Naudin *et al*., 2010). Thus, in organic crop productions prone to N deficiencies, the legumes might fix large amounts of N and enrich the soil-plant system. As soil organic matter (SOM) levels increase in legume-based systems over time, the mineralization of N from larger SOM pools may suppress BNF (Waterer and Vessey, 1993). The nitrogen available to plants is enhanced by the addition of inorganic fertilizers and biological

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fixation via legumes by the rhizobia bacteria (Naudin et al., 2010). Factors such as high soil temperature (Giller, 2001), low levels of soil moisture (Boonkerd and Weaver, 1982), low pH (less than 5.5), low organic matter (Dudega and Khuraha, 1989) affect the rhizobia survival.

Currently, progress has been made in understanding soybean production and improving its yields. Despite this, yields still remain less striking as compared to crops like corn, wheat, rice and sorghum in Africa (Muthuri, 2013). Approaches such as inoculation with proven and compatible strains, microbial screening for improved strains, host-plant screening and breeding and adoption of cropping systems and cultural practices have been found to enhance biological nitrogen fixation (Van Kessel and Hartley, 2000).

1.2   Problem statement

Soybean production in Kenya has remained low, partly due to soil nutrient depletion and degradation which have been considered serious threats to agricultural productivity. Studies have shown that productivity of soils in western Kenya is limited by a deficiency of nutrients such as nitrogen, phosphorous and potassium a problem compounded by low organic matter. Intensification of land use especially by small-scale farmers with minimal nutrient inputs has led to declining crops yields and increased nutrients removal (De Rider *et al.,* 2004). In addition, the rapid population growth rate in Kenya has put pressure on land making most farmers practice continuous cropping to meet their day to day food requirements.

Soybean production in most sub-Saharan countries and Kenya in particular, is faced with different constraints such as seed viability, poor nodulation with native rhizobia in the soil, inherent low levels of essential nutrients, low availability and awareness of inoculant use and lack of farmer market power information (Njira *et al*., 2013). Despite this, responses of soybean to inoculation with *Bradyrhizobium* spp have been studied and documented and found to increase soybean yields (Njira *et al*., 2013; Thuita *et al*., 2012). However, its full potential in sub-Saharan Africa is yet to be realized due to effects of varying soil limiting conditions. Critical levels of N below and above which response to rhizobial inoculation is or not guaranteed are unknown. Responses of soybean to N have been studied and documented in soybean growing areas of Kenya but little has been done to establish the scale of soil nitrogen at which response is guaranteed. A well-established fact is that when legumes are grown in low and high available nitrogen, the nitrogen

fixation rate is reduced (Solomon *et al*., 2012). In soils with low nitrogen, a moderate amount of 'starter nitrogen' would be required by the legume plants for nodule development, root and shoot growth before the onset of BNF (Herridge et al., 1984; Goi et al., 1993) which currently is not known in soybean production areas in western Kenya. This study therefore seeks to assess the critical levels of soil nitrogen at which response to inoculation are realized and suppressed in soybean production based on greenhouse and on-farm trials.

1.3   Justification

In Kenya about 80 % of soybean is consumed by the livestock industry with human consumption accounting for about 20 to 30 %. By the year 2016, demand was expected to rise to about 150, 000 tons per year (MOA, 2006; Karuga and Gachanja, 2004). This trend, therefore, calls for a need to increase soybean production to supply the deficit which is normally met through imports. Increasing the area of land under soybean to boost production is not feasible due to increasing population growth rate. The inclusion of soybean in the farming systems can contribute to an improvement in soil fertility since, as a legume, soybean fixes nitrogen (one of the plant nutrients lacking in most of Kenya's soils) from the atmosphere. In addition, three characteristics of soybean make it outstanding as a food security crop. First, soybean matures early (depending on the agro-ecological zone, with some varieties maturing in 90 to 100 days). Secondly, soybean's drought tolerant characteristic enables the crop to yield under water stress while many other crops fail due to drought and thirdly, soybean has a high commercial index (Chianu *et al*., 2008).

To improve soybean yield, biological nitrogen fixation through inoculation with efficient strains of bradyrhizobia has already been tested in several countries (McInnes and Haq, 2007; Houngnandan *et al*., 2009; Afzal *et al*., 2010; Hussain *et al*., 2011; Tairo and Ndakidemi, 2014) and this has become a usual practice in sustainable soybean production. Besides this, optimal soils conditions especially soil nitrogen levels is important for the success of the inoculant application. Deficiencies in nitrogen were observed during a survey conducted on integrated soil fertility in Siaya district Kenya. This was attributed to naturally low inherent levels in the soil, continuous cropping, lack of crop rotation, removal of crop residue from the fields, non-application of sufficient organic and inorganic fertilizers, reduction of fallow period and soil erosion (Mango, 1999).

A great demand is made on the alternative and inexpensive source of nitrogen (Mwangi 1994). In this case, soil amendment with Phymyx would act as a source of different macro and micronutrients and improve the soil organic matter coupled with reduced residual effects on the environment. Determining optimal soil nitrogen levels for soybean to respond to rhizobia inoculation and use of soil amendment at low fertile soils will therefore aid in guiding the best nitrogen management strategy to boost soybean production.

1.4   Objectives

1.4.1   General objective

To determine the optimal N and carbon levels for improvement of BNF of soybean with rhizobia inoculation in selected Kenyan soils.

1.4.2   Specific objectives

1. To determine the optimal range of soil nitrogen with rhizobia inoculation for effective BNF in soybean.
2. To assess the effect of organic amendment on soybean BNF within the optimal range of soil N.
3. To validate greenhouse results on the relationship between soybean BNF and soil N in field conditions.

1.5   Hypotheses

1. The response of soybean to rhizobial inoculation is optimal at certain soil nitrogen levels and carbon levels.
2. Soil organic amendment improves soybean response to rhizobial inoculation within the optimal range of soil N.
3. Greenhouse results on the relationship between BNF and soil N relate to results under field conditions.

1.6   Significance of the study

Optimal soil environment is an important component for an efficient BNF system. Available soil nitrogen and carbon influences the rates of BNF. However, optimal soybean response to rhizobial inoculants is expected within specific ranges of N. Hence, determining the actual ranges of N is important for improved soybean response to rhizobial inoculants, and consequently yield increase. This will be useful to determine when starter N or soil amendment with organic manure may be necessary to improve BNF. Importantly, the increased soybean yields will contribute to food security for small scale farmers, as well as improved income as soybean is also a cash crop.

1.7   Conceptual framework

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      │ Low soil fertility, poor BNF, poor        │
      │ nodulation, reduced soybean production     │
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           ▼                              ▼
┌────────────────────────┐    ┌────────────────────────┐
│ Use of rhizobial        │    │ Optimal nitrogen levels │
│ inoculants with         │    │ and plant nutrients     │
│ effective strains       │    │ supplements (organic)   │
└────────────────────────┘    └────────────────────────┘
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            ╲                            ╱
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          ┌──────────────────────────────┐
          │ Improved BNF                  │
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          │ Increased soybean yields      │
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          │ Food security, improved living │
          │ standards and increased income │
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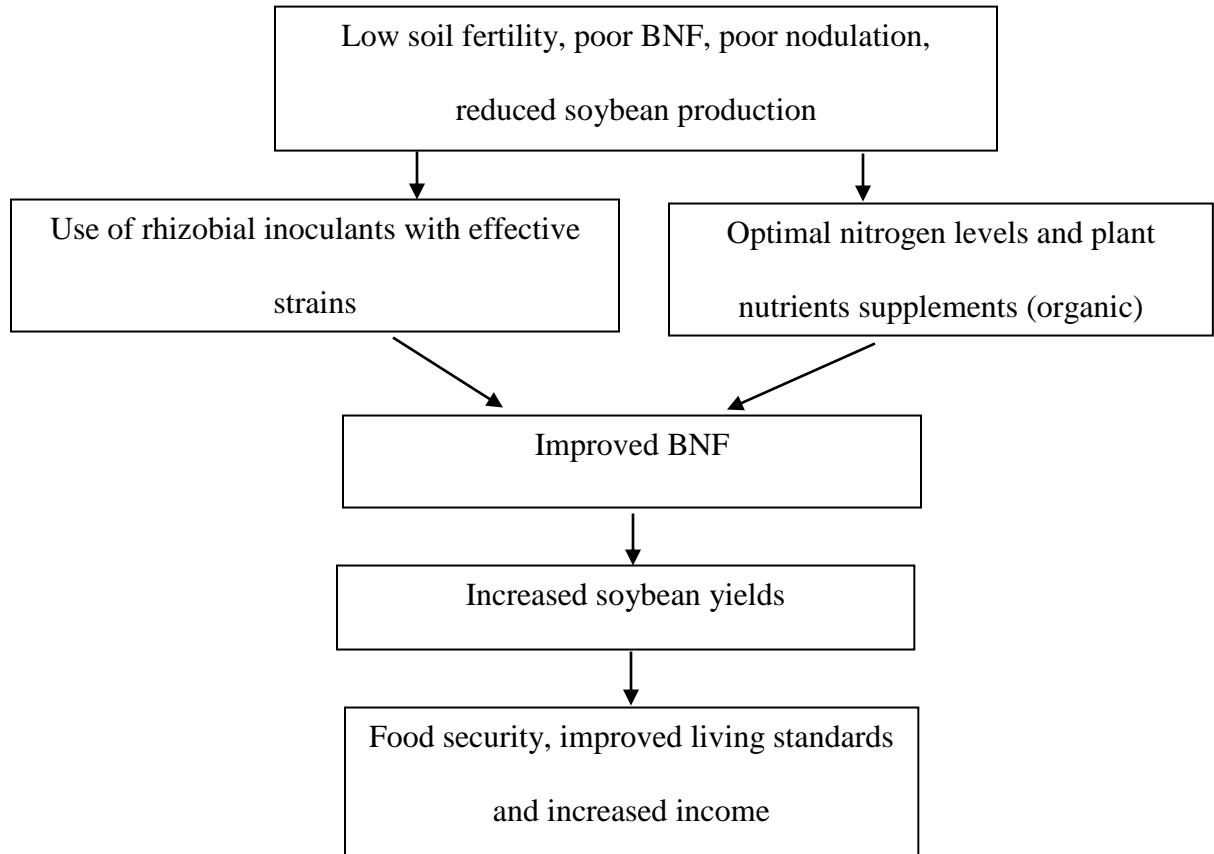
CHAPTER 2. LITERATURE REVIEW

2.1 Rhizobia

Rhizobia are rod-shaped, gram-negative and non-spore forming bacteria. They are aerobic and can be found free-living in soils, or cultured in agar (Vincent, 1982). They are genetically diverse and heterogeneous group of bacteria (Somasegaran and Hoben, 1994) and able to elicit nodule formation on legumes. Rhizobia comprise of the genera *Rhizobium*, *Bradyrhizobium*, Azorhizobium and *Sinorhizobium* (Denarie *et al*., 1996). *Sinorhizobium* consists of all fast growing acid producing rhizobia while *Bradyrhizobium* comprises of the slow growing alkali producing rhizobia. Rhizobia as bacteria have a unique ability to infect root hairs of legumes and induce effective nitrogen-fixing nodules to form on roots. They multiply by simple cell division and generation time ranges from two to four hours for fast growers which generally form relatively large colonies of 2-4 mm diameter within three to five days and for slow growers takes six to eight hours to form colonies of 1μ in diameter within 7 -10 days (Brockwell *et al.,* 1995). Rhizobia are part of the soil micro-flora in a free-living state in the rhizosphere of legumes (Allen and Allen, 1981) until the point where nodulation becomes possible (Rendig and Taylor, 1989). However, it is the nature and properties of soil that allows billions of microorganisms to coexist (Pepper and Upchurch, 1991). Despite the widespread distribution of leguminous crops, many soils remain void of rhizobial strains and under local conditions nodulation may not occur with introduced plants due to the lack of suitable soil conditions (Brockwell *et al*., 1995).

2.2 Nitrogen

Nitrogen is known as the most abundant element on earth. Despite this, it is one of the most limiting factors of growth and production of crops in sub-Saharan countries (Sanginga *et al*., 1997). Most nitrogen is naturally present in the soil as organic (Dashora, 2011) and can only be used when reduced to ammonia by nitrogen fixation. The reduction is either by chemical fixation through industrial production or biological fixation involving microorganisms. However, even in the presence of such process, nitrogen is one of the usually deficient plant nutrients in soils. Despite its abundance in the atmosphere as a gas, it cannot be utilized directly by plants. Most plants utilize nitrogen in its ionic forms ammonium (NH_4^+) and nitrate (NO_3^-) from soil (Solomon *et al*., 2012). Nitrogen is essential for all enzymatic reactions in a plant and also a major part of chlorophyll molecules playing a necessary role in photosynthesis (Tairo *et al*., 2013). In legumes, nitrogen improves the quality and quantity of dry matter (Uchida, 2000). Soil fertility decline has become a problem as a result of poor land management leading to continuous loss of soil nitrogen through the activities of soil erosion, leaching, and crop removal. Natural reserves of soil N are normally low in many African soils, measures must be taken to improve yield through BNF because when nitrogen is lost from soils; leguminous plants, however, are not able to convert atmospheric N into absorbable forms of NH_4^+ and NO_3^- before used by plants (Akpalu *et al*., 2014).

2.3   Biological nitrogen fixation

Biological nitrogen fixation is the biological process by which atmospheric N_2 gas is reduced to NH_4 (Salisbury and Ross, 1992). Biological nitrogen fixation (BNF) is of great importance in a number of environments, such as terrestrial, freshwater, marine and arctic (Salisbury and Ross, 1992). Raven *et al*. (1992) reported that the fixation of nitrogen is a process upon which all living organisms are dependent. Biological nitrogen fixation is estimated to be approximately 150 to 200 million tons annually on the earth's surface. It comprises of non-symbiotic and symbiotic systems. Non-symbiotic involves free-living organisms like *Azobactor, Klebsiella,* and *Clostridium* which fix atmospheric nitrogen independently while symbiotic involves a close association between the rhizobial bacteria and the leguminous plants. The symbiotic relationships between specific soil micro-organisms and plants are the most significant contributor of BNF in most terrestrial ecosystems (Boddey *et al*., 2000) believed to fix nitrogen within a range of 100 kg to 600 kg N/ha/year (Graham and Hubell, 1974). The fixation of nitrogen provides the plant with available ammonium, whilst the plant provides the rhizobia with simple sugars (Pepper, 1991). Estimates have shown that this symbiotic relationship contributes 40 million tons of nitrogen yearly to grain legumes (Hardy and Havelka, 1975). This makes BNF an important source of fixed nitrogen to productivity directly by increasing the production of legume and indirectly improving soil fertility (Mpepereki *et al*., 2000). However, constraints such as lack of knowledge on BNF information by most small-scale farmers have been identified as a major factor hindering the adoption of BNF technology (Woomer *et al*., 2003).

2.4   Factors affecting nitrogen fixation

2.4.1   Soil nitrogen

Nodule formation and nitrogen fixation are very sensitive to external nitrogen sources including fertilizers and available soil nitrogen. High available nitrogen is known to suppress nitrogen fixation (Halvin *et al*., 2005) while on the other hand low nitrogen levels also affect plant development and nitrogen fixation (Ajeigbe *et al*., 2010). When the supply of nitrogen available from the soil and fertilizer increases, the amount of nitrogen fixed by the plant decreases. Low levels of available nitrogen may have little impact on nodulation and fixation; however, when the combined levels of available soil and fertilizer nitrogen reach approximately 40 kg/ha (35 lb/acre), any additional nitrogen reduce nodulation and fixation. The application of combined nitrogen especially nitrate to soybean has shown to strongly inhibit nodule formation, growth and nitrogen fixation (Yamamoto *et al*., 2014). Furthermore, combined levels of soil and fertilizer nitrogen greater than 55 kg/ha (50 lb/acre) can dramatically delay nodulation and reduce or eliminate nitrogen fixation (Mcvicar *et al.,* 2007).

In a research conducted at the University of Arid Agriculture on evaluating the effects of different levels of nitrogen on inoculation, nitrogen levels of 15, 30 and 45 kg / ha, seed inoculation with rhizobia was found to have a significant increase in growth and nodules formation of green grams (Ahmed *et al.,* 2006). Highest increase on nodulation was reported at 0.05 and 0.15 % N with rhizobia inoculation (Musyoki *et al*., 2003).

Mendes *et al*. (2003) reported that soybean yield responses to starter N fertilization are extremely variable, depending on the efficiency of *Bradyrhizobium* strains, soybean

cultivars, soil NO_3^-N content and N rates. Furthermore, most effective rhizobia host plant symbiosis fixes little or no nitrogen at all if the soil nitrogen is sufficient to meet the nitrogen demand of the crop (Choudhny, 2012).

2.4.2 Soil organic matter

Soil organic matter provides a carbon source for primary producers like cyanobacteria that convert atmospheric nitrogen to plant-available N forms. Soil organic matter plays an important role in plants by keeping the nutrient reserves for microbial activities and plant growth, improving water holding capacity, enhancing the chelation and bioavailability of macro and micronutrients to soil microorganisms and plants (Njira *et al*., 2013). All organic matter contains carbon and nitrogen. The relative amount of each present (the carbon to nitrogen ratio) will determine whether nitrogen and sometimes other nutrients will be available for crops use. Carbon to nitrogen ratio of 20:1 is sufficient to supply of N for plants use (Miller and Gordiner, 2001). Bationo *et al.* (2006) reported that carbon content and status in the soil is closely associated with clay and silt contents and clay type, which is known to influence the stabilization of organic carbon and microorganisms. Soils with rich pools of soil organic carbon have reduced rates of BNF since they have a high percentage of available soil N (Waterer and Vessey, 1993). However, the amount of soil organic carbon can be increased in low fertile soils by use of organic sources to meet the crops demand. In a study to find out the effects of organic and inorganic sources of nitrogen with *Rhizobium* inoculation, nitrogen at the rate of 20 kg N / ha + seed inoculation with rhizobia showed a significant increase in plants height, dry matter and yield attributes. Furthermore, when the organic source of N + seed inoculation

was used better results were recorded. Vermicompost + inoculation produced the highest dry weight of nodules per plant compared to Urea + inoculation (Yadav and Malik, 2005). This implies that application of an organic source of organic matter can overcome deficiencies in the soil that might otherwise require amendment with inorganic fertilizers which are expensive and would lead to environmental degradation.

2.4.3 Other factors affecting N fixation

Many of the soil properties and agricultural management practices that affect nodulation also affect the genetic diversity of rhizobia found in a given site. The soil type influences the structure of microbial communities' especially bacterial populations in different textures (Fang *et al*., 2005). Soil texture, and specifically high clay soils, have been observed to reduce rhizobia diversity in soybean cropping (De Fatima Loureiro *et al*., 2007). Hamarashid *et al.* (2010) reported that fine-textured soils have the highest value of soil organic carbon and total nitrogen in the order clay loam, loam and silt loam as compared to course texture soils of loamy sandy and silty loam. Fine textured soils support more microbial biomass than coarse-textured soils and thus soils with high clay content would have more rhizobia populations than coarse soils (Carney and Matson, 2005). Silver *et al*. (2000) found that soil texture plays a key role in belowground carbon storage in soil ecosystems and strongly influences nutrient availability and retention, particularly in fine textural soils. Fine textured soils show more stable aggregates, which in turn may act as a media of greater amount of organic carbon and total nitrogen contents (Raiesi, 2006).

Nitrogen fixation is sensitive to the availability of phosphorous in the soil. P deficiency has shown reduced nodule mass and decreased ureide production (Sinclair and Vadez, 2002). Phosphorous is needed for biological nitrogen fixation as it is an energy driven process (Haru and Ethiopia 2012). Insufficient P restricts root growth, the process of photosynthesis and translocation of sugars which affects nitrogen fixation of plants directly or indirectly (Olivera *et al*., 2004).

Moisture stress affects the nodule functions. The drought conditions reduce nodule weight and nitrogenase activity. After exposure to the moisture stress for 10 days, the nodule cell wall starts to degrade resulting in senescence of bacteroids (Ramos *et al*., 2003).

Accumulation of Na^+ has been recorded to reduce the plant growth, nodule formation, and symbiotic N-fixation capacity under saline conditions (Kouas *et al*., 2010). High salt level directly affects the early interaction between the *Rhizobium* and legumes in nodule formation (Wahab *et al.,* 2002).

The plant nitrogenase activity reduces as a result of the formation of ineffective nodules at high temperature (40^0 C) (Hungria and Vargas, 2000). The N_2 fixing between legumes and their N_2 fixing bacteria is dependent upon many environmental factors and farm management practices (Peoples *et al*., 1995). This affects the plant growth and development. As a result, even the persistent *Rhizobium* strains fail to perform root infection and N fixation in their full capacity (Panchali, 2011)

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Nitrogen fixation is inhibited by low soil pH (Van Jaarsveld, 2002). The characteristics of highly acidic soils (pH < 4) are low levels of phosphorous, calcium, and molybdenum along with aluminum and manganese toxicity, which affects both plant and the rhizobia. As a result, under low soil pH conditions, nodulation and N fixation are more severely affected than the plant growth. Alkaline (pH > 8) soils tend to be high in sodium (Na^+), chloride (Cl^-), bicarbonate (HCO_3^-) and borate (BO_3^-) which reduces the N fixation (Anthraper and DuBois, 2003).

2.5   Soybean and rhizobia inoculation

Soybean is an important N_2-fixing leguminous crop, due to its high-quality protein and input of combined N_2 into the soil. The symbiotic relationship of rhizobia and soybean roots and the subsequent fixation is among the vital physiological processes which occur in the growth and development of soybean (Tairo *et al*., 2013). Soybean is a new crop in the tropics and it is important to inoculate the seed with the appropriate *Rhizobium* if no soybean crop has previously been grown in the field (Hungria *et al.,* 2006). Inoculation of legumes is especially critical when compatible rhizobia are absent, when population densities are low, or when native rhizobia are not effective (Catroux *et al*., 2001). The goal of inoculation is to introduce a large number of viable host-specific Rhizobia in order to increase infection rates, which ultimately leads to higher yields (Deaker *et al*., 2004). In this case, the addition of an inoculant with the host-specific Rhizobia can increase the BNF of the legume. The increase of rhizobia numbers in the rhizosphere is a response to the release of nutrients by the host legume (Somasegaran and Hoben, 1994). Studies have shown that BNF which is enhanced by inoculation of rhizobia to compatible

host legume leave residual nitrogen in the soil which improves soil organic matter for the following cropping seasons of cereals and other legumes (Tairo *et al*., 2013). BNF is thus considered to yield economic and ecological benefits (Ndakidemi *et al*., 2006).The maximization of biological nitrogen fixation has been obtained by inoculating legumes seeds with efficient *Bradyrhizobium* inoculants in low N soils without N fertilizer application (Egamberdiyeva *et al.,* 2004). Use of rhizobia inoculants coupled with phosphorous supplements on legumes plays a great role in cropping systems which in return increase plant productivity and soil fertility (Abbasi *et al*., 2008). Phosphorous is an essential ingredient for *Rhizobium* bacteria to convert atmospheric N_2 to ammonium, a form usable by plants (Dakora and Keya, 1997). Rhizobia inoculation and phosphorus among the factors that contribute to soybean success have shown prominent effects on nodulation, growth, and yield (Shahid *et al*., 2009). Soybean among the legumes is an important N_2 fixing crop and obtains nitrogen directly from the soil and indirectly through symbiotic fixation. Inoculation of soybeans with efficient strains of *Bradyrhizobium japonicum* have shown to increase plant dry matter, nitrogen concentration, nitrogen accumulation and grain yields (Javaid *et al*., 2010; Hungria *et al*., 2006).

Abbasi *et al*. (2008), reported that significant yield increases were obtained by inoculation of soybean with appropriate bacteria. *Bradyrhizobium* inoculation increased soybean seed yield by 85 % over control. Similarly, Egamberdiyeva *et al.* (2004) and Okereke *et al*. (2004) reported that nodule number, nodule dry weight, and soybean shoot yield were increased when seeds were inoculated with *Bradyrhizobium*. However, inoculation may not be required in fields where soybeans have been previously grown

and inoculated for many years (Li *et al*., 2008). Inoculation of legumes with *Bradyrhizobium* strains have a significant effect on the soil chemistry and enhances nutrients uptake of P, K, Ca, Mg, S, Mn, Fe, Cu, Zn, B, and Mo by plants (Ndakidemi *et al*., 2011 and Makoi *et al*., 2013). Seeds inoculation with beneficial rhizobia bacteria could be an alternative for use of expensive commercial nitrogen fertilizers and realization of optimal productivity in legumes (Hussain *et al*., 2011).

2.6 Nodule occupancy

The competition for nodule occupancy between strains of rhizobia in soybean is a complex area of study of legume-*Rhizobium* symbiosis. The intrinsic characteristics of the rhizobia, environmental variables and genetic determinants of the host contribute to the failure or success of rhizobia strains to occupy a significant portion of nodules formed (Thies *et al*., 1992). To identify rhizobia occupying nodules, and assess competitiveness, a reliable and sensitive method of identifying specific strains in nodules is required. This can be achieved by a number of procedures (Aouani *et al*., 1997). Enzyme-linked immunosorbent assay (ELISA) and Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) are the most used methods for studying the symbiotic effectiveness of different rhizobia strains (Thies *et al*., 2001). Serological identification of rhizobia strains involves the use of antibodies raised against surface antigens of the test strain to detect the presence (or absence) of that strain in a suspension through agglutination, immunodiffusion, immunofluorescence or the enzyme-linked immunosorbent assay (ELISA) (Kishinevsky and Gurfel, (1980). The ELISA technique is highly specific, reproducible, and commonly used to detect rhizobial strains directly from

nodules. Additionally, the method is sensitive, can detect antigens in small nodules, uses small quantities of reagents, is relatively quick, and permits the rapid screening of large nodule samples. It can also detect double strain occupancy of nodules. However, the immunofluorescence technique has also been successfully used to rapidly identify rhizobial strains though this requires expensive equipment and large quantities of labeled antibody (Spriggs and Dakora, (2009).

The PCR-RFLP method of the IGS between rDNA 16S and 23S is a tool often used in molecular characterization. It has yielded many results with isolated strains in culture (Laguerre *et al*., 1996) and recently with nodules for diversity studies (Sarr *et al*., 2005) The PCR-RFLP technique has a great advantage over ELISA to the study of rhizobial competition as it requires no prior preparation of individual strains, such as preparation of antisera, selection of screening for antibiotic resistances or introduction of a marker gene (Richardson *et al*., 1995). Moreover, the procedure does not necessarily require a detailed prior knowledge of the individual strains, and there is no need to specifically mark target strains. Therefore, PCR-based fingerprinting techniques allow useful information pertaining to any particular Rhizobia strain or isolate, or the rhizobial population itself, to be readily obtained (Richardson *et al*., 1995).

CHAPTER 3. MATERIALS AND METHODS

3.1 Study sites

The experiments were performed in three sets; two pot trials under greenhouse conditions at International Centre of Insect Physiology and Ecology (ICIPE), Duduville campus and one under field conditions Ugunja Division, Siaya County for the selected soil nitrogen levels (0.058 % and 0.080 %). Siaya County lies at latitudes of $0°26´$ North to $0°$ 90´ South and longitudes $33°$ 58´ East and $34°$ 58´ West. The area receives average bimodal rainfall of 1700 and 1450 mm per annum. The long rains during March to July and short rains from September to December. Mean minimum temperatures of $15°C$ and mean maximum temperatures of $30°$ C are experienced. The area has soils ranging from fine to course texture (Mango, 1999). Dominant types of soil are Acrisols, Nitisols and Lixisols.
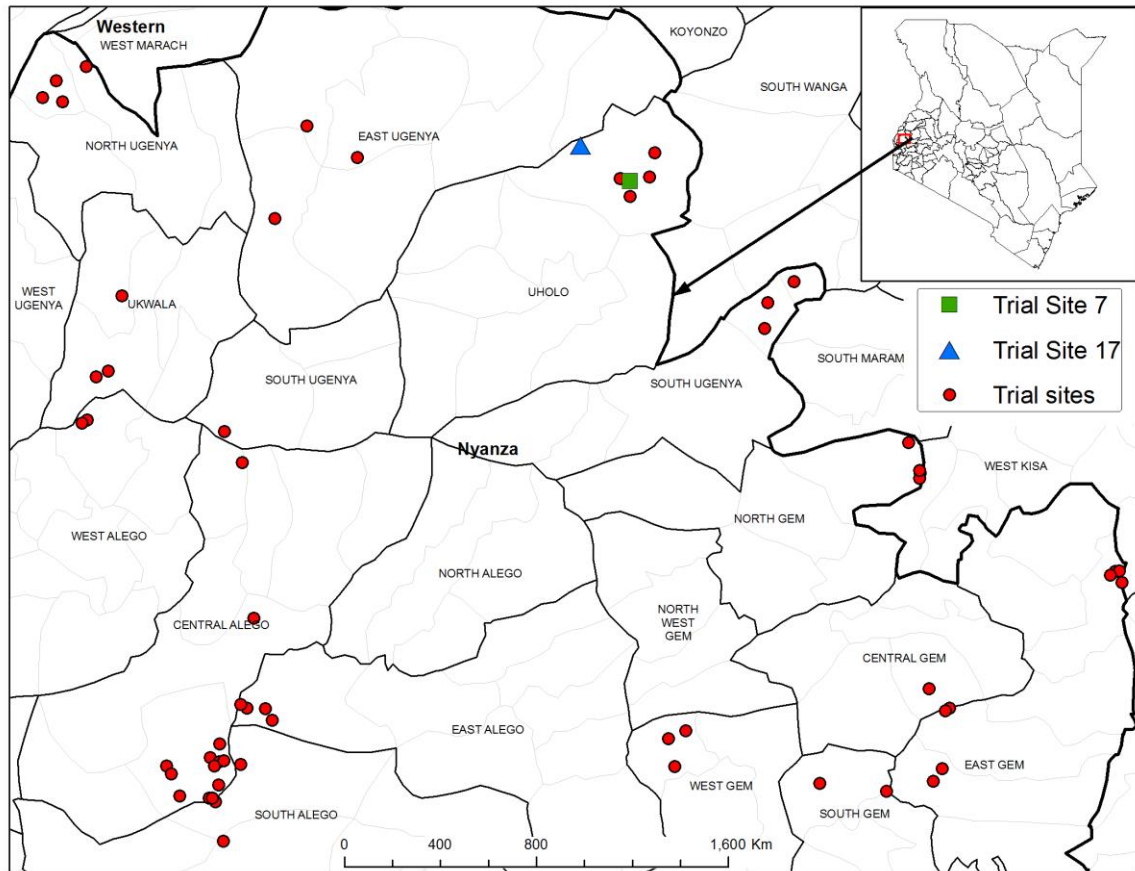
Figure 3.1: Map showing the sites where the soils used in the 1st greenhouse experiment were collected including Trial Site 17 (0.08 % N) that was also used in both the 2nd greenhouse experiment and the field trial, and Trial Site 7 (0.06 % N) used also in the field trial.

3.2 Soil sampling and analysis

Soils were collected at a depth of 0-20 cm in sixty selected farms in different locations of Siaya County. The selected farms had nitrogen levels ranging 0.029 % to 0.21 % according to IITA soil analysis database. The farms had a history of either soybean production or maize production and intercropping of the two crops. Sub-samples were

analyzed for physical and chemical properties. Organic Carbon was determined by chromic acid digestion and spectrophotometric analysis (Heanes, 1984), while total N determined from a wet acid digest (Buondonno *et al.,* 1995) and N analyzed by colorimetric analysis (Anderson and Ingram, 1993). Soil texture was determined using the hydrometer method and a textural triangle used to place them in textural classes. Soil pH in water and electrical conductivity were determined in a 1:2.5 (w/v) soil:water suspension. Total P - determined from a wet acid digest (Buondonno *et al.,* 1995) and analyzed using the Murphy and Riley procedure. Available P extracted using the Mehlich-3 procedure (Mehlich, 1984), the resulting extracts analyzed using the molybdate blue procedure described by Murphy and Riley (1962). Exchangeable cations (Ca, Mg, K, and Na) and microelements (Cu, Zn, Mn, and Fe) extracted using the Mehlich-3 procedure and determined by atomic absorption spectrophotometry.

3.3 Determination of indigenous rhizobia population

The most-probable-number (MPN) technique was used to estimate the indigenous rhizobia population (Brockwell et al., 1975). Sand was washed thoroughly dried, autoclaved and used as the growth medium for the experiment. Pots of 1 kg capacity were filled with sand and placed on saucers. Macronutrients N, P, K, Mg, Ca, and S, and micronutrients Mn, Zn, Cu, B, Mo, and Co were applied as solutions of KH_2PO_4, $CaCl_2$, $MgSO_4$, K_2SO_4, $MnSO_4$, $ZnSO_4$, $CuSo_4$, $CoSo_4$, H_3BO_3, Fe-citrate and Na_2MoO_4 (Somasegaran and Hoben, 1994). Five (5) ml of stock solutions were diluted in 5L of distilled water and added to plants once a week.

Seeds were surface-sterilized by soaking in 3.3 % NaClO solution for 2 minutes and then thoroughly washed and pre-germinated at 28^0 C for 48 hrs. Three pre-selected healthy seeds of uniform size were then planted per pot and thinned to one plant per pot of comparable height and vigor between 1 – 2 weeks after planting.

Ten grams of soil were suspended in 90 ml of physiological water and shaken at 200 RPM for 10 min in the shaker. One (1) ml of the suspension was then be pipetted into 4ml of physiological water and shaken for 5 min, this second step was repeated three more times to obtain the final dilution. Dilutions obtained as followed 1:50, 1:250, 1:1250, and 1:6250. Four control pots were un-inoculated for each soil. Watering was done daily under greenhouse conditions for a period of 8 weeks prior to harvesting. Presence (+) and absence of nodules (-) were recorded for each dilution and the results used to estimate the rhizobia population using the MPN tables (Fisher and Yates, 1963; Brockwell et al., 1975).

Table 3.1: Treatment structure for (MPN) experiment

Factor	levels	Description of levels
Crop	1	Soybean (TGx1740-2F)
Media	1	Sand
Treatment	5	Soil dilutions 1:50, 1:250, 1:1250, 1:6250
Soils	12	Different sites in Siaya
Replicates	4	
Total number of pots	240	

3.4 Determining the optimal range of soil nitrogen with rhizobia inoculation for effective BNF in soybean.

3.4.1 Experimental design and treatments

The experiment was laid as Completely randomized design (CRD) including: (i) 60 soils of variable N (that is, covering the whole range of N in soils with ≤ 2.1 % organic carbon, identified during soil characterization), (ii) two treatments (that is. with and without inoculation) and (iii) replicated 3 times for a total number of 360 experimental units.

Table 3.2: Treatment structure for greenhouse experiment 1.

Factor	Levels	Description of levels
Soil	60	Whole range of N within soil org C ≤ 2.1 %
Treatments	2	With and without inoculation
Replicates	3	CRD

Total number of pots 30 * 2*2* 3=360

3.4.2 Seeds preparation, inoculation, planting and thinning

A germination test was done for Soybean variety (TGx1740-2F) prior to planting in the greenhouse to determine the germination percentage. The variety is early maturing and promiscuous bred to nodulate with indigenous *Bradyrhizobium* spp. It is a cross of specific nodulating North American genotype and non-specific Asian soybean genotype (Kueneman *et al*., 1984). TGx1740-2F has better nodulation with a range of rhizobia than local varieties in different parts of Kenya (Wasike *et al*., 2009). Seeds were surface-sterilized by soaking in 3.5 % NaClO solution for 2 minutes and rinsed thoroughly 5 times to remove any disinfectant with sterile distilled water. Soils were air dried under shade, sieved and weighed to fill in perforated 2 kg pots. Sympal (P: K 23:15 + 10CaO + 4S + 1MgO + 0.1Zn) at a rate of 30 kg P ha^{-1} was thoroughly mixed with the soil for

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supplement of other essential nutrients. The seeds were later inoculated with Legumefix (*Bradyrhizobium japonicum* strain 532c) from Legume technology, UK, at a rate of 1 g for 100 soybean seeds. Healthy (2-3) seeds of uniform size were then planted per pot and thinned to one plant per pot of comparable height and vigor at 2 weeks after planting. Routine management practices such as watering were carried out till 50 % podding.

3.4.3   Harvest and nodulation assessment

The plants were harvested at 11 weeks after planting (at 50 % podding). Shoots were cut using a clean, sharp knife at 1 cm above the soil surface. The pots were emptied on a 2 mm sieve and soil washed to isolate the roots and nodules. Nodules fresh weight and fresh biomass weights were recorded per plant. Fresh nodules per plant were then surface sterilized and stored in glycerol for nodule occupancy determination. Fresh shoots were dried at $60\ ^0C$ for 48 hours to obtain the dry weight.

3.4.4   Nodule analysis

The nodules stored in the glycerol were surface sterilized using 70 % ethanol for 30 minutes and 3.3 % Ca $(ClO)_2$ for 2 minutes and rinsed with sterile distilled water thrice. One nodule was crushed in 150 µl of sterile water and DNA extracted as by Krasova-Wade *et al.* (2003) protocol.

3.4.5   Nodule occupancy analysis

The nodule occupancy was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. This involved amplification and restriction of the 16S-23S rDNA intergenic spacer region. The DNA amplification was done using

rhizobia specific primers derived from 3'end of the 16S (FGPS 1490-72; 5-TGCGGGTTTCCCCATCCTT-3' (Navarro *et al.,* 1992) and from the 5' end of the 23S rDNA (FGPL 132-38; 5'-CCGGGTTTCCCCATTCGG-3') (Ponsonet and Nesma, 1994). The PCR amplification was done in a Bio-Rad iCycler^{TM} thermal cycler at initial denaturation for 5 min at 94° C, 35 cycles of denaturation (30 s at 94° C), annealing (30 s at 58^0 C) and a final extension (7 min at 72° C). The PCR products were later visualized by electrophoresis of 3 µl of the amplified DNA on a 2 % horizontal agarose gel in TBE buffer (1.1 % Tris-HCl, 0.1 % Na$_2$ETDA.2H$_2$O, and 0.55 % boric acid). The gel was pre-stained with 0.033 mg ml^{-1} of ethidium bromide and photographed under UV illumination with Gel Documentation system (BIO-RAD) software (USA). Ten µl aliquots of PCR products were digested with restriction endonucleases *Msp*1 (5µ) in a total volume of 15µl for 2 h at 37° C. The restriction fragments were then separated by horizontal electrophoresis in 1× TBE buffer with 3 % agarose gel prestained with 0.033mg ml^{-1} of ethidium bromide. Gel was run at 100V for 3 h and photographed under UV illumination with Gel documentation system (BIO-RAD) software USA. The stain with identical fragment size and number were classified into the same profile and the profiles used to score the inoculant (Legumefix) efficacy in percentages.

3.5 Assessing the effect of the organic amendment on soybean BNF at 0.06 % N and 0.08 % N under greenhouse conditions.

3.5.1 Soil preparation and addition of organic matter

Phymyx, an organic fertilizer from Phytomedia International Ltd was used to raise the level of soil N. Phymyx was analyzed for pH, N, C, P, K, Mg and Ca prior to soil amendment. Soils with N-0.06 % and N-0.08 % which gave shoot biomass of 5 - 10 g plant^{-1} before inoculation and 10 - 15 g plant^{-1} after inoculation were selected for this experiment. The two soils were amended with 5 rates of Phymyx (including a zero) of even intervals, with the maximum rate increasing the tested soils N level to 0.21 % (Table 3.3). The highest rate of Phymyx was equivalent to the upper limit (0.21 % N) of the soil N (%) range obtained in experiment 1.

Table 3.3: Treatment structure for greenhouse experiment 2

Factor	levels	Description of levels
N levels	2	0.06 and 0.08 % N
Phymyx rate	5	0, 2.5, 5, 7.5 and 10 t ha^{-1}
Inoculation	2	with and without inoculation
Replicates	3	Completely randomized design (CRD)

3.5.2 Seeds preparation, inoculation, planting and thinning

A germination test was done for Soybean variety (TGx1740-2F) prior to planting in the greenhouse to determine the germination percentage. Seeds were surface-sterilized by soaking in 3.5 % NaClO solution for 2 minutes and rinsed thoroughly 5 times to remove any disinfectant with sterile distilled water. The two soils with N-0.06 % and N-0.08 % which gave shoot biomass of 5 g-10 g plant^{-1} before inoculation and 10-15 g plant^{-1} after

inoculation in the first greenhouse experiment were air-dried, sieved and 2.5 kg was weighed to fill 2 kg pots. Sympal and Phymyx were thoroughly mixed with the soil. The seeds were later inoculated with Legumefix at a rate of 1 g for 100 soybean seeds. Healthy (2-3) seeds of uniform size were then planted per pot and thinned to one plant per pot of comparable height and vigor at 2 weeks after planting. Routine management practices were carried out till 50 % podding.

3.5.3   Harvest and nodulation assessment

The plants were harvested at 12 weeks after planting. Shoots were cut using a clean, sharp knife at 1 cm above the soil surface. The pots were emptied on a 2 mm sieve and soil washed to isolate the roots and nodules. Nodules fresh weight and fresh shoot weights were recorded per plant. Fresh nodules were then surface sterilized and stored in glycerol for nodule occupancy determination. Fresh shoots were dried at 60° C for 72 hours to obtain the dry weight. The shoots were then ground and analyzed for their nitrogen concentrations using the modified Kjeldahl method as described by Jackson (1975). Finally, nitrogen uptake was determined as the product of SDW yield and the respective nitrogen content in the straw and reported as g nitrogen plant^{-1}. Nodule analysis and nodule occupancy were carried out as described in sections 3.4.4 and 3.4.5 above.

3.6 Assessing the effect of the organic amendment on soybean BNF at 0.06 % N and 0.08 % N under field conditions.

3.6.1 Experimental site

The experiment was carried out in two farmers' fields in Ugunja division of Siaya County where the soils for greenhouse experiment were collected.

3.6.2 Experimental designs

The treatments were laid out following a split plot arrangement in a randomized complete block design (RCBD) (full factorial) with three replicates for each of the two sites. The main plot was Sympal at two levels (0 and 30 kg P ha^{-1}) and the sub-plots as the interaction of the Phymyx levels and inoculation with Legumefix. The main plot size was 6 m by 3 m and subplots 3 m by 3 m and 0.5 m alley between the plots and 1m between the blocks. Soybean was planted at a spacing of 50 cm by 5 cm at the onset of long rains season (April 2016). The seed rate used was 50 kg ha^{-1}. Phymyx was used as the organic amendment at a rate of 10 t ha^{-1}. The rates were PI-0, P2-2.5, P3-5, P4-7.5 and P5-10 t ha^{-1} of Phymyx. Sympal was applied at a rate of 30 kg P ha^{-1}. This rate also supplied other nutrients (0:23:15+10CaO+4S+1MgO+0.1Zn).

Table 3.4: Field experiment treatments

1. Control (P1)	2. Sympal (P1)
3. Phymyx (P2)	4. Sympal + Phymyx (P2)
5. Phymyx (P3)	6. Sympal + Phymyx (P3)
7. Phymyx(P4)	8. Sympal + Phymyx (P4)
9. Phymyx (P5)	10. Sympal + Phymyx (P5)
11. Legumefix (P1)	12. Legumefix + Sympal (P1)
13. Phymyx (P2) + Legumefix	14. Legumefix + Sympal + Phymyx (P2)
15. Phymyx (P3) + Legumefix	16. Legumefix + Sympal + Phymyx (P3)
17. Phymyx (P4) + Legumefix	18. Legumefix + Sympal + Phymyx (P4)
19. Phymyx (P5) + Legumefix	20. Legumefix + Sympal + Phymyx (P5)

3.6.3   Land preparation and planting

Land preparation in each of the two sites was done by ploughing to a depth of 15-20 cm followed by harrowing to a moderate seedbed tilth using an ox-plough. Furrows were made at a spacing of 50 cm apart. Phymyx and Sympal were then applied in the furrows and mixed with soil before placement of seeds to avoid direct contact with the seed. Seeds were surface-sterilized by soaking in 3.5 % NaClO solution for 2 minutes and rinsed thoroughly to remove any disinfectant with sterile distilled water. Inoculation was done with Legumefix from Legume technology, UK, containing *Bradyrhizobium japonicum* at the rate of 10 g kg^{-1} of seed. Inoculum (10 g) was eventually added to the surface sterilized seeds and carefully mixed to minimize the death of the *Bradyrhizobium* bacterium mechanically. The control treatment without inoculant was planted first to

avoid contamination. The trials were kept weed free by weeding using hand hoes to reduce competition for space, moisture, nutrients, and light.

3.6.4 Biomass assessment

At 50 % podding, 8-10 plants were taken from one of the inner rows about 50 cm from the beginning of the line and nodules were dug out and washed for nodules fresh weight determination and shoots collected for drying and weighing. A sample (10 %) of the total number of nodules counted per treatment was taken and used for nitrogen fixation effectiveness determination. The nodules were cut into half and observations made for the presence of any color. Characterization was based on the following colors: red, pink and brown as effective while white and green as ineffective. The effective nodules were expressed in percentages of the total for each treatment. The remaining nodules were surface sterilized and preserved for nodule occupancy analysis as described in section 3.4.5. Plant shoots were oven dried at 60°C for 48 hours to constant weight for dry matter determination. The shoots were later ground for total N analysis by the modified Kjeldahl method as described by Jackson (1975). Nitrogen uptake was determined as the product of SDW and the respective nitrogen content in the straw and reported as g nitrogen plant^{-1}.

3.6.5 Harvest

At maturity (when 95 % of the pods had turned golden yellow), all plants were harvested from the net plot excluding the outer rows. Number and weight of all plants were recorded from each plot and grains and haulms separated and weighed. The grains were

later oven-dried to a constant weight, ground and total N analyzed as described in section 3.6.4 above. Grain nitrogen uptake was determined as the product of grain yield and the respective nitrogen content in the grains and reported as nitrogen kg ha^{-1}.

3.7 Data analysis

Pearson correlation was used to determine the relationship of the different variables while ANOVA was used to assess the effects of the various factors using mixed procedures of the SAS System statistical software version 9.4. The effects of the various factors and their interactions were compared by computing least square means and standard errors of difference (SED); significance of difference evaluated at $P<0.05$.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Determining the optimal range of soil nitrogen with rhizobia inoculation for effective BNF in soybean

4.1.1 Soil chemical and microbiological properties

The selected chemical analysis of the experimental soils carried out before planting are presented below in Table 4.1. The soil nitrogen and organic carbon ranged between 0.029 - 0.21 % and 0.53 - 2.1 % respectively which shows the soils were very low to moderate (Tekalign, 1991). Calcium, magnesium, and potassium had a similar trend (FAO 2006). Phosphorus ranged from very low to very high (Antonio *et al*., 2013). The rhizobia populations in the sixty soils were below 1.0×10^3 CFU g^{-1} of soil, which has been reported as the minimal population of native rhizobia for a response to inoculation to be achieved for legume crops like soybean (Thies et al. 1991). The application of nutrients, such as N and P, was thus needed to sustain crop productivity in these soils.

Table 4.1: Summary of the sixty soil chemical and microbiology properties

Parameter	Units	Mean	Minimum	Maximum	SD	CV %
Available P	mg kg^{-1}	15.49	1.02	156.57	31.9	205.9
pH(H$_2$O)	-	5.76	4.25	7.03	0.66	11.46
Total N	%	0.11	0.029	0.21	0.04	36.36
Organic C	%	1.39	0.53	2.1	0.43	30.94
Ca	cmol kg^{-1}	5.48	0.42	18.32	3.88	70.80
Mg	cmol kg^{-1}	2.33	0.23	8.56	1.74	74.68
K	cmol kg^{-1}	0.71	0.07	3.6	0.69	97.18
MPN	CFU g^{-1}	41	0	283	87.6	208.6

P, phosphorus; K, potassium; Ca, calcium; Mg, magnesium; N, nitrogen; Org C organic carbon; MPN, most probable number; CFU, colony forming units; SD, standard deviation of the means and CV, coefficient of variance

4.1.2 Effect of soil nitrogen levels and inoculation on nodule fresh weight

NFW was negatively correlated to the soil total N (r: 0.29-0.44) in inoculated and uninoculated soils respectively (Table 4.2). Soil fertility must have been primarily responsible for the poor correlation of NFW to N levels despite the increasing levels of N.

Table 4.2: Correlation summary of the different variables for greenhouse experiment 1

Parameters	1	2	3	4	5	6
1. Organic carbon	1					
2. N levels	0.94***					
3. SDW inoculated	0.47***	0.55***				
4. SDW uninoculated	0.32**	0.37**	0.85***			
5. NFW inoculated	0.28**	0.29**	0.71***	0.66***		
6. NFW uninoculated	0.42**	0.44**	0.65***	0.75***	0.71***	1

*Significance levels: p ≤ 0.05; **: ***: p ≤ 0.001, NFW Nodule fresh weight SDW Shoot dry weight

Inoculation had a significant effect on NFW (P<0.001). Inoculation increased NFW by 43.83 % over the control which is in conformity with Rahmani and Saleh-Rastin, (2001) and Bai *et al*. (2002) who observed an increase in nodulation when soybean was inoculated with *Bradyrhizobium* in low N soil.

Table 4.3: Summary of analysis of variance for greenhouse experiment 1

Effect	Nodule fresh weight (NFW)	Shoot dry weight (SDW)
Soil × Inoculation	***	***
Soil	***	***
Inoculation	***	***

*Significance levels: ***: p ≤ 0.001

Moreover, significant effects of inoculation were recorded by Neveen (2008) and Hungria *et al*. (2015), who reported that inoculation of soybean significantly increased the nodule number over the control. The highest nodules fresh weight was recorded at soil nitrogen level of 0.17 % and lowest at 0.029 % (Figure 4.1). Responses in soils with N ≥ 0.17 % were steady and relatively better than in the low N soils which could be as a result of sufficient nitrogen for the soybean plants. The level of increases varied among different levels of soil nitrogen. However, at soil N of 0.102, 0.141, 0.153 and 0.176 % the un-inoculated treatments gave higher nodules fresh weight of 1.71, 2.80, 1.99 and 1.93 g plant^{-1}as compared to the inoculated which gave nodule fresh weight of 1.67, 1.93, 1.83 and 4.38g plant^{-1} respectively. This was attributed to the larger size of nodules from the indigenous rhizobia which was not translated to high SDW as the nodules were largely ineffective in BNF. The nodules from the uninoculated treatments had green inner parts, indicating the absence of leghaemoglobin making the nodules ineffective. Soil N of

0.0141 % gave higher NFW than in the inoculated treatment which confirms that indigenous rhizobia present in the soil were more efficient and competitive than the introduced strain. The NFW was generally low in the soils of poor fertility, which calls for further investigation to reduce the spatial variability.
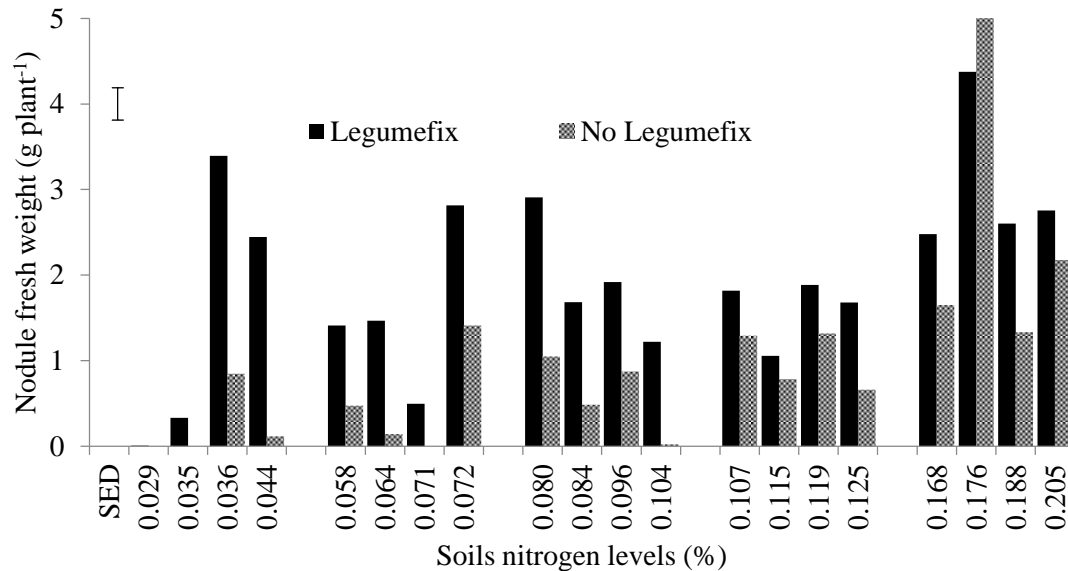
Figure 4.1: Effect of inoculation with Legumefix in different soil nitrogen levels on soybean nodule fresh weight for selected 20 soils used in the greenhouse experiment 1. The error bars represent the standard error of the difference (SED).

4.1.3 Effect of soil nitrogen levels and inoculation on nodule shoot biomass

Shoot dry weight was negatively correlated to the soil total N (r: 0.55-0.37) inoculated and uninoculated soils respectively (Table 4.2). The variation in SDW was also attributed to variation in the physical and chemical properties of the sixty soils (Table 4.1). In addition, differences due to soil variability may be attributed to the previous cropping system (Peoples *et al*., 1995), symbiotic properties of indigenous rhizobia populations

(Sanginga *et al*., 1996) and the available soil N content (Thies *et al*., 1991). Inoculation of soybean increased shoot dry weight by 38.35 % over the control, but the improvement of shoot biomass following inoculation significantly varied across soils. The increase in SDW of the inoculated treatment over the control concurs with a study conducted by Stefanescu and Palanciuc, 2000; Janagard and Ebadi-Segherloo, 2016 who revealed that shoot dry matter of the inoculated treatments was significantly greater than that of the control as a result of an increase in nodulation.
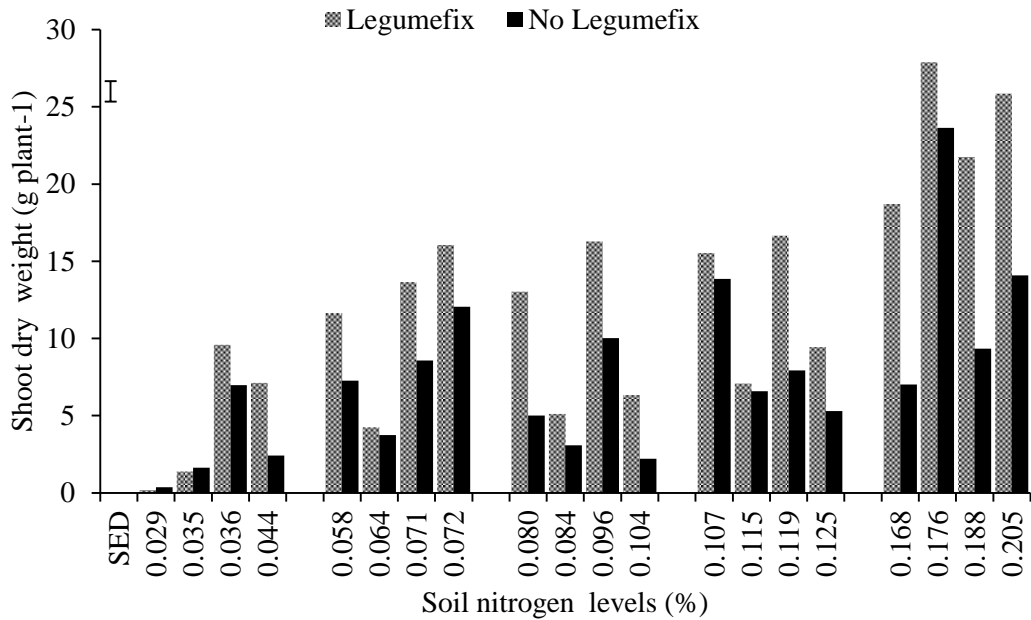
Figure 4.2: Effect of inoculation with Legumefix in different soil nitrogen levels on soybean shoot dry weight for selected 20 soils used in the greenhouse experiment 1. The error bars represent the standard error of the difference (SED).

4.1.4 Nodule occupancy

Nodule occupancy (IGS profiles as a function of total number of nodules with PCR-RFLP (930-1050 bp) (Figure 4.3) results after PCR-RFLP gave three IGS profile groups Figure 4.4). The IGS profile I (inoculant strain) was dominant in the inoculated soils, while IGS profile II and III together (indigenous strains) were dominant in the non-inoculated soils (Table 4.4). A nodule occupancy of 90.8 % was attained for the inoculated soils. This revealed that a larger number of nodules in the inoculated treatments were occupied by Legumefix confirming the effectiveness of the inoculant.

Table 4.4: Summary of nodule occupancy by Legumefix for greenhouse experiment 1 using PCR-RFLP method.

Treatment	Soils analyzed	Number of profiles analyzed	Nodule occupancy by IGS group (%)		
			I	II	III
Legumefix	32	184	91	2	7
No Legumefix	18	75	47	13	40

IGS group I represent the strain in Legumefix while II and III represent indigenous rhizobia strains
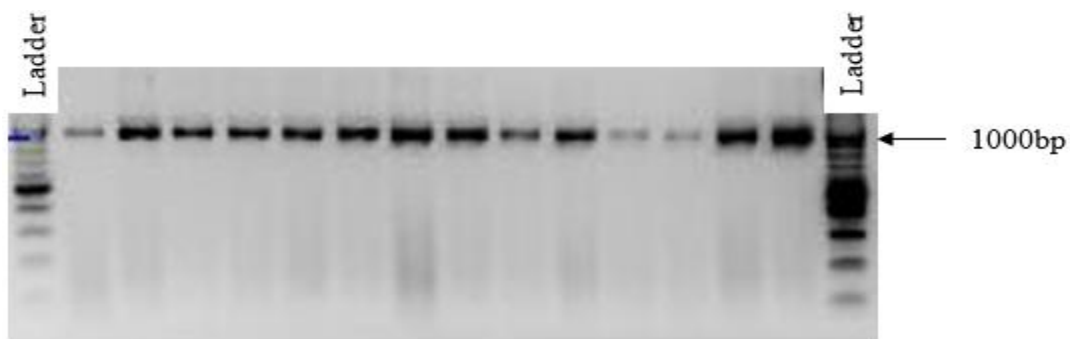
Figure 4.3: Bands (1000bp) obtained from DNA amplification using rhizobia specific primers derived from 3'end of the 16S (FGPS 1490-72; 5-TGCGGGTTTCCCCATCCTT-3'and from the 5' end of the 23S rDNA (FGPL 132-38; 5'-CCGGGTTTCCCCATTCGG-3').
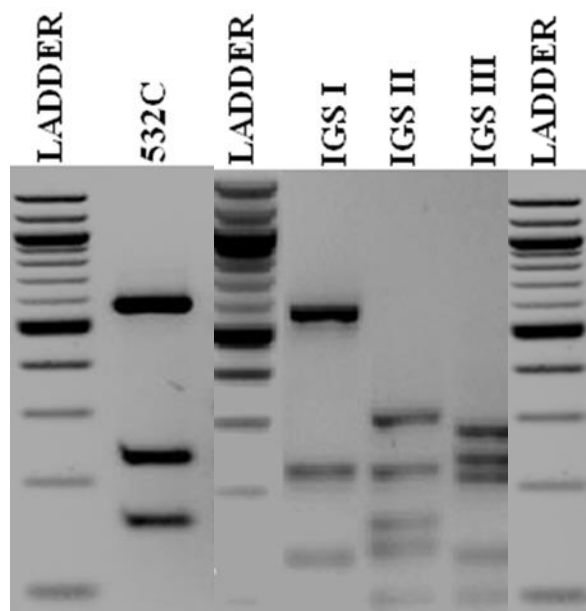
Figure 4.4: The IGS profiles of rhizobia inoculant strain 532c obtained from the DNA of pure cultures after isolation from the commercial product and the three IGS profiles of rhizobia obtained from the DNA of nodules after inoculation using *Msp 1* enzyme.

4.2 Assessing the effect of organic amendment on soybean BNF at 0.06 % N and 0.08 % N under greenhouse conditions

4.2.1 Effect of soil N amendment with Phymyx and inoculation with Legumefix on nodule fresh weight at 50 % podding of soybean

Soil × Phymyx, soil × Legumefix, Phymyx × Legumefix interactions had significant (P<0.05) effects on NFW for both soils. Phymyx significantly increased NFW (P<0.001) while soil had significant effects on NFW (P<0.05) (Table 4.5).

Table 4.5: Statistical assessment of the various treatment effects for greenhouse experiment 2**.**

Effect	Nodule fresh weight	Shoot dry weight	Biomass N uptake
S × P × I	*	NS	NS
S × P	NS	***	*
S × I	*	***	***
P × I	*	NS	NS
Soil (S)	**	***	***
Phymyx (P)	***	***	***
Inoculant (I)	NS	***	***

Significance levels: NS: p≥0.05; *: p<0.05; **: p<0.01; ***: p<0.001

The significant interaction effect of the soil and the inoculant confirmed that the success of legume inoculation depends on soil conditions. The soil with N = 0.08 % performed better, which was consistent with the better chemical/ fertility level when compared to the soil of N = 0.06 % (Table 4.6).

Table 4.6: Chemical and microbiology properties of the two soils and Phymyx used in the second greenhouse experiment

Property	Units	Soil 1	Soil 2	Phymyx
pH(H$_2$O)		4.52	5.43	6.7
P	mg kg^{-1}	3.06	119.77	0.39
Ca	mg kg^{-1}	0.85	5.49	0.29
Mg	mg kg^{-1}	0.33	1.51	0.1
K	mg kg^{-1}	0.21	0.26	0.22
Organic C	%	0.80	1.05	7.31
Total N	%	0.06	0.08	0.88
MPN[a]	CFU[b] g^{-1}	65	23	-

[a]MPN - most probable number of rhizobia
[b]CFU- colony forming units

The highest nodules fresh weight was observed at nitrogen level of 0.17 for both soils (0.06 and 0.80 % N) with a mean weight of 10.1 and 12.5g plant^{-1} respectively. However, this observation contradicts the results of Musyoki *et al*. (2003) with the highest increase on nodulation was at 0.05 and 0.15 % N. Above 0.17 % nitrogen level, the nodules fresh weight slightly decreased with further increase of nitrogen for the inoculated treatment. The decrease in NFW was attributed to inhibition of the formation of infection threads or suppression of nitrogen fixation by nodules (Ahmed *et al*., 2016) when N rates were increased (Solaiman and Rabbani, 2006). The uninoculated treatment did not show any significant difference on nodule fresh weight when nitrogen level was raised from 0.17 to 0.21 % (Table 4.7). The inoculated treatments had a relatively higher NFW than the uninoculated treatments.

Table 4.7: Effect of soil N amendment with Phymyx and inoculation with Legumefix on nodule fresh weight, shoot dry weight and N uptake at 50 % podding of soybean for second greenhouse experiment.

Soil N (%)	Soil 1 (0.06 % N)		Soil 2 (0.08 % N)	
	Legumefix	No Legumefix	Legumefix	No Legumefix
Nodule fresh weight (g plant^{-1})				
0.06/0.08[a]	1.2 ± 0.8	0.4 ± 0.3	3.1 ± 0.5	0.5 ± 0.5
0.10	5.5 ± 2.9	7.8 ± 0.8	7.9 ± 1.3	5.9 ± 1.1
0.14	6.4 ± 1.0	9.4 ± 0.2	7.9 ± 0.5	10.5 ± 0.7
0.17	10.1 ± 0.6	10.4 ± 1.0	12.5 ± 0.9	11.7 ± 2.4
0.21	9.8 ± 1.2	10.6 ± 0.5	11.2 ± 2.7	11.1 ± 3.4
Shoot dry weight (g plant^{-1})				
0.06/0.08[a]	1.69 ± 0.8	0.96 ± 0.8	15.76 ± 0.7	4.9 ± 0.5
0.10	12.64 ± 0.4	15.2 ± 0.6	24.72 ± 2.5	20.09 ± 3.2
0.14	18.41 ± 0.1	18.15 ± 1.8	23.89 ± 1.6	18.24 ± 1.5
0.17	19.33 ± 0.9	20.11 ± 1.2	25.35 ± 3.4	21.56 ± 4.0
0.21	20.34± 0.5	19.88± 1.1	25.14± 2.0	19.91± 4.4
N uptake (g plant^{-1})				
0.06/0.08[a]	0.03 ± 0.01	0.01 ± 0.01	0.27± 0.01	0.08 ± 0.01
0.10	0.22± 0.01	0.22 ± 0.01	0.44 ± 0.04	0.36± 0.06
0.14	0.32 ± 0.00	0.31 ± 0.03	0.42 ± 0.03	0.32 ± 0.03
0.17	0.34 ± 0.02	0.35 ± 0.02	0.45± 0.06	0.38 ± 0.07
0.21	0.36 ± 0.01	0.35 ± 0.02	0.35 ± 0.04	0.44 ± 0.01

[a]0.06/0.08 initial soil nitrogen levels

4.2.2 Effect of soil N amendment with Phymyx and inoculation with Legumefix on shoot dry weight at 50 % podding of soybean

Soil, Phymyx, Legumefix, soil × Phymyx and soil × Legumefix interactions had significant effects on SDW for both soils (P<0.001) (Table 4.5). Highest shoot dry weight was observed at 0.17 % for all the treatments except for the non-inoculated with initial soil N of 0.06 % N (Table 4.7). Initial soil N of 0.08 % gave a relatively higher SDW than initial soil N of 0.06 %. A positive correlation between NFW and SDW was recorded

(r=0.94 and r=1) for soil N of 0.06 % (Table 4.8) and similar results for the soil N=0.08 % (r=0.89 and r=0.87) (Table 4.9).

Table 4.8: Correlation of different parameters greenhouse in experiment 2 (0.06 % N)

Parameters	1	2	3	4	5	6
1. NFW inoculated	1					
2. NFW uninoculated	0.94**	1				
3. SDW inoculated	0.94**	0.99***	1			
4. SDW uninoculated	0.93**	1***	0.99**	1		
5. N uptake inoculated	0.95**	0.94**	0.98**	0.94**	1	
6. N uptake inoculated	0.96**	0.95**	0.98**	0.94**	1***	1

Significance levels: p ≤ 0.05; **: ***: p ≤ 0.001

Similar findings have been reported previously (Lamptey *et al*., 2014; Lawson and Quainoo, 2008; Revellin *et al*., 2000) who observed increases in nodules weight resulted in higher plant biomass. In addition, the significant positive correlation of NFW to SDW confirms that the nodules were effective and this was translated to increase in the SDW in both soil nitrogen levels. A slight decrease in SDW was observed when the soil N nitrogen was amended from 0.17 to 0.21 % N.

4.2.3 Effect of soil N amendment with Phymyx and inoculation with Legumefix on biomass N uptake at 50 % podding of soybean in greenhouse conditions

Soil, Phymyx, Legumefix and soil × Legumefix interaction had significant effects on N uptake for both soils (P<0.001) while soil × Phymyx interaction had significant effects on N uptake (P<0.05). Initial soil N of 0.06 % gave highest N uptake at 0.21 % of 0.36 g plant^{-1}, while initial N of 0.80 % gave a mean of 0.45 g plant^{-1} at 0.17 % N. Initial soil N of 0.08 % recorded a higher N uptake as compared to initial soil N of 0.06 % with mean

average of 0.38 g plant^{-1}. Soil with N=0.06 % N uptake correlated positively with SDW (r=0.98 and r=0.94) and similar results with soil N=0. 08 % (r=1 and r=0.99). A positive correlation between the NFW and N uptake (r=0.94 and 0.94) at 0.06 % N (Table 4.8) and (r=0.90 and r=0.86) at 0.08 % N significant at p<0.05 were recorded (Table 4.9).

Table 4.9: Correlation of different parameters in greenhouse experiment 2 (0.08 % N)

Parameters	1	2	3	4	5	6
1. NFW inoculated	1					
2. NFW uninoculated	0.91**	1				
3. SDW inoculated	0.89**	0.88**	1			
4. SDW uninoculated	0.89**	0.87**	0.99**	1		
5. N uptake inoculated	0.90**	0.88**	1***	1***	1	
6. N uptake uninoculated	0.92**	0.86**	0.97**	0.99**	0.99**	1

Significance levels: p ≤ 0.05; **: ***: p ≤ 0.001

The positive correlation of the NFW and N uptake confirms that increase in nitrogen resulted in the high accumulation of N in the plant's biomass through biological N_2 fixation (Tesfaye, 2015). Amended soils with inoculation gave the highest means of N uptake while the non-amended soils gave low N uptake levels. A slight decline in N uptake was recorded at 0.08 % N for the inoculated treatment when soil N was amended from 0.17 to 0.21 %.

4.2.4   Nodule occupancy

At 0.06 % N, the highest number of nodules occupied by Legumefix strain was recovered at 0.17 and 0.1. The high recovery of the indigenous rhizobia strains at 0.14 and 0.21 % N was attributed to high level of the indigenous rhizobia which could be more competitive than the introduced strain. Additionally, at 0.21 % the nitrogen level could be high suppressing nodules occupancy by the introduced strain. At 0.08 % N nodules occupied

by the Legumefix strain were consistent with 100 % in all nitrogen levels except the control which was in line with better soil properties at this level.

Table 4.10: Summary of nodule occupancy for greenhouse experiment 2 using PCR-RFLP method.

Initial soil N	N levels	No of Profiles	Nodule occupancy by IGS group (%)		
			I	II	III
0.06 % N	0.06	0	0	0	0
	0.1	2	100	0	0
	0.14	3	0	0	100
	0.17	5	100	0	0
	0.21	4	25	0	75
0.08 % N	0.08	0	0	0	0
	0.1	9	100	0	0
	0.14	6	100	0	0
	0.17	5	100	0	0
	0.21	6	100	0	0

IGS group I represent the strain in Legumefix while II and III represent indigenous rhizobia strains.

4.3 Assessing the effect of organic amendment on soybean BNF at 0.06 % N and 0.08 % N under field conditions

4.3.1 Effect of soil N amendment with Phymyx and inoculation with Legumefix on nodule fresh weight of soybean in field conditions

Inoculation with Legumefix significantly increased nodule fresh weight at 0.06 % N $p < 0.05$ while Sympal + Phymyx, Phymyx + Legumefix, Legumefix and Phymyx significantly increased nodule fresh weight at 0.08 % N (Table 4.11).

Table. 4.11: Analysis of variance table for field experiment (0.06 % N)

Effect	Nodule weight	Effective nodules	biomass N uptake	Grain N uptake	Yields
0.06 % N					
L × S × P	NS	NS	NS	NS	NS
S × P	NS	NS	*	NS	NS
S × L	NS	NS	NS	NS	NS
P × L	NS	NS	NS	NS	NS
Legumefix(L)	NS	***	*	NS	***
Phymyx(P)	*	**	***	***	***
Sympal (S)	NS	NS	NS	NS	NS
0.08 % N					
I × S × P	NS	NS	NS	NS	NS
S × P	***	NS	NS	NS	NS
S × L	NS	NS	NS	NS	NS
P × L	**	NS	NS	NS	NS
Legumefix (L)	***	***	**	***	***
Phymyx (P)	NS	***	NS	NS	NS
Sympal (S)	NS	NS	NS	NS	NS

Significance levels: NS: $p \geq 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

This interactive effect was attributed to the balanced application of Phymyx (organic fertilizer) and Sympal (inorganic fertilizer) which favorably increased the other nutrients and root density hence providing infection sites of the introduced rhizobia (Devi *et al*., 2013). The nodule weight in the control plots reflects the presence of indigenous rhizobia. However, the nodules formed in the control plots were small in size, of the lateral roots and ineffective (white in color) (Tahir *et al*., 2009). The highest nodule fresh weight was recorded with a combination of Phymyx (organic fertilizer), P (Sympal) and rhizobia inoculation (Tahir *et al*., 2009). The highest nodules fresh weight was recorded with a combination of 10 t ha^{-1} Phymyx + Sympal + Legumefix for both sites 0.06 % N and 0.08 % N with 1.5 and 2.1 g plant^{-1} respectively (Figure 4.5). The highest NFW with a combination of N source, P source along with inoculation is in line with (Mishra *et al*., 2010; Tahir *et al*., 2009). Application of sole Sympal, sole Legumefix, and sole Phymyx had a significantly higher nodule fresh weight than the control for both sites. The increase of NFW due to sole application of Sympal which is a P source confirms the importance of phosphorous in nodule formation. Generally, P has been reported to improve nodule weight as it improves the overall performance of the plant leading to a better nodulation (Yoseph and Worku, 2014). A steady increase in NFW at 0.06 % N was observed as the level of Phymyx increased. However, at 0.08 % N a slight decrease in NFW at 0.08 % N was observed when Phymyx was increased from 5 t to 7 t ha^{-1}. There was no significant difference in NFW with 2.5 t and 5 t ha^{-1} + Legumefix and Sympal for the two sites. Higher NFW was recorded at 0.08 % N as compared to 0.06 % N. At 0.08 % N NFW increased by 74% with a combination of Phymyx + Legumefix as compared to the sole application of Phymyx. On the other hand combination of Sympal + Phymyx +

Legumefix increased the NFW by 69 % over Phymyx + Sympal at 0.06 % N 29 % and 23 % increases were also recorded at 0.08 % N respectively.
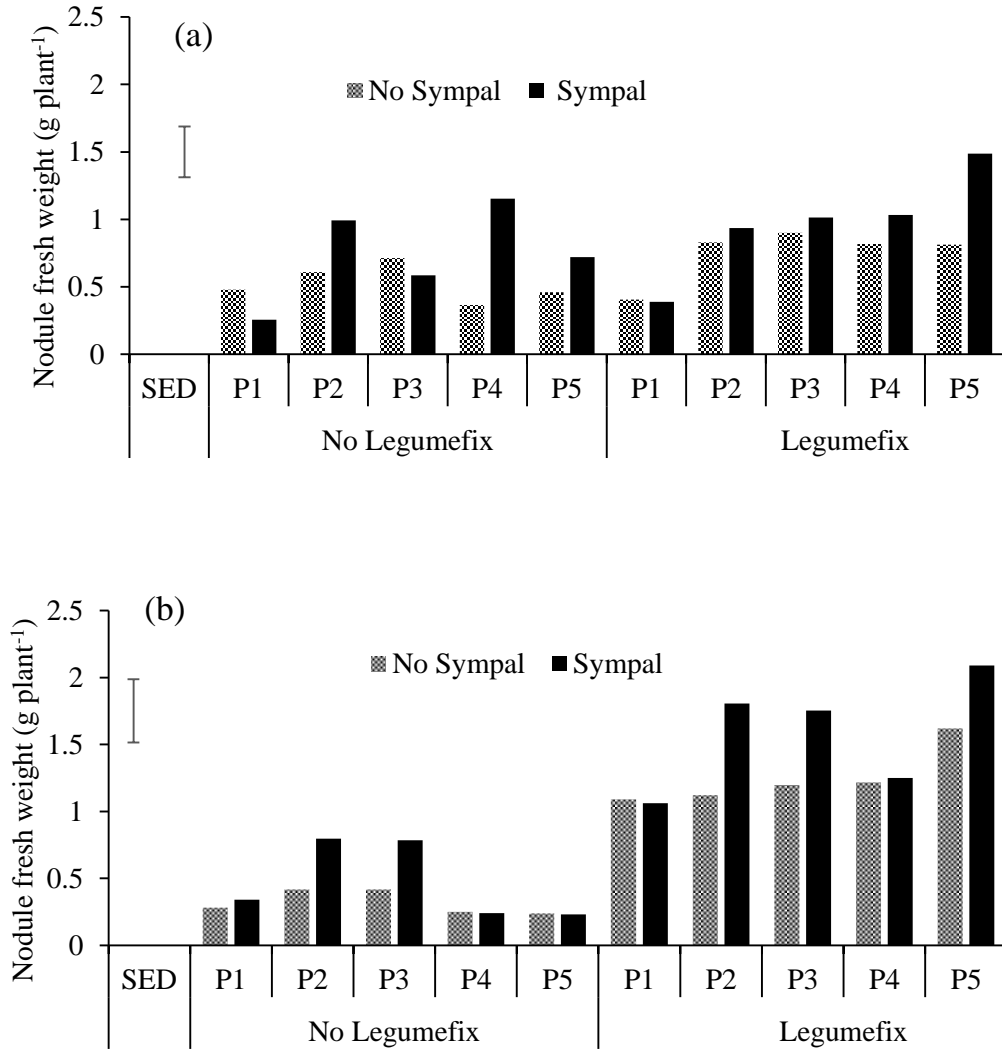
Figure 4.5: Interactive effect of Sympal, Legumefix and soil N amendment with Phymyx on nodules fresh weight of soybean under field conditions (a) 0.06 % N and (b) 0.08 % N. P1, P2, P3, P4, and P5 represents 0 t, 2.5 t, 5 t, 7.5 and 10 t ha^{-1} of Phymyx respectively. The error bars represent the standard error of the difference (SED).

Table 4.12: Correlation of different parameters for field experiment at 0.06 % N and 0.08 % N

0.06 % N

Parameters	1	2	3	4	5	6
1. NFW	1					
2. Effective nodules	0.72***	1				
3. SDW	0.76***	0.70***	1			
4. Biomass N uptake	0.82***	0.75***	0.95***	1		
5. Grain N uptake	0.35NS	0.38NS	0.36NS	0.44*	1	
6. Yields	0.63**	0.47*	0.60**	0.62**	0.61**	1

0.08 % N

Parameters	1	2	3	4	5	6
1. NFW	1					
2. Effective nodules	0.92***	1				
3. SDW	0.73***	0.65**	1			
4. Biomass N uptake	0.74***	0.66***	0.99***	1		
5. Grain N uptake	0.67***	0.76***	0.45*	0.44*	1	
6. Yields	0.66***	0.74***	0.42NS	0.42NS	0.98***	1

Significance levels: $p \leq 0.05$; *$p \leq 0.01$: ***: $p \leq 0.001$

4.3.2   Effect of soil N amendment with Phymyx and inoculation with Legumefix on nodule effectiveness of soybean in field conditions

Phymyx and Legumefix significantly enhanced nodule effectiveness ($p < 0.05$) at both sites (Table 4.11). Highest nodule effectiveness was recorded with 10 t ha^{-1} Phymyx + Sympal + Legumefix in both sites (Figure 3.4). However, nodules effectiveness was relatively higher at 0.06 % than at 0.08 % N. Nodules effectiveness increased by 51 % with the application of Phymyx + Legumefix over sole Phymyx application at 0.06 % N while application of Phymyx + Sympal + Legumefix increased nodules effectiveness by 55 % over Sympal + Phymyx. At 0.08 %, a combination of Phymyx and Legumefix increased effectiveness by 51 % over Phymyx alone while the combination of Phymyx, Legumefix and Sympal increased the average number of effective nodules by 26 % over application of Sympal and Phymyx alone. Phymyx alone increased nodules effectiveness at both N levels, however, a slight decrease in nodules effectiveness was observed with application of 7.5 t ha^{-1}. A steady increase of the average number of effective nodules was observed with a combination of Phymyx, Legumefix, and Sympal at both N levels. Nodules effectiveness is an important feature for an efficient soybean rhizobia symbiosis. The nodules from the inoculated plots were pink in colour which ascribes the presence of leghaemoglobin. This is in agreement with Adjei and Chambeiss (2002), Butler and Evers (2004), and Tasfaye (2015) that legume nodules with inner pink or red parts is an indication of the effectiveness of the strain used. The recovery of a significantly high percentage of nodules occupied by Legumefix at 7.5 t ha^{-1} and the decline of the nodules

effectiveness when the Phymyx was increased to 10 t ha^{-1} confirms that high levels of nitrogen in this study supplied by Phymyx suppressed the nodules formation. This was attributed to the inhibitory effects of N on nodulation and N_2 fixation of soybean which were clear at high concentrations, but far less at lower concentrations (Weisany *et al*., 2013).
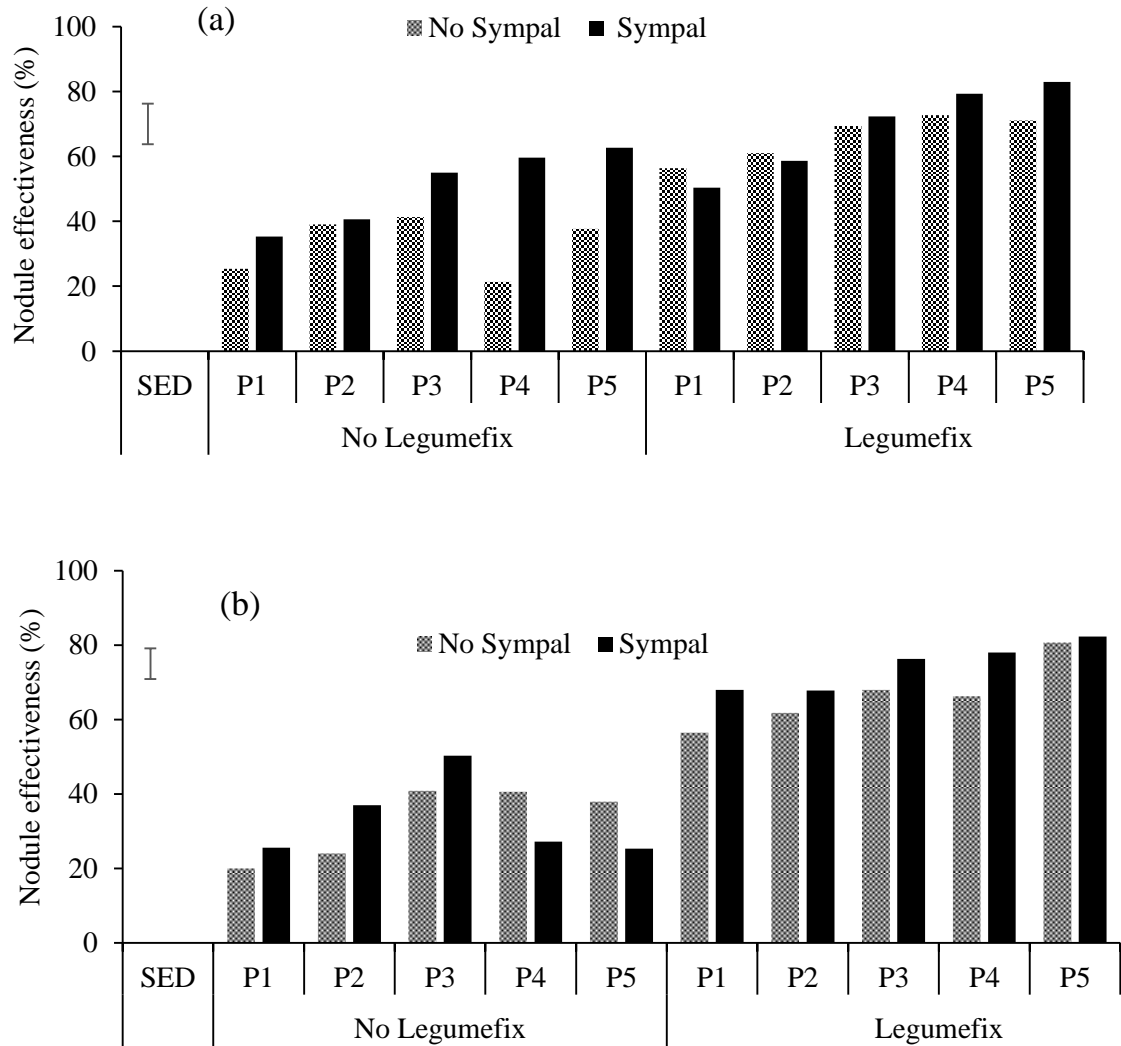
Figure 4.6: Interactive effect of Sympal, Legumefix, and soil N amendment with Phymyx on nodules effectiveness of soybean under field conditions (a) 0.06 % N and (b) 0.08 % N. P1, P2, P3, P4, and P5 represents 0t, 2.5 t, 5 t, 7.5 and 10 t ha^{-1} of Phymyx respectively. The error bars represent the standard error of the difference (SED).

4.3.3 Nodule occupancy

Highest Legumefix recovery was observed in the combination of 5 t and 7.5 t ha^{-1} Phymyx, Legumefix and Sympal with 94 % and 100 % at 0.06 % and 0.08 % N respectively (Table 4.15). This shows the importance of nitrogen, phosphorous and rhizobia for an efficient BNF system. The addition of 7.5 t to 10 t ha^{-1} of Phymyx reduced the recovery of Legumefix from the nodules at both nitrogen levels. This confirms high nitrogen levels reduced the recovery of Legumefix strain from the nodules. This could be as a result of inhibitory effects of high dose of nitrogen on nodule formation, reduced N$_2$ fixation and acceleration of nodules senescence (Ohyama *et al*., 2011). There was no significant difference in nodules occupancy by Legumefix at both nitrogen levels when Phymyx was increased from 5 t to 7.5 t ha^{-1}. This suggests that 5 t of Phymyx was sufficient for N$_2$ fixation.

Table 4.13: Nodule occupancy for the field experiment with different Phymyx levels using PCR-RFLP method.

Initial soil N	Treatment	Number of profiles	Nodule occupancy by IGS groups (%)		
			I	II	III
0.06 % N	P3 + S + L	18	94	0	6
	P3 + S	2	0	0	100
	P3 + L	2	50	0	50
	P4 + S + L	18	94	0	6
	P4 + S	1	0	100	0
	P4 + L	0	0	0	0
	P5 + S + L	14	79	0	21
	P5 + S	1	0	0	100
	P5 + L	4	50	0	50
0.08 % N	P3 + S + L	14	100	0	0
	P3 + S	4	0	50	50
	P3 + L	6	100	0	0
	P4 + S + L	20	85	0	15
	P4 + S	1	0	100	0
	P4 + L	7	0	0	100
	P5 + S + L	20	85	0	15
	P5 + S	4	0	25	75
	P5 + L	5	100	0	0

P3, P4 and P5 represent 5 t, 7.5 t and 10 t ha^{-1} of Phymyx respectively; S-30 kg ha^{-1} of Sympal and L- Legumefix. IGS group I represent the strain in Legumefix while II and III represent indigenous rhizobia strains.

4.3.4 Effect of soil N amendment with Phymyx and inoculation with Legumefix on biomass N uptake of soybean in field conditions

Legumefix, Phymyx and Sympal × Phymyx interaction had significant effects on biomass N uptake (p<0.5) at 0.06 % N while only Legumefix had significant effects on biomass N uptake at 0.08 % N (P<0.05) (Table 4.11). Similar significant effects of rhizobia inoculation on N uptake of soybean has been reported by (Tahir *et al*., 2009). However, a decrease of N uptake was recorded when the Phymyx level was increased from 7.5 t ha^{-1} to 10 t ha^{-1} with Sympal for both nitrogen levels (Figure 4.7). The reduced N uptake due

to increased nitrogen application justifies the importance of nitrogen to plants for increased N uptake (Tahir *et al*., 2009), especially at low soil levels.

At 0.06 % N highest biomass N uptake was achieved with a combination of 10 t ha^{-1} of Phymyx + Sympal + Legumefix. There was no significant difference in N uptake when a combination of 7.5 t ha^{-1} of Phymyx + Sympal and 7.5 t ha^{-1} of Phymyx + Sympal + Legumefix were used. At 0.08 % N highest biomass N uptake was recorded with a combination of 2.5 t ha^{-1} of Phymyx + Sympal + Legumefix closely followed by 10 t ha^{-1} of Phymyx + Sympal + Legumefix. These findings are in agreement with Tahir *et al*. (2009) who reported highest N uptakes of soybean in a combination of Phosphorous + Rhizobial inoculation + nitrogen source. This could be as a result of increased nitrogenase activity and nodule mass translating to high N uptake. Biomass N uptake was positively correlated to NFW, effective nodules and SDW with r=0.82, 0.75 and 0.95 respectively (p<0.001) at 0.06 % and r=0.74, 0.66 and 0.99 respectively (p<0.001) at 0.08 % N (Table 4.12). Similar findings have been reported by Tahir *et al*. (2009), Sarr *et al*. (2005), Zhang *et al*. (2002) and Seneviratne *et al*. (2000).
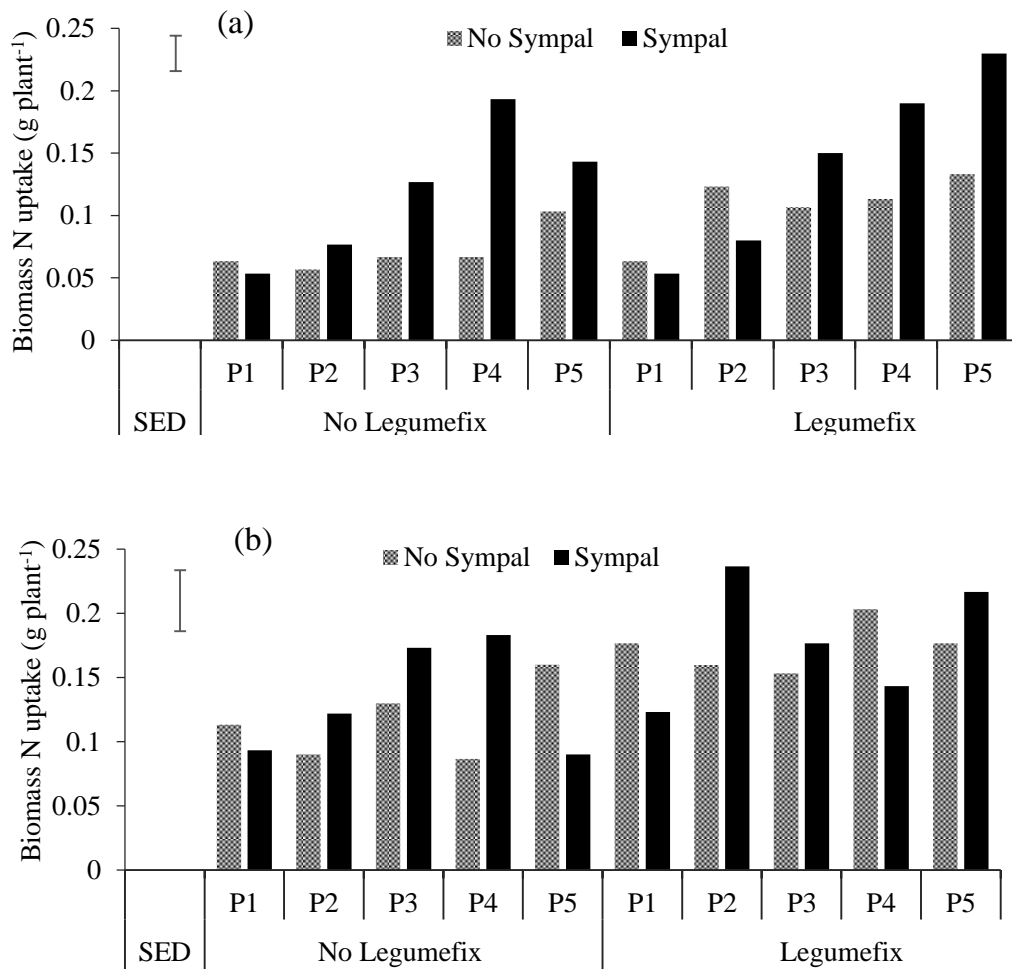
Figure 4.7: Interactive effect of Sympal, Legumefix and soil N amendment with Phymyx on biomass N uptake of soybean under field conditions (a) 0.06 % N and (b) 0.08 % N. P1, P2, P3, P4 and P5 represents 0 t, 2.5 t, 5 t, 7.5 and 10 t ha^{-1} of Phymyx respectively. The error bars represent the standard error of the difference (SED).
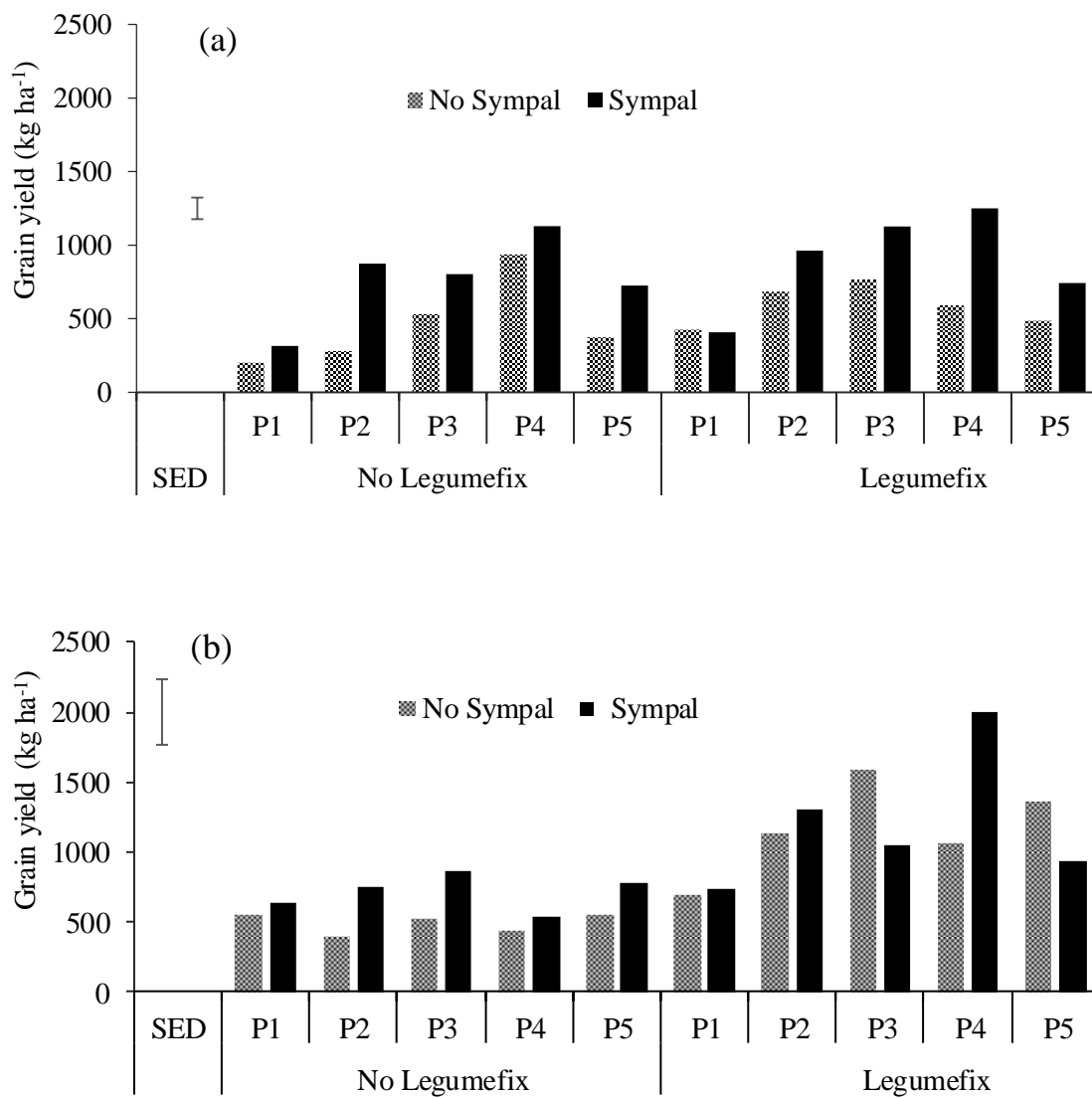
4.3.5 Effect of soil N amendment with Phymyx and inoculation with Legumefix on grain yield of soybean in field conditions

Legumefix and Phymyx significantly increased grain yields at 0.06 % N while only Legumefix significantly increased grain yield at 0.08 % N (p<0.05) (Table 4.11). This positive significant effect of inoculation on grain yields confirms the success of the rhizobial inoculation which has been previously reported (Hussan *et al.*, 2011). The significant increase of grain yield with application of Phymyx which was an organic fertilizer was in conformity with the findings of (Mpepereki *et al.*, 2000) who observed yield increase due to the application of organic fertilizers. The yields at 0.08 % N were relatively higher than at 0.06 % N which was consistent with greenhouse experiment 2 results (based on biomass production). The higher yields at 0.08 % were as a result of better soil properties as shown in (Table 4.3). An increase of Phymyx from 7.5 t to 10 t ha^{-1} with Sympal and Legumefix significantly decreased the average grain yield at both N levels. The decrease in grain yields in both sites when Phymyx was increased from 7.5 t to 10 t ha^{-1} had a similar trend with the results obtained in greenhouse experiment 2. This was attributed to a high dose of nitrogen which inhibits nodules formation and N$_2$ fixation translating to reduced grain yields (El-Shaarawi, 2011). All the parameters correlated significantly to grain yields in both sites except for SDW and biomass N uptake at 0.08 % N (Table 4.12). This confirms good nodulation translates to increased grain yields due to efficient BNF.
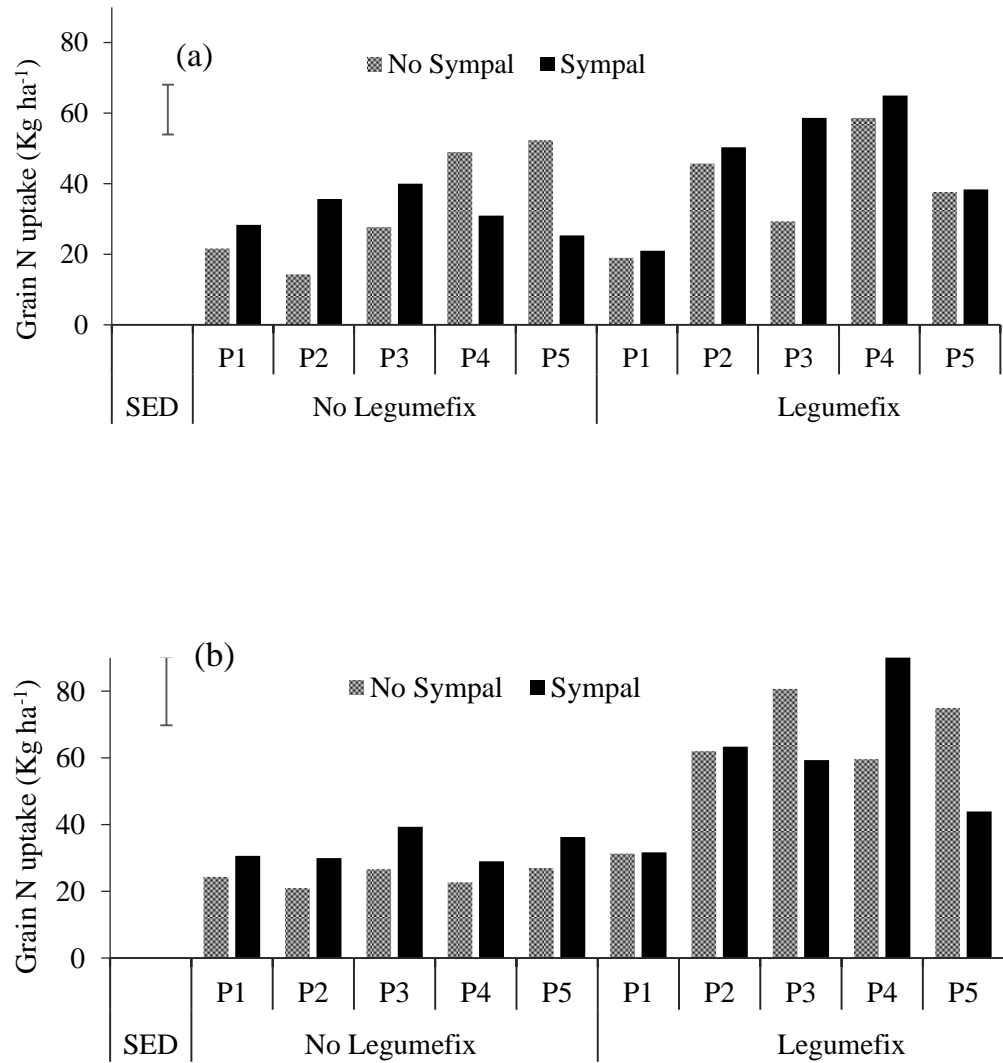
59

Figure 4.8: Interactive effect of Sympal, Legumefix, and soil N amendment with Phymyx on grain yields of soybean under field conditions (a) 0.06 % N and (b) 0.08 % N. P1, P2, P3, P4, and P5 represents 0 t, 2.5 t, 5 t, 7.5 and 10 t ha^{-1} of Phymyx respectively. The error bars represent the standard error of the difference (SED).

4.3.6 Effect of soil N amendment with Phymyx and inoculation with Legumefix on grain N uptake of soybean in field conditions

Sympal $\times$ Phymyx and Phymyx $\times$ Legumefix interactions significantly affected the grain N uptake ($p<0.01$, $p<0.05$) respectively at 0.06 % N while Legumefix significantly affected grain N uptake ($p<0.001$) at 0.08 % N (Table 4.11). The interactive effect of Sympal (P source) and Phymyx (N source) confirms their specific roles in symbiotic N_2 fixation leading to increased N grain uptake (O' Hara $et\ al.$, 2002; Rathke $et\ al.$, 2005). Inoculation of soybean with Legumefix increased grain uptake which was attributed to increases in nodulation and effective nodules resulting in high accumulation of nitrogen due to atmospheric N_2 fixation (Tahir $et\ al.$, 2009).

A combination of Legumefix + Sympal + 7.5 t ha^{-1} Phymyx gave the highest grain N uptake for both nitrogen levels with 96 kg ha^{-1} at 0.08 % N). The increase of Phymyx from 7.5 t ha^{-1} to 10 t ha^{-1} reduced the grain N uptake in both soil nitrogen levels suggesting that 7.5 t ha^{-1} of Phymyx was optimal when the N soybean requirements were sufficiently reached (Adeli $et\ al.$, 2005). Similarly, reduced N concentration of soybean due to increased nitrogen source has also been reported (Osborne and Riedell, 2006). At 0.08 % N a significant correlation of NFW (r=0.67) and efficient nodules (r=0.76) with grains N uptake was recorded. (Table 4.12). This is in line with the findings of Sarr $et\ al.$, (2005) who reported increases of grain N uptake due to an increase in nodulation. However, at 0.06 % N NFW and number of effective nodules did not correlate to grain N uptake. Soil N of 0.08 % gave relatively higher grain N uptake which is consistent with the grains yields than at 0.06 % N.

Figure 4.9: Interactive effect of Sympal, Legumefix and soil N amendment with Phymyx on grains N uptake of soybean under field conditions (a) 0.06 % N and (b) 0.08 % N. P1, P2, P3, P4 and P5 represents 0 t, 2.5 t, 5 t, 7.5 and 10 t ha^{-1} of Phymyx respectively. The error bars represent the standard error of the difference (SED).

CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

When soils of different composition are used like in the case of the first greenhouse experiment, it would be difficult to determine the critical levels of N for effective soybean inoculation. Soil amendment with Phymyx increased soybeans traits and yield under greenhouse and field conditions. However high levels of the organic source of N suppressed the BNF leading to the decline of the soybean traits. The study indicates that application of 7.5 t ha^{-1} of Phymyx and Sympal at 30 kg ha^{-1} was the potential threshold level above which soybean response to inoculation would not be achieved. In low N soils, the use of an organic N source would not hinder the performance of rhizobia inoculants in soybean when proper rates are used; it would conversely improve nodulation. Development initiatives focusing only on legume inoculation or co-application of rhizobia inoculants and phosphorus fertilizers only, without proper soil fertility diagnosis, must be revised to optimize the benefits expected from inoculation including biological nitrogen fixation.

5.2 Recommendations

Critical N levels for effective soybean inoculation with rhizobia is of importance in increasing soybean productivity. In this respect;

1. Physical soil properties should be considered when assessing critical N levels for soybean response to rhizobia inoculations.

2. Amendment of low nitrogen soils with Phymyx to boost soybean response to inoculation should be adopted in soybean growing areas in Ugunja, western Kenya.

3. Application of 7.5 t ha^{-1} of Phymyx and sympal at 30 kg ha^{-1} could be used as potential threshold level above which soybean response to inoculation would not be achieved in clay soils.

4. There will be a need to investigate further data points around 0.17 % N so as to confirm it as the threshold value above which, application of N source would hinder soybean response to inoculation.

5. Further investigation should be carried out on more sites and seasons with the same soil properties with respect to other textural classes.

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