EFFECTS OF LIME AND PHOSPHORUS ON BIOLOGICAL NITROGEN FIXATION BY SOYBEANS (*Glycine max* (L.) Merrill) IN AN ULTISOL, MOROGORO, TANZANIA

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ABSTRACT

Low soil pH and phosphorus are among the major soil constraints which limit BNF and soybean production in sub-Saharan Africa. Most Probable Number (MPN) plant infection technique and glasshouse pot experiment were conducted at SUA to estimate the population of native *Bradyhizobium japonicum* and determine the effect of lime and phosphorus on soil pH and BNF by soybean in an Ultisol. The pot experiment was a 3^3 factorial in a split plot experimental design replicated two times. The main plots were inoculation, non-inoculation and inorganic N fertilizer at the rate of 200 kg N ha^{-1}. The levels of lime were 0, 5 and 10 ton/ha and the levels of phosphorus were 0, 50 and 100 kg/ha, whose combinations constituted the sub-plots. The soil was acidic (pH 4.6) and contained 2-79 cells of indigenous *Bradyhizobium japonicum* per gram of soil, which is low and appeared to limit BNF by soybean. The soil pH and N content in soybean plants increased significantly ($P < 0.05$) with increase in liming rate. The interaction of lime and phosphorus on nodulation, N_2 fixed, DMYs, P content in inoculated plot and on Ca contents of soybean plants in inorganic N applied plot was significant. It was concluded that for soybean to be grown in the studied area should be inoculated before planting due to the low *Bradyhizobium japonicum* population. The application of 5 tons lime/ha and 100 kg P/ha would serve as the optimum rates for the Ultisol used in this study. It is recommended that the study be validated under field conditions before conclusive decisions can be made.

DECLARATION

I, Alinafe Kachiguma, hereby declare to the Senate of Sokoine University of Agriculture that this is my original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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LIST OF ABBREVIATIONS AND SYMBOLS

AAS	Atomic absorption spectrophotometer
AGRA	Alliance for Green Revolution in Africa
atm	Atmosphere
BNF	Biological nitrogen fixation
CEC	Cation exchange capacity
cmol	Centimole
DMY	Dry matter yield
ECEC	Effective cation exchange capacity
FAO	Food and Agriculture Organisation
IITA	International Institute for Tropical Agriculture
MPN	Most Probable Number
SSA	Sub-Saharan Africa
TFRA	Tanzania Fertilizer Regulatory Authority
UNESCO	United Nations Educational, Scientific and Cultural Organization
USA	United States of America
USDA	United States Department of Agriculture

CHAPTER ONE

1.0 INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is a leguminous plant of the Pea family, *Fabaceae* (Singh and Shivakumar, 2010), which grows under a wide range of environments, from tropical, subtropical to temperate climates (IITA, 2009). Soybean is one of the most efficient legumes in biological nitrogen fixation (BNF), which is an important process in the development of cropping systems such as crop rotation keyed to limited inputs of N fertilizer (Chianu *et al.*, 2011). It has been reported that environmental conditions such as changes in photoperiod, temperature and precipitation have a negative effect on soybean plant development and grain yield (Hu and Wiatrak, 2012), thus jeopardising enhanced and sustainable soybean production.

Soybean, like other food legumes, complements cereal crops as a source of protein and minerals while agronomically serving as a rotation crop with cereals. It reduces soil pathogens by disturbing their life cycles and supplies nitrogen to the subsequent cereal crop through biological nitrogen fixation (BNF) and by incorporating its residues, which have high % total N content, into the soil (Akibode and Maredia, 2011). Soybean also serves as a feed crop in many farming systems and fetches higher prices as compared to cereals and are thus increasingly grown to supplement farmers' incomes. Although legume crops like soybean have many desirable characteristics in terms of nutrition and environmental benefits, in most countries especially in the developing world such as sub-Saharan Africa (SSA), they are considered as minor or secondary crops and thus do not receive the same level of attention by the countries' agricultural statistical units in the documentation and reporting protocols like it is for the important cereal crops, namely wheat, maize and rice (Akibode and Maredia, 2011). As a secondary crop category,

pulses do not receive investment resources and policy attention from governments as do the cereal crops, which are often considered as food security crops. About seventy five percent of the area under pulse production falls under rainfed-low input production system (Akibode and Maredia, 2011).

Trends show that acreage under soybean production in SSA had increased by 19% from 426.5 thousand hectares as of mid-1990s to 528.5 thousand hectares in 2008 (Akibode and Maredia, 2011). The increase had been due to realization of the soybean potential in supplementing diets to the farmers, other members of the community, livestock, and its ability to fix nitrogen through symbiotic association with *Bradyrhizobium japonicum* (Chianu *et al.*, 2011; Rao and Reddy, 2010; Thuzar *et al.*, 2010).

Low soybean yield is a general problem facing most farming systems in SSA. In spite of the increase in acreage over the years, the yield increase per unit area has remained marginal, ranging from 1.16 t/ha in 1990s to 1.2 t/ha in 2008 (Akibode and Maredia, 2011), compared to the potential average yield which ranges from 3.31-4.41 t/ha under optimal growth conditions (Aniekwe and Mbah, 2014). These low yields are pronounced in soybean grown on acidic soils like Ultisols and Oxisols which cover more than half of the land area of the SSA (Giller and Wilson, 1991). Ultisols and Oxisols are often associated with low soil fertility attributed to low pH and P, and Al toxicity, which are unfavourable conditions for the symbionts hence reduced N_2 fixation (Chianu *et al.*, 2011).

Biological nitrogen fixation by soybean-*Bradyrhizobium* symbiosis is less effective in Ultisols and Oxisols because of their low percentage base saturation and low levels of available P (Rao and Reddy, 2010). This has led to low soybean yields hence contributing

to food insecurity. Binang *et al.* (2013) reported that although soybean cultivars differ in their tolerance to soil acidity and aluminum toxicity, optimal yields could not be attained in acid soils of pH less than 5.1.

Research has been conducted to reduce the impact of acidity in Ultisols and Oxisols so as to increase soybean yield production (Binang *et al*., 2013). It has been reported that nitrogen fertilization increased yields of soybean seeds as compared to control treatment, which produced low yield due to poor growth (Macák and Candráková, 2013).

Lime applications and seed inoculation with *Bradyhizobium japonicum* strains were effective in increasing root nodulation, plant vegetative growth and grain yields in Nigeria (Binang *et al.,* 2013) because of raised pH and increase in *Bradyrhizobium* population. Similar results were obtained by Bekere (2013), who reported that when calcite and seed inoculation with *B. japonicum* were used simultaneously in Ethiopia, the yield increased as compared to the control treatment.

P fertilization on soybean production dependent on BNF for its N supply instead of mineral N has been reported to have positive effect on soybean nodulation and yield (Abdul-Aziz, 2013). Mahamood (2008) reported that application of P to soybean, where effective strains of *Bradyrhizobium* were present in high numbers, increased nodule numbers, N_2 fixed and the yields in an Ultisol in Nigeria.

Biological nitrogen fixation (BNF) enhancement in Ultisols and Oxisols through liming with calcite, phosphorus application and pre-sowing seed inoculation constitutes one of the potential solutions in sustainable soybean production (Chianu *et al*., 2011; Njeru *et al*., 2013). However, information on the optimum levels of liming materials and

phosphorus for BNF and soybean production in SSA is scanty, hence the need to undertake the current research. The main goal of this research was to increase soybean production by optimizing BNF, through the application of the appropriate levels of lime and phosphorus, in acidic Ultisols in Morogoro, Tanzania.

The overall objective of the study was to increase soybean yield by enhancing and capitalizing on the synergistic effect of lime ($CaCO_3$), phosphorus and rhizobial inoculation on the biological nitrogen fixation in an Ultisol. The specific objectives of the study were to:

i. Assess the fertility status of the Ultisol,
ii. Estimate the native rhizobial population in the Ultisol of the study area,
iii. Determine the effects of lime and phosphorus on the pH of the experimented soil, the Ultisol,
iv. Determine the effects of lime and phosphorus on nodulation and the amount of N fixed by the soybean-*Bradyrhizobium* symbiosis,
v. Determine the effects of lime and phosphorus on soybean dry matter yields (DMY) and
vi. Determine the effects of lime, phosphorus on N, P and Ca contents in soybean shoots.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin of Soybean

Soybean originated from China in the 11th century (IITA, 2009). For centuries, soybean was considered as the staple food of the people of East Asia (Martino *et al*., 2011). In the USA the first reports of cultivation of soybean date from 1765 (Martino *et al*., 2011). Unlike in East Asia, in the USA soybean was initially cultivated as a forage plant and not used as a source of edible oil (Johnson and Bernard, 1963). In Brazil, soybean was introduced in 1882; however, the soybean crop became important in Brazil as from 1960, initially in the south where it showed better adaptation because of the similarity with regions of cultivation in southern United States (Martino *et al*., 2011). With the development of new cultivars adapted to different agro-climatic regions of the country, Brazil has become the second largest soybean producer in the world, after USA (USDA, 2014). Soybean was introduced to Africa in the 19th century by Chinese traders along the east coast of Africa (IITA, 2009). Nigeria is the largest producer in Sub-Saharan Africa (SSA) with about 625 000 ha of land under soybean cultivation, followed by South Africa (IITA, 2009).

2.2 Botanical Aspects of Soybean

2.2.1 Development of the soybean plant

Soybean plant undergoes two different developmental stages, whose understanding is an important factor in evaluating its yield potential (Mahamood, 2008). The soybean developmental stages are referred to as vegetative (V) and reproductive (R) stages. Plant development stages are determined by classifying leaf, flower, pod, and or seed development, as well as node identification (Naeve, 2011). The stages can overlap, as

such when determining the growth stage of the crop, a growth stage begins when 50 % or more of the plants are in or beyond that stage (Pedersen, 2007).

2.2.2 Classification of soybean cultivars

Soybean cultivars are classified by their morphological (form and structure) growth habit (Mahamood, 2008). They exhibit either a determinate, an indeterminate or semi-determinate growth pattern. Growth of these types is similar during the vegetative growth phase, and at the time of flowering (R1) (Svec, 1979). Once flower initiation has been attained, the indeterminate and determinate plants differ dramatically in stem growth habit (Pedersen, 2007).

Indeterminate cultivars extends the main stem growth by developing leaves on the main stem and branches after initial flowering throughout the flowering period, which can last as long as 40 days, while main stem growth in determinate types terminates shortly after initial flowering but leaves continue to develop on branches until the beginning of seed (R5) development stage (Mahamood, 2008). Indeterminate types flower sequentially beginning at the lower nodes then up the main stem and can have well-developed pods on lower nodes with newly developed flowers on top nodes (Svec, 1979). Determinate types flower more uniformly at all nodes on the main stem. The semi-determinate types have indeterminate stems that terminate vegetative growth abruptly after the flowering period. Their growth habits and flowering lie between the growth habits of determinate and indeterminate cultivars of soybean (Mahamood, 2008).

2.3 Nutritional and Economic Importance of Soybean

Soybean is one of the food legumes which play an important and diverse role in the farming systems and in the diets of poor people in developing countries such as in SSA.

Soybean is an ideal crop for simultaneously achieving three developmental goals in targeted population, namely reducing poverty, improving human health and nutrition and enhancing ecosystem resilience (Akibode and Maredia, 2011).

Soybean is a rich source of proteins both for human beings and animals for low income households, compared to other sources such as meat and eggs (Binang *et al.*, 2013; IITA, 2009). Soybean contains 38–40% proteins, more especially the amino acids lysine and methionine, which complements the low proteins content (6-10%) in cereal, root and tuber crops (Chianu *et al.*, 2011). It is also a rich source of dietary fibers, such as cellulose, minerals such as iron and zinc, and bioactive compounds such as isoflavones which are good for human health (Macák and Candráková, 2013). Incorporating soybean flour to replace wheat flour in the formulation of bakery products increases the nutritional value of these products, when compared to conventional product (Martino *et al.*, 2011). It has been reported that frequent consumption of legumes, four times or more weekly, reduces the risk of coronary heart disease (CHD) by 22% and cardiovascular disease (CVD) by 11% (Bouchenak and Lamri-Senhadji, 2013). According to Martino *et al.* (2011), this is so because soybean or its bioactive compounds contribute significantly to reducing cholesterol and triglyceride levels in animals and humans.

Legume foods such as soybean and cowpeas are the main sources of protein in the diet of vegetarians, and feature prominently in the traditional cuisine of virtually every region of the globe (Akibode and Maredia, 2011). Soybean increases income levels for the farmers (Odendo *et al.*, 2011) as it can be sold as food grain and/or in processed forms like soy nuts, soy milk, soy pulp and oil (Akibode and Maredia, 2011). Soybean accounts for 36.65 million tons of oil which is used for the production of edible oils, printing ink and biodiesel, more than any other field crops raised for oil extraction. Soybean, thus, serves

as a major foreign exchange earner for countries that produce it (Aniekwe and Mbah, 2014; Singh and Shivakumar, 2010).

Agronomically, soybeans serve as a rotation and/or inter-mixed crop with cereals where it serves as a barrier to organisms such as bud worm that attack maize from being transmitted from one maize crop to another (Akibode and Maredia, 2011; Seran and Brintha, 2010). Soybean also improves soil porosity and structure through its deep and proliferated tap root system, thereby improving aeration which encourages microbial activities in the soil. Organic matter obtained upon decomposition of soybean residues helps in soil and soil moisture conservation (Singh and Shivakumar, 2010). Furthermore, soybean contributes to soil nitrogen enrichment when its residues are incorporated into the soil and through biological nitrogen fixation by its roots in symbiotic association with rhizobia (Singh and Shivakumar, 2010).

2.4 Factors that Influence the Growth Performance of Soybean

The performance of soybean crops growing in any given area depends on biotic and abiotic factors such as the variety of the soybean, appropriate soil and crop management practices, as well as the climate (Mlahagwa, 2001).

2.4.1 Soil factors

Some of the soil factors that account for the growth performance of soybean plants include, for examples, soil reaction, soil texture and structure (USDA, 2014).

2.4.1.1 Soil reaction

Soybean performs well in moderately acidic to slightly alkaline soil conditions of pH 5.5-7.5 (Njeru *et al.*, 2013; Soil Survey Division Staff, 1993). Many tropical soils are

associated with highly acidic soil conditions which are stressful to the growth of soybean due to toxicity of Al and Mn (Mlahagwa, 2001). At very acidic soil conditions macro nutrients including phosphorus and magnesium which are essential for metabolic processes such as photosynthesis in plant tend to be available in low quantities (Giller and Wilson, 1991).

2.4.1.2 Soil texture and structure

Soil texture and structure determine the amounts of water and nutrients that can be retained, supplied by the soil and lost from the soil (Mlahagwa, 2001). Good soil structure and texture also facilitate aeration which promotes the growth of plant roots and microorganisms, which are involved in the decomposition of organic matter thereby releasing plant nutrients in the soil (Merumba, 2004). Soybean performs well on nearly all soil types except on extremely deep sands because of poor water and nutrient retention (Howell, 1963). However, soybean can grow more satisfactorily even on soils with low fertility than maize provided *Bradyrhizobium japonicum* infecting soybean are present (Howell, 1963).

2.4.2 Climatic factors

Environmental conditions such as drastic changes in photoperiod, temperature and precipitation have a negative effect on soybean plant development and grain yields (Hu and Wiatrak, 2012).

2.4.2.1 Photoperiod

Soybean plants are classified as short-day plants (Svec, 1979), requiring 8-10 hours of daylight with one hundred foot-candles of intensity for optimal growth. Light is the important source of energy, which controls numerous growth processes such as flowering

(Howell, 1963). The length of light hours is the function of latitude. Soybean varieties are regarded as full-season crops (Johnson and Bernard, 1963). The onset of flowering in soybeans takes place when the critical day length is reached that triggers the flowering response (Svec, 1979), otherwise soybeans are completely incapable of flowering and would remain vegetative almost indefinitely when the daylights are less than 8-10 hrs. Longer hours of daylight result in increased duration of each developmental stage resulting in delayed maturity (Howell, 1963). Howell (1963) also observed that soybeans subjected to darkness or long day light hours also contain less protein and starch.

2.4.2.2 Temperature

Temperature is one of the important factors that control plant growth and development. The suitable temperature for soybean growth is 15-22°C at emergence, 20-25°C at flowering, and 15-22 °C at maturity (Liu *et al.*, 2008). Daily or seasonal temperatures above optimum and temperature extremes, has been reported to be a major factor limiting crop production when it coincides with critical stages of plant development (Thuzar *et al.*, 2010).

The reproductive stage of plants is more sensitive to high temperatures than vegetative stage, hence the metabolic and physiological processes, for example the plant reproductive organs are more vulnerable to changes in short episodes of high temperatures prior to and during early flowering stage (Reddy and Kakani, 2007). Conditions such as high temperatures prior to flowering, affect yield mainly by causing ovary or seed abortion, or by changing seed filling duration (SFD) or seed filling rate (SFR) (Thuzar *et al.*, 2010). High temperatures hasten crop development especially the reproductive stage and therefore shortens the SFD, however, the seeds that do develop are often small although SFR may be stimulated by the high temperatures (Svec, 1979).

2.4.2.3 Precipitation

Soil moisture is important for the successful growth of soybean from germination to maturity (Howell, 1963). Drought stress during reproductive stages reduces photosynthesis, sugar production for both the soybean plant and *Bradyhizobium* and flow of metabolites to the expanding cells, which in turn results into increased flower and pod abortion and decreased vegetative growth, duration of the seed filling stage, seed number, and seed size (Svec, 1979). Pod formation and the seed-filling periods are critical for high yields of soybeans (Svec, 1979). Heavy rainfall immediately after planting can cause compaction and crusting on some soil types, such as sand and clay soils due to splashing of the soil particles making seed emergence difficult, resulting into low yields due low plant population (Howell, 1963). Excessive water fills the pore spaces in the soil thus reducing the amount of oxygen required for seed respiration, hence little or no seed emergence (Howell, 1963).

2.4.3 Agronomic factors

Agronomic factors that contribute to the growth performance of soybean include liming, fertilizer application, inoculation, early planting, breeding and weeding.

2.4.3.1 Lime application

Liming refers to the application of calcium-containing material such as calcite ($CaCO_3$) with the aim of improving the chemical properties of acid soils. Fageria *et al.* (2007) reported that lime application significantly improved the soil chemical properties such as soil reaction, base saturation and cation exchange capacity. In acid soils there is high levels of soluble Al and Mn which tend to react with P to form insoluble compounds, rendering P unavailable to plants. Liming decreases the solubility of Al and Mn in acid soils such as Ultisols and Oxisols (Buni, 2014) thereby increasing the availability of P and

providing conducive environment for microbial activities. Adding lime to the soil helps to neutralise acidity and increase the pH thereby increasing the availability of the essential plant nutrients such as Ca and P (Fageria *et al*., 2007). However, in high pH soils (pH 8 and above), phosphorus reacts with calcium and/or magnesium to form insoluble compounds such as dicalcium phosphate which limit their availability (Merumba, 2004). Landon (1991) recommends that pH should be raised to about 5.5-6.5 to avoid the formation of these insoluble compounds.

2.4.3.2 Fertilizer application

The application of moderate levels of mineral fertilizers especially N and P have been shown to increase soybean plant growth and N_2-fixation compared with the unfertilized control, thereby improving grain yields (Chianu *et al*., 2011). Nitrogen facilitates soybean growth because though soybean obtains 65-85% of its N needs through the symbiotic N fixation process, the soybean plants start to fix substantial amounts of atmospheric nitrogen approximately 4 weeks after germination (Rao and Reddy, 2010). A modest N fertilizer application of 30-50 kg ha^{-1} at sowing is recommended (Singh and Shivakumar, 2010). Some researchers have noted a favourable effect of N applied at the time of sowing on nodule weight, rhizobia activity and biological N-fixation hence increase in plant growth compared with the unfertilized control (Chianu *et al*., 2011; Rao and Reddy, 2010).

The application of phosphorus (P) at the rate of 60 kg ha^{-1} to P deficient soils has been reported to positively affect soybean plant height, biomass and pod production (Njeru *et al*., 2013). Soybeans require P for the biological N_2 fixation processes and growth. In acidic soils with very low pH such as Ultisols, P tends to be fixed hence liming and P

fertilization are required to increase its availability, so as to enhance N fixation (Giller and Wilson, 1991).

Many SSA countries have the desire to increase the use of mineral fertilizers to enhance legume crop yields because of decline in soil fertility over the years (Chianu *et al*., 2011). However, majority (about 60%) of African smallholder farmers are unable to afford the high prices of mineral fertilizers as such the grain legumes are rarely fertilized (Njeru *et al*., 2013). For the few farmers who can afford to buy mineral fertilizers, most of them do not use fertilizers for their grain legumes like soybean due to lack of awareness of the associated economic returns (Chianu *et al*., 2011).

2.4.3.3 Inoculation

Inoculation of the seeds or the soil with rhizobia also helps to boost the yield of grain legumes, leading to land savings, and cheap assurance for higher yields. Studies have shown that rhizobia inoculation is needed for most of the grain legumes in all agricultural lands deficient in N such as marginal lands, arid and semiarid lands, and where N supply is a key limiting factor in crop production (Chianu *et al.*, 2011). Inoculating soybean seeds with *Bradyrhizobium japonicum* was reported to increase the rate of their establishment, root nodulation, biomass and biomass N yields (USDA, 2014). Inoculation helps to increase the number of effective *Bradyrhizobium japonicum* and especially, for soybean, which has been newly introduced into an area, to form effective symbiosis. Inoculation is also important in soybean production because indigenous *Bradyrhizobium japonicum* do not always meet the full demand for N by soybean (USDA, 2014). Poor yields may occur whenever effective *Bradyrhizobium japonicum* population in the soil is low (< 20–50 cells g^{-1} soil) or even when available in adequate numbers (> 20–50 cells g^{-1} soil) if they are not infective (Chianu *et al.*, 2011).

2.4.3.3 Early planting

Since flowering in soybean is controlled by photoperiod, planting date/time affects the plant size attained before flowering begins (Svec, 1979). Soybeans, especially for the indeterminate types, when planted late have less time to develop vegetatively and thus become shorter culminating into low yields.

2.4.3.4 Weed control

Weeds interfere with crop growth by competing for nutrients, water and light resulting into poor growth and reduced yields (Akobundu and Poku, 1987). Yield components that are good indicators of weed competition in soybean production include leaf area index (LAI), dry matter weight and crop growth rate, all of which become reduced under unattended weed competition with soybean (Howel, 1963). The damage from weed competition is severe when soil moisture is limiting during the reproductive stages of soybeans (Akobundu and Poku, 1987), because most weeds are more efficient in soil moisture absorption than soybeans. Little or no reduction in yield occurs if the soybeans are kept weed free for the first 4 weeks because of its dense canopy which suppresses the growth of the weeds (Akobundu and Poku, 1987).

2.4.4 Plant factors

Soybean varieties have different growth habits, which range from highly branching types to thin-line types which produce a single, main stem (Svec, 1979). Tall varieties are generally more susceptible to lodging during heavy storms which might lead to low yields (Svec, 1979). Highly branched and tall varieties compete well against weeds unlike the shorter varieties by shading the weeds from light, hence good growth performance (Akobundu and Poku, 1987).

Soybean varieties that can resist pod shattering and major pests and diseases of their target environments have been developed through genetic manipulations (Dashiell *et al*., 1987). Some of the diseases for which soybean genes of resistance have been incorporated into improved lines include bacterial pustule caused by *Xanthomonas campestris*, frogeye leaf spot (*Cercospora sojina*) and soybean mosaic virus (Dashiell *et al*., 1987). Soybean varieties that can resist pod shattering and these major diseases in their target environment are likely to perform better than non-resistant varieties (Johnson and Bernard, 1963).

The growth performance of soybean plants in N deficient soils depends on the availability of infective *Bradyhizobium* in such soils for effective BNF process. Soybean varieties that can form nodules with *Bradyhizobium* indigenous to Sub-Saharan Africa soils have been developed through genetic manipulations to enhance soybean performance since most farmers have no access to inoculants (Dashiell *et al*., 1987).

Viability of seeds used for planting determine the growth performance of soybean. Seeds usually lose their viability when stored under ambient conditions hence the need for storage in cold environments/conditions (2-16 ^{o} C) (Stahl, 2014), which is impractical for poor resource farmers. However, some soybean varieties have been bred whose seeds remain viable when stored and grown in the tropics (Dashiell *et al.*, 1987).

2.4.5 Pests and diseases

There are many diseases that affect soybean plants, which include seed-borne and soil-borne diseases (Svec, 1979). Selection of seeds for planting that are free of these diseases is important in reducing loss of production attributed to these diseases.

Soybean seed diseases include purple seed stain, which is caused by the fungus *Cercospora kikuchii* (Hershman, 2009). The infected seeds have purple to brown discoloration and the seed coats may be roughened and cracked (Svec, 1979). This disease usually would not affect seed quality for processing, but the use of such seed for planting is not recommended because the embryo may be infected hence fail to germinate (Svec, 1979). Treatment of seed with fungicides like for examples trifloxystrobin ($C_{20}H_{19}F_3N_2O_4$) and azoxystrobin ($C_{22}H_{17}N_3O_5$) before planting may reduce early seed-borne infection (Hershman, 2009).

Grey moldy seed-borne disease caused by the fungus, *Diaporthe phaseolorum* var. *sojae*, causes soybean stem blight (Pedersen, 2007). The fungus invades the plant and pod late in the season, especially under high humidity conditions. Seeds infected with this fungus become grey to black in colour, appearing moldy and shrunken (Svec, 1979). This disease may be controlled by the use of clean seeds for planting, adopting crop rotation and seed treatment fungicide (Svec, 1979; Pedersen, 2007). Environmental conditions such as high humidity at the time of seed development increases the occurrence of this disease (Pedersen, 2007).

Phytophthora root rot, is a soil-borne disease caused by fungus *Phytophthora megasperma* var. *sojae*, which affects soybean growth in poorly drained and heavy soils (Svec, 1979). Damping off is common in seedlings infected by the fungus, while older plants turn yellow, their leaves wilt and their roots rot until the plant dies (Pedersen, 2007). Use of resistant or tolerant soybean varieties is the best prevention method of this disease (Pedersen, 2007).

Use of resistant or tolerant varieties and production practices, such as crop rotation, are the best methods of disease control (Svec, 1979). Soybeans can tolerate fairly high levels of defoliation (10-35%) due pests attack during the vegetative development stages without reduction in yield (Svec, 1979). After blooming, loss of up to 20% foliage will not be detrimental to the yield of soybean.

2.5 Nitrogen Fixation

Nitrogen fixation refers to the natural or artificial process in which the gaseous form dinitrogen (N≡N) is converted into forms such as ammonia (NH_3), which is usable by plants (Wojciechowski and Mahn, 2008). There are three types of nitrogen fixation, namely; atmospheric, industrial and biological.

2.5.1 Atmospheric nitrogen fixation

Atmospheric nitrogen fixation takes place through lightning during thunder storms, where N reacts with O_2 to form NO_2 or NO_3. Nitrate (NO_3^-) or nitrite (NO_2^-) are formed through the following stepwise reactions (Kumar, 2010):

i. $N_2 + O_2 \xrightarrow{\text{Lightning}} 2NO$ (Nitric oxide)

ii. $2NO + O_2 \xrightarrow{\text{Lightning}} 2NO_2$ (Nitrogen dioxide)

iii. $2NO_2 + H_2O \xrightarrow{\text{Lightning}} HNO_3 + HNO_2$

The overall reaction:

$$N_2 + 2O_2 + H_2O \xleftrightarrow{\text{Lightning}} HNO_3 + HNO_2 \longrightarrow 2H^+ + NO_3^- + NO_2^-$$

The nitrite and nitrate can be readily utilized by plants and soil microorganisms for metabolic processes such as respiration.

2.5.2 Industrial nitrogen fixation

Industrial nitrogen fixation is carried out through the Harber process where the anhydrous ammonia (NH_3) is artificially manufactured from hydrogen and nitrogen gases (Barker and Bryson, 2006). The sources for the nitrogen and the hydrogen are the air and natural gas (CH_4) and water, respectively (Barker and Bryson, 2006). The reaction takes place at high temperature (400-500 ^{o} C) and high pressures of 300-1000 atm in the presence of a catalyst, like iron or other metals (Berg *et al*., 2002) according to the following reaction:

$$N_2 + 3H_2 + \text{catalyst (i.e. CaO)} \xrightarrow[\text{high pressure}]{\text{high temperature}} 2NH_3 \quad (\Delta H = -92.4\ \text{kJ}\cdot\text{mol}^{-1}).$$

Industrial nitrogen fixation is the major source of N fertilizers used by farmers today, which may be manufactured through the reaction of the ammonia mineral (NH_3) and acids and salts such as sulphuric acid (H_2SO_4) to form ammonia sulphate according to the reaction:

$$2NH_3 + H_2SO_4 \longleftrightarrow (NH_4)_2SO_4$$

2011, the world annual production of ammonia fertilisers was estimated to be 137.7 million tonnes (FAO, 2012).

2.5.3 Biological nitrogen fixation

Biological nitrogen fixation (BNF) is the process whereby atmospheric nitrogen (N_2) is reduced to ammonia (NH_3) through the association of roots of legume plant and the rhizobia or through free-living bacteria (Metuzals, 2014). The conversion of N_2 to NH_3 occurs in the presence of nitrogenase which is a biological catalyst (enzyme) for

biological N-fixation found naturally in rhizobia (Metuzals, 2014). The reduction of ammonia occurs through the following stepwise reactions (Berg *et al*., 2002).

i. $N_2 + 6\ H^+ + 6\ e^- \xrightleftharpoons{\text{Nitrogenase}} 2NH_3$

Two molecules of ATP are hydrolyzed for each electron transferred, thus, at least 16 molecules of ATP are hydrolyzed for each molecule of N_2 reduced. The overall reaction for BNF process is:

ii. $N_2 + 8\ H^+ + 8\ e^- + 16\ ATP + 16\ H_2O \xrightleftharpoons{\text{Nitrogenase}} 2NH_3 + H_2 + 16\ ADP + 16\ P_i$

It is estimated that about 11.1 million metric tons of nitrogen are fixed annually through BNF in developing countries (Chianu *et al.*, 2011). Biological nitrogen fixation can be non-symbiotic carried out by free-living bacteria or symbiotic carried out by mutualistic bacteria (Wojciechowski and Mahn, 2008).

2.5.3.1 Non-symbiotic biological N fixation

In non-symbiotic BNF, the free-living bacteria such as *Azotobacter* and *Azospirillum* live outside or on the roots of the plant hence is not parasitic on it (Kennedy *et al.*, 2004). The free-living bacteria depend on their own source of energy, by oxidizing organic molecules released by other organisms or from decomposition of organic matter (Wagner, 2011). There are some free-living bacteria that have chemolithotrophic capabilities and can thereby utilize inorganic compounds such as ammonia and hydrogen as their source of energy. The free-living bacteria convert free nitrogen into soluble compounds such as ammonia which are then released into the soil and subsequently absorbed from the soil by cereal plants (Kennedy *et al*., 2004). Non-symbiotic BNF is usually associated with cereals like maize, wheat, sugarcane and rice. Several researchers have reported significant amounts of N fixed from the non-symbiotic BNF pathways. Kennedy *et al*. (2004) reported that *Acetobacter*-sugarcane system fixed significant amount of N which

supplemented the recommended dose of 275 kg urea-N ha^{-1} in India. It has been reported by Tra ˆn Van *et al*. (2000) that *Burkholderia*-rice relationship fixed about 25–30 kg N ha^{-1} in Vietnamese soil.

2.5.3.2 Symbiotic biological N fixation

Symbiotic biological N-fixation occurs through the associations of plant roots with nitrogen-fixing bacteria called rhizobia. An example of such association is between soybean and *Bradyrhizobium*. *Bradyrhizobium* infect root hairs of the soybean plants and produce the nodules which become the site for the BNF in the soybean-*Bradyhizobium* symbiotic association (Van *et al*., 2013). The *Bradyhizobium japonicum* obtain energy from the soybean plant and take free nitrogen from the atmosphere and convert it to soluble nitrogen which is available for the soybean growth (Morel *et al*., 2012). *Bradyrhizobium* lives in association with soybean plant roots and in the absence of the compatible host plant (soybeans), the population decreases due to lack of source of energy for survival, hence low biological N_2 fixation. Artificial seed inoculation is often needed to restore the population of effective strains of the rhizobia in the rhizosphere to hasten and increase BNF (Chianu *et al.,* 2011; Mishra *et al.,* 2013). The likelihood of response to inoculation by soybean is strongly influenced by the number of effective *Bradyrhizobium japonicum* in the soil and the soil chemical conditions such as soil reaction. Soybean would likely respond to inoculation if the small number of effective *Bradyrhizobium japonicum* in the soil is within 20–50 cells g^{-1} soil (Mishra *et al.*, 2013). Under appropriate conditions, about 80% of the above-ground N accumulation in soybean is due to N fixation by the *Bradyrhizobium japonicum* (Chianu *et al.*, 2011).

2.6 Factors Influencing the Extent and Magnitude of BNF

The biological nitrogen fixation is affected positively and/or negatively by both edaphic and crop factors (Mabrouk and Belhadj, 2012).

2.6.1 Edaphic factors

The edaphic factors that influence the extent and magnitude of BNF include soil reaction, levels of soil N, phosphorus and calcium contents, native rhizobia population in the soil and the soil climate, as outlined below.

2.6.1.1 Soil reaction

Saline-sodic, sodic ($pH \geq 9.0$) and very strongly acidic ($pH \leq 5.0$) soil conditions reduce the survival of rhizobia thus inhibit nodulation and biological N fixation (Mabrouk and Belhadj, 2012). This is because rhizobia are neutrophiles in nature preferring slightly acidic to slightly alkaline (near neutral) soil reaction in the range of pH 5.1-8.9 for their optimum growth and activity (Slonczewski *et al*., 2009). Under acidic or alkaline conditions the high concentration of Al^{3+}, Mn^{3+}, Cu^{2+} and Fe^{3+} and salts, respectively may become toxic to the rhizobia (Coskan and Dogan, 2011). Acidic conditions decrease the availability of molybdenum (Mo) which is a structural component of nitrogenase, the enzyme involved in N fixation by root-nodule bacteria of leguminous plants (Tisdale *et al*., 1993). Liming reduces soil acidity and increases availability and uptake of plant nutrients such as Mo, phosphorus and calcium (Fageria *et al*., 2007). According to Njeru *et al.* (2013), BNF in soybean production is effective in soils with a pH range of 5.5–7.5.

2.6.1.2 Soil N

Soils rich in plant available nitrogen (≥ 30 mg N kg^{-1} in the 0-30 cm layer) have been found to subdue the biological nitrogen fixation process through retarding root infection,

nodule development and the extent of nodulation (Chianu *et al.*, 2011; Singh and Shivakumar, 2010). Nitrogen fixation is inhibited at high levels of NO_3^- because legumes use these mineral sources of N since it requires less energy for the plant to take up N directly from the soil than to fix N through BNF (Silva and Uchida, 2000). Most soils under soybean cultivation have 5-10 mg N kg^{-1} in the 0-30 cm soil layer, which is favourable range for BNF by soybean.

2.6.1.3 Phosphorus

Phosphorus (P) enhances the symbiotic nitrogen (N) fixation process in legume crops through its effect on the growth of the leguminous roots which are the centres for nodulation and N fixation.

The biological nitrogen fixation is an energy intensive process (Metuzals, 2014) which requires a readily available source of energy and phosphorus provide that energy when adenosine triphosphate (ATP) is converted to adenosine diphosphate (ADP) during the reduction of N_2 molecule to NH_3 (Armstrong and Griffin, 1999). According to Armstrong and Griffin (1999), the concentration of P in the tissue of healthy, active nodules is often two to three times higher than that in the roots on which they are formed. Phosphorus provides the mechanism for storage of energy sugars (photosynthate) in the form of ATP and the translocation of this energy from leaves to the roots for the rhizobia (Weisany *et al.*, 2013). In acidic soils with very low pH such as Ultisols, P tends to be fixed hence P fertilization is required so as to enhance N fixation (Giller and Wilson, 1991). The critical level of available P for grain legumes including soybean has been reported by Mahamood (2008) to be 10.8 mg P kg^{-1} soil.

2.6.1.4 Calcium

Plant available calcium facilitates the attachment of rhizobia to the root hairs, nodulation and nodule development (Franche *et al*., 2008; Weisany *et al*., 2013), hence increase in N fixation. If calcium is low, a situation common in most acidic soils, addition of appropriate liming materials such as calcite ($CaCO_3$) that will provide Ca and raise the soil pH at the same time is mandatory.

2.6.1.5 Native rhizobia in the soil

Nitrogen fixation is enhanced if the soil has high populations of infective native rhizobia that are compatible with the legume crop (Silva and Uchida, 2000). If native rhizobia strains are non-infective, inoculation with infective rhizobia which are aggressive colonizers is necessary so as to effectively outcompete the native strains (Metuzals, 2014; Chianu *et al.*, 2011; Singh and Shivakumar, 2010). Chianu *et al.* (2011) reported that inoculant rhizobial numbers of up to 1000 cells g^{-1} soil need to be applied to soybean seed to obtain greater nodule occupancy by inoculant *Bradyrhizobium japonicum* than the indigenous strains.

2.6.1.6 Soil climate

Symbiotic N fixation is highly sensitive to soil moisture stress, a major limiting factor to legume production in the semi-arid tropics (Chianu *et al.*, 2011; Morel, *et al.*, 2012). Rhizobia require moisture (water) to carry out some metabolic processes such as hydrolysis in which energy is obtained by the breakdown of the larger molecules into smaller ones. In low soil moisture condition (below field capacity) nodule and rhizobial respiration decreases (Coskan and Dogan, 2011) thereby promoting nodule decay and reduced rhizobia numbers, respectively, hence affecting the efficiency of BNF activity (Singh and Shivakumar, 2010). Heavy downpours results in waterlogging conditions

which affect respiration of aerobic bacteria (Singh and Shivakumar, 2010). Loamy and clay soils favour nitrogen fixation as compared to sandy soils due to poor microbial activity and low water retention capacity of the latter type of soil.

Very high or very low ambient temperatures affect soil temperature which affect the survival of rhizobia in the soil and hence inhibition of nodulation and N fixation (Chianu *et al.*, 2011; Morel *et al.*, 2012). The optimum temperature range for growth, for soybean-*Bradyrhizobium* association is 28 to 31 ℃ (Mabrouk and Belhadj, 2012). Temperature below or above the optimum range usually results in the formation of ineffective nodules.

2.6.2 Crop factors

Genetic constitution

Different varieties of soybean have different genetic constitution which affect their compatibility with nitrogen-fixing microbes and hence their influence on the activity of the microbes on nodulation and nitrogen fixation (Singh and Shivakumar, 2010). Promiscuous soybean genotypes like Hernon 147, nodulate and fix nitrogen effectively with diverse indigenous rhizobia available in the soil while non-promiscuous genotypes form symbioses with specific rhizobia strains thus limiting their N-fixing ability (Njeru *et al.*, 2013). However, Sanginga *et al.* (2000) noted that promiscuous soybean types are incapable of nodulating effectively with indigenous rhizobia in all locations in the moist savanna zone of Nigeria. Similarly, Bala (2008) concluded that it is also not clear whether promiscuous soybean cultivars are effectively nodulated by indigenous rhizobial populations in all soils and under all conditions. Therefore, it has been concluded that it may be safer to rely on effective inoculant strains rather than breed for the ability to nodulate with indigenous rhizobia strains of unknown potential (Eaglesham, 1989).

2.6.3 Crop management factors

Various agronomic practices have profound influence on microbial activities and in turn the rate of BNF (Singh and Shivakumar, 2010). For instance, seed inoculation with efficient strains of *Bradyrhizobium,* timely sowing, small doses of nitrogen through fertilizers, light irrigation to avoid water logging, avoiding the use of pesticides and herbicides that harm microbes and proper tillage operations have positive influence on BNF and lead to increased biological nitrogen fixation (Abdul-Aziz, 2013). Singh and Shivakumar (2010) have reported higher number of nodules, nodule dry weight, biological nitrogen fixation and nitrogen balance in soils with limited tilled condition compared to tilled ones. Tillage stimulates decomposition of organic matter in the soil which results in the availability of high levels of nitrate which may depress nodulation and N fixation.

2.7 BNF in Various Soybean-based Cropping Systems

Cropping system refers to the spatial and temporal arrangement of different crops to exploit natural resources and enhance crop productivity per unit area and time (Singh and Shivakumar, 2010). Soybean is suitable and practiced in many mixed and sequential cropping systems because of its moisture stress tolerance (Aniekwe and Mbah, 2014), contribution to soil fertility and lesser pest and disease incidences. Inclusion of a cereal in soybean-based cropping systems is very common feature in many areas (Chianu *et al*., 2011).

2.7.1 Mono-cropping

Mono-cropping or sole-cropping refers to the practice of growing one crop in a field at given period of time. The total amount of N fixed in soybean sole cropping tends to be higher compared to intercropping or mixed cropping systems (Metuzals, 2014).

Matusso *et al*. (2013) reported that sole soybean accumulated 14.95 mg N $kg^{-1}$of soil compared to intercropping which accumulated 8.70 mg N kg^{-1} of soil. This is because of the increased competition for light and nutrients as well as decreased legume populations densities in the latter system (Njira *et al*., 2012).

2.7.2 Intercropping

This is the spatial arrangement of crops in the field whereby crops of varying morphological features, rooting pattern, growth phenology and type of economic yield are grown on the same piece of land in the same growing period (Singh and Shivakumar, 2010). Planting geometry is planned so as to reduce mutual competition for resources and enhance complementarities to increase overall productivity. It helps in effective utilization of land, soil moisture, nutrients and solar radiation. Studies show that with soybean-maize and soybean-sorghum cropping system, maize and sorghum obtain 65 and 80 kg N ha^{-1} respectively from BNF (Singh and Shivakumar, 2010). However, the success of intercropping depends on the efficiency of BNF. Matusso *et al*. (2013) reported that the number of compatible rhizobia in the rhizosphere and the degree of infection of the root by the bacteria are important factors for BNF which are controlled by environmental conditions such as soil moisture. Thus, in cereal-legume intercropping, without adequate BNF, the N demand of each intercrop may increase N competition, particularly when relatively low amount of fertilizer N are used.

2.7.3 Rotational cropping

Rotational cropping system refers to a temporal arrangement of crops which aims at growing crops one after another in sequence to exploit the congenial conditions of the different seasons (Singh and Shivakumar, 2010). Under optimal conditions, soybean-*Bradyrhizobium japonicum* symbiotic relationship fixes up to about 200 kg N ha^{-1} of

atmospheric nitrogen (Metuzals, 2014). A substantial portion of this is used by the growing soybean, but some is left unused in the soil and some in the nodules. The left over nitrogen is available to the next crop after soybean is harvested (Morel *et al*., 2012). The extent of this left over nitrogen depends on the efficiency of BNF process, utilization by the crop and the environmental conditions such as rainfall which may leach down the nitrogen. Studies in India showed that maize crop grown after soybean required about half of nitrogen fertilizer required by continuously cropped maize, as soybean added the equivalent of 150 kg fertilizer N ha^{-1} (Singh and Shivakumar, 2010). Wheat planted after soybean required 21kg N/ha less than wheat planted after grain sorghum. According to Aniekwe and Mbah (2014), soybean is capable of increasing grain yield of succeeding maize or wheat by 1.3 t/ha.

Soybean crop residue incorporation also has a positive impact on nitrogen fixation through decomposition of the residue and N-release to the soil pool, which is beneficial for the subsequent cereal crop like maize. Kihara *et al*. (2011) reported that soybean residues contain total N up to 30 kg N ha^{-1} which should be returned to the soil after grain removal to optimize on BNF.

2.8 Determination of the Quantities of N Fixed by BNF Process

Growing legumes in agricultural systems enhances soil fertility because of their potential capacity to fix large amounts of atmospheric N_2, through the association between legume and *Rhizobium* spp. (Peoples *et al*., 1989; Marandu *et al*., 2010). Ineffective symbiosis in a soil with low mineral-nitrogen (N) results in reduced legume production, and application of N-fertiliser is required to achieve seed yields similar to those of a well-nodulated crop. On the other hand, in soil with higher mineral-N content, the legume may

compensate for poor N fixation by scavenging N from the soil, however, the net result of cropping in this case is an exploitation of N reserves (Peoples *et al*., 1989).

The overall benefits of including N-fixing legumes in cropping systems can only be assessed with a reliable and accurate field measurement of the levels of fixation achieved (Peoples *et al.*, 1989; Giller and Wilson, 1991). This enables the farmer to determine the amounts of N that will have to be supplemented as mineral fertilizer to optimize yields under inter and/or rotation cropping systems (Marandu *et al*., 2010). Any other parameter, like nodule number and legume biomass provides only a qualitative and descriptive assessment of BNF (Peoples *et al*., 1989). Different methods are used for measuring N fixation and no one technique provides an accurate measure of N fixation for all legumes grown in any soil under diverse environmental conditions (Peoples *et al*., 1989; Abdul-Aziz, 2013). Each technique has its own unique advantages and limitations. Some of the methods used for measuring N fixation are discussed below.

2.8.1 The acetylene reduction assay

The acetylene reduction assay is used for the detection of nitrogenase activity (Peoples *et al*., 1989; Weaver and Danso, 1994). It has been widely used in all areas of N fixation research because of its high sensitivity and simplicity. However, its reliability is now questioned as the acetylene reduction provides only an instantaneous measure of nitrogenase activity under the prevailing assay conditions (Weaver and Danso, 1994). Its accuracy has always been restricted by the requirement for many repeated determinations to adjust for marked diurnal and seasonal changes in N-fixing activity (Peoples *et al*., 1989). Errors in the field can also arise due to the use of an inappropriate calibration factor to relate ethylene production to N fixation, incomplete recovery of a plant's total nodule population, nodule detachment or damage prior to assay, plant

disturbance, or an acetylene-induced decline in nitrogenase activity during assay (Peoples *et al*., 1989; Weaver and Danso, 1994).

2.8.2 Total N accumulation

The total N accumulation in a whole plant reflects the amount of N originating from N fixation (Peoples and Giller, 1996). This is a cumulative evaluation for N fixation from planting until harvest. It is necessary to determine the amounts of total plant and crop N in order to quantify inputs of N by N fixation in terms of kg N/ha (Peoples *et al*., 1989). Evaluation and interpretation of N fixation data can also be assisted by measurements of soil N status. There are two methods upon which all commonly used analyses for total plant nitrogen are based. The first is the oxidative method in which organic materials are oxidised in the presence of copper oxide to produce N_2 gas, the volume of which is measured (Peoples *et al*., 1989). Incomplete combustion can be a problem with this method and it may not be suitable for very precise determinations of low levels of N, particularly if there is variation in N abundance or dry matter N contents between different plant tissues and insufficient care taken in subsampling (Peoples *et al*., 1989). The common alternative to the oxidative method is the 'wet' Kjeldahl digestion (Peoples *et al*., 1989). In Kjeldahl digestion, organic and mineral N is reduced to NH_3 in hot, concentrated sulphuric acid in the presence of a catalyst. The NH_3 is recovered by distillation or diffusion and estimated by titration or colorimetrically.

2.8.3 Xylem-solute technique

The roots and the shoots of legumes contain xylem sap, which carries N-containing compounds from nodules as assimilation-products of N fixation, and soil mineral N taken up by the roots (Peoples *et al*., 1989). Xylem saps of fully symbiotic plants and non-nodulated plants which are totally dependent upon soil N are analysed to establish

differences in xylem N-solute composition between the two, so as to assess the extent to which plants rely on N fixation or soil mineral N.

Collected xylem exudates samples are then analysed for the N-components (i.e. ureides, a-amino N and nitrate) by colorimetric assays in a test tube (Peoples *et al*., 1989). The method is not destructive technique as sufficient sap can be collected from stem segments and laterals of mature plants for complete analysis. Since sampling is confined to the accessible aerial parts of the plant, the solute method may potentially overcome many problems associated with measuring N fixation by twining ground-cover or forage legumes, or woody perennial legumes (Peoples *et al.*, 1989).

The major disadvantage of the N-solute technique is that it provides a short-term rather than time-integrated measure of symbiotic dependence (Peoples *et al*., 1989). If an estimate of seasonal fixation is required rather than a comparative measure of treatment effects on N fixation, repeated measurements must be combined with sequential sampling from the crop for dry matter and total N contents.

2.8.4 Nitrogen-difference technique

Field estimates of N fixation obtained by measuring total amount of N in the legume crop are based on the arbitrary assumption that these crops derive all their N from symbiotic fixation (Peoples *et al*., 1989; Weaver and Danso, 1994). This suggestion is unrealistic and unsubstantiated and values calculated on this basis of legume N yield almost always overestimate fixation (Peoples *et al*., 1989). A true measure of fixation based on crop N accumulation can be obtained only when the contribution of soil N to the total legume N is determined (Giller and Wilson, 1991). This is estimated by growing a companion non-N-fixing control crop in the same soil and under identical conditions as the legume (usually in an adjacent plot). A non-N-fixing control plant may be a non-legume, an

uninoculated legume of the same species (requires soil to be devoid of effective *Rhizobium* spp) or non-nodulating legume genotype (Peoples *et al*., 1989). The difference in total shoot N accumulated on a per-plant or per-unit-area basis between the legume and control crop is then generally regarded as the contribution of symbiotic fixation to the legume (Weaver and Danso, 1994). Generally the quantity of legume N derived from N fixation (Q) is calculated as:

Q = N yield (legume) -N yield (control)

2.8.5 15N-isotopic techniques

There are two stable isotopes of nitrogen, ^{14}N and ^{15}N which are widely used in agricultural research (Weaver and Danso, 1994). The heavy isotope, ^{15}N, occurs in atmospheric N_2 at a constant abundance of 0.3663 atoms % (Peoples *et al*., 1989). If the ^{15}N abundance in plant-available soil N is higher than this, an estimate of the proportions of legume N derived from each source can be made (Peoples *et al*., 1989). For these estimates, the abundance of ^{15}N in plant-available soil nitrogen is obtained by analysing a non-N-fixing reference plant which is totally dependent on soil N for growth (Weaver and Danso, 1994).

2.9 Contribution of BNF to Sustainable Agriculture

Earles (2005) defined sustainable agriculture as one that produces abundant food without depleting the earth's resources or polluting its environment. Farmers usually depend on mineral fertilizers as source of nitrogen. The use of N fertilisers, although effective, they are expensive and substantially contributes to environmental degradation through groundwater pollution by nitrates (Chianu *et al*., 2011). This leaching of N into waters is an economic flaw since only a part of the fertilizer is used by the plants. It has been reported by Van *et al*. (2013) that 30-50% of nitrogen fertilizer applied is lost through

leaching and run off which cause the eutrophication of waterways such as lakes and other significant environmental problems. Agriculture sustainability mandates that alternatives to N-fertilizers be sought (Northrop and Connor, 2013; Metuzals, 2014). The process of biological nitrogen fixation is important to agriculture sustainability, as it offers one of the alternatives to the reduced use of N fertilizers (Chianu *et al.*, 2011). Optimizing biological nitrogen fixation processes has the potential to increase crop yields and enhance soil fertility whilst simultaneously reducing farming costs and harmful environmental impacts. Therefore, to achieve food security through sustainable agriculture, the requirement for nitrogen could be met by BNF rather than by industrial nitrogen fixation (Mishra *et al.*, 2013).

2.10 The scope of BNF in SSA

A study carried out in Uganda showed that BNF contributes 22% of nitrogen inflows for perennial crops and 44% for annual crops (Nkonya *et al.*, 2008). Chianu *et al.* (2011) reported that despite the potential of inoculants to address low N and its cost effectiveness, the demand and adoption of BNF and inoculant technologies in the farming systems of SSA is low. Some of the reasons for the low adoption include absence of or very weak institutions, policy and budgetary support for biotechnology research, limited knowledge of inoculation responses of nodulating soybean varieties, hence a weak basis for decision-making on biotechnology issues in SSA (Chianu *et al.*, 2011). Other reasons according to Njeru *et al.* (2013) include poor quality inoculants, inadequate and inefficient marketing outlets, as well as inadequate extension services covering soybean management (production, processing, and use) and inoculant use, and limited involvement of the private sector in the distribution of inoculants. Chianu *et al.* (2011) reported that limited training in participatory research skills, tools and methodologies has limited the success of various approaches like participatory rural appraisal (PRA),

participatory learning and action research (PLAR) and participatory agro-ecosystem management (PAM) to BNF adoption in SSA. Addressing the constraints to the use of BNF technologies in most Sub-Sahara African countries could reduce expenditures on fertilizer imports through a full exploitation of BNF.

Few interventions in soybean Rhizobium inoculation have been carried out, in SSA in order to understand how best to effectively advise on future investments to popularize soybean enterprises and enhance production and adoption of BNF and inoculant technologies so as to improve the farming systems, household nutrition, income and overall welfare in SSA (Chianu *et al*., 2011). Microbiological Resource Centers (MIRCENs) were established by UNESCO in developing countries such as SSA whose responsibilities included the collection, identification, maintenance and testing of strains as well as preparing inoculants and distributing cultures compatible with local crops (Odame, 1997). The Nairobi MIRCEN also developed a marketable bio-fertilizer called Biofix which was distributed by NGOs whose active involvement in Biofix promotion over-stretched their financial and human resources and waned (Bala, 2008).

In the 1990s Sokoine University of Agriculture developed a biofertilizer (Nitrosua) for use in soybean production (Mugabe, 1994). In collaboration with the Ministry of Agriculture and some NGOs, SUA also established extension activities to disseminate Nitrosua to local farmers. These activities also waned over time due to lack of resources (Bala, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of the Study Area

The study was based on a Ultisol sampled from Magadu section of the SUA farm which is located at the foot slopes of Uluguru Mountain in Morogoro municipality, Morogoro region, Tanzania. Magadu section of SUA farm lies between 06^0 50' 24.7" S and 37^0 38' 59.8" E and at an elevation of 526 m above sea level (Msanya *et al*., 2003). The rainfall distribution is bimodal with the first rainy season running from March to May while the second rainy season runs from November to January. According to Msanya *et al*. (2003) the onset and distribution of the rainfall are irregular and unreliable. The annual rainfall range between 800 and 950 mm (semi-arid area). The mean monthly maximum temperature ranges from 27.5°C, during the coldest months of May-July, to 32^o C, during the hottest months of October-December (Kaaya *et al*., 1994). The mean monthly minimum temperature ranges from 15.1°C to 21.5° C, during the coldest months of May-July and the hottest months of October-December, respectively. The dominant soil of the study area is Ultisol which is well drained and dominated by kaolinitic 1:1 layer silicate clay minerals (Kaaya *et al*., 1994).

3.2 Soil Sampling and Preparation for Laboratory Analysis and Glasshouse Pot Experiment

Prior to gathering the composite soil sample for the soil fertility evaluation and the glasshouse pot experiment, reconnaissance survey was undertaken using transect walks to establish the extent of horizontal and vertical soil variations of the study site based on the local indicators of soil fertility (LISF) such as soil colour, texture, structure and vegetation. Upon completion of the reconnaissance survey, 20 sampling points were

established based on the simple random sampling plan (Pennock *et al*., 2007) from an area covering about 3 ha. Soil samples of about 12 kg each were collected from the established sampling points and mixed to constitute a composite sample of about 240 kg. The samples were collected at the rooting depth of 0 - 30 cm using hoes and spades. The bulk composite soil sample was air dried, ground and sieved through a 6 mm sieve. From the 6 mm sieved bulk composite sample about one kilogram soil sample was randomly gathered and sieved through a 2 mm sieve for laboratory analysis to characterise the fertility of the soil from the study area.

3.3 Soil Analysis for the Soil Fertility Evaluation

The representative composite soil sample was used for the determination of the following soil properties: particle size distribution, pH, organic carbon, total N, available P, cation exchange capacity, exchangeable bases (Ca^{2+} Mg^{2+}, K^{+} and Na^{+}), soil extractable micronutrients (Fe, Cu, Zn, Mn and B) and exchangeable acidity.

Particle size distribution was determined by the hydrometer method after dispersion with 5% sodium hexametaphosphate (Gee and Bauder, 1986) and soil textural class was determined using the USDA soil textural class triangle (USDA, 1975). Soil pH was determined electrometrically in 1:2.5 soil:water and soil:0.01M $CaCl_2$ suspensions (Thomas, 1996). CEC and exchangeable bases (Ca^{2+} Mg^{2+}, K^{+} and Na^{+}) were determined by the NH_4-acetate saturation equilibrium method followed by displacing adsorbed $NH4^{+}$ using 1 M KCl. CEC was then determined by Kjeldahl digestion-distillation method (Chapman, 1965) while exchangeable Ca^{2+} and Mg^{2+} were quantified using an atomic absorption spectrophotometer (AAS) and exchangeable K^{+} and Na^{+} were determined by the use of a flame photometer (Thomas, 1996). Organic carbon was determined by the Walkely and Black wet oxidation method (Nelson and Sommers, 1996). Total N was

quantified by the Kjeldahl digestion-distillation method as described by Bremner (1996). Available P was quantified by the Bray-1 method (Shio-Kuo, 1996) spectrophotometrically at 884 nm. Soil extractable micronutrients (Fe, Cu, Zn and Mn) were determined by the DTPA extraction method (Lindsay and Norvell, 1978) while boron was determined by hot water extraction method (Bremner, 1996). Exchangeable acidity was determined by the use of titration method (Thomas, 1996).

3.4 Preparation of the Soybean Seeds for MPN and Glasshouse Pot Experiment

Sufficient number of uniform undamaged soybean seeds were selected, rinsed in tap water then with 95 % alcohol for about 10 seconds. Thereafter, the seeds were surface sterilized by immersing in 3% sodium hypochlorite solution for 3 minutes, then rinsed six times with sterile distilled water. The seeds were pre-germinated on sterile water agar in a petri dish, incubated at room temperature until the radicles were developed.

3.4.1 Assessment of rhizobia in the Ultisol

3.4.1.1 Presumptive test for rhizobia

In order to assess the presence of the native rhizobia in the study soil, a presumptive test for rhizobia was carried out. Standard isolation of rhizobia from the soil was done by conducting a serial dilution of the test samples. Ten grams of air-dried soil were diluted in 90 ml of sterile water. One milliliter of the soil suspension was drawn and poured into a vial containing 9 ml water to make the second dilution (10^{-2}). One ml aliquot was drawn from 10^{-2} after hand shaking into another vial containing 9 ml sterile distilled water to make third dilution (10^{-3}) and finally forth dilution (10^{-4}). The three dilutions (10^{-2}, 10^{-3} and 10^{-4}) were inoculated on separate yeast mannitol agar containing Congo red and incubated for isolation of rhizobia as described by Somasegaran and Hoben (1985). Daily observations were made for the appearance of colonies typical of rhizobia.

After 5 days of incubation, colonies typical of rhizobia were milky to translucent, showing very little or no absorption of the dye. Fresh soil was not used because the suggestion to enumerate rhizobial numbers came long after the soil had already been collected and air-dried. Collecting and using new sample (in rain season) might not have reflected the condition of the first sample (collected in hot dry season) as the seasons were different (Wollum, 1994).

3.4.1.2 Authentication test for rhizobia as soybean-*nodulating Bradyrhizobium*

To confirm that the isolates included soybean-nodulating *Bradyrhizobium*, plant infection technique was used. Soybean plants grown in Leonard jars were inoculated with a soil solution from the experimental soil samples to induce nodulation. The soil inoculated plants were reared in a screen house until flowering. At flowering, the plants were carefully uprooted and the presence or absence of nodules observed.

3.4.1.3 Enumeration of indigenous rhizobia by MPN plant infection technique

Estimation of indigenous *Bradyrhizobium* population specific to soybean was determined using the Most Probable Number (MPN) plant infection technique (Woomer, 1994). Modified Leonard jar assemblies as described by Somasegaran and Hoben (1985) were used as the growth containers while the growth medium was nitrogen-free nutrient solution. The nitrogen-free nutrient solution was composed of four stock solutions namely, $CaCl_2.2H_2O$ 294.1 g/l; KH_2PO 136.1 g/l; Fe-citrate 6.7 g/l, $MgSO_4.7H_2O$ 123.3 g/l, K_2SO_4 87 g/l, $MnSO_4.H_2O$ 0.338 g/l; and H_3BO_3 0.247 g/l, $ZnSO_4.7H_2O$ 0.288 g/l, $CUSO_4.5H_2O$ 0.1 g/l, $CoSO_4.7H_2O$ 0.056 g/l and $Na_2MoO_4.2H_2O$ 0.048 g/l (Somasegaran and Hoben, 1985). The nutrient solution was prepared by diluting 0.5 ml of each stock solution per 1 liter of distilled water. There were three basic treatments; inoculated, a plus-nitrogen control with no inoculation and a non-inoculated control with no nitrogen. Three germinated seeds were planted and inoculated 2 days after emergence.

The soil from Magadu section of SUA farm was used to make dilutions for inoculating soybean seedlings. Serial dilutions were done by weighing 10 g of soil and placed into 90 ml of diluent, sterile distilled water. The soil and the diluent were mixed thoroughly by rapid hand shaking to make suspension, this was the first dilution (10^{-1}). Before the soil settled, 1 ml of the suspension (10^{-1}) was drawn and poured into a vial containing 9 ml water to make the second dilution (10^{-2}). One ml aliquot was drawn from 10^{-2} after hand shaking into another vial containing 9 ml sterile distilled water to make third dilution (10^{-3}) and finally forth dilution (10^{-4}). The three dilutions (10^{-2}, 10^{-3} and 10^{-4}) were used to inoculate soybean seedlings grown in the modified Leonard jars in 2 replications arranged in the completely randomized design (CRD).

To estimate the population of the soybean-nodulating *Bradyrhizobium* in the experimental soil (Ultisol), the most probable number-plant-infection technique was employed. The root system of soybean plants in the Leornard Jars were assessed for nodulation at flowering stage and results were recorded as positive for nodulation and negative for no nodulation. The most likely number of *Bradyrhizobium* specific to soybean was estimated by locating the experimental results on appropriate MPN table, according to the method by Woomer (1994). To establish the lower and upper confidence limits at $P = 0.05$, the confidence factor 6.61, was divided into the population estimate and multiplied by the same population estimate.

3.4.2 Experimental design and the treatments for the glasshouse pot experiment

The pot experiment was a 3 x 3 x 3 factorial with 27 treatment combinations. The experiment was laid down in a split plot experimental design replicated 2 times. The main plots were factor A with inoculation, no inoculation and inorganic N fertilizer at the rate of 200 kg N ha^{-1}. No inoculation plot was used as an absolute control and inorganic N fertiliser was used as positive control for the experiment. Sub-plots were

combinations of lime and phosphorus (factor B) as in Appendix 1. The three levels of lime (L) applied were 0, 5 and 10 ton/ha and the three levels of phosphorus (P) were 0, 50 and 100 kg/ha. The treatment combinations were L_0P_0, L_0P_{50}, L_0P_{100}, L_5P_0, L_5P_{50}, L_5P_{100}, $L_{10}P_0$, $L_{10}P_{50}$ and $L_{10}P_{100}$. Pure calcite ($CaCO_3$) was used as the agricultural liming material and triple super phosphate ($Ca(H_2PO_4)_2.H_2O$) was used as the source of phosphorus.

Four kilogram soil sample portions of the 6 mm sieve composite soil sample were mixed with desired combination levels of lime and P and placed into 5 litre capacity plastic pots with holes at the bottom loosely plugged with cotton wool to facilitate drainage. A plastic plate (saucer) was placed under each pot for the collection of any excess soil solution which was returned into the appropriate pots. The pots were appropriately labelled according to the treatment combinations.

Water was added to the pots to attain field capacity moisture status and to allow reaction for 40 days. After the equilibration period (40 days), pre-germinated soybean seeds were sown at a depth of about 15 mm. The seedlings in inoculated plots were inoculated with the TFRA approved commercial inoculant Legumefix by pipetting 1 ml of the inoculant to each seedling 2 days after emergence. Nitrogen fertilizer (urea) was applied to inorganic N applied main plot one week after seedling emergence. All standard agronomic practices were carried out accordingly.

3.4.2.1 Determination of effects of lime and phosphorus on the pH of the Ultisol

To determine the effect of lime and phosphorus on the pH of the Ultisol, soil samples were taken from the pots forty days after the equilibration with calcite and phosphorus.

The pH was determined electrometrically in 1:2.5 soil:water suspensions as described by Thomas (1996).

3.4.2.2 Determination of the effects of lime and phosphorus on nodulation, shoot total N and the amount of N fixed by the soybean-*Bradyrhizobium* symbiosis

The effects of lime and phosphorus on the nodulation by the soybean-*Bradyrhizobium* symbiosis were determined at the flowering growth stage. The growth medium containing the roots was washed through a small sized sieve to expose and avoid the nodules being washed away. The roots were then washed thoroughly with water to remove soil particles and organic debris. Nodulation parameters collected were the number of nodules per plant, nodule fresh volume per plant, nodule dry weight per plant and N fixed per plant.

The number of nodules per plant was obtained by counting the number of nodules from all the sampled plants per pot and then averaged as per plant, nodule fresh volume was determined using the volume displacement technique. In the displacement technique, the roots were washed with distilled water and blotted dry with paper towels and immersed in a 10 mL capacity plastic measuring cylinder which was filled up to 5 mL with water (Solomon *et al.*, 2012). The difference in volume of water displaced by the nodules was recorded as volume of nodules per pot, and the average was considered as nodule volume per plant. Nodule dry weight was determined by drying the nodules at 70 ˚C to constant weight, using an electronic balance.

The above ground portions of the sampled plants were used to determine N fixed by estimating the total N accumulation in the shoots. The dried plant samples were milled into powder, sieved to pass through a 0.5 mm sieve and wet digested by Kjeldahl digestion and the total N accumulation was determined by distillation-titration method as

described by Peoples *et al*. (1989). N_2 fixed was estimated as total N balance according to Unkovich *et al.* (2008) as follows:

Total N balance = (final N in soil + N in plant material) – (initial N in soil)

Where initial (before the experiment) and final (at the end of the experiment) soil N were determined by distillation-titration method whereby 0.5 g of soil sample was put in digestion tube in which a mixed catalyst and 10 ml of H_2SO_4 were added. These were digested for 1 hr and allowed to cool, after which, 50 ml of distilled water was added and then distilled with boric acid. The distillate was titrated with 0.02 N H_2SO_4 solution.

3.4.2.3 Determination of the effects of lime and phosphorus on soybean dry matter yields (DMY)

At flowering growth stage, the above ground shoot of the sampled plants was used to determine dry matter yields (DMY). Plant samples were cleaned of any debris and then packed in envelopes and dried in ventilation oven at 60-70℃ to constant weight. The dry matter yields were determined using an electronic balance.

3.4.2.4 Determination of the effects of lime and phosphorus on P and Ca contents in soybean shoots

To determine the effects of lime and phosphorus on P and Ca contents in soybean shoots, the harvested plant samples were subjected to total plant analysis to determine the concentration of phosphorus and calcium in soybean plant tissues. The samples were oven-dried, milled and sieved as explained above. Dry combustion (ashing) method was used in the analysis as described by Anderson and Ingram (1989). Phosphorus was determined by spectrophotometer and atomic absorption spectrometer analysis was used for Ca determination.

3.5 Statistical Analysis

The data collected, that is nodule numbers, nodule fresh volumes, nodule dry weights, N fixed, dry matter yields (DMYs), concentration of N, P and Ca in soybean plants were subjected to analysis of variance (ANOVA). Data on nodule count, nodule dry weights and shoot dry matter yields were not normally distributed and therefore, were transformed according to Gomez and Gomez (1984). Statistical analyses were done using both excel and GenStat 14th edition. Treatment means were separated using Duncan's New Multiple Range Test (DNMRT) at 0.05 probability level. The general statistical model used for the analysis was:

$Y_{ijk} = \mu + \beta_i + \alpha_j + \gamma_{ij} + \rho_k + (\alpha\rho)_{jk} + \varepsilon_{ijk}$

Where:

Y_{ijk} = Response

μ = Overall mean

β_i = The *i*th block (replication) effect

α_j = The *j*th main plot factor (factor A) effect

γ_{ij} = The ith random effect (error a) associated with whole plot factor

ρ_k = The kth subplot factor/treatment (factor B) effect

$(\alpha\rho)_{jk}$ = The jth interaction effect of the main plot and subplot treatments

ε_{ijk} = The random error effect (error B)

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Assessment of the Fertility Status of the Ultisol

Some of the chemical, physical and biological properties of the composite soil sample from the study site, Magadu section of Sokoine University of Agriculture (SUA) farm are as presented in Table 1. The soils of the studied area were classified by a previous study as Ultisols according to USDA Soil Taxonomy (Msanya *et al*., 2003). The textural class of the Ultisol from the study area was determined in the current study as loam (USDA, 1975). Texture is the most stable physical characteristic of the soil which influences other soil properties such as structure, aeration, moisture and nutrient retention (Landon, 1991). According to Howell (1963), soybean plants perform well on nearly all soil textural classes except on extremely sandy soils. Accordingly, the loamy texture of the soil at the study area would enhance the growth and production of soybean for its good aeration, moisture and nutrient retention capacities among others.

The pH of the soil in the study area was low (4.6), which corresponds to very strongly acidic soil reaction (pH 4.5-5.0) (Landon (1991). Very strong acidic soil are not suitable for most annual crops as the condition may limit availability of various plant nutrients such as phosphorus and the bases such as calcium and magnesium (Marschner, 1995). Nitrogen fixation by legumes such as soybean could be adversely impaired as N fixers prefer slightly acidic (pH 5.1) to slightly alkaline (pH 8.9) soil conditions indicating the need to raise the soil pH through liming.

The total N was very low (< 0.1 %) (Landon, 1991), indicating the requisite soil N conditions for the legumes to amply fix N. Very low nitrogen levels could probably be

due to low pH which reduces the breakdown of organic matter by microorganisms in the soil.

Table 1: Chemical, physical and biological properties of the soil (Ultisol) from the study area

Soil parameter	Value	Rating (Landon, 1991)
Particle size distribution		
Clay (%)	25	
Silt (%)	30	
Sand (%)	45	
Soil textural class	Loam	
pH in water, (H_2O)	4.6	Low
pH in $CaCl_2$, (0.01M $CaCl_2$)	4.2	Low
Organic carbon (%)	0.64	Very low
Organic matter (%)	1.1	Very low
Total N (%)	0.08	Very low
Available P Bray 1 (mg kg^{-1})	7.42	Low
Cation exchange capacity (cmol (+) kg^{-1})	13.51	Low
Exchangeable bases (cmol (+) kg^{-1})		
Ca^{2+}	1.6	Low
Mg^{2+}	0.81	High
K^{+}	0.4	High
Na^{+}	0.07	Low
Exchangeable acidity (cmol(+)kg^{-1})	3.2	High
ECEC	6.08	
Base saturation %	21.32	Medium
Extractable micronutrients (mg/kg)		
Fe	32.65	High
Cu	1.39	High
Zn	1.22	High
Mn	42.48	Low
B	1.65	Medium

Low N may also be attributed to the dynamic/mobility nature of nitrogen of being lost from the soil either by leaching and/or volatilization. The organic carbon was very low (< 2 % OC) according to Landon (1991). The organic carbon influences the physical, chemical and biological properties of the soil, such as soil structure, water retention, nutrient content and retention and microbiological activities in the soil. Very low OC could be due to reduced activity of soil microorganisms, the main decomposers of organic matter, mainly caused by the unfavourable low pH conditions. It could also be due to low organic matter input through crop residues and manure to the soil, hence less organic matter decomposed.

The site had low available P (< 15 mg kg^{-1} soil) which necessitated application of P fertilizer to enhance soybean N_2 fixation. The low available P could be due to the very acidic soil reaction which enhanced the chemical reaction of phosphate ions with iron and/or aluminium to form compounds which are not readily available to plants. The nature of the parent material could also had low P reserves (Landon, 1991). Application of agricultural lime materials is considered as necessary to raise the pH to favourable levels to increase P availability and N fixation.

Exchangeable Ca and Na were low (< 4 cmol (+) kg^{-1} soil and < 1 cmol (+) kg^{-1} soil), respectively. Landon (1991) reported that optimal growth of most crops including soybean is attained in soils with exchangeable calcium between 0.2 and 4 cmol (+) kg^{-1}. Crops usually respond to calcium fertilisation when the exchangeable calcium is less than 0.2 cmol (+) kg^{-1} soil. However, application of Ca fertiliser and/or calcium-containing materials when Ca levels are higher than 0.2 cmol (+) kg^{-1} soil, can lead to improved yield if it is the pH and not the plant nutrient Ca which is the limiting factor. Sodium is not an essential plant nutrient thus it is not usually detrimental to plant nutrition when present in

small amount (Landon, 1991). However, when present in the soil in significant quantities (>1 cmol (+) kg^{-1} soil), it can have an adverse effect on the physical conditions of the soil such as dispersion.

The exchangeable Mg and K were high (> 0.5 cmol (+) kg^{-1} soil and ≥ 0.4 cmol (+) kg^{-1} soil), respectively (Landon, 1991). Sanchez (1976) reported that 0.2 to 0.64 cmol (+) kg^{-1} levels of exchangeable magnesium are sufficient for most crops. Therefore, the level of exchangeable magnesium in the Ultisol is sufficient for soybean production. The exchangeable potassium is also adequate for soybean growth and production as crop response to K fertilization occurs when the exchangeable potassium is below 0.2 cmol (+) kg^{-1} soil (Landon, 1991). The relatively higher values of exchangeable magnesium and potassium in the Ultisol could be attributed to their high contents in the parent materials from which the soil was formed and developed. The high levels of exchangeable magnesium and potassium could also indicate low leaching and washing away by run-off water.

Cation exchange capacity (CEC) of the soils under study was low (5-15 cmol (+) kg^{-1} soil). Low CEC, which usually is a function of the type of clay and organic matter levels is unsatisfactory (Landon, 1991) for most agricultural crops such as soybean, due to low nutrient reserves. Low nutrient reserves are attributed to the fact that the Ultisol is dominated by 1:1 layer silicate clays and the hydrous oxides of Al and Fe, which have very low capacity to hold basic cations like Ca, hence the prerequisite for liming the soil in this study so as to raise the pH. This is an indication that the Ultisol is highly weathered.

Extractable micronutrients, Fe, Cu, and Zn were on the high side (> 4.5 mg kg^{-1} soil, > 0.75 mg kg^{-1} soil and > 1 mg kg^{-1} soil), respectively, while Mn was low (< 65) (Landon, 1991). According to Landon (1991), B falls within medium or sufficient levels (1.5-3 mg kg^{-1} soil) for most crops. The high levels of Fe, Cu, and Zn could be due to low pH and the nature of the parent materials. Landon (1991) reported that Mn is usually high or toxic in soils with pH less than 5.5, and that its low availability such as in the studied Ultisol could be due high level of iron. The low Mn level (10-64 mg kg^{-1}) could be suitable for N fixation since Schulte and Kelling (1999) recommends application of manganese if the soil test is < 10 mg kg^{-1}, the threshold at which most crops responds to manganese treatment. Coskan and Dogan (2011) reported that at high levels (> 65 mg kg^{-1}), manganese may become toxic to the rhizobia, hence low N fixation.

The percent base saturation (PBS) of the Ultisol was medium (20-60 %) hence dystric (PBS < 50 %) or less fertile according to Landon (1991). The medium PBS of the soil could be attributed to leaching of bases such as Ca down the profiles of which probably is caused by high rainfall intensity and low soil pH, hence poor base retention by the soil colloids.

Overall, based on the soil analytical data (Table 1), the Ultisol of the study area could be rated as of low fertility status for soybean production. The soil fertility limitations being the soil pH, total N and available P among others. Enhanced and sustainable soybean production at the Magadu section of SUA farm could be attained if the following are taken on board, namely liming the soil, application of phosphorus and/or N fertilizers and inoculation of soybean seeds before planting. Liming the soil to increase the soil pH to at least 5.5 could enhance cation exchange capacity of the soil and increase mineralization of soil N through decomposition of organic matter as the microbial activities increase.

Application of phosphorus fertilizer could also enhance and sustain soybean production through BNF (Mahamood, 2008). Inoculation of soybean seeds would enhance biological nitrogen fixation, hence reducing the use of inorganic N which are expensive for poor resource farmers and may have adverse environmental conditions such as pollution of water bodies like rivers.

4.2 Indigenous Rhizobia Numbers in the Test Soils

Presumptive, Congo-red tests for the presence of rhizobia in the Ultisol enabled the observation of colonies typical of rhizobia, with milky to translucent appearance, showing very little or no absorption of the dye. Authentication test confirmed these isolates as soybean rhizobia, as such nodulation could be observed on soybean root system grown in the pots without inoculating the soybean seeds. The most probable number of rhizobia specific to the soybean bean was calculated from the MPN results as shown in Table 2.

Table 2: MPN counts of rhizobia in the Ultisol

	Replication		
Dilution level	**1**	**2**	**Total**
10^{-2}	-	+	1
10^{-3}	-	-	0
10^{-4}	+	-	1

The experimental results obtained 12 cells g^{-1} soil. Dividing and multiplying the population estimate of 12 cells g^{-1} soil by the confidence factor 6.61, estimated the number of rhizobia capable of nodulating soybeans in the Ultisol in the range of 2 to 79 cells per gram of soil, expressed as 12(2 to 79, $P = 0.05$). The small number of estimated rhizobial cells could be attributed to the effect of drying the soil sample, which reduces the number of microbes (Wollum, 1994). However, Morel *et al.* (2012) reported that the

soil might not contain any rhizobia if it has never been under soybean cultivation or other legumes, which is their source of energy, and this is the case with Magadu section of the SUA farm hence the estimated rhizobial population might reflect the actual most probable number of rhizobial cells despite the fact that air-dried soil was used to estimate the population. Mishra *et al*. (2013) reported that soybean will likely respond to inoculation if the number of *Bradyrhizobia* in the soil is < 50 cells g^{-1} soil, hence the need to inoculate the soil of Magadu section of SUA farm to avoid the use of mineral N for environmental and economic sustainability by capitalising on the biological nitrogen fixation by soybean.

4.3 Effects of Lime ($CaCO_3$) and Phosphorus ($Ca(H_2PO_4)_2.H_2O$) on the pH of Ultisol

The effect of the lime and phosphorus on the pH of the soil after forty days of equilibration is presented in Table 3. The results show that lime had significant ($P < 0.05$) effect on the soil pH. The pH increased as the rate of liming was increased from 0 to 5 to 10 tons/ha. Similar results on pH increase due to lime application have been reported elsewhere (Ayodele and Shittu, 2014; Bekere, 2012). The rise in pH could enhance *Rhizobium* activities in otherwise low pH soils, hence increase in nodulation and N fixed by soybean plants due to increase in BNF. Soil pH remained unchanged with the application of 50 kg P/ha. But as the rate of P application increased from 50 to 100 kg/ha, the soil pH decreased by 0.1-0.2 units, although the change was not statistically significant at 0.05 significant ($P < 0.05$).

Table 3: Effect of the lime and phosphorus on the pH of Ultisol

	Phosphorus (kg) applied		
Lime (Tons)	**0**	**50**	**100**
0	4.6a	4.6a	4.5a
5	6.0b	5.9b	5.7b
10	6.8c	6.8c	6.7c
CV % = 1.7			

Values in the same column followed by different letters are significantly different according to Duncan's New Multiple Range Test at $P = 0.05$.

4.4 Effects of Lime and Phosphorus on the Extent of Nodulation and Amount of N2 Fixed by the Soybean-*Bradyrhizobia* Symbiosis

4.4.1 Effects of lime and phosphorus on nodule numbers

The effects of lime and phosphorus on nodule numbers tested under inoculated, non-inoculated and inorganic N applied conditions are as presented in Fig. 1 and Appendix 2. The raw data for nodule numbers in Appendix 2 was transformed to $\log_{10}$ to normalize it. The results indicate that there was an interaction effect of lime and phosphorus on nodule numbers in inoculated and non-inoculated. Increasing P applied from 0 to 50 kg/ha while holding lime at 0 tons/ha resulted in a slight increase in nodule numbers in inoculated main plot. As P applied was further increased to 100 kg/ha, there was a sharp decrease in nodule numbers. The decrease in nodule numbers as P increased could be due to increase in hydrogen ions released by the TSP which may have lowered the soil pH to 4.5, of the Ultisol, hindering root infection by the inoculant. Several researchers have reported significant increase in the nodule numbers on the application of phosphorus fertilizer (Mahamood, 2008; Abdul-Aziz, 2013), but especially so when low pH was not part of the problem. Mahamood (2008) reported that application of P fertilizer at recommended rate of 30-80 kg P ha^{-1} (depending on variety) to P deficient soils increased the nodule numbers.

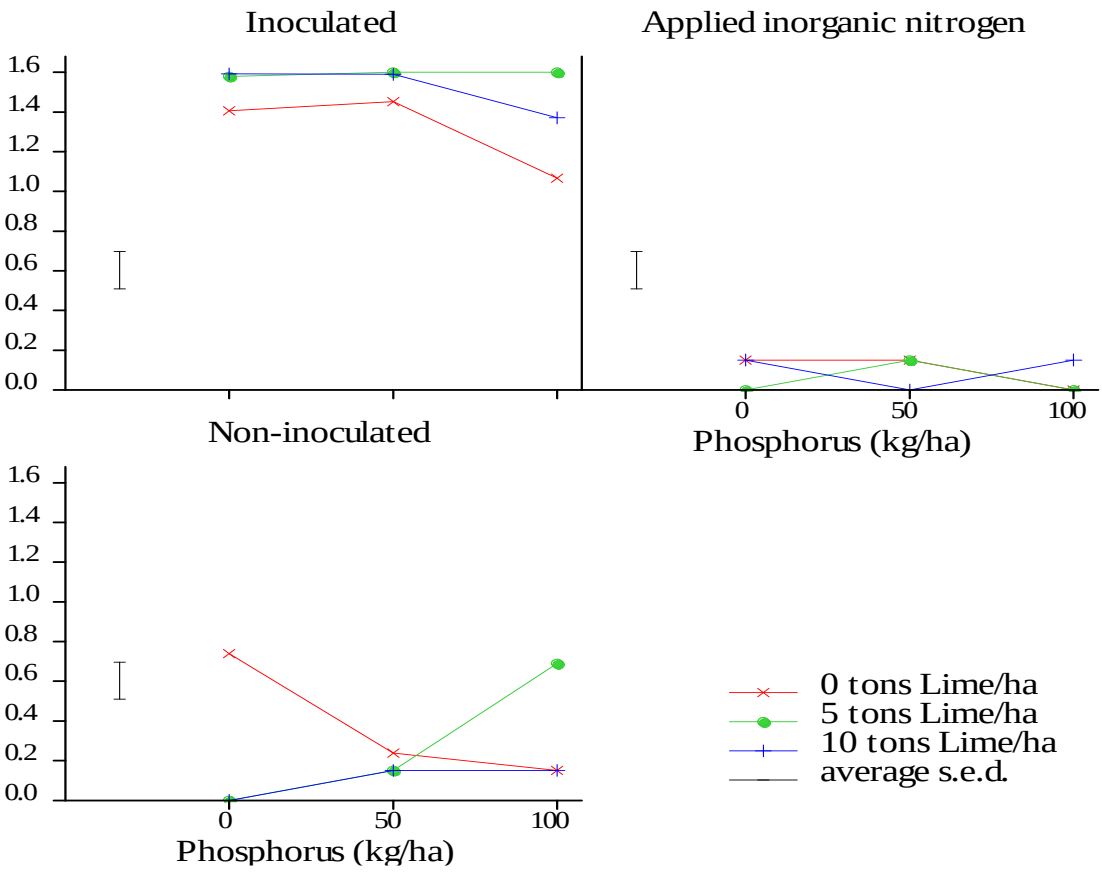

Figure 1: Interaction of lime and phosphorus on nodule numbers

The same trend was observed when liming was held at 10 tons/ha while increasing P applied from 0 to 50, and later to 100 kg/ha. This could be attributed to the fact that at high rate of liming a chemical reaction between calcium and phosphate ions does occur, leading to formation of compounds that would render both calcium and phosphorus not readily available to the plants (Landon, 1991). The results showed a slight increase in nodule numbers when lime was held constant at 5 tons/ha, and P applied was increased from 0 to 50 and later to 100 kg/ha in inoculated and non-inoculated plots. Nodule numbers decreased sharply in non-inoculated plot as P applied was increased from 0 to 50 tons/ha while holding lime at 0 tons/ha. Further increase in P applied to 100 kg/ha, still holding lime at 0 tons/ha, continued to decrease the nodule numbers but slightly.

The decrease could be due to a low pH and rhizobial count which retarded root infection by the bradyrhizobia and nodule development (Chianu *el al.*, 2011; Singh and Shivakumar, 2010). The interaction in inorganic N applied plot on nodule numbers was inconsistently small, probably due to high plant available N content in the soil solution which also retarded root infection by the bradyrhizobia and nodule development. Overall, interactions indicated that best nodule numbers were obtained when the soil was limed at 5 tons/ha and P applied to 100 kg/ha in inoculated plot compared to non-inoculated plots.

4.4.2 Effects of lime and phosphorus on the nodule fresh volume

The results for the effect of lime and phosphorus on the nodule fresh volume tested under inoculated, non-inoculated and inorganic N applied conditions are shown in Fig. 2 and Appendix 2. The nodule fresh volume increased with increasing amount of P, holding lime at 0 tons/ha, within the inoculated and non-inoculated plots. Increasing P applied from 0 to 50 kg/ha, while holding lime at 0 tons/ha did not increase or decrease the nodule fresh volume in the inoculated plot. However, as 100 kg P was applied still holding lime at 0 tons/ha, a sharp decrease in nodule fresh volume was observed. The decrease could probably be due to low pH, since lime which could raise the pH was not applied. In non-inoculated under similar treatments the nodule fresh volume decreased sharply as P was increased from 0 to 50 and later to 100 kg/ha**.** Increasing liming rate from 0 to 5 tons/ha and holding it, while increasing phosphorus application from 0 to 50 and later to100 kg/ha resulted in a slight and later significant increase in nodule fresh volume in inoculated plot.

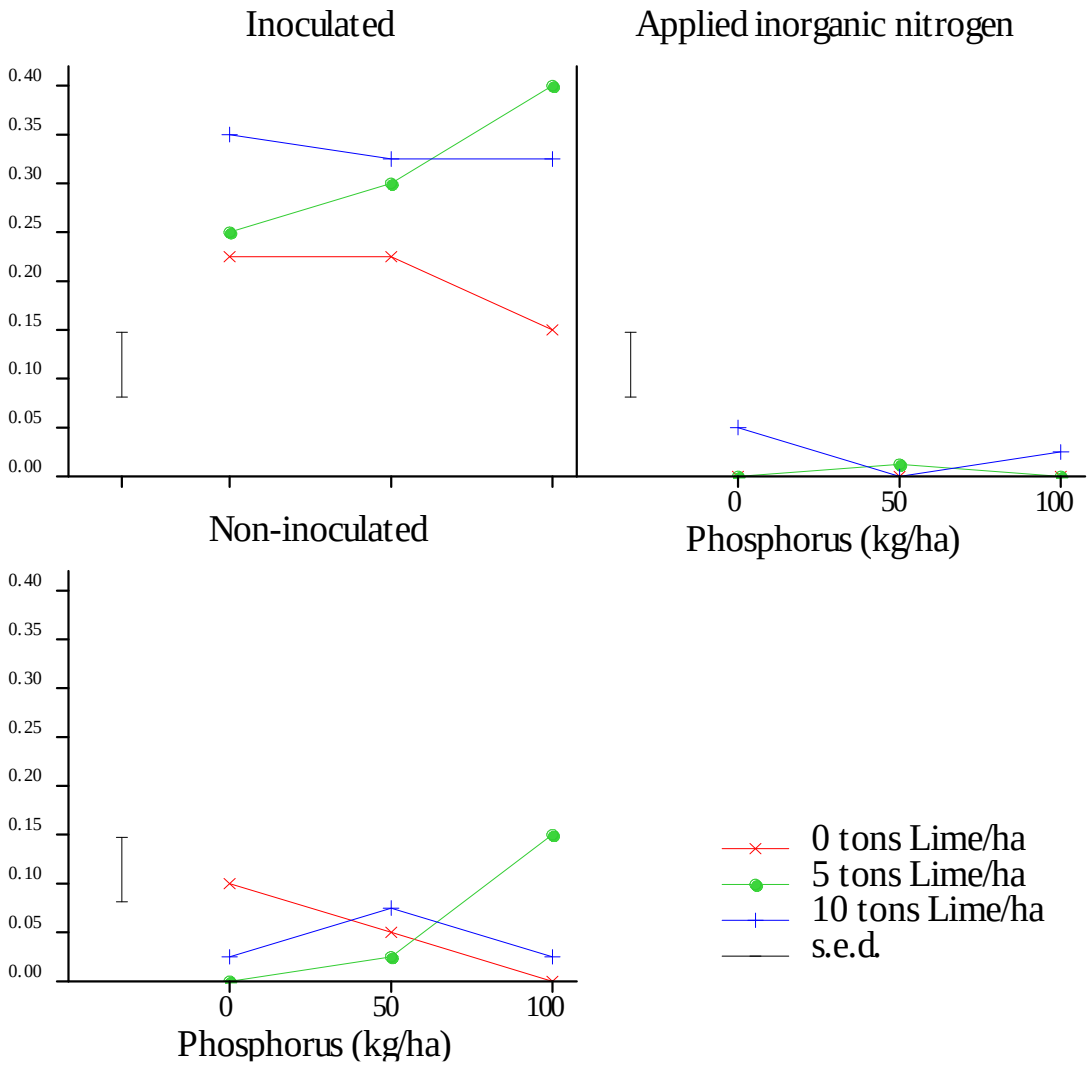

Figure 2: Interaction of lime and phosphorus on nodule fresh volume

Similar trend was observed in non-inoculated plot. Similar observations have been reported by Bekere and Hailemariam (2012). Results showed further that holding lime at 10 tons/ha as P applied was increased from 0 to 50 and later to 100 kg/ha decreased the nodule fresh volume slightly, and later it remained constant in inoculated plot, while in non-inoculated plot it initially increased and decreased at the application of highest rate of P.

In terms of nodule fresh volume overall, the interactions showed that the best results were obtained when the soil was limed at 5 tons/ha and P applied at 100 kg/ha in the inoculated plot compared to non-inoculated and applied inorganic N plots. Low nodule fresh volume in non-inoculated plants could be attributed to low rhizobia population in the Ultisol. Solomon *et al*. (2012) observed similar results on the effect of inoculation on the nodulation parameters such as nodule fresh volume.

4.4.3 Effects of lime and phosphorus on the nodule dry weights

Figure 3 and Appendix 2 show the results of the effects of lime and phosphorus on the nodule dry weight per plant tested under inoculated, non-inoculated and inorganic N applied conditions. A slight increase in nodule dry weight could be observed in inoculated plot when lime was held constant at 0 tons/ha while increasing phosphorus applied from 0 to 50 kg/ha.

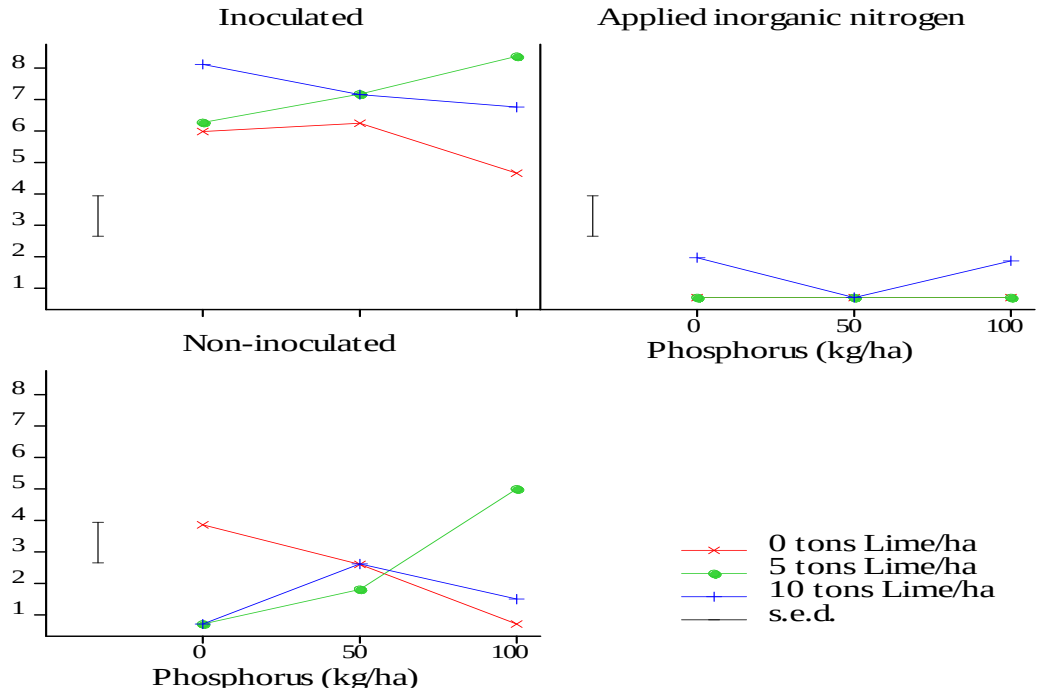

Figure 3: Interaction of lime and phosphorus on nodule dry weights

Further increase of P applied to 100 kg/ha resulted in sharp decrease in nodule dry weight due to low pH contributed by hydrogen ions released by TSP. In non-inoculated plot only a sharp decrease was observed when P applied was increased from 0 to 50 and later to 100 kg/ha, while holding lime at 0 tons/ha. Holding the rate of liming at 5 tons/ha and increasing P applied from 50 and later to 100 kg/ha, showed an increase in nodule dry weight in inoculated plot. Similar trend was observed in non-inoculated main plot. Similar findings were reported by Mahamood (2008), who observed that application of P fertilizer at recommended rate of 30-80 kg P ha^{-1} (depending on variety) to P deficient soils increased nodule dry weight in soybean. The results further show decrease in nodule dry weight in inoculated plot as the rate of liming held constant was increased to 10 tons/ha, while varying application rates of P. In non-inoculated plot, nodule dry weights increased as P applied was increased from 0 to 50 kg/ha, holding lime at 10 tons/ha. Further increase in P applied to 100 kg/ha resulted in sharp decrease of nodule dry weights.

The general observation was that nodule dry weights were significantly ($P < 0.05$) different between the inoculated and non-inoculated plants. Overall, the interactions showed that the best nodule dry weights were obtained when the soil was limed at 5 tons/ha and P applied at 100 kg/ha in inoculated plot compared to non-inoculated plants. Bhuiyan *et al*. (2008) reported similar results on the positive effect of inoculation on nodule dry weight compared to non-inoculated plants.

4.4.4 Effects of lime and phosphorus on the amount of N2 fixed

Figure 4 and Appendix 2 show the results for the effects of lime and phosphorus on the amounts of N_2 fixed by the soybean-rhizobia symbiosis tested under inoculated and non-inoculated conditions. The results indicate that increasing phosphorus application from 0

to 50 and later to 100 kg/ha, while holding liming rate at 0 tons/ha slightly increased and later decreased N_2 fixed in inoculated plot.

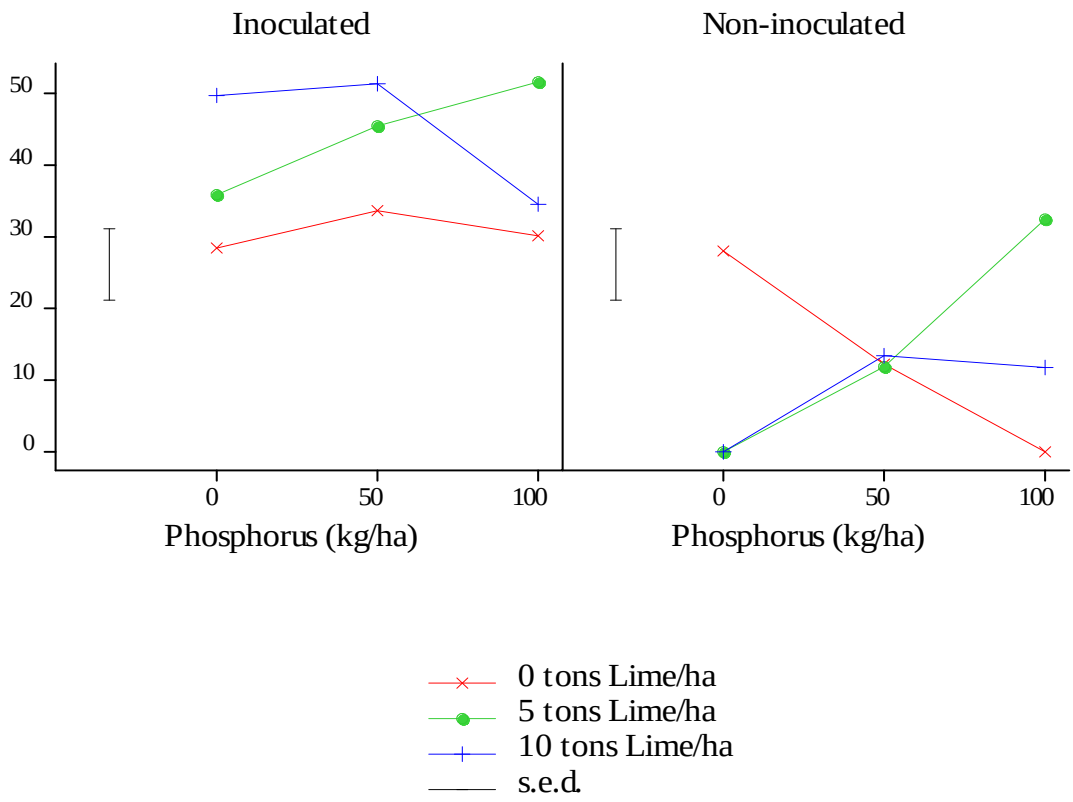

Figure 4: Interaction of lime and phosphorus on amount of N_2 fixed

In non-inoculated plot a sharp decrease was observed under similar conditions. The decrease could be attributed to low rhizobia population, hence no root infection and low N_2 fixed. Increasing the rate of liming held constant from 0 to 5 tons/ha, and varying P applied from 0 to 50 and later to 100 kg/ha, resulted in an increase in N_2 fixed in both inoculated and non-inoculated main plots. A similar trend was observed in both inoculated and non-inoculated plots when lime was held constant at 10 tons/ha and P applied was increased from 0 to 50 kg/ha. However, results showed reverse trend when P was increased to 100 kg/ha.

The general observation was that the inoculated plot had higher amounts of fixed N_2 compared to non-inoculated plot. In similar studies elsewhere, Mahamood (2008) also observed increase on N_2 fixed in inoculated plants with application of lime, P and the presence of adequate numbers of *Bradyrhizobium japonicum*. However, Giller (2001) reported non-significant results and concluded that the ability to form nodules is not always enough to obtain an effective nitrogen fixation symbiosis. Overall, interactions indicated that best N_2 fixed were obtained when the soil was limed at 5 tons/ha and P applied at 100 kg/ha in inoculated plot compared to non-inoculated plots.

4.5 Effects of Lime and Phosphorus on the Soybean Shoot Dry Matter Yields

The results for the effects of lime and phosphorus on soybean shoot dry matter yields tested under inoculated, non-inoculated and inorganic N applied conditions are presented in Fig. 5 and Appendix 2. The results show that there was an interaction effect between lime and phosphorus in inoculated, non-inoculated and inorganic N applied plots. Increasing P applied from 0 to 50 kg/ha, holding lime at 0 tons/ha, increased DMYs in inoculated plot. Further increase in P applied to 100 kg/ha, resulted in reduction of DMYs. Under similar conditions, a gradual decrease in DMYs was observed in both non-inoculated and inorganic N applied plots. Non-significant results of soybean to P rates above 20 kg P ha^{-1} have been reported by Mahamood (2008). This researcher suggested that the non-significant response might be due to limitation of other nutrients or attributed to P occlusion. Similar results were observed by Singh and Saxena (1972) who reported that P had little or negative effect on dried matter yields but it significantly increased the total P content in the shoot (section 4.6.2 on P content in shoot).

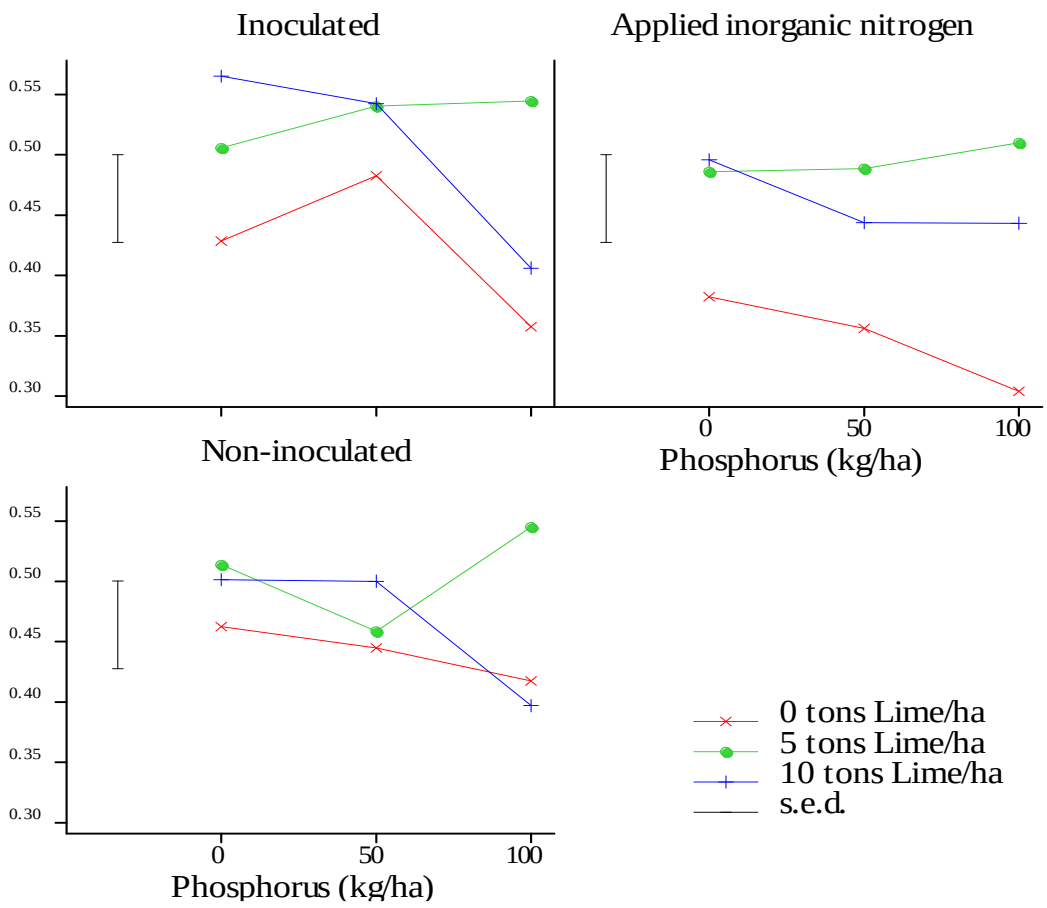

Figure 5: Interaction of lime and phosphorus on shoot dry matter yields

Holding lime at 5 tons/ha, while increasing P from 0 to 50 and later to 100 kg/ha yielded gradual increase in DMYs in inoculated and inorganic applied N plots. Under similar conditions, a sharp decrease in DMYs was observed in non-inoculated plot, where further increase of P to 100 kg/ha sharply increased DMYs. Dry matter yields decreased gradually in inoculated, non-inoculated and inorganic applied N plots as P applied was increased from 0 to 50 and later to 100 kg/ha, while holding lime constant at 10 tons/ha. The decrease could be due to chemical reaction between Ca and P to form insoluble compounds not readily available to plants.

Overall, interactions indicated that best shoot dry matter weights were obtained when the soil was limed at 5 tons/ha and P applied to 100 kg/ha in inoculated plot compared to non-inoculated plots

4.6 Effects of Lime and Phosphorus on N, P and Ca Contents in Soybean Shoots

4.6.1 N content

The results presented in Table 4 and Appendix 2 show the effects of lime and phosphorus on N content in soybean shoots tested under inoculated, non-inoculated and inorganic N applied conditions. Increasing the rate of liming from 0 to 10 tons/ha decreased significantly ($P < 0.05$) N content in inorganic N applied main plot. This could be due to nutrient imbalance as phosphorus was not applied.

Table 4: Effect of lime and phosphorus on N content in soybean shoots

		Shoot total N (%) in the main plots	
Treatments	**Inoculated**	**Non-inoculated**	**Inorganic N**
L_0P_0	1.54ab	1.207a	4.06fgh
L_0P_{50}	1.61ab	1.575ab	4.725h
L_0P_{100}	2.118abcd	1.26a	4.323gh
L_5P_0	1.593ab	1.137a	3.132def
L_5P_{50}	1.768abc	1.347a	3.36efg
L_5P_{100}	1.978abc	1.277a	3.377efg
$L_{10}P_0$	1.855abc	1.365a	2.765cde
$L_{10}P_{50}$	1.995abc	1.435ab	2.782cde
$L_{10}P_{100}$	2.135abcd	1.697abc	2.538bcde
F Pr. (Lime) = 0.015			
F Pr. (P) = 0.246			
F Pr. (L x P) = 0.951			
CV % = 6.2			

Values in the same column followed by different letters are significantly different according to Duncan's New Multiple Range Test at $P = 0.05$.

The results further showed that increasing the rate of liming from 0 to 10 tons/ha increased N accumulation in inoculated plot but the increase was not significant, probably could be due to low N fixation. Similarly, in non-inoculated plot, N content increased non-significantly as the rate of liming was increased from 0 to 10 tons/ha.

In the inoculated, non-inoculated and inorganic N applied plots, N content increased non-significantly as the rate of phosphorus application was increased from 0 to 100 kg/ha, holding lime at 0 tons/ha. In similar studies Bekere *et al*. (2012) reported non-significant difference between inoculated and non-inoculated plots on the N content when phosphorus application rate was increased up to 30 kg/ha in Ultisol. However, Abdu-Aziz (2013) reported a significant increase in N content in plants of inoculated than non-inoculated plants with increase in the rate of phosphorus application.

4.6.2 P content

The results on the effects of lime and phosphorus on P content in soybean shoots tested under inoculated, non-inoculated and inorganic N applied conditions are as shown in Fig. 6 and Appendix 2. The results indicate that increasing P application from 0 to 50 kg/ha while holding lime at 0 tons/ha, increased P content in all the main plots, with inoculated and non-inoculated plots having gradual increases and inorganic N applied having a sharp increase. As P applied was increased further to 100 kg/ha, a sharp increase was observed in inoculated plot while in non-inoculated plot there was a gradual decrease. In inorganic N applied plot, a gradual increase in P contents was observed when P was increased to 100 kg/ha.

In the inoculated plot P content gradually increased as phosphorus application was increased from 0 to 50 kg/ha and later to 100 kg/ha, holding lime at 5 tons/ha applied.

Similarly, in non-inoculated and inorganic N applied plots, P content gradually increased as amount of P applied was increased from 0 to 50. Further increase of P to 100 kg/ha, showed a reverse trend in P content whereby in non-inoculated plot it sharply decreased, while in inorganic N applied plot it decreased gradually.

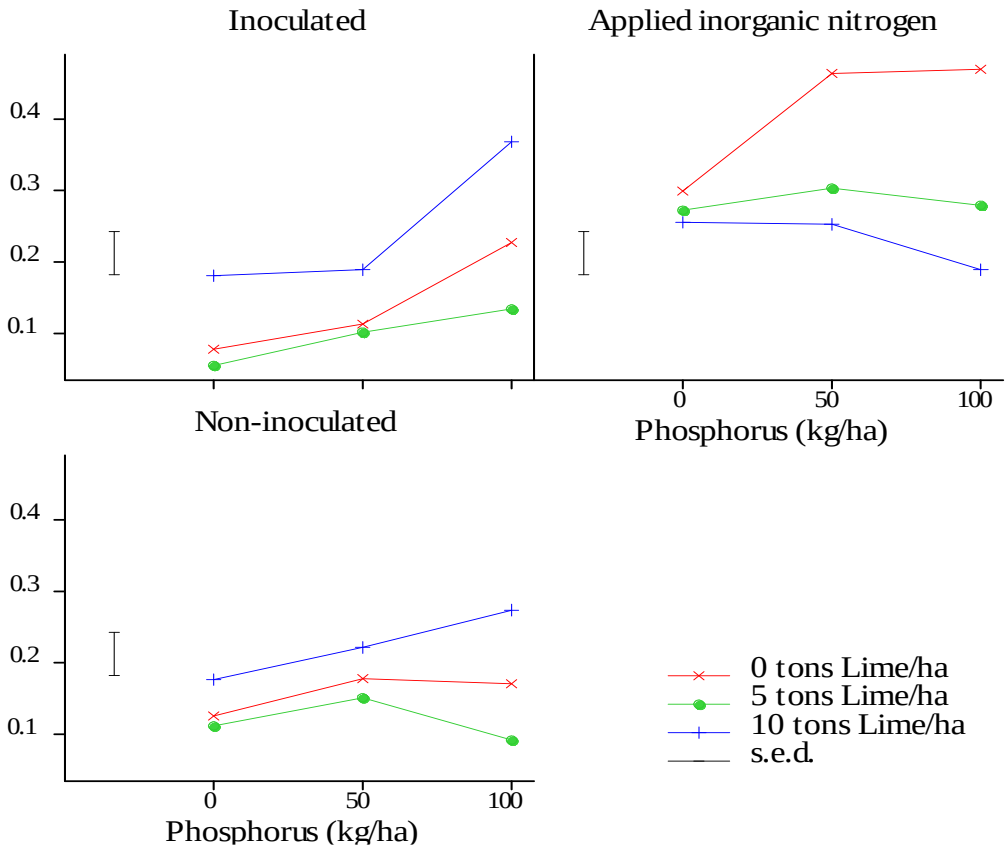

Figure 6: Interaction of lime and phosphorus on P contents in soybean shoots

The results further showed that holding lime constant at 10 tons/ha, while increasing P applied from 0 to 50 kg/ha gradually increased P content in inoculated plot. As P was increased to 100 kg/ha later, a sharp increase was observed. Under similar conditions, in non-inoculated plot the P contents gradually increased with increase in P applied from 0 to 50 and later 100 kg/ha. A reverse trend was observed in inorganic N applied plot where there was a gradual and sharp decreases when P was increased to 50 kg/ha, and later to 100 kg, respectively.

Under all conditions, the general observation was high P content in inorganic N applied plot, followed by non-inoculated then inoculated plot. Similar results have been reported by Adams and Odom (1982) who observed that accumulation of P in soybean shoots was increased by the increase in application of phosphate fertilizer from 0 to 60 kg/ha. Singh and Saxena (1972) also reported significant response of P content by soybean with application of phosphorus fertilizer.

In comparison the non-inoculated plants accumulated higher P content than the inoculated plants. This observation also affirms the findings of Basu *et al*. (2008) who reported that non-inoculated control accumulated the highest P than the inoculated treatments. This could be due to the fact that P was also used for energy storage and transfer from the leaves to the roots for the symbionts in the inoculated plot (Weisany *et al*., 2013). Overall, interactions indicated that best P contents were obtained when the soil was limed at 0 tons/ha and P applied to 100 kg/ha in inorganic N applied plot compared to inoculated plot.

4.6.3 Ca content

The effects of lime and phosphorus on Ca content in soybean shoots tested under inoculated, non-inoculated and inorganic N applied conditions is as presented in Fig. 7 and Appendix 2. Calcium content in soybean shoots increased with increasing the rate of P applied from 0 to 50 and later to 100 kg/ha while holding lime at 0 tons/ha, in inoculated main plot. Similar results have been reported by Adams and Odom (1982), who observed that the accumulation of Ca in soybean shoots increased by the application of phosphate fertilizer at the rate of 30 kg P ha^{-1}, more especially where there were adequate rhizobia numbers. Under similar conditions, in non-inoculated plot there was a sharp increase in Ca contents when 50 kg P/ha was applied and a reverse trend when the

highest rate of P was applied. In inorganic applied nitrogen the increase was very gradual at 50 kg P/ha and decreased in the same way when P was applied at the highest rate of 100 kg/ha.

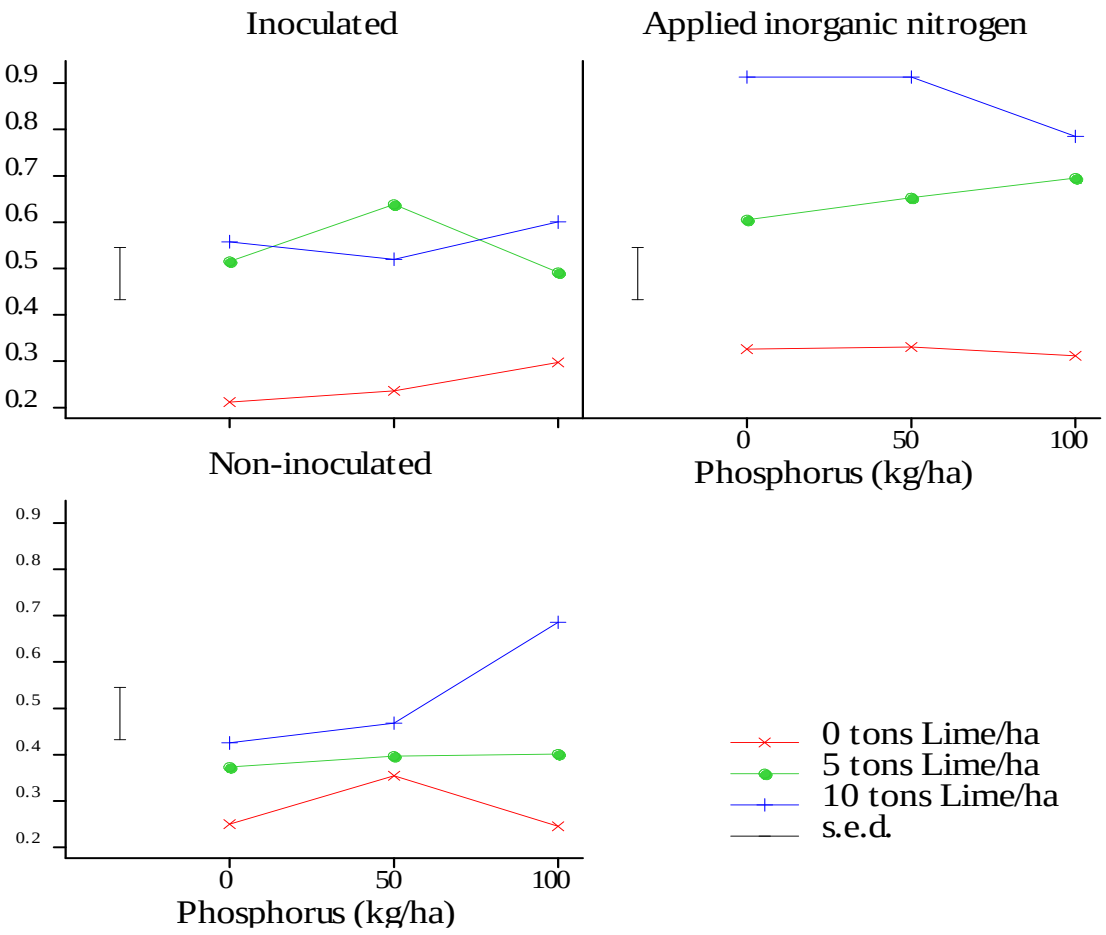

Figure 7: Interaction of lime and phosphorus on Ca contents in soybean shoots

Holding lime at 5 tons/ha, while increasing P applied from 0 to 50 kg/ha, showed a sharp increase in Ca contents in inoculated plot. Further increase in P applied to 100 kg/ha resulted in sharp decrease in Ca contents. Under similar conditions, gradual increase was observed in non-inoculated and inorganic N applied plots. Holding lime at 10 tons/ha and increasing P applied from 0 to 50 kg decreased Ca contents in inoculated plot.

Further increase in P increased the Ca contents. In non-inoculated plot, a gradual increase was observed when P applied was increased from 0 to 50 kg/ha. Further increase in P to 100 kg/ha sharply increased the Ca contents. In nitrogen plot, Ca contents was initially constant when P was increased from 0 to 50 kg/ha, while holding lime at 10 tons/ha. However, a significant decrease was observed with increase in P applied to 100 kg/ha.

Under all conditions, the general trend was that Ca contents of soybean shoots, was significantly different among the main plots. The inorganic N applied plants had higher Ca content than non-inoculated followed by inoculated plants, more specially at the highest rate of lime, 10 tons/ha and P applied at 100 kg/ha. Ayodele and Shittu (2014) reported similar results in which lime significantly increased Ca content in soybean plants.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This study shows that the Ultisol has low population of the native *Bradyrhizobium*, therefore, sustainable soybean production at the Magadu section of SUA farm require inoculation of the seeds with *Bradyrhizobium jopanicum* to capitalize on BNF.

The soil pH of the experimental soil (Ultisol) increases with increase in the rate of liming. Liming at the rate of 10 tons per hectare can increase the pH of the soil from 4.6 to about 6.8, in which microbial activity for BNF could be enhanced.

No advantage is obtained on BNF by applying P on this soil in the absence of limimg. Increasing liming rate to 5 tons/ha and phosphorus to the rate of 100 kg/ha increases nodulation parameters, N_2 fixed, shoot DMYs and nutrients contents in soybean plants.

Therefore, lime and phosphorus application at the rate of 5 tons/ha and 100 kg/ha respectively, combined with inoculation are optimum for BNF on the study soil at Magadu section of SUA farm that would ultimately increase soybean production.

5.2 Recommendations

i. Soybean planted on the Ultisol at Magadu should be inoculated with *Bradyrhizobium jopanicum*.
ii. The soil should be limed and P fertilised at application rate of 5 tons/ha and 100 kg P/ha, respectively.
iii. Field experiment should be conducted to validate the rate of lime and phosphorus established in this study under field conditions.

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APPENDICES

Appendix 1: Lay out of the glasshouse pot experiment

Replicate 1	MAIN PLOTS								
	INOCULATED			UNINOCULATED			NITROGEN		
	L_0P_0	L_0P_{50}	L_0P_{100}	L_0P_0	L_0P_{50}	L_0P_{100}	L_0P_0	L_0P_{50}	L_0P_{100}
SUB-PLOT	L_5P_0	L_5P_{50}	L_5P_{100}	L_5P_0	L_5P_{50}	L_5P_{100}	L_5P_0	L_5P_{50}	L_5P_{100}
	$L_{10}P_0$	$L_{10}P_{50}$	$L_{10}P_{100}$	$L_{10}P_0$	$L_{10}P_{50}$	$L_{10}P_{100}$	$L_{10}P_0$	$L_{10}P_{50}$	$L_{10}P_{100}$

Replicate 2	MAIN PLOT								
	INOCULATED			UNINOCULATED			NITROGEN		
	L_0P_0	L_0P_{50}	L_0P_{100}	L_0P_0	L_0P_{50}	L_0P_{100}	L_0P_0	L_0P_{50}	L_0P_{100}
SUB-PLOT	L_5P_0	L_5P_{50}	L_5P_{100}	L_5P_0	L_5P_{50}	L_5P_{100}	L_5P_0	L_5P_{50}	L_5P_{100}
	$L_{10}P_0$	$L_{10}P_{50}$	$L_{10}P_{100}$	$L_{10}P_0$	$L_{10}P_{50}$	$L_{10}P_{100}$	$L_{10}P_0$	$L_{10}P_{50}$	$L_{10}P_{100}$

L = Lime, P = Phosphorus.

L_0, L_5 and L_{10} = Rates of lime applied in tons ha^{-1}

P_0, P_{50} and P_{100} = Rates of phosphorus applied in kg ha^{-1}

Appendix 2: Response of soybean to lime and phosphorus in an Ultisol

Rep	Main plot	Lime (Tons)	P (kg)	pH at planting	Nodules/ plant	Fresh nodules vol/plant	Nodule DW/plant	N_2 fixed (mg N/plant)	DMY (g)	%N in shoot	% P	% Ca
1	Inoculated	0	0	4.53	26	0.3	47.25	37.64	2.46	1.4	0.052431	0.079545
1	Inoculated	0	50	4.35	31	0.25	40	30.74	1.97	1.54	0.117266	0.126894
1	Inoculated	0	100	4.3	7	0.1	12.75	25.47	0.98	2.415	0.339958	0.164773
1	Inoculated	5	0	6.4	35	0.2	29	35.07	2.11	1.61	0.077801	0.325758
1	Inoculated	5	50	6.16	43	0.3	49	43.29	2.46	1.715	0.077801	0.25
1	Inoculated	5	100	6.53	65	0.5	92.75	57.52	3.15	1.68	0.103171	0.278409
1	Inoculated	10	0	6.84	33	0.35	66.25	51.55	2.73	1.925	0.184919	0.354167
1	Inoculated	10	50	7.01	35	0.2	30.5	45.02	2.33	1.855	0.145455	0.306818
1	Inoculated	10	100	6.48	28	0.4	56.25	37.24	1.66	2.135	0.30895	0.363636
1	Uninoculated	0	0	4.71	4	0.1	16.75	33.44	1.76	1.4	0.103171	0.183712
1	Uninoculated	0	50	4.43	2	0.1	19.75	24.42	2.14	1.155	0.111628	0.268939
1	Uninoculated	0	100	5.13	0	0	0	0	2.21	0.945	0.089077	0.107955
1	Uninoculated	5	0	6.68	0	0	0	0	1.95	1.085	0.111628	0.25
1	Uninoculated	5	50	6.12	5	0.05	8	23.72	1.41	1.505	0.117266	0.268939
1	Uninoculated	5	100	5.64	11	0.2	43.25	32.17	1.93	1.61	0.103171	0.306818
1	Uninoculated	10	0	6.76	0	0	0	0	2.66	1.26	0.193376	0.287879
1	Uninoculated	10	50	6.94	0	0	0	0	1.9	1.82	0.162368	0.363636
1	Uninoculated	10	100	6.46	3	0	0	20.68	1.73	1.645	0.25821	0.429924
1	Nitrogen	0	0	4.16	2	0	0	21.07	1.88	3.57	0.241297	0.145833
1	Nitrogen	0	50	5.01	1	0	0	0	1.28	5.355	0.449894	0.193182
1	Nitrogen	0	100	4.13	0	0	0	0	1.07	4.41	0.494996	0.193182
1	Nitrogen	5	0	5.8	0	0	0	0	2.35	2.765	0.201832	0.410985
1	Nitrogen	5	50	6.12	1	0.025	0	0	2.24	2.87	0.235659	0.373106
1	Nitrogen	5	100	5.55	0	0	0	0	2.42	3.325	0.280761	0.467803
1	Nitrogen	10	0	6.71	1	0.1	10	0	2.73	2.1	0.289218	0.808712
1	Nitrogen	10	50	6.69	0	0	0	0	2.27	2.52	0.184919	0.55303
1	Nitrogen	10	100	7.04	1	0.05	8.75	0	2.17	1.715	0.173643	0.619318
	CV %			1.7	10.9	8.6	6.7	2.1	15.2	6.2	12.9	21.5
	s.e.			0.0959	1.676	0.00982	1.57	0.0137	0.1537	0.1375	68.5	0.2470

Appendix 2 cont.

Rep	Main plot	Lime (Tons)	P (kg)	pH at planting	Nodules/ plant	Fresh nodules vol/plant	Nodule DW/plant	N_2 fixed (mg N/plant)	DMY	%N in shoot	% P	% Ca
2	Inoculated	0	0	4.72	**23**	**0.15**	25	19.24	1.08	1.68	0.103171	0.344697
2	Inoculated	0	50	4.51	**24**	**0.2**	37	36.55	2.11	1.68	0.108809	0.344697
2	Inoculated	0	100	4.43	**16**	**0.2**	31.75	34.78	1.62	1.82	0.114447	0.429924
2	Inoculated	5	0	5.2	**39**	**0.3**	50	36.63	2.3	1.575	0.032699	0.704545
2	Inoculated	5	50	5.59	**35**	**0.3**	53	47.64	2.48	1.82	0.125722	1.026515
2	Inoculated	5	100	5.34	**23**	**0.3**	49.75	45.69	1.96	2.275	0.165187	0.704545
2	Inoculated	10	0	6.73	**44**	**0.35**	64.5	47.87	2.62	1.785	0.176462	0.761364
2	Inoculated	10	50	6.93	**41**	**0.45**	76	57.68	2.65	2.135	0.23284	0.732955
2	Inoculated	10	100	7.2	**18**	**0.25**	35.25	31.84	1.44	2.135	0.427343	0.837121
2	Uninoculated	0	0	4.37	**5**	**0.1**	12.25	22.61	2.05	1.015	0.148273	0.316288
2	Uninoculated	0	50	4.44	**0**	**0**	0	0	1.47	1.995	0.244116	0.439394
2	Uninoculated	0	100	4.8	**3**	**0**	0	20.32	1.13	1.575	0.252572	0.382576
2	Uninoculated	5	0	5.94	**0**	**0**	0	0	2.61	1.19	0.111628	0.496212
2	Uninoculated	5	50	6.19	**3**	**0**	0	22.02	2.43	1.19	0.184919	0.524621
2	Uninoculated	5	100	5.41	**9**	**0.1**	11	32.74	3.2	0.945	0.08062	0.496212
2	Uninoculated	10	0	6.95	**0**	**0**	0	0	1.75	1.47	0.159549	0.5625
2	Uninoculated	10	50	6.55	**1**	**0.15**	20	0	2.45	1.05	0.280761	0.57197
2	Uninoculated	10	100	6.67	**4**	**0.05**	4.75	23.50	1.28	1.75	0.289218	0.941288
2	Nitrogen	0	0	4.88	**0**	**0**	0	0	1.02	4.55	0.356871	0.505682
2	Nitrogen	0	50	5.1	**0**	**0**	0	0	1.26	4.095	0.478083	0.467803
2	Nitrogen	0	100	4.23	**0**	**0**	0	0	0.96	4.235	0.444257	0.429924
2	Nitrogen	5	0	5.53	**0**	**0**	0	0	1.8	3.5	0.342777	0.799242
2	Nitrogen	5	50	5.83	**0**	**0**	0	0	1.93	3.85	0.370965	0.931818
2	Nitrogen	5	100	5.68	**0**	**0**	0	0	2.06	3.43	0.277942	0.922348
2	Nitrogen	10	0	6.8	**0**	**0**	0	0	1.63	3.43	0.221564	1.017045
2	Nitrogen	10	50	6.52	**0**	**0**	0	0	1.36	3.045	0.320226	1.272727
2	Nitrogen	10	100	6.48	**0**	**0**	0	0	1.43	3.36	0.204651	0.950758
	CV %			**1.7**	**10.9**	8.6	6.7	2.1	15.2	6.2	12.9	21.5
	s.e.			**0.0959**	**1.676**	0.00982	1.57	0.0137	0.1537	0.1375	68.5	0.2470