MOLYBDENUM-RHIZOBIA-PHOSPHORUS MANAGEMENT FOR AMELIORATION OF BIOLOGICAL NITROGEN FIXATION IN COMMON BEAN ON A FERRALSOL

BY

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DECLARATION

I hereby declare that this is my original work and has never been submitted for any other degree award to any other University. All sources of the materials are duly acknowledged.

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To my late mother Joweria Nalumansi and my family

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ABBREVIATIONS AND ACRONYMS

ADP:	Adenosine Diphosphate
ARA:	Acetylene Reduction Assay
ATP:	Adenosine Tri-Phosphate
BNF:	Biological Nitrogen fixation
CF:	Conversion factor
CIAT:	Center for International Tropical Agriculture
CRD:	Completely Randomized Design
CRSP:	Collaborative Research Support Program
FAO:	Food Agricultural Organization
MUARIK:	Makerere University Agricultural Research Institute Kabanyolo
NARO:	National Agricultural Research Organization
PGPR:	Plant Growth Promoting Rhizobacteria
SNF:	Symbiotic Nitrogen Fixation
SSA:	Sub-Saharan Africa
USA:	United States of America

ABSTRACT

Common bean (*Phaseolus vulgaris* L.) is among the primary food security crops in Sub-Saharan Africa (SSA), whose production almost exclusively depends on natural Biological Nitrogen Fixation (BNF) as the source of nitrogen. The process is constrained by various biotic (e.g. low BNF capacity of indigenous rhizobia strains) and abiotic (e.g. inadequate levels of soil Mo and P) stresses. It is, therefore, imperative that BNF processes are enhanced through or revisiting rhizobia strains and amelioration of BNF relevant soil nutrient profiles. A study was conducted with the objectives of: (i) determining the response of BNF in common bean to different regimes of molybdenum application, treated with a local and imported (BioStacked) rhizobia inoculant in a Ferralsol; and (ii) determining the influence of intervention with P on BNF in common bean treated with Mo and rhizobia inoculants.

A screenhouse study was conducted at Makerere University Agricultural Research Institute Kabanyolo (MUARIK), Uganda, during the December to March 2016; using a Ferralsol obtained from previously cropped fields with different types of legumes and cereals. The study was arranged in two sets namely; (i) molybdenum verses rhizobia inoculants and (ii) molybdenum x rhizobia inoculants x phosphorus. Specific treatments in (i) included Mo applied at rates of 0, 0.6, 1.3 and 2.5 mg kg^{-1} of soil, equivalent to 0, 0.5, 1 and 2 kg ha^{-1}; and rhizobia inoculants namely BioStacked (code named “stress tolerant inoculant” by USA manufacturer) and Mak-Bio-fixer (indigenous from Makerere University). The second experimental setup, which tested the effectiveness of (ii) with P intervention, comprised of three treatments namely Mo and rhizobia inoculants as described above, and P at rates of 0, 38 and 76 mg kg^{-1} of soil, equivalent

to 0, 15 and 30 kg ha^{-1}. The experiment was laid out in a Completely Randomized Design (CRD) with 3 replicates and two repeats. The experimental crop was common bean, NABE 4 variety.

Results revealed that joint application of Mo and the two types of rhizobia inoculants in setup (i) had a significant ($P<0.005$) effect on the number of effective nodules and their dry weights, total shoot N and shoot dry weight. Molybdenum rates of 0.5 to 1 kg ha^{-1} yielded the best overall. However, estimated values for biologically fixed nitrogen (total shoot N from rhizobia inoculated pots discounted for rhizobia uninoculated pots) were not significantly ($P>0.005$) influenced by Mo application, rhizobia inoculation and/or both treatments. Additionally, intervening with P application in the Mo-rhizobia inoculant setup (setup ii) had no significant ($P>0.005$) effect on the status of estimated biologically fixed nitrogen generated by the common bean; implying that energy requirement for biological fixation of N was not the key limiting factor for the process. Overall, the BioStacked rhizobia inoculant (imported) tended to perform better in terms of number of effective nodules and their dry weights without Mo and/or P application than its Mak Bio Fixer counterpart.

CHAPTER ONE: INTRODUCTION

1.1 Background

Common bean (*Phaseolus vulgaris* L.) is the second most important food crop in Sub-Saharan Africa (SSA) after maize, being a major source of proteins, especially for the socio-economically vulnerable communities (Snapp *et al.*, 2018). Unfortunately, common bean production in SSA, is way below the desired levels based on regional statistics. For instance, Larochelle *et al.* (2014) estimated the current per *capita* availability for consumption of dry beans in SSA to be 262.12 kg year^{-1}, with countries like Uganda and Rwanda having their annual per *capita* consumption at 11 and 29 kg, respectively.

One of the major causes of the low common bean production is decline in soil fertility, most especially nitrogen and phosphorus (Dogbe *et al.*, 2002); and micro-nutrients such as molybdenum (Mo) (Vieira *et al.*, 2005, 2011; Wurzburger *et al.*, 2012) that play very important roles in nitrogen fixation processes. In Sub-Saharan Africa, soil N is primarily obtained from natural biological N fixation (BNF) mechanisms by soil microorganisms commonly known as rhizobium bacteria, which are largely inefficient, especially in common beans (Giller, 2001). Therefore, rhizobia inoculation (augmenting number and quality of rhizobia bacterial cells) plays a key role for good nodulation, nitrogen fixation, plant growth and achievement of high yields of legumes including common bean (Somasegaran and Hoben, 1994).

Most of the soils in the SSA region are highly degraded and therefore, there is need to optimise BNF in beans (Bekunda and Kaizzi, 2008). Furthermore, the initial micro-symbiont populations in the soil are often inadequate, yet this is a precursor to achieving a rigorous BNF process. The other important attribute that causes BNF failure is the ineffective infestation capacity of

rhizobia strains due to incompatibility with the bean plant and inadequate capacity of a strain to generate sufficient nitrogenase enzyme required for substantial BNF. Hence, there is often need for inoculation with appropriate rhizobia strains previously screened for effectiveness. More efficient inoculants for beans have been produced in various parts of the world (CIAT, 2008). Recently, in USA developed a "versatile" inoculant for beans, with capacity to perform well even under stressful conditions (Anonymous, 2009). This BioStacked inoculant, also known as RHIZO-STICK® comprises of viable cells of *Rhizobium leguminosarum bv. phaseoli*, in packages of 2.5 kg produced by Becker Underwood Company, in the United States of America. This package is sufficient for dressing about 900 kg dry bean seeds, and is also estimated to deliver about 1.0×10^9 cells g^{-1} of self-sticking peat-based inoculant (Anonymous, 2009).

Molybdenum is an integral part of the nitrogenase enzyme which is responsible for effecting symbiotic nitrogen fixation (Hoffman *et al.*, 2014). It is a constituent of component I (MoFe-protein) of nitrogenase enzyme complex. The MoFe protein is known to contain two metal clusters, namely the iron-molybdenum cofactor (FeMo-co), which provides the active site for substrate N_2 binding and reduction; and the P-cluster, which is involved in electron transfer from the Fe protein to FeMo-co (Hoffman *et al.*, 2014). Great effort has been made to understand the biochemistry of BNF and significant role of Mo in effecting the process. However, despite this hardly any effort has been made to monitor and ascertain the sufficiency and functional environment of Mo for BNF in the soils of SSA, especially in Ferralsols that predominate the agricultural soils in the region. Hence, the erratic observations made with the use of different rhizobia strains and leguminous crops may partly be attributed to inadequacy of Mo for the function or the soil environment being unfavorable for the uptake and utilisation of Mo for BNF. Efforts are therefore, necessary to consider mainstreaming Mo in the rhizobia inoculants

intervention schemes in SSA.

The other key element to the functioning of nitrogenase enzyme is soil phosphorus, which is primarily responsible for providing substantial amounts of available energy required by the N fixation process. It is estimated that 16 moles of ATP are required to produce 2 molecules of ammonia, as the product of nitrogen fixation by the enzyme (Hubbell and Kidder, 2009; Hoffman *et al.*, 2014). Hence, this implies that for effective N fixation to occur, there must be sufficient Mo and P in the soil. Unfortunately, majority of the agricultural soils in SSA are over-weathered, thus containing low P mineral reserves, and yet comprising of soils of sesquioxides chemistry typically high in P fixation (Bationo *et al.*, 1998; Ssali, 2000; Nziguheba *et al.*, 2016). It is, therefore, possible that the high P deficiency that is endemic in most SSA soils, directly affects BNF, even where there would-be-effective inoculants are administered.

1.2 Statement of the problem

The potential of common bean to fix nitrogen for plant consumption is through forming a symbiotic relationship with soil rhizobia. The inoculation technology has long been recommended and new inoculants are produced for farmers as a sustainable and cheaper means for improvement of soil fertility for increased common bean yields. However, the technique is limited by a number of factors, including among others; inadequate population of effective rhizobia strains (Somasegaran and Hoben, 1994). Therefore, it is necessary that we evaluate other candidate strains available elsewhere to upgrade the capacity provided by the indigenous strains to cope with the increasing stresses imposed by ecological factors, such as climate change.

The other plausible causes of the occurrence of BNF failure in the SSA is inadequacy of relevant soil nutrients such as molybdenum and phosphorus. Legume plants that depend on biological N_2 fixation for their N supply require more Mo and P for conversion of atmospheric nitrogen by the nitrogenase enzyme. Molybdenum is the main component of the nitrogenase enzyme, which provides the active site for substrate atmospheric nitrogen binding and reduction. It also acts as an electron acceptor from the Fe protein component. On the other hand, phosphorus is needed in legume plants for energy transformation in the nodules in the form of Adenosine triphosphate (ATP) to the nitrogenase enzyme activity. But most of the agricultural soils have inadequate amounts of P to support efficient BNF, as it exists in stable chemical compounds which are least available to plants. Therefore, these nutrients are very important for the functioning of the plant nodule, a strong sink of these nutrients to guarantee adequate nitrogen fixation. Furthermore, in tropical soils where low natural fertility and high acidity are quite common, both molybdate and phosphate can be rapidly adsorbed to soil colloids of positive charge and bound to organic matter, thereby reducing their availability to the legume crops. The deficiency of Mo and P causes significant yield reduction in leguminous crops.

For farmers to realise the importance of the P application in BNF improvement, an optimal amount is required, and this is yet to be ascertained. The performance of the BNF in common bean is partly attributed to Mo levels in the soils. Unfortunately, Mo levels in SSA soil have not been widely studied (Peyue, 1963). However, there is evidence of Mo deficiency as illustrated by some available literature. For example, Sillanpaeae (1982) found Mo to be low in the soils of Ghana, Nigeria, Sierra Leone, Malawi, Tanzania and Zambia. More recently, low BNF in the soils of some parts of South Africa, was suspected to be due to Mo deficiency and also ineffective indigenous rhizobia strains (Bambara and Ndakidemi, 2010).

1.3 Justification of the study

In Sub-Saharan region, farmers practice agriculture without any external input like inorganic N fertilizers; thus, the observed low yields. The need to increase yields has met a number of challenges, especially the escalating international inorganic fertilizer prices limiting their use by small scale farmers. There is evidence that use of the rhizobia inoculation technology as an alternative to N fertilizers boosts soil nitrogen and soil fertility resulting into two-fold increment in crop yields (Rhawhia *et al.*, 2010).

Low nutrient availability and soil acidity, typical of weathered soils, constitute serious restrictions to nitrogen fixation in the common bean grown in the tropics (Graham and Vance, 2000). It is believed that supplementing these soils with P and Mo greatly improves grain yields of common bean. This was realised when supplied P showed increased growth, nodulation and N accumulation, particularly at low soil P levels (Chagas *et al*. 2010). On the other hand, soils supplemented with Mo had higher accumulation of plant biomass and N in shoots, and higher root nitrogenase activity (Bambara and Ndakidemi, 2010); and also produced more grains in soil with low N content. With such supplementation, rhizobia inoculation is likely to be improved as these nutrients provide energy and also forms main composition of the enzyme in the nodule.

However, for this technology to benefit the farmers, selective measures should be put in place to ensure that farmers use appropriate rhizobia strains that are efficient in respective environmental conditions. A number of rhizobia inoculants exist on the market, but their nitrogen fixing efficiency is unknown. Additionally, there is a new rhizobia inoculant developed in USA by Becker Underwood called BioStacked inoculant which is believed to be superior to other inoculants (Anonymous, 2009). This superiority is yet to be determined under local conditions.

1.4 Objectives of the study

The main objective of this study was to improve the performance of BNF in common bean growing areas in East Africa, through rhizobia inoculation and management of soil P and Mo. The specific objectives were to:

(i) determine the response of stress tolerant rhizobia inoculant (BioStacked) on common bean BNF to the different regimes of soil molybdenum in a Ferralsol; and

(ii) determine the performance of a temperate renowned stress resistant rhizobia inoculant (BioStacked) plus P and Mo concentration regimes in the soil on common bean BNF.

1.5 Hypothesizes

(i) Application of Molybdenum and phosphorus on common beans can improve Biological Nitrogen Fixation grown on ferralsol;

(ii) BioStacked as novel inoculant is superior to CIAT 899 the locally available rhizobia strain of common beans.

CHAPTER TWO: LITERATURE REVIEW

2.1 Biological nitrogen fixation

The dinitrogen (N_2) gas represents almost 80% of the earth's atmospheric gases; plays an important role in the plant nutrient systems and later as dietary proteins for animals. However, it is not directly available to plants as a nutrient because it is relatively inert. The main artificial (Haber–Bosch) and natural (biological nitrogen fixation) processes where atmospheric nitrogen is converted to a form utilizable for plants; the latter being natural process in the soil, it has for long supported small scale farmers in the SSA to increase their legume crop yields (Giller, 2001). The ability to fix atmospheric N comes from the symbiotic relationship between legumes and rhizobia, bacteria in soil, through which the legume supplies energy and carbon to rhizobia through the products of photosynthesis, and rhizobia provides the legume with N, mainly in the form of ammonium (Howard and Rees, 1996; Giller, 2001; Garg and Geetanjali, 2007).

Biological Nitrogen fixation (BNF) is the process whereby a number of bacterial species use the enzyme nitrogenase to convert atmospheric N_2 into ammonia (Shiferaw *et al.*, 2004). This process is found active not independently but rather specific micro-organisms in nature. The conversion process is accomplished by organisms through asymbiotic and symbiotic relationships. However, the symbiotic relationship is a beneficial one where two organisms are involved in reducing atmospheric N into a biologically useful combined form; for example protein and nucleic acids, of the bacteria and associated plants (Smil, 2004).

2.1.1 Working of biological nitrogen fixation

In symbiotic relationship, the macro symbiont (legume plant) and micro symbiont (bacteria) form an association to fix atmospheric nitrogen. Symbiosis between the aerobic bacteria rhizobia and roots of Leguminosae; the bacterium are host specific (for example common bean is nodulated by *Rhizobium tropici*) and development of root nodule that house the process and the bacteria. The process starts with root infection as precursor to nodulation which is a multi-step process that involves specific plant and bacterial gene expression (Hungria and Stacey, 1997). Before nodulation, the two symbionts each exists as individual organisms. Prior to nodulation, multiplication of the bacteria in the rhizosphere will start ensuring bacterial population to cause an infection (Laguerre *et al.*, 1997). Prior to infection, the plant releases molecular signals (hormones) recognized by the compatible rhizobia and later attach themselves to the surface of root hairs (Sprent, 2001; Garg and Geetanjali, 2007).

The process begins when the rhizobia are attracted to flavonoids released by the host legume's roots referred to as infection. Infection and nodule organogenesis occur simultaneously during root nodule formation. The invading rhizobia attached to the emerging root hairs release Nod factors that induce a pronounced curling of the root hair cells (Garg and Geetanjali, 2007). The rhizobia become enclosed in a small compartment formed by the curling of the root hair and a leghemoglobin component which is the active form of the nodule. This is part of the nitrogenase enzyme that is actively responsible for the reduction of the atmospheric nitrogen into ammonia utilizing the energy provided in the form of photosynthates from the plant host. In 2003, Lodwig and colleagues reported that the metabolic dependence of the two symbiotic partners is more complex than a simple exchange of products of photosynthates and ammonium.

2.1.2 Nitrogenase enzyme

Nitrogenase enzyme in the legume nodule has been determined to consist of a two-component system composed of the MoFe protein (dinitrogenase or component I) and the electron transfer Fe protein (dinitrogenase reductase or component II) (Somasegaran and Hoben, 1994; Kaiser *et al.*, 2005; Hoffman *et al.*, 2014). Reduction reactions occur while N_2 is bound to the nitrogenase enzyme complex; Fe protein is first reduced by electrons donated from ferredoxin. The reduced Fe protein binds Adenosine Tri Phosphate (ATP) and this cause molybdenum-iron protein to be reduced, which donates electrons to N_2, producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to $H_2N\text{-}NH_2$ and then to $2NH_3$ (Hoffman *et al.*, 2014).

$N2 + 8e^- + 16ATP + 8H^+$ ----------> $2NH3 + H2 + 16\ ADP + 16P_i$ …… eqn. (1)

The MoFe protein contains two metal clusters: the iron−molybdenum cofactor (FeMo-co), which provides the active site for substrate binding and reduction, and P-cluster, involved in electron transfer from the Fe protein to FeMo-co (Ma *et al.*, 1996). In fact, the molybdenum found in the protein is directly responsible for the site activation. Hoffman and colleagues (2013) continue to emphasize that a reducing source and MgATP are required for catalysis. The Fe protein and MoFe protein associate and dissociate in a catalytic cycle involving single electron transfer and MgATP hydrolysis (Canfield *et al.*, 2010). This MgATP is a magnesium adenosine triphosphate enzyme where the Mg is a carrier and ATP is an energy source from photosynthates to the nodule for BNF accomplishment.

2.1.3 Indicators of Biological Nitrogen Fixation

The actual percent N fixed and amounts of N fixed by individual crops are influenced by environment and management effects, including soil nitrate levels at planting. Importantly, number of effective nodules, weight of effective nodules, shoot N content and shoot dry weight must be considered when estimating performance of fixed N and used by the plant for growth.

Leghemoglobin pigment. Leguminous plants form nitrogen fixing root nodules, these contain an oxygen carrier and hemoprotein called leghemoglobin. In response to symbiotic interaction between plant and *Rhizobium* species; roots colonized with nitrogen-fixing bacteria do synthesize leghemoglobin (Nadler and Avissar, 1977). This protein has a close chemical and structural similarities to hemoglobin in human beings and is red in colour. The host plant intensely expresses heme biosynthesis genes within nodules, and that activation of those genes correlates with leghemoglobin gene expression in developing nodules (Nadler and Avissar, 1977; Sangwan and O'Brian, 1993;1999).

Plants nodulated with *Rhizobium*, for example common bean, the presence of oxygen in the root nodules would reduce the activity of the oxygen-sensitive nitrogenase (Balestrasse *et al.*, 2006), the enzyme responsible for the fixation of atmospheric nitrogen. This protein buffers concentration of oxygen in the cytoplasm of the plant cells to ensure proper and efficient functioning of the nodules; it is also said to have a high affinity for oxygen than the β chain for human hemoglobin (Minchin *et al.*, 2008). This provides an oxygen concentration high enough to the bacteria for respiration and also a very low concentration for optimal functioning of the nitrogenase enzyme in the nodule (Marino *et al.*, 2009). Its function is to help provide oxygen to the respiring symbiotic bacterial cells in a manner analogous to hemoglobin transporting oxygen

to respiring tissues in animals. Therefore, an active nodule possess pink/ red pigment, is confirmatory for presence of BNF in the nodule.

Acetylene reduction assay (ARA). The ARA is the most common method for measuring BNF in different ecosystem types. It is based on the nitrogenase enzyme preferential reduction of acetylene (C_2H_2) to ethylene (C_2H_4) that involves just two electron transfer, instead of reducing N_2 which involves eight, when C_2H_2 is present at relatively high concentrations (10% v/v; Schollhorn and Burris, 1967; Hardy *et al.*, 1968), according to the following equation (Staal *et al.*, 2001):

$C_2H_2 + 2H^+ + 2e^- \rightarrow C_2H_4$ ----------eqn (2)

Despite its simplicity ARA is an indirect method and a conversion factor (CF) is needed to estimate BNF rate equivalents based on the number of moles of C_2H_4 produced. The theoretical CF obtained from the formulas (1) and (2) relating the number of reducing equivalents is 4:1 (moles of C_2H_4 produced per mole of nitrogen fixed) (Zehr and Montoya, 2007). Empirical measurements in *in vitro* experiments of nitrogen fixing bacteria (*Azotobacter* and *Clostridium*) as well as *in situ* have found that the ratio of C_2H_4 produced to N fixed was between 3 and 4.5 (Hardy *et al.*, 1968). These method requires sophisticated tools and chemicals to apply it, hence its limitation for use in this study.

Ureid analysis. Tropical legume crops for example *Phaseolus vulgaris* exports fixed nitrogen from the root nodules to the shoots mostly as ureides. These ureides are translocated in the xylem and provides the major supply of nitrogen to these plants. One technique which can be easily applied in the field and which uses comparatively simple equipment, is based on the analysis of ureides and nitrate in the xylem sap (McClure *et al.*, 1980). The principal products of BNF in the

nodules are ureides, allantoin and allantoic acid; the technique exploits the fact that many tropical legumes including forage legumes of genera *Calapogonium*, *Centrosema* and *Desmodium*, although not of *Arachis* and *Stylosanthes* are exported in the above forms. If it is assumed that most N derived from the soil is absorbed as nitrate and little of this is reduced until it is translocated to the leaves, then the ratio of ureide-N to ureide-N + nitrate-N + amino acid-N in the xylem stream should represent the ratio of N derived from BNF to that derived from soil at the time of sampling.

This ureide technique has been widely applied to the quantification of BNF contributions to soybean (Wang *et al.*, 1993), common bean (Kabahuma, 2013) and even some woody species (Peoples *et al.*, 1996) and in many cases has been shown to be well calibrated with ^{15}N isotope based techniques. The problem with many tropical forage legumes is the difficulty in extracting xylem sap. Boddey *et al.* (2000), reported that with experience of several species, that it is almost impossible to obtain significant quantities of sap even when using suction of cut stems as suggested by Herridge *et al.* (1988).

2.2 Molybdenum forms

In soils, Mo can be found in four major fractions: (i) dissolved Mo in soil solution (water soluble), (ii) Mo occluded with oxides (e.g. Al, Fe and Mn oxides), (iii) Mo solid phases (e.g. molybdenite (MoS_2), powellite ($CaMoO_4$), ferrimolybdite ($Fe(MoO_4)_3$), wulfenite ($PbMoO_4$), and (iv) Mo associated with organic compounds (Reddy *et al.*, 1997). However, MoO_4^{2-} anion is a planted available species. Molybdenum availability is affected by factors such as soil pH, soil organic matter, clay drainage, nutrient interaction and crop sensitivity. Even wulfenite ($PbMoO_4$), the least soluble of soil Mo compounds, becomes more soluble as pH increases (Vlek

and Lindsay, 1974). The MoO_4^{2-} anion exists in an exchangeable form in the soil. Thus, the fact that Mo availability to plants increases with increasing pH, may be explained by anion exchange of the type $2OH^- \Leftrightarrow MoO_4^{2-}$ (Goldberg, 2010).

2.2.1 Role of molybdenum in BNF

In addition to the inorganic nutritional requirements of legumes growing on combined nitrogen, nodulated legumes have been shown to require increased amounts of molybdenum (Bogino *et al.*, 2006). Molybdenum is a constituent of component I (MoFe-protein) of nitrogenase enzyme in the bacteria (Madigan *et al.*, 2000). The ammonia produced in BNF process undergo transamination reactions within the nodules and are then translocated rapidly to the xylem stream to the growing parts of the host plant. In order to improve common bean production for small holder farmers, nutrient like Mo that highly support the process of BNF and physiological processes of bacteria are required in the vicinity of the root nodule.

Molybdenum availability is not routinely assessed anywhere, due to the difficulty of accurately determining its very small quantities. For total Mo, few data are available on its content and distribution in African soils. Efforts of Peyue (1963), reported very low total Mo contents (0.44 – 0.75 ppm) for Malian surface soils while Cottenie *et al*. (1981) found a range of total Mo contents (3 – 4.2 ppm) for selected Nigerian soil profiles. Mwakatundu (1977), concluded that Mo is deficient in soils derived from sedimentary and metamorphic rocks and from Kainozoic rocks in Ivory Coast. In another study carried out by Cottenie *et al*. (1981), who studied the micronutrient status of soils from the humid zone of southern Nigeria and Togo, reported low levels of ammonium oxalate-extractable Mo. Similarly, Ibrahim in 1982, reported low levels of extractable Mo in the soils of Gezira scheme in Sudan.

2.3 Role of phosphorus in biological nitrogen fixation

A positive correlation has been observed between BNF and P availability in natural soils (Pearson and Vitousek, 2002; Labidi *et al.*, 2003). Phosphorus is an energizing nutrient for the BNF process in legumes; it plays a key role in nodule activity through increased formation and availability of Adenosine Tri-Phosphate (ATP); a resource material for the energy intensive N reduction process in root nodules enhancing increased growth of the plant (Hubbell and Kidder, 2009). Phosphorus becomes involved as an energy source when 16 molecules of ATP are converted to Adenosine Diphosphate (ADP) as each molecule of atmospheric N_2 is reduced to NH_3. The ATP is generated during the process of photosynthesis, when light energy is transformed and stored in the form of ATP for later use; in case of legume plants, processes like BNF utilize this stored energy (Hubbell and Kidder, 2009).

Common bean is a renowned consumer of nitrogen and phosphorus; although as a legume, the crop is endowed with potential for replenishing soil N through BNF, the continuous production process results in a net depletion of the other nutrients, especially P which is required in fairly large quantities by the process. The research carried out by Robson *et al*. (1981), concluded that P nutrition increased BNF in Subterranean clover (*Trifolium subterraneum* L.) through stimulating host plant growth rather than by exerting specific effects on rhizobial growth or on nodule formation and function.

2.4 Rhizobia strains

The family Rhizobiaceae is a group of genetically diverse and physiologically heterogeneous soil microorganisms collectively called rhizobia. Rhizobia is a term used to describe a range of soil bacterial genera including *Rhizobium, BradyRhizobium, SinoRhizobium, MesoRhizobium,*

AlloRhizobium, and *AzoRhizobium* that are able to enter into symbiotic relationship and nodulate members of the plant family Leguminosae (Howieson and Ballard, 2004). There is a great variation in the specificity of interaction with Rhizobia observed among legume species. Some Rhizobia–legume associations are very specific and the legume will only form nodules when infected with a specific Rhizobia; while other legumes will form nodules with a range of rhizobia (Broughton, 2000; Graham and Vance, 2000). Specificity involves the recognition of the bacterium by host and of the host by a bacterium through the exchange of signals compounds, which induce differential gene expression in both partners (Broughton, 2000).

2.4.1 Counting rhizobia by plant infection method

The most probable number (MPN), is also known as the plant infection count, is used to determine the number of viable and infective rhizobia cells present in the soil or inoculant peat. The trap legume used must belong to the same cross-inoculation group of the legumes nodulated by the rhizobia under investigation. It is a direct method commonly used to determine the quality of inoculants produced from nonsterile or sterilized carrier materials. It is also used to determine the number of rhizobia population in the soil (Monza *et al.*, 2019). The description of the MPN method is well elaborated in the Handbook for rhizobia a laboratory manual for methods in Legume-Rhizobium technology by Somasegaran and Hoben (1994) and has been validated by metagenomic and metatranscriptomic datasets in soil (Mauchline *et al.*, 2014).

2.4.2 Rhizobia species nodulating common bean

The bacteria, which are able to form root nodules on common bean are classified into five species of the genus *Rhizobium*, *R. leguminosarum biovar* (*bv.*) *phaseoli* (Jordan D. C., 1984), *R. tropici* (Martinez-Romero *et al.*, 1991), *R. etli bv. phaseoli* (Segovia *et al.*, 1993), *R. gallicum*

bvs gallicum and *phaseoli* (Amarger *et al.*, 1997), and *R. giardinii bvs giardinii* and *phaseoli* (Amarger *et al.*, 1997). Others still unclassified but already genetically well characterised rhizobia such as *Rhizobium sp.* BR816 (Hungria and Phillips, 1993), *Rhizobium sp.* NGR234 (van Rhijn *et al.*, 1994) and *Rhizobium sp.* GRH2 (Herrera *et al.*, 1985) can also symbiotically infect *Phaseolus vulgaris*.

Specifically, the strain CIAT 899 of *Rhizobium tropici sp.* is commonly used on the common bean in soils within Uganda; the strain is packaged from Makerere University and promoted among common bean farmers in the region. This strain was sourced from International Tropical Center of Agriculture (CIAT) who identified and isolated it from the tropical soil. The *R. tropici* strains are tolerant to high temperatures and high levels of acidity in culture and are symbiotically more stable.

In the recent past, Ormeno-Orrillo *et al*. (2012), reported interesting findings about the genome of CIAT 899: it has large numbers of genes encoding drug-efflux systems, which may explain their high resistance to antimicrobials. They further said, genome analysis also revealed a wide array of traits that may allow this strain to be a successful rhizosphere colonizer; including surface polysaccharides, uptake transporters and catabolic enzymes for nutrients, diverse iron-acquisition systems, cell wall-degrading enzymes, type I and IV pili and novel T1SS and T5SS secreted adhesins.

Recently, in USA developed a “versatile” inoculant for beans, with capacity to perform well even under stressful conditions (Anonymous, 2009). This BioStacked inoculant, also known as RHIZO-STICK® comprises of viable cells of *Rhizobium leguminosarum bv. phaseoli*, in packages of 2.5 kg produced by Becker Underwood Company, in the United States of America.

This package is sufficient for dressing about 900 kg dry and snap bean seeds, and is also estimated to deliver about 1.0 x 10^9 cells g^{-1} of self-sticking peat-based inoculant (Anonymous, 2009).

2.4.3 Stress factors influencing performance of Rhizobia strains

Environmental and management constraints regulate the amount of N fixed by legume bacteria, besides influencing plant growth associated with soil nutrients, water supply, diseases and pests (Peoples and Herridge, 2000). Legumes commonly fix around 20-25 kg of shoot N for every tone of shoot dry matter accumulated over a range of environments unless local practices (no inoculation, poor inoculants quality, excessive tillage, extended fallows, fertilizer N, and rotations) curtail their fixing capacity and rotations (Peoples and Herridge, 2000). Legume roots may contribute up to 0.7 kg of fixed N accumulated in the dry matter of the shoots (Peoples and Herridge, 2000).

Therefore, strategies which influence either the N derived from the atmosphere or legume growth will affect the total input of fixed N (Segovia and Young, 1993). The values show the potential biological N_2 fixation of legume bacteria ranging from 200 to 300 kg N ha^{-1} per crop. The contribution of fixed N to the agricultural production system would be further reduced if legume crop residues were removed from the field for use as animal fodder, organic mulch or fuel, and if the trash and stubble remaining after seed harvest were as well burnt (Wani *et al*., 1995).

In addition, desertification causes disturbance of plant-microbe symbioses, which are a critical ecological factor in helping further plant growth in degraded ecosystems. Among several environmental conditions, which are limiting factor such as salinity, temperature extremes and pH stress are probably the most problematic. A competitive and persistent rhizobial strain is not

expected to express its full capacity for nitrogen fixation as the limiting factors (e.g. salinity, unfavorable soil pH, temperature extremes, nutrient deficiency etc.) impose limitations on the vigour of the host legume. Inoculation of stress tolerant strains of rhizobia may enhance the nodulation and nitrogen fixation ability of plants under stress conditions (Damodara *et al.*, 2018).

Plant growth promoting rhizobacteria (PGPR) can improve plant growth and productivity by several mechanisms. Few strains from genera such as *Bacillus, Pseudomonas, Erwinia, Caulobacter, Serratia, Arthrobacter, Micrococcus, Flavobacterium, Chromobacterium, Agrobacterium, Hyphomycrobium, Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium and Allorhizobium* are well known PGPR. They aid in improving plant stress tolerance to drought, salinity and metal toxicity. The underlying mechanisms of plant growth promotion by PGPR have been comprehensively described in several articles (Kloepper *et al.*, 2004; Yang *et al.*, 2015).

CHAPTER THREE: MATERIALS AND METHODS

3.0 Nature of study

A screenhouse experiment was conducted at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK) in Uganda, for two consecutive rounds (December 2015 to April 2016). This study was conducted in two parts based on the study objectives as described below.

3.1 Part 1: Molybdenum verses rhizobia inoculants

This study investigated the interactive effect of Mo rates and rhizobia inoculants (local and imported) on BNF related and plant growth parameters on a Ferralsol. Treatments included molybdenum applied at four levels 0, 0.6, 1.3 and 2.5 mg kg^{-1} soil, equivalent to 0, 0.5, 1.0 and 2 kg ha^{-1}. Sodium molybdate (analytical grade) was used as the source of Mo. The specified quantities of Mo were each dissolved in distilled water and then applied around the planting hole in respective treatment pots. The local rhizobia inoculant used was Mak Bio Fixer, obtained from the Department of Agricultural Production, Biological Nitrogen Fixation Laboratory at Makerere University in Uganda. The inoculant contained a *Rhizobium* strain CIAT 899 packaged in unsterile peat with estimated cells per gram (Table 2). The recommended sticker is a tea-spoonful of cane sugar in 300 ml of clean water. On the other hand, the imported rhizobia inoculant BioStacked from Becker Underwood in USA, was only known to contain a high population of rhizobia cells per gram (Table 2), sterilized peat with inbuilt sticker and antifungal agents. The rhizobia inoculants were coated onto the seed prior to sowing. The design used in this study was a completely randomised design (CRD), with 3 replicates and the study was repeated twice.

For each rate of Mo, the total amount required for the pots with Mak Bio Fixer (3 pots per rate) and BioStacked (3 pots per rate) inoculants was established and the quantity required computed.

For instance, for Mo rate at 0.6 mg kg^{-1} of soil, this was multiplied by 2 to obtain an amount of Mo required per pot (2x0.6 mg pot^{-1}) and subsequently multiplied by 3 to carter for the replicates (3x2x0.6 mg the three pots in the replicate). This total amount of Mo in form of sodium molybdate was dissolved in distilled water in container to form 100 ml solution. Then 10 ml of this solution was taken to contain the required rate of Mo per pot; and was administered around the planting hole using a syringe. This procedure was performed shortly after introducing the seed in the planting hole. Then, the planting hole was manually covered with soil. The procedure was repeated for all Mo rates and inoculant types.

Prior to administering the Mo treatments to the pots, the seeds were dressed with respective rhizobia inoculants, carefully, to avoid cross contamination. For this purpose, the inoculation process started with control pots, where no rhizobia inoculation was applied. This was followed by administration of respective pots with Mak Bio Fixer. The strain used in this inoculant is technically known as CIAT 899, obtained from tropical soils by Center for International Tropical Agriculture (CIAT) in the early 1990s. The strain is known to be stress tolerant, especially in terms of soil acidity and high temperatures (Martinez-Romero *et al.*, 1991; Ormeno-Orrillo *et al*. 2012).

In order to dress the seeds with inoculants, a sticker material comprising of cane sugar (tea spoonful), dissolved in distilled water was prepared. Specifically, one tea spoonful (approximately 20 g) was dissolved in 300 ml of distilled water using a coca cola soda bottle. Then, 1 kg of common bean seeds (NABE 4 variety) was put in a clean plastic basin and fully

dressed with the sticker, by manually sprinkling the bottle contents while hand-mixing the beans. The powdered inoculant was sprinkled on the sticking seeds, gently hand mixed to uniformly dress rhizobia inoculant around the seed. The dressed beans were planted at the rate of 3 seeds per pot. A day before planting, all bean seeds were soaked in distilled water for seeds to imbibe uniform moisture seed content prior to planting.

The entire process of inoculation followed with Mak Bio Fixer, was repeated in the case of BioStacked inoculant. All the dressing was done prior to planting to enhance the bacteria-seed adherence and also increase survival of the bacterial cells (Giller, 2001). However, because this inoculant already contained inbuilt/ engrained sticker material, the process of sticker administration was omitted in this case. The administration of this inoculant involved sprinkling of distilled water, just sufficient enough to wet the seeds surface. Then the inoculant was sprinkled on the wet seeds and gently hand mixed to ensure uniform distribution of the material around the seed.

Prior to administration of both inoculants, a test of cell counts on the rhizobia inoculants was performed to ensure an informed starting point of rhizobia cell population. The method used was the most probable number (MPN) described in a laboratory manual by Somasegaran and Hoben (1994). The results of the cell count assessment are presented in the table 1.

Table 1: Inoculant population estimated using Most Probable Number (MPN) prior to inoculation

Rhizobia inoculant	**Population counts (cells g^{-1})**
Mak Bio fixer	6.7×10^6
BioStacked	4.6×10^7

The experimental setup was kept on the benches in the screenhouse and watered with distilled water at 2 to 3 days intervals. One week after germination, the seedlings were thinned to one plant per pot. Weeding was done by hand whenever weeds appeared in the pots. The plants were routinely inspected for pests and diseases symptoms.

Data collection. Data were collected on effective nodules and plant growth. Data on root nodules involved uprooting of the plant at 50% flowering stage, root nodule collection and enumeration (counts per plant). The nodules were cleaned per plant using distilled water. Then a surgical blade was used to dissect each nodule cross-sectionally to assess for the presence of leghemoglobin. The presence of leghemoglobin gives a pink/ red color to the nodule interior, depictive of effective nodules for BNF functioning (Somasegaran and Hoben, 1994).

Each nodule that displayed pink/ red colour was considered for oven drying and these were dried in aluminium foil for oven dry weight (grams per plant). The drying process was done at 60 ^{0}C for 48 hours. After cooling in a desiccator for about 12 hours, the materials were weighed using Denver Instrument Company Model No. 201169.1 weighing scale.

The plant shoot part remaining after harvesting the root nodules were also separately oven dried at 60 ^{0}C for 48 hours. The materials were cooled under desiccator conditions and weighed for shoot dry weight (grams per plant). Thereafter, each plant sample was grounded into fine powder

using porcelain mortar and pestle. The powder was used for analysis of total shoot nitrogen (percent) content.

3.2 Part 2: Molybdenum x rhizobia inoculant x phosphorus

Based on the observation that Mo rates and rhizobia inoculant types investigated in Part 1, had an effect on the number and weight of effective nodules, but not on BNF related proxy indicators, it was suspected that energy required for BNF accomplishment was a limiting factor. For that matter, P which is central in energy processes was considered to subsequently part of the treatments.

For that matter, therefore, treatments in this part of the study included; all the Mo rates and inoculant types and procedures described in Part 1 of this study. Furthermore, P was included at rates of 0, 38 and 76 mg P kg^{-1} soil, equivalent to 0, 15 and 30 kg P ha^{-1}.

All other experimental procedures, including data collection as described in Part 1 were also adopted for this part of the study. The design used in this study was a CRD using split plot experimental arrangement with 3 replicates and the study was repeated twice.

3.3 Data analysis

All data collected from Part 1 and Part 2 of the study were entered in Excel Microsoft software before statistical analysis. The data were then subjected to a two-way analysis of variance (ANOVA) using GenStat software (version 13.0, VSN International, 2008) to generate mean values. A Fisher's Protected Least Significant Difference (LSD) was used to separate different treatment means that were found significant at $P \leq 0.05$ level. Correlations of number of

effective nodules and weight of effective nodules, with shoot nitrogen content and shoot dry weight were evaluated.

To obtain estimated BNF performance of the respective Mo and P application treatments; the estimated BNF values were obtained by subtracting control values from total shoot N and shoot dry weight in uninoculated pots from inoculated pots to estimate the biologically fixed nitrogen values of the experiment.

3.4 Soil and plant analysis

Soil analysis. Bulk sampling was done using hand hoes in the fields of Makerere University Agricultural Research Institute Kabanyolo (MUARIK) in Uganda. The source fields had been subjected to continuous cropping including cereals (maize) and legumes (beans and soybean), occasionally grown with inoculants. The bulk soil samples (0 – 20 cm soil depth) were bagged in gunny bags lined with polythene, in quantities of 50 kg and totaling to 500 kg. The bags were emptied on polythene sheets in shades, and the soil dried for seven days prior to further sampling to obtain 5 sub-samples which were used for quarter sampling for further routine analysis.

Soil analysis: A soil sample obtained from the bulk was used to obtain baseline soil properties prior to the experiment. The sample was air dried for 7 days and then pulverized before sieving using 2 mm wire mesh. It was subjected to routine physico-chemical analysis for soil pH, a soil water solution ratio of 1 : 2.5, available phosphorus by Bray I method, Kjeldahl nitrogen and exchangeable bases from an ammonium acetate extract by flame photometry (Na^+ and K^+) and atomic absorption spectrophotometer (Ca^{2+}, Mg^{2+}); and particle size distribution (soil texture) using the Bouyoucos (hydrometer) method. All were done following procedures as described by

Okalebo *et al.* (2002). In addition to the above tests, available molybdenum content was analysed using the calorimetric thiocyanate method (Stewart *et al.*, 1974).

Study soil results before the experiment are presented in Table 2. The pH of the soil was within the suitable agronomic range (5.5 – 6.8). The soil was low in organic matter, phosphorus, total nitrogen and exchangeable potassium. However, the soil had moderately low levels of available molybdenum, with a high exchangeable calcium. The soil textural class was sandy clay, with a bulk density of 1.36 g cm^{-3} and field capacity of 18.5% soil water (by SPAW software).

Table 2: Some Physico-chemical properties of experimental study soil from Kabanyolo

Soil properties	**Test values**	**Critical values***
Soil pH (1:2.5 H2O solution)	5.65	5.50
Soil Organic Matter (%)	1.06	6.80
Total N (%)	0.10	0.25
Available Mo (mg kg^{-1})	0.12	0.20
Available P (mg kg^{-1})	3.57	15.00
Potassium (cmol. kg^{-1})	0.26	0.38
Magnesium (cmol. kg^{-1})	1.83	0.20
Calcium (cmol. kg^{-1})	3.60	5.00
Sandy (%)	50.00	
Silt (%)	42.00	
Clay (%)	8.00	
Soil textural class	Sandy clay	

*Critical values for most crops in East Africa.

Plant analysis. The dried plant samples were grounded using a porcelain mortar and pestle into fine powder. About 0.5 g of plant sample was taken into digestion tube and the digestion mixture added; the solution was digested at 360 ^{0}C following Kjeldahl N method described in the laboratory manual by Okalebo *et al.* (2002).

3.5 Pot preparation

Before establishing the experiment, the remaining bulk soil samples were freed of visible plant materials and stones before it was sterilized in drums using firewood. The sterilization process lasted approximately 8 hours. Then after cooling, 2 kg of soil were taken per pot; following a layer of gravel arranged at the bottom of the pot with three centimeter thickness of sand. The pots had been labelled with treatment identification, in readiness for treatment administration. The pots had earlier been perforated at the bottom (four holes) and on the sides (six holes) using hot 6 inch nail, to prevent water logging and to allow smooth aeration in the soil.

CHAPTER FOUR: RESULTS

4.1 Part 1: Molybdenum and rhizobia inoculants

4.1.1 Number of effective nodules

There was a dramatic increase ($P<0.05$) in the number of effective nodules with increase in Mo doses, in the order of 35, 60 and 77% for the respective rates of Mo applied (Table 3). The number of effective nodules was superior with BioStacked inoculant at the lower rates of Mo application ($\leq$0.5 kg Mo ha^{-1}), to that of the Mak Bio Fixer inoculant. However, the reverse was true¸ that the higher rates of Mo application (1 kg Mo ha^{-1}), effective nodules were more for Mak Bio Fixer than for its BioStacked inoculant counterpart.

Table 3: Performance of number of effective nodules in response to Mo application in common bean on Ferralsols in Uganda

	Mo Applied (kg ha^{-1})			
Rhizobia Inoculants	0	0.5	1.0	2.0
No inoculation	0	0	0	0
Mak Bio Fixer	3	5	30	49
BioStacked	16	23	16	31
$LSD_{0.05}$		18.00		

4.1.2 Effective nodule dry weight

Application of the two rhizobia inoculant types, that is, Mak Bio Fixer and BioStacked rhizobia inoculants caused a significant ($P<0.05$) difference in the dry weight of effective nodules (Table 4). However, there was no significant ($P>0.05$) different between the two inoculant types. The control presented no data for this parameter.

Table 4: Performance of rhizobia inoculants on effective nodule dry weight in common bean on Ferralsol in Uganda

Rhizobia Inoculants	**Effective nodule dry weight (g plant^{-1})**
No inoculation	0.00
Mak Bio Fixer	0.19
BioStacked	0.17
$LSD_{0.05}$	0.09

4.1.3 Shoot total nitrogen content

Results for total shoot N and total N minus the control (uninoculated) are presented in Table 5. Discounting for the control from total N values was considered in order to obtain BNF representative values accruing from the study. It is clear that Mo application, with or without rhizobia application, enhanced total shoot N content of the plants. However, the increase in response was greater with rhizobia inoculation than without. Overall, Mak Bio Fixer inoculant maintained superiority over the BioStacked inoculant, ranging from 0.8 folds in 2 kg Mo ha^{-1} to 6.9 folds in the 0.5 kg Mo ha^{-1}. In fact, the no Mo control performed better than 1 and 2 kg Mo ha^{-1} rates.

In terms of the balance after accounting for the control values in total shoot N (values in parentheses in Table 5), which is presumed to be biologically fixed N, there is no definitive response pattern to Mo with or without rhizobia inoculation (Table 5). There was, though a slight decrease in the fixed shoot N fraction in the case of Mak Bio Fixer inoculant, in response to Mo application. For BioStacked inoculant in the case of Mo applied at 1 and 2 kg Mo ha^{-1} rates. It is also important to note application of 0.5 kg Mo ha^{-1} together with BioStacked inoculant decreased total shoot N and ultimately had no detectable fixed N.

Table 5: Molybdenum x Rhizobia inoculants performance on shoot N in common bean

	Mo Applied (kg ha^{-1})			
Rhizobia Inoculants	0	0.5	1.0	2.0
No inoculation	1.15	1.25 (0.10)	5.45 (4.30)	3.98 (3.98)
Mak Bio Fixer	3.02	5.45 (2.43)	5.26 (2.24)	3.46 (0.44)
BioStacked	1.19	0.79 (-0.40)	4.85 (3.36)	4.56 (3.37)
$LSD_{0.05}$	2.17			

*Values in parentheses represent response values after subtracting the control values

4.1.4 Shoot dry matter

There was no significant (P>0.05) interaction effect between Mo rates and rhizobia inoculant types on bean shoot dry matter yield. However, the effect of Mo on shoot dry weight per se was positively significant; application of molybdenum at 1 kg Mo ha^{-1} increased dry weight by 2.7 g $plant^{-1}$, before declining with subsequent Mo application rates.

Table 6: Performance of Mo application on shoot dry weight and plant response differentials without control values

Mo rates (kg ha^{-1})	Shoot dry weight (g $plant^{-1}$)
0	3.18
0.5	2.76 (-0.42)
1	5.86 (2.68)
2	4.04 (0.86)
$LSD_{0.05}$	1.84

*Values in parentheses represent response values after subtracting the control values

4.2 Part 2: Molybdenum, phosphorus and rhizobia inoculants

4.2.1 Number of effective nodules

The 3 factor interaction (P, Mo and rhizobia inoculants) was not significant (P>0.05) on the number of effective nodules per plant. However, application of P with rhizobia inoculants had a significant effect on the number of effective nodules (Fig. 1).

Phosphorus application had a significant effect on the number of effective nodules per plant (Fig. 1); whereby addition of 15 kg P ha^{-1} increased number of effective nodules by 27% for both Mak Bio fixer and BioStacked rhizobia inoculants. Application of P had an overall improvement on nodule number. However, application of P beyond 15 kg ha^{-1} suppressed the effect of the BioStacked inoculant (54%) to a level beyond control, leaving Mak Bio fixer inoculant insignificantly stable (Fig. 1).

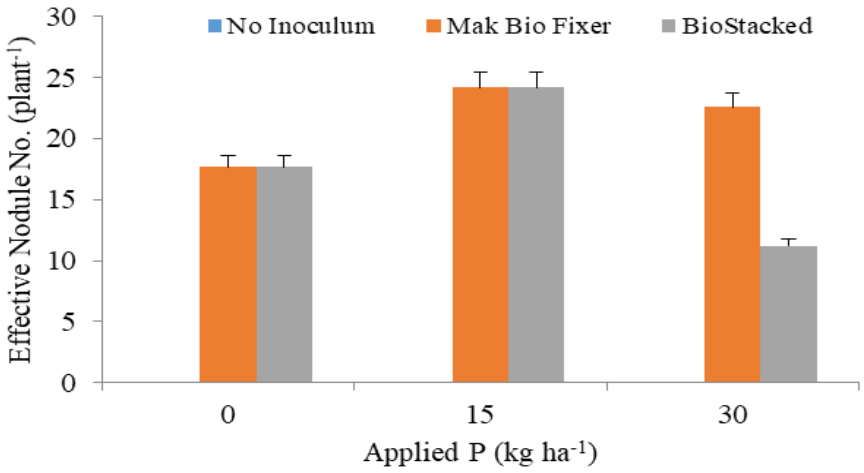

Figure 1: Phosphorus x inoculant types on the number of effective nodules per plant.

4.2.2 Effective nodule dry weight

The 3 factor interaction (P, Mo and rhizobia inoculants) was not significant at (P>0.05) on the effective nodule dry weight per plant. However, application of P with rhizobia inoculants and Mo with rhizobia inoculants had a significant effect on effective nodule dry weight.

Phosphorus application had a significant effect on effective nodule dry weight per plant (Fig. 2); whereby addition of 15 kg P ha^{-1} increased effective nodule dry weight by 65 and 67% of Mak Bio fixer and Biostack rhizobia inoculants, respectively. Although the BioStacked inoculant effect was suppressed (45%) to a level below the control, contrasting with Mak Bio fixer inoculant whose effect remained stable (Fig. 2).

Mak Bio fixer inoculant performed better ($P<0.05$) than the BioStacked inoculant for effective nodule dry weight, for all P application rates (Fig. 2). Addition of P to 15 kg ha^{-1} and Mak Bio fixer inoculant increased nodule dry weight 67%. However, P rate 30 kg ha^{-1}, effective nodule dry weight for Mak Bio Fixer inoculant maintained other than the BioStacked inoculant effective nodule dry weight which declined even below the control.

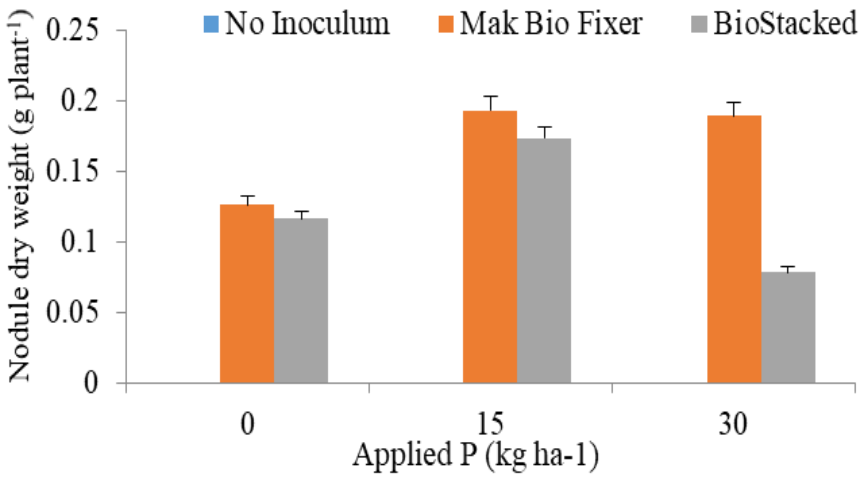

Figure 2: Phosphorus x inoculant types on Effective nodule dry weight (g) per plant.

4.2.3 Plant shoot N content

Total shoot N content with the three factor interaction (P, Mo and rhizobia inoculants) was not significant at ($P>0.05$) on the shoot N content per plant. However, application of P with rhizobia inoculants had a significant effect on shoot nitrogen content per plant (Fig. 3). When 15 kg P ha^{-1} was added, this increased the content of N in the shoot by 54% without rhizobia inoculation. With Mak Bio fixer and BioStacked inoculants, application of 15 kg P rate increased shoot N by 29 and 41%, respectively. Further increase in P rate significantly suppressed shoot N content (15

to 19%), irrespective of the inoculant type. There was a general decline in shoot N content per plant when 30 kg P ha^{-1} was applied in all inoculated and uninoculated treatments (Fig. 3).

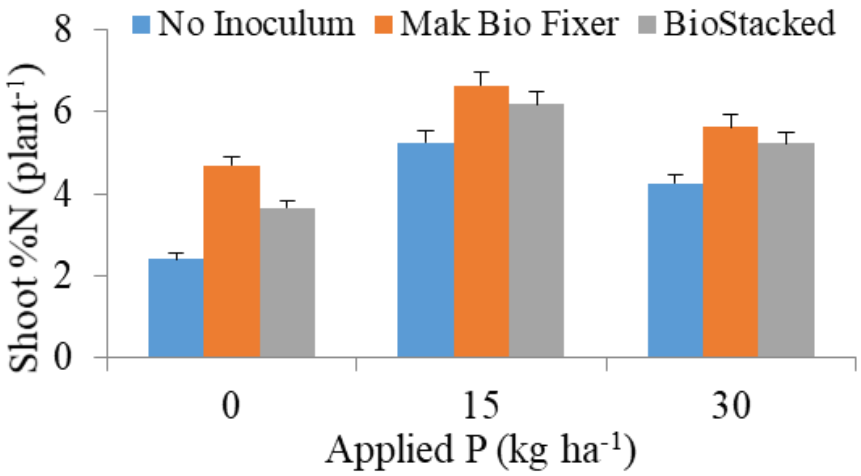

Figure 3: Influence of applied phosphorus and rhizobia inoculants on shoot N on NABE 4.

With regard to the estimated BNF related to shoot N content (total shoot N from inoculated pots minus total shoot N from uninoculated pots), it is clear that application of P elevated shoot N content slightly for all inoculated pots (Fig. 4). Overall, the BioStacked inoculant was more responsive to P application than its Mak Bio Fixer counterpart. On the other hand, application of P at the level beyond 15 kg ha^{-1}, tended to suppress the estimated balance of shoot N by >2 folds in the case of Mak Bio Fixer; and by approximately 1.5 folds for BioStacked inoculant, and uninoculated control.

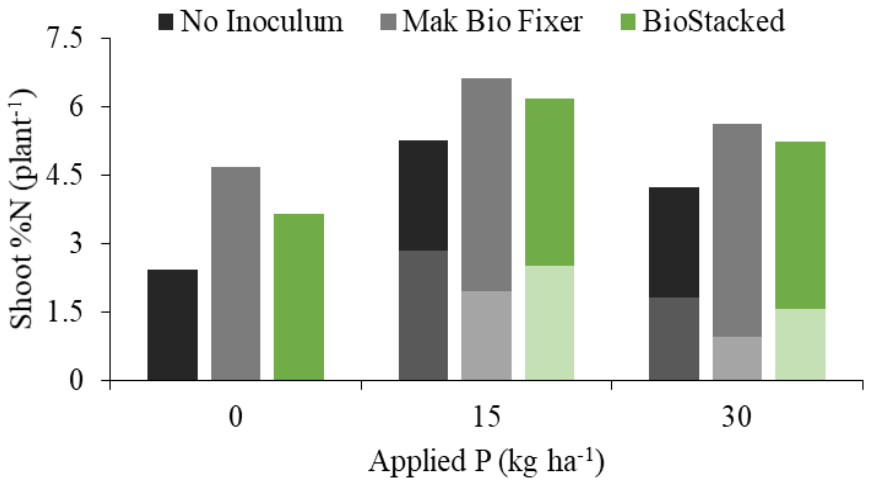

Figure 4: Influence of applied phosphorus and rhizobia inoculants on shoot N on NABE 4. Stacked graph represents estimated N due to BNF, obtained by subtracting total shoot N in uninoculated pots from inoculated pots.

4.2.4 Shoot dry matter

The shoot dry matter with the three factor interaction (P, Mo and rhizobia inoculants) was not significant (P>0.05) on the shoot dry matter per plant. However, application of P with rhizobia inoculants had a significant effect on shoot dry matter per plant (Fig. 5). Application of phosphorus increased shoot dry matter by 32%, when 15 kg P ha^{-1} was applied with Mak Bio fixer rhizobia inoculant. Further increase in P rate suppressed shoot dry matter with both Mak bio Fixer and BioStacked rhizobia inoculants.

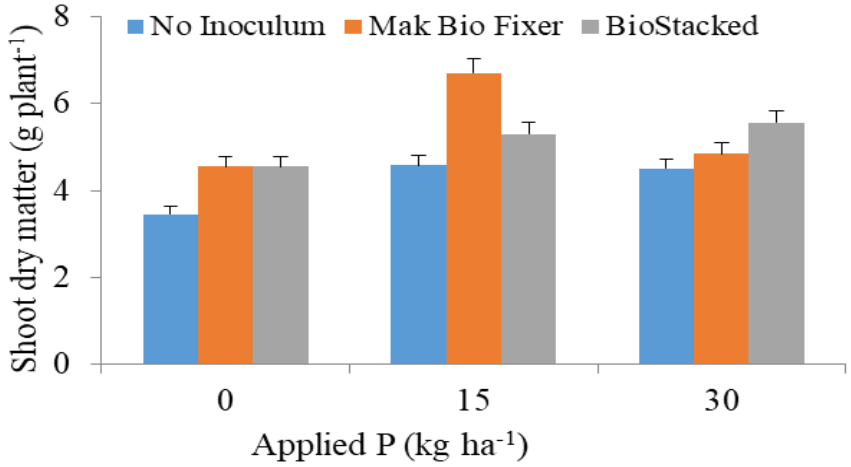

Figure 5: Phosphorus x rhizobia inoculants on shoot dry matter weight (g plant^{-1}) in NABE 4.

With regard to the estimated biologically fixed nitrogen related to shoot dry matter (shoot dry matter from inoculated pots minus shoot dry matter from uninoculated pots), it is clear that application of P elevated shoot dry matter slightly for all inoculated pots (Fig. 6). However, Mak Bio Fixer inoculant was more responsive to P application at lower rate than its BioStacked counterpart. Then again, application of P at the level beyond 15 kg ha^{-1}, tended to suppress estimated balance of shoot dry matter by less than 1.8 folds in the case of Mak Bio Fixer; by approximately a 0.25 folds for BioStacked inoculant, and by 0.08 folds uninoculated control.

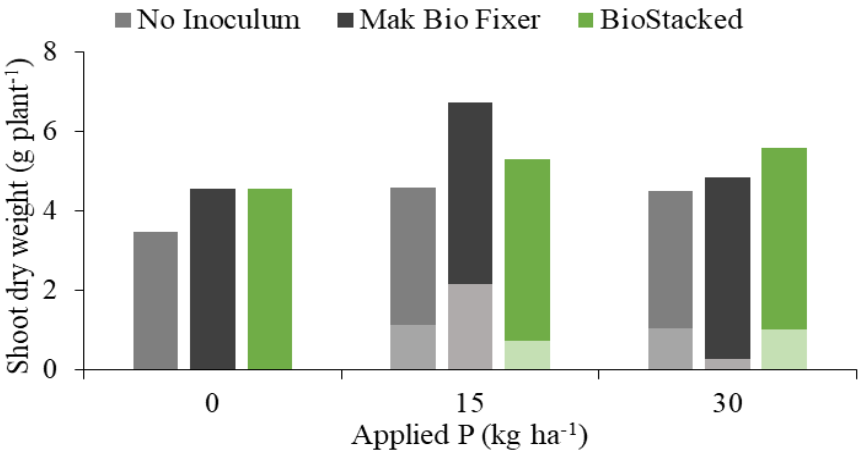

Figure 6: Influence of applied phosphorus and rhizobia inoculants on shoot dry matter on NABE 4. Stacked graph represents estimated shoot dry weight due to BNF, obtained by subtracting total shoot dry matter in uninoculated pots from inoculated pots.

CHAPTER FIVE: DISCUSSION

This section presents a discussion of results in two components, namely Part 1: the effect of Mo under two rhizobia inoculants regimes (Mak Bio Fixer and BioStacked): and Part 2: phosphorus plus molybdenum plus the two inoculants.

5.1 Part 1: Molybdenum versus rhizobia inoculants

5.1.1 Number of effective nodules

The dramatic increase in number of effective nodules per bean plant observed with application of Mo on the study soil (Table 2) is a proxy evidence that this nutrient is insufficient for biological nitrogen fixation. This is the first time soil Mo assessment has been done in Uganda in relation to BNF in grain legumes production. This also explains the inconsistent response results obtained as feedback from farmers who use rhizobia inoculants from BNF Laboratory at Makerere University. In order to promote BNF for common bean production, therefore, there is need for inclusion of Mo in the inoculants package supplied to farmers.

The superiority of number of effective nodules with BioStacked inoculant at the lower rates of Mo application ($\leq$0.5 kg Mo ha^{-1}), compared to Mak Bio Fixer inoculant, is difficult to explain from the study results. However, because the exact components of BioStacked inoculant package were not disclosed at the time of inoculant specimen acquisition from the mother company (Becker Underwood), it may be speculated that Mo could have been included or could have been a contaminant during its processing stages. On the other hand, it is possible that the rhizobia

strain in the BioStacked inoculant could be tolerant to lower concentrations of Mo in the soil to meet its BNF requirements (Paudyal *et al.*, 2007).

The contrasting superior performance of Mak Bio Fixer at the higher rates of the Mo application, compared to its BioStacked counterpart, further demonstrates the sensitivity of Mak Bio Fixer as a native rhizobia strain to the sufficiency of Mo in the soil resulting from its application (Ormeno-Orrillo *et al.*, 2012). The bacterial strain code named CIAT899 (*tropici strain*) in Mak Bio Fixer inoculant has been previously characterized to be tolerant to high temperatures, high levels of acidity and also is symbiotically more stable (Martinez-Romero *et al.*, 1991; Ormeno-Orrillo *et al.*, 2012).

Overall, this study has demonstrated that BioStacked inoculant promotes production of effective nodules more than Mak Bio Fixer, especially at lower levels of Mo application in a Ferralsol. However, in the absence of the BioStacked inoculant, Mak Bio Fixer will benefit greatly from application of molybdenum.

5.1.2 Effective nodule dry weight

The significant effect of applied rhizobia inoculants on effective nodule dry weight (Table 4) is difficult to explain from this study. However, it can be theorized that the inoculant enhancement of BNF in the effective nodules, resulting in better growth of the host plant and greater photosynthetic activity, which in return supplied more photosynthates to the N source nodules, thus to becoming denser and possibly larger in size. It can also be speculated that the expanded nodule size became a sink for further uptake of nutrients from the soil, which possibly also added to the weight of the recipient nodule. Literature related to this phenomena is hard to find, although Thamir *et al.* (1998); Njira *et al.* (2013); Alberton *et al.* (2018), in their studies on BNF

inoculants in soybean observed increases in nodule dry weight with rhizobia inoculation. However, they did not reveal whether the effect was linked with effective nodules as such; neither did they articulately explain their relationships. The superiority of Mak Bio Fixer inoculant over the BioStacked in promoting effective nodule dry weight requires further investigation. But it could be attributed to the former being more adopted to the study soil conditions than the later. Several authors (Ormeno-Orrillo *et al.*, 2012; Sohlenkamp *et al*., 2018), have observed that CIAT 899, the strain used in the present study was versatile at stressful pH values (pH 4.5) with genes encoding response regulators and membrane transporters, plus enzymes involved in amino acids and carbohydrates metabolism and proton extrusion. It also implies that BioStacked inoculant is not as stress tolerant under study soil conditions as has been prescribed by its manufacturers.

5.1.3 Total shoot nitrogen

The significant positive response of total shoot N to Mo application (Table 5), in the absence of evidence of BNF, suggests that the effect of Mo on total N was *via* a path different from that BNF. Molybdenum, apart from being a direct plant nutrient, is known to be part of two enzymes related to nitrogen utilization in plants; namely nitrogenase and nitrate reductase enzymes (Hoffman *et al.*, 2014). Nitrogenase is a complex enzyme responsible for biological nitrogen fixation, either symbiotically or asymbiotically. It comprises of component I (FeMo protein) and component II (Fe protein) within which molybdenum and iron are central of electron transport chain (Hoffman *et al.*, 2014).

On the other hand, nitrate reductase which is largely resident in the roots of higher plants, is responsible for reducing nitrate ions at uptake, to amino forms which are readily and safely

transported within the plants for ultimate utilization (Srivastava, 1980). Although nitrates ions account for the bulk of mineral form absorbed by the plant, they are toxic to plant cells and therefore, cannot be allowed to accumulate in plant tissues. Hence, with or without BNF occurrence in leguminous plants, nitrate reductase remains operational and accounts for most of the nitrogen found in plant tissues (Beevers and Hageman, 2003). It is, therefore, possible that the situation observed in the present study was primarily caused by the nitrate reductase path rather than its nitrogenase counterpart. Although both nitrogen metabolism paths can be dually operational, the nitrate reductase path usually suppresses its counterpart for reasons postulated to be heavy demand for energy of the latter, which the plant often avoids in a bid to save energy for other essential functions (Cannell and Thornley, 2000; Liu *et al.*, 2011).

What is difficult to explain though is the sustained source of nitrate ions in a soil that tested low for total N before establishment of the experiment (Table 2). Other researchers have alleged that increased availability of Mo in the soil boosts the plant's ability to mobilize nitrates from the soil (Mendel and Haensch, 2002; Williams and Frausto da Silva, 2002; Sauer and Frebort, 2003; Kaiser *et al.*, 2005). The occurrence of this phenomenon is difficult to conceptualize based on existing knowledge. On the other hand, the absence of effect of rhizobia inoculants on total shoot N values may be attributed to unfavorable soil conditions for BNF such as inadequate supply of key nutrients like available P which was critically low in the soil before the experiment (Table 2). It is estimated that up to 16 ATP molecules are utilized in BNF process to produce two molecules of ammonia Hence, low supply of P as well as other nutrients greatly impact on the performance of BNF in legume plants (Abdulameer, 2011; Weldu and Habtegebrial, 2013; Habtegebrial *et al.*, 2015). Empirical evidence related to this phenomenon is discussed in sub-section 5.2.1 of this thesis.

With regard to the presumed BNF fraction (balance after discounting for control values in total shoot N, values in parentheses in Table 5), the limited effect of Mo application with or without application of rhizobia inoculants, also echoes the reasons presented above; that is soil P deficiency (Table 2) which could have impacted the performance of nitrogenase enzyme, and subsequently causing a dysfunction in BNF. This observation implies that although Mo was able to increase the number and dry weight of effective nodules with application of rhizobia inoculants (Tables 4 and 5), the functionality of nitrogenase enzyme whose capacity was augmented by Mo may have been impaired by deficiency of P in the soil (Gidago *et al.*, 2011). Again, whether interventions with P has a significant effect on the performance of BNF under the influence of Mo and rhizobia inoculants is discussed in the sub section 5.2.3 of this thesis.

5.1.4 Shoot dry weight

The lack of significant interaction effect of Mo and rhizobia inoculant types on bean shoot dry weight (Table 6), is further evidence that Mo effect had no influence on BNF. In light of this, therefore, the significant effect of Mo per se on shoot dry weight (Table 6) must have been through the nitrate reductase pathway rather than the nitrogenase-BNF pathway. Other studies by Turuko and Mohammed (2014); Fageria and Baligar (2016); Chikowoa *et al.* (2018); suggested that phosphorus application consistently showed a positive response to shoot dry matter on common bean. However, more investigations are necessary to confirm these observations because the authors did not explain the mechanism through which P affected the plant.

5.2 Part 2: Phosphorus plus Molybdenum plus rhizobia inoculants

5.2.1 Number of effective nodules

The lack of the 3 factor interaction (P, Mo and rhizobia inoculants), and yet the presence of a significant effect of Mo by inoculant type on number of effective nodules per plant (Table 3) raises interesting results, in light of the earlier speculation that P was a possible constraint to the performance of nitrogenase enzyme to result in BNF (sub-section 5.1.3). This suggests that the limitation to the performance of nitrogenase enzyme may have been something else rather than P per se. It can also be speculated that the positive interaction effect between P and rhizobia inoculants, with or without Mo (Fig. 1), may have been due to increased efficiency of the performance of nitrogenase, despite the limited quantities caused by low Mo supply under natural soil (Bhuiyan *et al.*, 2008; Bekere and Hailemariam, 2012; Mfilinge *et al.*, 2014; Samago *et al.*, 2018). Nevertheless, further investigation are recommended to verify these inferences.

5.2.2 Effective nodule dry weight

The significant effect of joint application of P and rhizobia inoculants on effective nodule dry weight (Fig. 2), is difficult to explain in the absence of Mo application to this soil. The effect of rhizobia strains manifests through nitrogenase enzyme, which is also a function of Mo (Hoffman *et al.*, 2014). The observation could be explained by the possibility that rhizobia inoculants contained Mo as an intended ingredient, which in part catered for Mo deficiency in the study soil (Table 2). Various inoculant carriers have been reported to contain various levels of Mo, naturally born or deliberately impregnated by human activity (Zhou *et al.*, 2017). Because Mo is required in minute quantities by plants including for BNF, the quantities delivered by the inoculants sometimes over shadow the deficiency of Mo in the soil.

The present study did not consider chemical and biological analysis of the inoculant carriers for levels of Mo. The effect of the interaction between P and rhizobia types on effective nodule dry weight could have been through enhanced performance of the limited quantities of nitrogenase formed as a result of increased Mo availability in the soil. Literature available mainly alludes to the effect of P on number of nodules formed, but not BNF indicators (Samago *et al.*, 2018). Therefore, further research on this subject is necessary to reach logical conclusions related to Mo, P and rhizobia inoculants in this particular soil.

5.2.3 Estimated BNF related to shoot nitrogen content

The slight detectable increase in proxy (estimated) BNF values based on shoot N could be attributed to the explanation provided earlier (sub-section 5.2.1) that increased levels of P due to its application, increased efficiency of the performance of nitrogenase enzyme despite the limited quantities caused by low Mo supply under natural soil . The greater response to P application displayed by the BioStacked inoculant treated plants, suggests that this strain is not as tolerant to abiotic stresses as prescribed by the supplier company. On the other hand, the more stable performance of Mak Bio Fixer inoculant in terms of this subject seems to mark the fact that the inoculant is more adopted to the study conditions than its BioStacked counterpart (Martinez-Romero *et al.*, 1991).

5.2.4 Estimated BNF related to shoot dry weight

The insignificant response of estimated BNF values of the shoot dry weight could be attributed to the effect of the applied phosphorus. The phosphorus in soil is known to influence plant nutrient uptake and hence, increase the vigor of the plant parts (Mfilinge *et al.*, 2014); plants grow vegetatively high when nitrogen and phosphorus are available for biochemical processes.

The positive response to P application with Mak Bio Fixer rhizobia inoculant treated plants, gives an impression that this strain is adapted to conditions presented in this study environment (Ormeno-Orrillo *et al.*, 2012). On the other hand, the slight response to P application of BioStacked rhizobia inoculant is not as tolerant to abiotic stresses as prescribed by the supplier company.

CHAPTER SIX: CONCLUSIONS, RECOMMENDATIONS AND FUTURE RESEARCH

6.1 Conclusions

From the study, the following conclusions can be drawn:

(i) Common bean grown on a Ferralsol in Uganda, under rhizobia inoculation with a local and imported strain respond to Mo application through increase in number of effective nodules and their dry weights. Application of Mo at rates of 0.5 to 1 kg ha^{-1} yields best results related to BNF overall. However, discounting total shoot N of control pots from Mo treated pots indicates no evidence of biological nitrogen fixation.

(ii) Intervening in case of Mo in 1 above, with P application does not significantly change the status of estimated BNF generated by common bean grown under rhizobia inoculation with a local and imported strain. This implies that energy requirement for biological fixation of N is not the key limiting factor for BNF process in this study.

(iii) In terms of stress resistance, the BioStacked rhizobia inoculant tends to perform better without Mo and/or P application than its Mak Bio Fixer counterpart.

(iv) The consistent response of total shoot N to Mo application can be attributed to active operationisation of the nitrate reductase pathway which in effect directly benefited the crop while possibly shutting down the high energy demanding BNF pathway. However, it is difficult to account for the source of nitrate when the level of Kjeldhal N was below the critical value for plant growth in this Ferralsol use.

6.2 Recommendations

This study was conducted in a screen house mostly under controlled conditions; hence, there is need for field studies to validate the results before recommendations are made for farmer application. Nevertheless, the results for this study point to a beneficial practice of Mo and rhizobia inoculant application; the former of which can be administered while producing the rhizobia packages. Application Mo at rates of 0.5 to 1 kg ha^{-1} yielded best results related to BNF overall.

6.3 Future research

During the implementation of this study, a number of issues that were beyond the scope of this study emerged and these need to be considered.

(i) Further research is necessary to include measurement of mineral N (nitrate and ammonium) at the start of the study. Also a direct method measuring biologically fixed N for instance the ureid test will be necessary. This was not possible in the present study due to limitations with resources. Hence, we used proxy methods for estimating BNF; namely effective nodule number and weight and difference between total plant N in the inoculated plot and that uninoculated control.

(ii) The need for application of molybdenum requires evaluation of the different methods of Mo administration to achieve best results without harming rhizobia cells.

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