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EFFECT OF RHIZOBIUM INOCULATION, MOLYBDENUM AND LIME ON THE GROWTH AND N_2 FIXATION IN *P. VULGARIS* L.

By

BAMBARA SYLVIE KARUMEYI

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Supervisor: Assoc. Prof. Patrick A. Ndakidemi

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DECLARATION

I declare that this thesis is my own work. It is being submitted for the Master degree in Horticulture in the Faculty of Applied Sciences, Cape Peninsula University of Technology. It has not been submitted for any degree or examination at any other University.

Signed Date: December 2009

DEDICATION

I dedicate this thesis to my family especially those lost during my quest for knowledge.

Rwakigarama G. Gervais

Father

(1 August 1939-23 March 2007)

Karumeyi Christelle

Daughter

(12 January 2009-18 April 2009)

ABSTRACT

The study used common bean (*P. vulgaris* L. variety Provider) in a spilt-split-plot design involving 2 levels of *Rhizobium* inoculation (with and without rhizobia), 3 levels of lime (0, 2 and 3 $t.ha^{-1}$) and 3 levels of molybdenum (0, 6 and 12 $g.kg^{-1}$ of seeds) in a glasshouse experiment. The glasshouse experiment was then verified in the field during 2008 and 2009 cropping seasons. The aim was to assess the effects of *Rhizobium* inoculation, molybdenum and lime supply on: i) yield and yield components of the *P. vulgaris* L. ii) Changes in soil pH and the concentrations of selected plant-available nutrients in the rhizosphere, iii) photosynthesis and chlorophyll formation in *P. vulgaris* L. and (iv) plant growth and N_2-fixation in *P. vulgaris* L.

The results showed that *Rhizobium* inoculation had significant effects in increasing yield components and ultimately the final seed yield. Rhizobial inoculation also significantly increased the levels of chlorophyll content in leaves, improved all photosynthetic parameters, increased dry matter yield of different organs and decreased $\delta^{15}N$ values in all organs assessed. As a result, % nitrogen derived from atmosphere (%Ndfa) in all organs as well as the amount of N derived from fixation was improved. In the field, the whole plant level of N-fixation of *P. vulgaris* L. from *Rhizobium* inoculation accounted for approximately 33 kg $N.ha^{-1}$. Furthermore, soil pH and the concentration of mineral nutrients (P, K, Ca, Mg, Na, Fe, Cu, Zn and Mn) in the rhizosphere were significantly increased with *Rhizobium* inoculation when compared with the control.

Molybdenum supply also differentially affected many parameters measured. For example, Mo supply significantly increased the number of $pods.plant^{-1}$, number of $seeds.plant^{-1}$, 100-seed weight and seed yield. In general, these parameters were significantly increased with molybdenum supplied at the highest rate of 12 $g.kg^{-1}$ of seed. With regard to plant growth, some growth parameters in the glasshouse and field experiment (i.e. dry matter yield for shoots, pods and whole plant) were significantly greater at different levels of Mo supply compared with zero control. Isotope analysis showed that the $\delta^{15}N$ values of roots, shoots, pods and whole-plant of both glasshouse and field experiments were reduced with Mo supply. Relative to zero control treatment, Mo supplied at 6 and 12 $g.kg^{-1}$ of seed significantly decreased the $\delta^{15}N$ of their roots, shoots, pods, and whole plants. The lowest $\delta^{15}N$ values were always recorded in the treatment supplied with 12 g $Mo.kg^{-1}$ of seed. This pattern resulted into more N fixation in *P vulgaris* L.

For example, relative to the zero control in the field study, the application of Mo at 6 and 12 $g.kg^{-1}$ of seeds increased significantly the N fixed (kg $N.ha^{-1}$) by 45% and 71% respectively.

In this study, application of lime significantly increased leaf chlorophyll content (Chl), the photosynthesis (A), the intercellular CO_2 concentration (Ci), transpiration rate (E), number of $seeds.pod^{-1}$ and the final seed yield. Highest significant values were observed on treatments with lime rate of 3 $t.ha^{-1}$ compared with the control and 2 t $lime.ha^{-1}$. The soil pH and the exchangeable Ca and Mg level in the rhizosphere were also significantly increased with lime application when compared with the control treatment. Significant interactions were also noted between different combinations of *Rhizobium* inoculation x Mo x Lime.

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CHAPTER 1

THE POTENTIAL ROLES OF LIME AND MOLYBDENUM ON THE GROWTH, N_2 FIXATION AND ASSIMILATION OF METABOLITES IN NODULATED LEGUME: A SPECIAL REFERENCE TO *P. VULGARIS* L.

1.1 Introduction

The common bean (*P. vulgaris* L.) is a major vegetable legume grown and consumed in Southern Africa. *P. vulgaris* L. yields in Southern Africa are reported to be very low (Mukoko *et al*., 1995; Mloza-Banda *et al*., 2003) and the average yield for the African continent being only 650 $kg.ha^{-1}$ (Singh, 1999). The poor yields are partly due to infertility caused by acidic soils which have low nutrient contents including Ca^{2+} (Lunze *et al*., 2007; Wortman *et al*., 1995 and 1998) and Mo content (Liebenberg, 2002). Research efforts at improving bean yields in Africa have increased over the past few decades, where the main emphasis focused on improving nitrogen and phosphorous nutrition (Anderson, 1974; Choudhury *et al.*, 1983; Ssali and Keya, 1986; Smithson *et al*., 1993; Amijee and Giller, 1998; Giller *et al*., 1998; Lunze *et al*., 2007). Therefore, it is important to assess effects of other limiting nutrients to plant growth such as Ca^{2+} and Mo.

Soil acidity may affect all stages of growth and specifically the legume- rhizobium symbiosis, from strain survival in soil and on the seed, to root-hair infection, nodule initiation and nitrogen fixation (Munns, 1978; Keyser and Munns, 1979; Graham *et al*., 1982; Wood *et al*., 1984). Higher concentrations and contents of hydrogen ion, aluminium and manganese in acidic soils are known to be the major causes of poor growth to plants due to their toxicity effects to plants and micro organisms such as N fixing bacteria (Munns, 1978; Graham *et al*., 1982, 1992; Peoples *et al*., 1995).

The most common management practice to ameliorate acid soils is through the surface application of lime (Bolan *et al*., 2003). The major influence of lime when applied in the soil is on its ability to supply Ca^{2+} which is essential for plant growth (White and Broadley, 2003) and neutralizing the toxicity effects of H^{+}, Al^{3+} and Mn^{2+} in the soil (Staley and Brauer, 2006). Lime may also increase soil pH resulting in negative charges on soil particles and therefore, decreasing the activities of iron and aluminium oxides which are good sinks for Mo in soils (Mandal *et al*., 1998). It is therefore justifiable to introduce lime in acidic soils with low Ca^{2+} levels such as those found in some parts of Southern Africa.

Molybdenum is a trace element found in the soil and is required for growth of most biological organisms including plants (Anderson, 1956; Agarwala *et al*., 1978; Gurley and Giddens, 1969; Franco and Munns, 1981; Graham and Stangoulis, 2005; Purvis, 1955). Similar to other metals required for plant growth, molybdenum has been utilized by specific plant enzymes as a co-factor that participate in reduction and oxidative reactions in plants (Mendel and Hänsch, 2002; Williams and Frausto da Silva, 2002). Generally, molybdenum is an essential micronutrient for plants and bacteria. In some parts of southern Africa, several cases of Mo deficiency have been identified in a variety of crops including maize, Lucerne, fruits,

vegetables and other crops (Pienaar and Bartel, 1968; Tanner, 1978 and 1982; Rhodes; and Kpaka, 1982; Kang and Osiname, 1985; Thibaund, 2005).

Molybdenum deficient plants exhibit poor growth and low chlorophyll content (Gupta and Lipsett, 1981; Gupta *et al*., 1991; Marschner, 1995). Molybdenum is also a component of some bacterial nitrogenase, and therefore is especially important for plants that live in symbiosis with nitrogen-fixing bacteria (Gupta *et al*., 1991; Hale *et al*., 2001) such as (*P. vulgaris* L.) that is widely grown as a vegetable crop in Southern Africa. Experiments with soybean have shown that molybdenum fertilization in deficient soils enhanced nitrogen-fixation through increased nitrogenase activity rates and increased nodule sizes (Parker and Harris, 1977; Adams, 1997).

In some parts of Southern Africa, reports indicate that soils are acidic, and hence both Ca^{2+} and Mo are inadequate to support good plant growth (Lunze *et al*., 2007; Ndakidemi, 2005; Thibaund, 2005; Pienaar and Bartel, 1968; Rhodes and Kpaka, 1982 and Tanner, 1978 and 1982). In agricultural soils, molybdenum is strongly held into positively charged metal oxides in acidic soils of up to pH 5.5 (Smith *et al*., 1997). Research evidence indicates that as the soil solution becomes more alkaline, the MoO_4^- availability to plants and other forms of life increases (Lindsay, 1979; Brady and Weil, 2008). Consequently, the application of lime to agricultural soils may be an important tool to adjust soil pH and increase soluble molybdate (Kaiser *et al*., 2005). Therefore, it is important to understand the fundamental reactions of these important mineral nutrients (lime and molybdenum) at different stress levels. This review outlines how leguminous plants such as *P. vulgaris* L. plants may respond and benefit to lime and Molybdenum in relation to growth, yield, N_2 metabolism and in the production of metabolites such as phenolics compounds and phosphatase enzymes in their tissues and the rhizosphere.

1.2 Effects of selected mineral nutrients on phenolic compounds metabolism

Phenolic compounds such as flavonoids and anthocyanins are diverse group of phytochemicals that are produced by various plants in high quantities (Dixon and Steele, 1999). They exhibit a wide range of biological activities mainly from their antioxidant properties and ability to modulate several enzymes or cell receptors (Hodek *et al*., 2002).

Flavonoids play an important role in plant growth and development, and in defence of plants against micro organisms and pests serving as means of plant-animal warfare (Dixon and Harrison, 1990; Dixon and Steele, 1999; Ndakidemi and Dakora, 2003; Makoi and Ndakidemi, 2007).

The pathway of phenolic compounds biosynthesis in plant species is highly regulated (Hasegawa and Maier, 1981). For instance, constitutive levels of flavonoids are produced during normal growth and development, but additional formation of specific compounds can be induced by wounding, attack by pathogens and other mineral nutritional stresses (Stafford, 1990; Ndakidemi and Dakora, 2003; Makoi and Ndakidemi, 2007) such as those involving Ca^{2+}, Mg^{2+} and molybdenum.

Nutrient stress has a marked effect on phenolic levels in plant tissues (Rengel, 1999; Makoi and Ndakidemi, 2007). Phosphorus, sulphur, iron, calcium or magnesium starvation stimulates the production of phenolics in plant tissues (Gerschenzon, 1983; Ndakidemi and Dakora, 2003; Makoi and Ndakidemi, 2007). Therefore, phenolic compounds may be considered as an essential factor for a plant's adaptive success in diverse environments (Ndakidemi and Dakora, 2003, Makoi and Ndakidemi, 2007) such as those stressed with calcium or magnesium and molybdenum.

Phenolic compounds are known to prevent microbial degradation of ectoenzymes (phosphatases) and/or organic acids released by the roots as the response to the nutritional deficiencies (Neumann and Römheld, 2001). The exudation of phenolic compounds from the roots of nutrient- starved plants seems to be an important way by which plants can respond to their environment. By modifying the biochemical and physical properties of the rhizosphere, plants increase nutrient availability and buffer the effect of hostile surroundings (Makoi and Ndakidemi, 2007). Although the fate of exuded phenolics in the rhizosphere and the nature of the reactions they are involved in within soils remain poorly understood, phenolics in the soil may clearly contribute significantly to plant growth and development.

Nutrient deficiencies may induce important modifications in several primary metabolic pathways such as in sugar metabolism and secondary metabolism (Gerschenzon, 1983; Ndakidemi and Dakora, 2003; Makoi and Ndakidemi, 2007) and finally influencing the final seed yield. The alteration of metabolism of phenolic compounds under nutrient stress seems to be a response that allows the plant to adapt and survive in harsh environments. Whatever physiological mechanism involved, the enhanced phenolic metabolism under nutrient scarcity may help plants to face the unfavourable environment (Aoki *et al*., 2000). As lime and molybdenum may play a crucial role in *P. vulgaris* L. grown in poorly depleted soils, their influence on the metabolism of phenolic compounds such as flavonoid and anthocyanin and the ultimate effects on plant growth needs to be further investigated and reported.

1.3 Effects of lime and molybdenum on phosphatase enzyme activity

Soil enzymes serve several important functions. They are involved in the cycling of nutrients, affect fertilizer use efficiency, reflect the microbiological activity in soil and act as indicators of soil change (Dick *et al*., 2000; Ndakidemi, 2005 and 2006). Enzyme activities in soil are known to serve as an indicator of soil health and to mediate and serve as a catalyst for soil functions such as organic matter decomposition, release of inorganic nutrients for plant growth, N_2 fixation, and detoxification of xenobiotics, nitrification, and denitrification (Dick, 1997; Nadiya *et al*., 2000; Makoi and Ndakidemi, 2008).

Phosphatase enzymes are believed to play a major role in transformation of organic phosphorous (P) into mineral P (Speir and Ross, 1978; Tabatabai, 1994; Makoi and Ndakidemi, 2008). They are produced when plants and soil micro organisms are subjected to stress such as P (Ndakidemi, 2005 and 2006; Makoi and Ndakidemi, 2008) Ca^{2+} (Speir and Ross, 1978; Bremner and Mulvaney, 1978) and Mo (Sugiura *et al*., 1981; Gellatly *et al*., 1994; Guo *et al*., 1998; Bozzo *et al*., 2002; Lopez *et al*., 2007).

Changes in soil pH can affect the activity of enzymes in the rhizosphere and plants (Dick *et al*., 2000). The pH can affect enzyme activity by influencing the concentration of inhibitors or activators in the soil solution and the effective concentration of substrate (Dick *et al*., 2000). From this background, the sensitivity of soil enzymes to pH should make it possible to evaluate the effective pH and the relative activity of phosphatase enzymes when lime and Mo are supplied in the cropping system.

Evidence in the literature shows that lime, organic and different forms and types of inorganic amendments added to soil influenced the levels of phosphatase enzyme activities (Bremner and Mulvaney, 1978; Speir and Ross, 1978; Ndakidemi, 2005). For instance, in acid soil, addition of lime generally increases sulfatase activities and decreases phosphatase activities in plants whereas the addition of phosphate fertilizers decreases activities of phosphatase, sulfatase, and urease (Haynes and Swift, 1988; Ndakidemi, 2005 and 2006). It is worth investigating if a similar trend is observed in *P. vulgaris* L. plants supplied with lime.

Molybdenum has been described as a potent inhibitor of acid phosphatase activity (Lopez *et al*., 2007). Some studies carried out with phosphatases extracted from different species of plants showed an inhibition with the addition of Mo (Sugiura *et al*., 1981; Gellatly *et al*., 1994; Guo *et al*., 1998; Bozzo *et al*., 2002; Lopez *et al*., 2007). In a study involving tomato, the addition of small amounts of molybdenum (0.0028 mM of molybdate) significantly inhibited the activity of acid phosphatase (Bozzo *et al*., 2002). In another study involving a legume, Guo *et al.* (1998) reported an inhibitory effect of micromolar concentrations of molybdate on the activity of phosphatase from the cytosolic fraction of pea plumules. Other studies have

observed a strong inhibition of acid phosphatase activity from Irish potato and sweet potato supplied with low levels of Mo (Sugiura *et al*., 1981; Gellatly *et al*., 1994).

Soil amendment through the addition of Mo and lime may result in the production of acid and alkaline phosphatases into ratios that will influence plant growth and development at different levels. Lack of adequate information on this aspect has prompted us to focus along this direction.

1.4 Effects of lime and molybdenum on photosynthesis and chlorophyll formation

Light is the environment factor that has most influence on growth and yield quantity and quality of crops through its influence on photosynthesis and chlorophyll formation (Montanaro *et al*, 2007). If other factors are not limiting, high light intensity generally stimulates photosynthesis and hence plant growth (Sattelmacher *et al*., 1993). Other factors such as macro and micro nutrients may also affect the metabolic reactions in photosynthetic apparatus (Marschner, 1995). It is well established that inadequate levels of any mineral nutrient in the growth media may limit photosynthesis due to their involvement in carbohydrate synthesis (Lambers *et al*., 1998). Calcium (Ca^{2+}) and Molybdenum (Mo) are essential plant nutrients; whose role has been well documented (Marschner, 1995; White and Broadley, 2003). Ca^{2+} is involved in several biochemical and physiological processes in plants (Hepler and Wayne, 1985). The structural role of apoplastic Ca^{2+} is particularly important in cell wall and on the shelf- life of plant tissues (Bauchot *et al*., 1999).

Calcium may function directly in several aspects of photosynthesis. It appears to modulate activity of the phosphatase enzymes in the carbon reduction cycle in the synthesis of different sugar components (Haupt and Weisenseel, 1976; Brand and Becker, 1984). Some research evidence also supports a calcium function in the water-splitting complex, and other evidence suggesting its role in a reaction centre in the photosystem II (Brand and Becker, 1984). As light is absorbed, this catalytic centre drives the photosynthesis process (Marschner, 1995). In the chlorophyll molecule embedded in a protein there is a catalytic centre of photosynthetic water oxidation, which is composed of a Mn^4Ca cluster. It is obvious that supply of Calcium through lime may have a significant influence on the photosynthesis process at cellular level in *P. vulgaris* L. plants.

In practice, calcium deficiency is corrected by supplying the agricultural lime or other sources of calcium. For instance, XiaoJun *et al.* (2004) showed that the photosynthesis and photosynthetic efficiency of the leaves of rice were significantly enhanced with the supply of Ca^{2+}. In this study, levels of net photosynthetic rate, stomatal conductance, the contents of chlorophyll, and soluble sugar of the leaves increased more significantly than the control treatment without Ca^{2+}. Furthermore, addition of Ca^{2+}

decreased the content of malondialdehyde and the permeability of cell membrane, but increased the superoxide dismutase activity (XiaoJun *et al*., 2004) and hence affecting photosynthesis.

Molybdenum (Mo) is an essential micronutrient for plants. It plays an important key role in chlorophyll synthesis. In plants, it is absorbed as MoO_4^{2-}. In Southern Africa, several cases of Mo deficiency have been identified in variety of crops (Kang and Osiname, 1985; Pienaar and Bartel, 1968; Rhodes; and Kpaka, 1982; Tanner, 1978 and 1982; Thibaund, 2005). Molybdenum deficiency of soil is a widespread agricultural problem that induces yield and quality losses in many crop species worldwide (Liu, 1991, 2001 and 2002). Molybdenum deficient plants exhibit poor growth and low contents of chlorophyll and ascorbic and shows reduced leaf blade formation, inter-veinal mottling and chlorosis around edges and tips of older leafs (Marschner, 1995; Liu, 2002).

It is generally accepted that legumes need more Mo than most of other plants (Mcbride, 2005) due to its key involvement in the Nitrogen fixation process. Various studies have reported that application of Mo enhances the yield in crops that grow in deficient soil (Liu, 2001; Min *et al*., 2005; Xue-Cheng *et al*., 2006).

Therefore, it is important to establish verify and quantify the influence of Ca^{2+} and Mo supplied as lime or Molybdenum salts or their interaction on different photosynthetic activities in *P. vulgaris* L. grown under different conditions in southern Africa.

1.5 Effects of lime and molybdenum on nitrogen fixation

The mineral nutrient nitrogen is a constituent of all proteins, nucleic acids and many other biomolecules and it is essential in all living organisms (Marschner, 1995 McCammon, and Harvey, 1987). In plants, nitrogen is the most limiting nutrient for growth (Verhoeven *et al*., 1996). Leguminous plants in partnership with *Rhizobium* have the ability to convert the atmospheric nitrogen into usable forms (Galloway *et al*., 1995). Nitrogen fixation involving symbiotic association between rhizobia in legumes is influenced by several factors including Ca^{2+} and Mo (Kucey and Hynes, 1989; Bottomley, 1992; Graham *et al*., 1992; Tu, 1992; Banath *et al.,* 1996; Andrade *et al*., 2002). According to established guidelines, some areas in the Southern Africa have been reported to be deficient in Ca^{2+} and Mo (Ndakidemi, 2005; Thibaund, 2005) and these may have N_2 fixation limitations.

In nutrient deficient soils, soil mineral distribution involving Ca^{2+} and Mo is mostly related to pH levels. For instance, calcium and molybdenum become scarce at acidic pH (Brady and Weil, 2008) and the exchange site are mainly dominated by aluminium (Al) and manganese (Mn) ions. At elevated levels, these ions may reduce N_2 fixation by injuring the host plant or interfering with nodulation or N_2 fixation

processes (Kamprath and Foy, 1985). Under such circumstances, exogenous supply of Ca^{2+} and Mo into the growth media is important.

Calcium supplied to plants through lime may perform multiple functions in plants. They are essential component in symbiotic N_2 fixation and nodule formation in legumes. Studies have indicated that Calcium deficiency in legumes depressed the calcium content of nodules, impairing nitrogen fixation due to inadequate calcium for nodule structure and/or metabolism (Banath *et al.,* 1996; Graham, 1992). In this context, Ca^{2+} deficiency in legume decreased the supply of fixed nitrogen from nodules to other organs, thus impairing plant growth.

With regard to Molybdenum, it is known to have a notable influence on nitrogen metabolism in N_2 fixing legumes (Vieira *et al*., 1998. Marschner, 1995; Parker and Harris, 1977; Franco and Munns, 1981). In nodulated legumes, Mo is necessary for the reduction of atmospheric nitrogen (N_2) to ammonia by nitrogenase. The symbiotic bacteria require about ten times more Mo for N_2 fixation than does the host plant (for protein synthesis). For this reason, Mo deficiency will commonly occur in legumes before it does in other plants, when grown in the same soil (Thibaund, 2005). Molybdenum is also essential for nitrate reductase and nitrogenase enzyme activity (Westermann, 2005). The symbiotic bacterial enzyme nitrogenase is comprised of MoFe protein which is directly involved in the reduction of N_2 to NH_3 (Lambers *et al*., 1998) during fixation process. Supply of Mo to bacteroids is therefore an important process and most likely a key regulatory component in the maintenance of nitrogen fixation in legumes that may influence plant growth (Kaiser *et al*., 2005).

When leguminous plants are grown under molybdenum deficiency conditions, phenotypes with hindered and/or retarded plant growth characteristics may develop. Most of these phenotypes may be associated with reduced activity of molybdoenzymes (Agarwala and Hewitt, 1954; Spencer and Wood, 1954; Afridi and Hewitt, 1965; Randall, 1969; Jones *et al*., 1976; Agarwala *et al*., 1978). These enzymes include the primary nitrogen assimilation enzymes such as nitrate reductase (NR), and the nitrogen-fixing enzymes nitrogenase found in bacteroids of legume nodules (Vieira *et al*., 1998). Other molybdoenzymes have also been identified in plants including xanthine dehydrogenase/oxidase involved in purine catabolism and ureide biosynthesis in legumes, aldehyde oxidase and sulfite oxidase (Mendel and Haensch, 2002; Williams and Frausto da Silva, 2002). Generally speaking, we can conclude that, Molybdenum deficiency is primarily associated with poor nitrogen health in plants and ultimately impaired growth.

Research reports have indicated the stimulating influence of Mo in N_2 fixation in legumes. In their research, (Gurley and Giddens, 1969; Franco and Munns, 1981; Ishizuka, 1982; Brodrick and Giller, 1991) showed that Mo supply in legumes increased molybdenum concentrations in nodules, improving N_2 fixation, development of seeds and other tissues. Experiments with soybean and common bean have

shown that molybdenum fertilization enhanced nitrogen-fixing symbiosis through increased nitrogenase activity rates and larger nodule formation (Parker and Harris, 1977; Adams, 1997; Vieira *et al*., 1998).

Despite of the existence of substantial evidence on the influence of lime and Mo on nitrogen fixation in pasture legumes and other related crops in Southern Africa, their effects and interaction on N_2 fixation in *Phaseolus vulgaris* in some parts of Africa is not documented.

1.6 Effects of lime and molybdenum on growth and yield of legumes

Plant needs some macro- and micronutrients for their normal growth. Some of these elements play vital roles in different growth process. For instance, research evidence suggests that Calcium and Mo deficiency in legumes can restrict plant growth through different mechanisms (Evans *et al*., 1950; Evans and Purvis, 1951; Marschner, 1995).

Calcium deficiency is known to restrict the amount of N_2 fixed in legumes, hence resulting into reduced plant growth due to inadequate nitrogen which is required as building blocks of proteins (Dutta, 2004). On the other hand, plants with severe Ca^{2+} deficiency have shown low levels of nitrogen in their tissues and this has always been associated with poor growth.

Studies by Lucrecia *et al.* (1987) demonstrated that supply of Ca^{2+} through lime significantly increased both nodule weight and plant productivity. In an experiment done by Hartley *et al*. (2004), lime supply increased nodulation and yield of Serradella (*Ornithopus compressus*). The beneficial effects of liming on nodulation and plant growth most likely resulted from the enhanced conditions for seedling growth and nodulation.

With regard to Mo, it is well known that leguminous plants are very sensitive to Mo deficiency, but excess Mo also may impair growth, decreases the biomass, seed yield and deteriorates the quality of production (Kevresan *et al*., 2001; Liu and Yang, 2000; Nautiyal and Chatterjee, 2004). During different growth processes in plants and in legumes in particular, Mo is involved in a number of different enzymatic processes (Marshner, 1995; Vieira *et al*., 1998). For example, molybdenum is a constituent of nitrogenase enzyme, and *Rhizobium* bacterium fixing nitrogen needs molybdenum during the fixation process (Vieira *et al*., 1998). Therefore, with this task, molybdenum has a positive effect on growth, yield, N content of foliage and roots, nodule forming in legume crops (Kliewer and Kennedy, 1978; Togay *et al*., 2008). With regard to other aspects of plant nutrition, molybdoenzymes are involved in nitrogen metabolism, improving qualities of ascorbic acid, soluble sugar, and chlorophyll concentrations (Zhao and Bai, 2001; Chen and Nian, 2004). Therefore, its deficiency may show overall reductions in plant growth and

development, expose the plant to susceptibility to pest damage, and poor pod and/or grain development (Graham and Stangoulis, 2005).

Although there is considerable literature on the beneficial effects of liming and Mo on legume growth in other parts of the world (Staley and Brauer, 2006), site specific factors can yield different results. As Ca^{2+} and Mo or their interaction may play important role(s) in legume growth, these mineral nutrients warrants further investigations both to ascertain their effects on plant growth and development in common legumes grown by farmers such as *P. vulgaris* L.

In conclusion, lime and molybdenum are essential nutrient for legumes growing in acidic soils deficient in Ca^{+2}, Mg and Mo. Liming application in particular, is recommended for most legume species to counter deleterious effects of soil acidity and the availability of mineral elements such as Ca^{+2}, Mg. Liming increase plant growth, accumulation of plant metabolites, nitrogen fixation, dry matter and final seed yield of legumes.

On the other hand, molybdenum nutrition is an essential component in legumes. Molybdate which is the predominant form available to plants is required at very low levels where it participates in various redox reactions in plants. In symbiotic legumes, the enzyme nitrogenase is comprised of MoFe protein that is directly involved in the reduction of N_2 to NH_3 and finally to other available forms of N to plants.

Much more research is required to ascertain the usefulness of this important mineral nutrients and how they may further be used in future to support the expanding legume cultivation in areas where soil Mo and Ca and/or Mg profiles limit plant growth and productivity, such as those found in acidic environments of Africa.

Thus, the main Objective of the study was to explore the effects of lime and molybdenum on growth and yield of the *P. vulgaris* L. The specific objectives were:

1) To assess the effects of *Rhizobium*, molybdenum and lime on the growth and yield in *P. vulgaris* L.
2) To assess the effects of *Rhizobium*, molybdenum and lime on the photosynthesis and chlorophyll formation in *P. vulgaris* L.
3) To assess the effects of *Rhizobium*, molybdenum and lime in the rhizosphere soil of *P. vulgaris* L.
4) To assess the effects of *Rhizobium*, molybdenum and lime on the nitrogen fixation in *P. vulgaris* L.

CHAPTER 2

P. VULGARIS RESPONSE TO *RHIZOBIUM* INOCULATION, LIME AND MOLYBDENUM IN SELECTED LOW PH SOIL IN WESTERN CAPE TOWN, SOUTH AFRICA.

2.1 Introduction

Soil acidity is a major factor that affects plant growth in many countries (Xu *et al*., 2002; Bolan and Hedley, 2003; Godsey *et al*., 2007). Soil acidity is therefore, a main hindrance to the availability of bases such as Ca and Mg and other nutrients such as Mo and N may secondarily influence the growth and yield in legumes (Munns, 1970; Bell *et al*., 1989). These constraints may be ameliorated by supplying lime, Mo and inoculation of legumes with the appropriate *Rhizobium*.

Leguminous plants needs Ca, N, and Mo for their normal growth. Some of these elements play vital roles in different growth process. For instance, research evidence suggests that Ca, Mo and N deficiency in legumes can restrict plant growth through different mechanisms. For example, calcium deficiency is known to restrict the amount of N_2 fixed in legumes, hence resulting into reduced plant growth due to inadequate nitrogen which is required as building blocks of proteins. On the other hand, plants with severe Ca deficiency have shown low levels of N in their tissues and this has always been associated with poor growth (Evans *et al*., 1950; Evans and Purvis, 1951; Marschner, 1995; Dutta, 2004).

Studies by Shoemaker *et al*. (1961); Adams and Evans (1962); Curtin *et al*. (1984); Edmeades *et al*. (1985); Lucrecia *et al*. (1987) demonstrated that supply of Ca^{2+} through lime significantly increased plant growth and productivity. In an experiment done by Hartley *et al*. (2004), lime supply increased nodulation and yield of Serradella (*Ornithopus compressus*). The beneficial effects of liming on nodulation and plant growth most likely resulted from the enhanced conditions for seedling growth and nodulation. Interestingly, a study by Phillips *et al*. (1999) has also reported that rhizobia inoculants can stimulate growth and final yield of leguminous plants.

With regard to Mo, it is well known that leguminous plants are very sensitive to Mo deficiency, but excess Mo also may impair growth, decreases plant biomass, seed yield and deteriorates the quality of production (Liu and Yang, 2000; Kevresan *et al*., 2001; Nautiyal and Chatterjee, 2004). During different growth processes in plants and legumes in particular, Mo is involved in a number of different enzymatic processes (Marshner, 1995; Vieira *et al*., 1998). For example, Mo is a constituent of nitrogenase enzyme, and is needed by *Rhizobium* bacterium during the fixation process (Vieira *et al*., 1998). Therefore, with this task, Mo has a positive effect on growth, yield, N content of foliage and roots, as well as nodule forming in legume crops (Kliewer and Kennedy, 1978; Yesim *et al*., 2008). With regard to other aspects of plant nutrition, molybdoenzymes are involved in N metabolism, improving qualities of ascorbic acid, soluble

sugar, and chlorophyll concentrations (Zhao and Bai, 2001; Chen and Nian, 2004). Therefore, its deficiency may show overall reductions in plant growth, poor pod and/or grain development as well as exposing the plant to pest damage (Graham and Stangoulis, 2005).

Although there is considerable literature on the beneficial effects of liming, Mo and *Rhizobium* inoculation on legume growth in other parts of the world (Staley and Brauer, 2006), site specific factors can yield different results. As Ca, Mo and N play important role(s) in legume growth, these mineral nutrients warrants further investigations to ascertain their effects on plant growth and development in common legumes grown by farmers such as *P. vulgaris* L.

2.2 Materials and Methods

2.2.1 Site location and description

The experiments were conducted in the glasshouse of the Cape Peninsula University of Technology, Cape Town Campus, Keizersgracht from August 2008 to January 2009 and the field experiment was conducted at the Agricultural Research council Nietvoorbij site (33°54'S, 18°14'E) in Stellenbosch, South Africa, during the summer seasons; from October 2008 to March 2009. The site lies in the winter rainfall region of South Africa at an elevation of 146 m above sea level. The mean annual rainfall on the farm is 713.4 mm and mean annual temperatures range from 22.6°C at 11°C at night.

The experimental site had a previous history of grape cultivation. The soil type was sandy loam (Glenrosa, Hutton form) according to the soil classification working group (SCWG, 1991), which is equivalent to skeletic leptosol according to FAO classification (FAO, 2001). Following land preparation, but prior to planting, soil samples were collected for nutrients analysis.

2.2.2 Experimental design

The experimental treatments consisted of 2 levels of *Rhizobium* inoculation (with *Rhizobium* and without *Rhizobium*), 3 levels of lime (0, 2 and 3 t of lime.ha^{-1}) and 3 levels of molybdenum (0, 6 g.kg^{-1} and 12 g.kg^{-1} of seeds). The experimental design followed a spilt-split-plot design with 4 replications per treatment. The field plots measured 4 m x 4 m with 4 rows spaced 0.5 m apart from one another. *P.*

vulgaris was sown with inter-row planting distance of 20 cm. The plots were interspaced by small terraces of 1 m to prevent contamination. The plant populations were around 200,000 plants.ha^{-1}.

Planting was done after ploughing, harrowing, and lime application was done 2 weeks before planting. Twelve hours before planting, seeds were soaked into molybdenum solution. The control was also soaked in a water solution containing zero Mo. To avoid contamination, all *Rhizobium* uninoculated treatments were sown first. *Rhizobium* inoculation was done manually by putting the inoculant (*Rhizobium leguminosarum* biovar phaseoli-bakteriee registrasie nr. L1795 wet 36/1947) in the planting hole. The inoculants used were obtained from the University of Pretoria.

2.2.3 Plant harvesting and analysis

At physiological maturity, the plants in the two middle rows of each plot were counted and harvested for assessing grain yield. The border plants within each row were excluded. For yield components, 10 plants were sub-sampled from each plot to determine the number of pod per plant and number of seeds.pod^{-1}. Both pods were manually threshed and allowed to dry to 13% moisture content. Grain yield was determined for each plot and 100-seeds weight recorded.

2.2.4 Statistical analysis

The data from this experiment was analysed using the software of STATISTICA programme 2008. When significant differences were detected by the analysis of variance (ANOVA), Fisher's least significant difference was used to compare treatment means at $P \leq 0.05$ level of significance (Steel and Torrie, 1980).

2.3 Results

2.3.1 Effect of *Rhizobium* inoculation on yield components of *P. vulgaris* L.

The results in Table 2-1 clearly demonstrate that *Rhizobium* inoculation had significant effects on yield and all the other yield components assessed in this study. All parameters measured (number of pods.plant^{-1}, number of seeds.plant^{-1}, 100-seed weight, and seed yield) were significantly increased with *Rhizobium*

inoculation. For instance, the number of pods.plant^{-1} for both glasshouse and field experiments were increased significantly with *Rhizobium* inoculation by 13% for the glasshouse and 8% for field experiment relative to the uninoculated treatment (Table 2-1). In field experiment, the number of seeds.pod^{-1} in the inoculated treatment was 8% greater compared with uninoculated control. The 100-seed weight (g) was increased by 17.3% in the treatments supplied with *Rhizobium* compared with control. The grain yield (kg.ha^{-1}) of *P. vulgaris* L. were also significantly greater by 122% in plots inoculated with rhizobia compared with the uninoculated control.

2.3.2 Effect of molybdenum on yield and yield components of *P. vulgaris* L.

There was a significant response in yield and other yield components of *P. vulgaris L.* supplied with Mo at 0, 6 and 12 g.kg^{-1} of seeds (Table 2-1). In general, all parameters measured were significantly increased with Mo application relative with control (zero-level of Mo).

In the glasshouse experiment, compared with the control, supplying 6 or 12 g Mo increased the number of pods.plant^{-1} by 17 and 23% respectively. In the field experiment, the number of pods.plant^{-1} were significantly greater in plots supplied with the highest rate of 12 g of Mo.kg^{-1} of seed and was followed by 6 g Mo.kg^{-1} of seeds (Table 2-1). Compared with the control treatment, applying 6 and 12 g Mo increase the number of pods.plant^{-1} by 20 and 763% respectively.

The value of 100-seed weight (g) also increased significantly by 13.6 - 17.7% with the supply of Mo at 6 or 12 g Mo.kg^{-1} seeds respectively compared with the control.

Results from this study also showed that application of Mo at any level significantly increased grain yield (kg.ha^{-1}) of *P. vulgaris* L. For instance, supplying Mo at 6 and 12 g significantly increased the seed yield by 41 - 76.5% respectively compared with zero-molybdenum control (Table 2-1).

2.3.3 Effect of lime on yield and yield components of *P. vulgaris* L.

In this experiment, the results in Table 2-1 demonstrate that lime had no significant effects on the number of pods.plant^{-1} and 100-seed weight. However, significant increases were recorded in number of

seeds.pod^{-1}, and seed yield. Application of lime at the highest rate (3 t lime.ha^{-1}) was significantly superior to the control and 2 t lime.ha^{-1}.

The values of final grain yield increase significantly at each level of lime application. These values increased gradually with the highest yield being recorded by supplying lime at 3 t lime.ha^{-1}. As compared with the control treatment, the increase by applying 2 and 3 t lime.ha^{-1} was 11 and 27% respectively (Table 2-1).

2.3.4 Interactive effects of *Rhizobium*, molybdenum and lime.

The results in Figure 2-1A-C show that significant interaction between *Rhizobium* and Mo were observed only in the number of seeds.pod^{-1}, the 100-seed weight, and the final grain yield of *P. vulgaris* L. The lowest number of seeds.pod^{-1} was recorded in the control treatment (Figure 2-1A), whereas highest seed yields were found in treatments supplied with *Rhizobium* and different levels of Mo (Figure 2-1C).

The *Rhizobium* x lime interaction was significantly different for the number of seeds per pod and the final grain yield of *P. vulgaris* L. (Figure 2-2A-B). Greater yields were recorded in treatments involving *Rhizobium* inoculation and lime. Increasing lime levels progressively resulted into increased seed yield (Figure 2-2B)

The results in Figure 2-3A-C shows that there was significant interaction between Mo and lime for the number of pods.plant^{-1}, the number of seeds.pod^{-1}, the 100-seed weight, and the final grain yield. Highest rates of Mo and lime resulted into greater grain yield values (Figure 2-3C).

The interaction between *Rhizobium*, Mo and lime was significant only in the final grain yield (Figure 2-4). More yields were obtained in treatments including the combination of all these resources.

2.4 Discussion

In this study, we combined lime, Mo and *Rhizobium* inoculation in order to maintain optimal soil pH and increase soil fertility and ultimately obtain a sustainable combination which will produce reasonable yield.

This study reiterated the fact that *Rhizobium* inoculation was helpful in improving yield and yield components of *P. vulgaris* L. The treatments supplied with *Rhizobium* inoculation had great positive response in yield and other yield components (Table 2-1). The number of pods.plant^{-1} for both glasshouse and field experiments, the number of seeds.pod^{-1}, 100-seed weight (g) and grain yield (t.ha^{-1}) of *P. vulgaris* L. increased significantly in the *Rhizobium* inoculated treatments as compared with the control. Such a significant effect of rhizobia inoculation on common bean has also been reported by other workers (Munns, 1978; Keyser and Munns, 1979; Graham *et al*., 1982; Wood *et al*., 1984; Graham *et al*., 1992; Galloway *et al*., 1995; Peoples *et al*., 1995; Ndakidemi *et al*., 1998). The higher yields obtained with inoculation indicates that the rhizobial technology is just as efficient in supplying N to legumes as inorganic-N fertiliser and a better option for resource-poor farmer who can't afford to purchase expensive inputs. It is well established that, leguminous plants in partnership with *Rhizobium* have the ability to convert the atmospheric nitrogen into usable forms (Ndakidemi *et al*., 2006). From this study, it is clear that *Rhizobium* inoculation was important and play crucial role in improving plant growth and increasing the grain yield of *P. vulgaris* L. in the study site.

Data collected from this study revealed that Mo played a significant role in improving some attributes of yield and yield components of *P. vulgaris* L. Results showed that plants supplied with 6 or 12 g of Mo.kg^{-1} seed significantly increased yield of *P. vulgaris* L. by 41 and 76.5% respectively compared with zero-control treatment (Table 2-1). Molybdenum is known to be a constituent of nitrogenase enzyme. Molybdoenzymes are also involved in N metabolism and in improving qualities of ascorbic acid, soluble sugar, and chlorophyll concentrations in plants (Kliewer and Kennedy, 1978; Vieira *et al*., 1998; Zhao and Bai, 2001; Chen and Nian, 2004; Yesim *et al*., 2008). Therefore, the supply of Mo in the study area which is known to be deficient in Mo (Thibaund, 2005) might have resulted into the observed positive effect on growth, yield and yield components (Table 2-1). Our results are consistent with other workers (Kliewer and Kennedy, 1978; De Yunda and Gonzalez, 1982; Vieira *et al*., 1998; Zhao and Bai, 2001; Chen and Nian, 2004; Yesim *et al*., 2008) who reported positive results on growth and yield in other related legume species (Kliewer and Kennedy, 1978; Yesim *et al*., 2008). The application of Mo in the study area was essential because it improved plant growth and increase the grain yield of *P. vulgaris* L.

In our experiment, the application of lime at 2 or 3 t.ha^{-1}, improved the number of seeds.pod^{-1} and the final grain yield of *P. vulgaris* L. Seed yield increased by between 11 and 27% compared with zero-lime control (Table 2-1). In similar studies involving other legumes by Shoemaker *et al*. (1961); Adams and Evans (1962); Curtin *et al*. (1984); Edmeades *et al*. (1985); Lucrecia *et al*. (1987) and Hartley *et al*. (2004), liming materials significantly increased plant productivity. The beneficial effects of liming on

acidic soils such as those used in this study is most likely from the improved soil conditions through the neutralization of soil acidity and the improved Ca and Mg supply in the soil media.

A significant interactive effect was observed between *Rhizobium* inoculation and the Mo on the number of seeds.pod^{-1}, 100-seed weight (g), and grain yield. Good results were reported in *Rhizobium* inoculated treatments in combination with highest rate of Mo (Figure 2-1A-C), suggesting significant additive results by mixing these inputs. Given that Mo has a crucial role in N_2 fixation (Kliewer and Kennedy, 1978; Vieira *et al*., 1998; Yesim *et al*., 2008), an important component of nitrogenase enzyme; it is possible that in combination with *Rhizobium* inoculation, it plays a critical function in N_2 fixation and finally resulting into the observed results.

The application of *Rhizobium* inoculation and lime interacted significantly in such a way that the number of seeds.pod^{-1} and the final grain yield of *P. vulgaris* L. were increased. Better results were recorded in treatments involving *Rhizobium* inoculation and lime. Increasing lime levels progressively resulted into increased seed yield (Figure 2-2B). It may be suggested that the observed benefits were due to ability of these treatments to improve the nutrition on N (from rhizobia) and Ca and/or Mg (from lime). It is well established that acidic soil has low capability to support plant growth and are deficient in N, Ca and Mg. Therefore, in this study there was a significant advantage of combining the two treatments together. Similar results were also reported in peanut by Simbajon and Duque (1987).

Significant interactions also occurred with respect to Mo and lime in number of pods.plant^{-1}, number of seeds.pod^{-1}, 100-seed weight, and grain yield. The greater grain yield values were recorded into the highest rates of Mo (12 g of Mo.kg^{-1} seed) and lime (3 t.ha^{-1}) (Figure 2-3C). It is evident that, the combination of Mo and lime was important in alleviating the Mo, Ca and/or Mg stress in the study area, given that they have important role(s) to play in plant growth and development (Marschner, 1995). Research reports suggest that one major function of lime in acidic soils is to make Mo more available to plants (Quaggio *et al*., 2004). Similar results have also been reported in other leguminous species (Sahu *et al*., 1995; de Oliveira *et al*., 1998; Quaggio *et al*., 1998; Bailey and Laidlaw, 1999; Quaggio *et al*., 2004).

In the presence of all three treatments (*Rhizobium*, Mo and lime), a significant interaction was observed on the final grain yield of *P. vulgaris* L. The greater grain yield was observed into the highest rates of Mo and lime with *Rhizobium* inoculation (Figure 2-4). The improvement observed could be related to the amelioration effects of the limiting nutrients which were supplied as treatments in this study. Increasing

levels of lime and Mo resulted into progressive increase in *P. vulgaris* L. seed yields. It is possible that supplying these inputs into the soil increased their content and finally improved the growth conditions. Coventry *et al.* (1985) also reported the ameliorating effects of *Rhizobium*, lime and Mo in clover in which the plant growth and yield were increased.

In conclusion, rhizobia inoculation and the supply of Mo significantly improved yield and all yield components reported in this study. Better results were recorded in plots supplied with the highest rate of 12 g of $Mo.kg^{-1}$ of seed and was followed by 6 g of $Mo.kg^{-1}$ of seed. Lime application alone significantly improved number of $seeds.pod^{-1}$ and the final seed yield. Significant interactive effects were reported by inoculating the soil with *Rhizobium*, and supplying Mo and lime, indicating the need for these inputs in the study area.

Table 2-1: Yield components of nodulated *P. vulgaris* L. supplied with rhizobia, lime and molybdenum.

Treatments	Glasshouse	Field			
	No of pods plant $^{-1}$	No of pods plant $^{-1}$	No Seeds pod $^{-1}$	100-seed wt (g)	Seed yield (kg/ha)
Rhizobium					
-R	3.7±0.14b	3.8±0.13b	3.7±0.10b	15.0± 0.40b	758±52.5b
+R	4.2±0.12a	4.1±0.14a	4.0±0.01a	17.6± 0.40[a]	1679±51.9a
Molybdenum (g.kg^{-1})					
0	3.5±0.18a	3.5±0.16c	3.5±0.13b	14.7 ± 0.56 b	875±102.9c
6	4.1±0.14a	4.2±0.18b	4.0±0.00a	16.7 ± 0.41a	1237±99.2b
12	4.3±0.13a	30.2± 0.73a	4.0±0.00a	17.3 ± 0.53a	1544±99.7a
Lime (t.ha^{-1})					
0	3.9±0.20a	3.9±0.17a	3.7±0.12b	16.04 ± 0.594	1082±115.9c
2	3.9±0.12a	4±0.1 a	3.8±0.07b	16.4 ± 0.4	1205±104.8b
3	4.27±0.16a	4 ±0.2 a	3.9±0.04a	16.4± 0.58	1369±117.9a
3- Way ANOVA (F-Statistic)					
R	11.4**	3.9*	51.9***	34.1***	7748.3***
Mo	10.3***	6.9**	51.9***	12.7***	1364.7***
L	1.882NS	0.292ns	8.1***	0.287 NS	252.7***

R: *Rhizobium*; -R: without *Rhizobium*; +R: with *Rhizobium*. Values presented are means ± SE. *, **, *** = significant at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ respectively, NS = not significant. Means followed by similar letter (s) in a column are not significantly different.

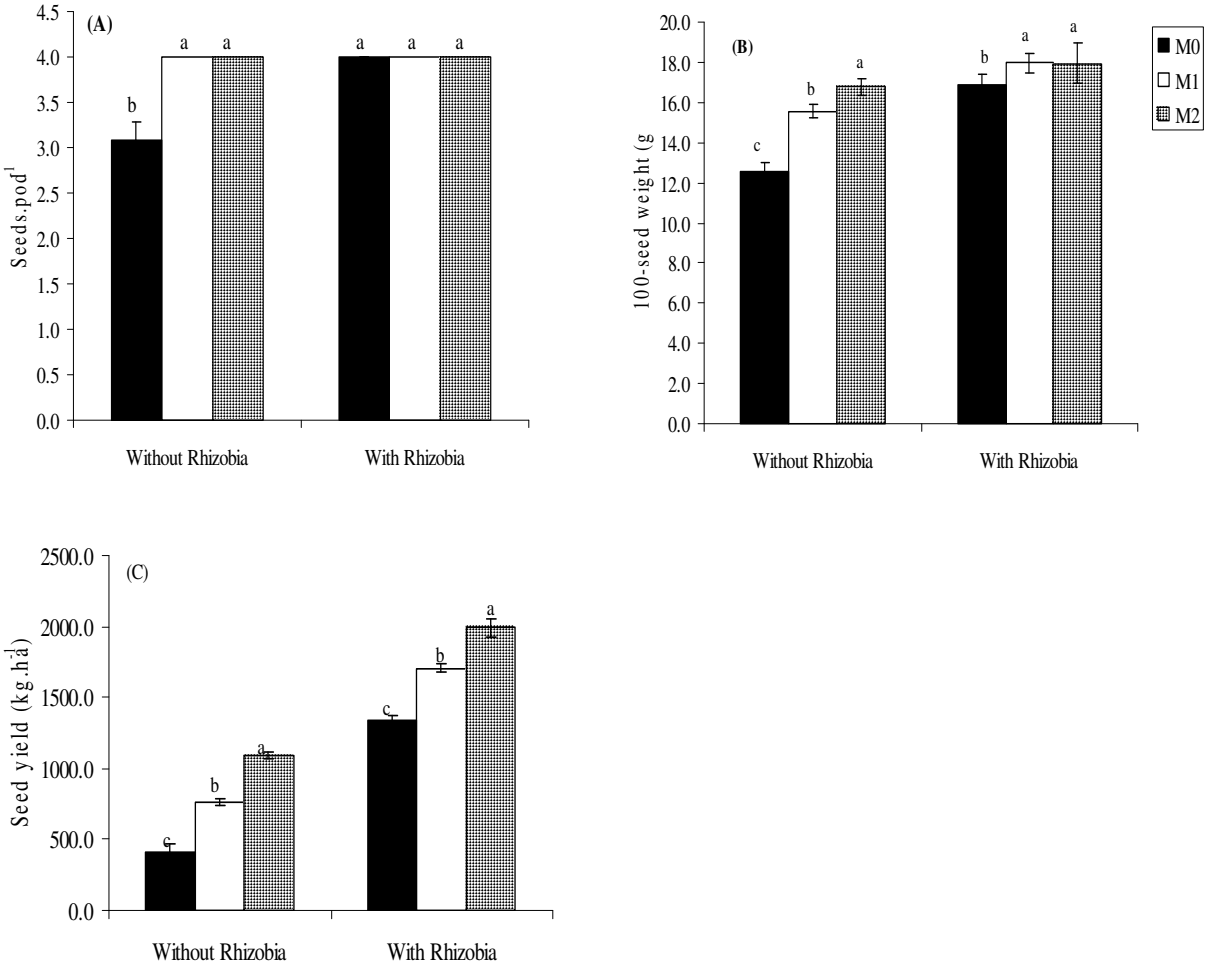

Fig. 2-1. Interactive effects of rhizobia and molybdenum (Mo) on: (A) No of seeds per pod, (B) 100-seed weight, and (C) Seed yield. M0 = Control, M1 = 6 g $Mo.kg^{-1}$ seed, M2 = 12 g $Mo.kg^{-1}$ seed. Bars followed by similar letter are not significantly different.

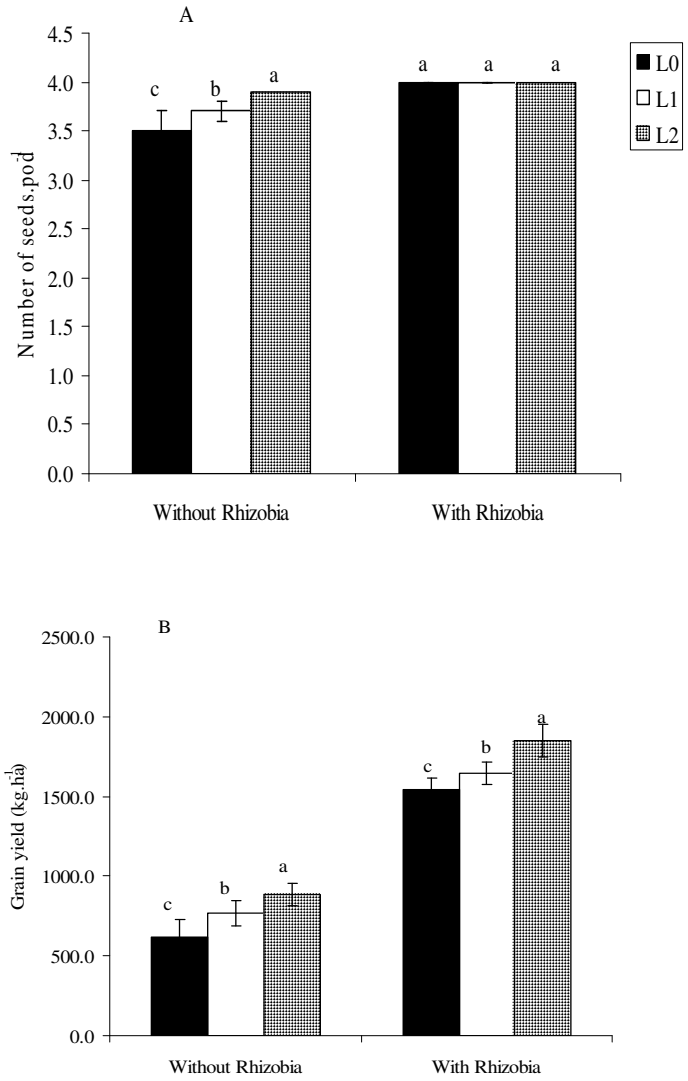

Fig. 2-2. Interactive effects of rhizobia and lime on: (A) No of seeds per pod, (B) Seed yield. L0 = Control; L1 = 2t lime per ha; L2 = 2t lime per ha. Bars followed by similar letter are not significantly different.

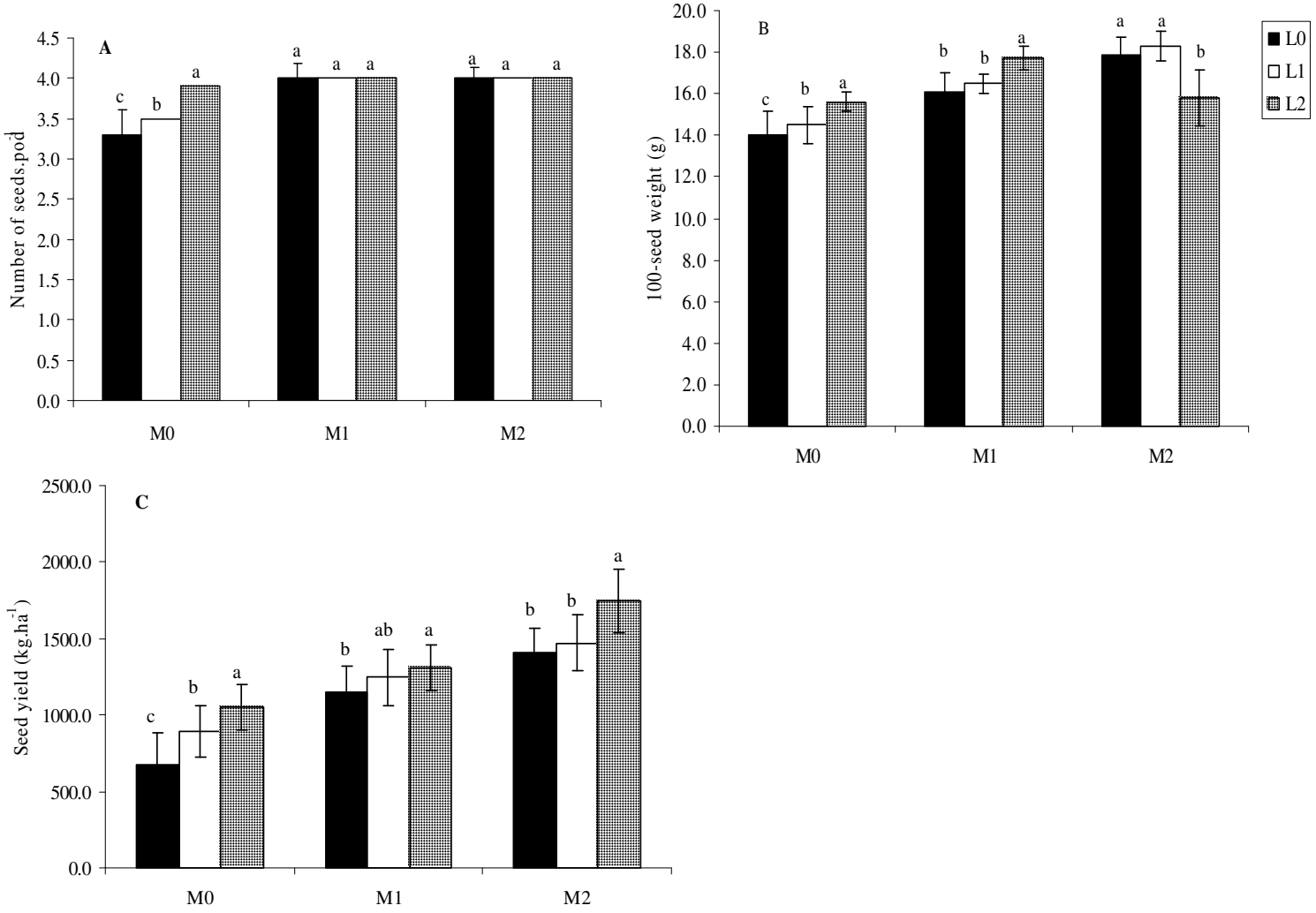

Fig. 2-3. Interactive effects of molybdenum (Mo) and lime (L) on: (A) No of seeds per pod; (B) = 100-seed weight; and C = Seed yield. L0 = Zero lime; L1 = 2t lime per ha; L2 = 2t lime per ha; M0 = Zero Mo; M1 = 6 g Mo per kg seed, M2 = 12 g Mo per kg seed. Bars followed by similar letter(s) are not significantly different.

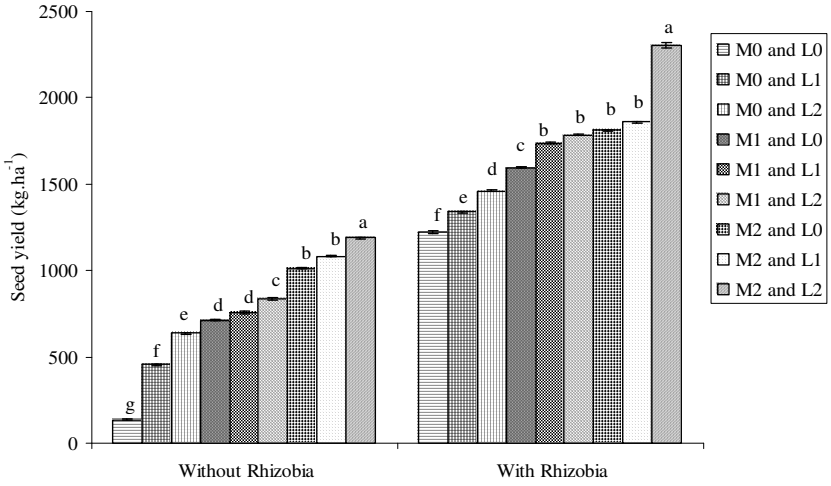

Fig. 2-4. Interactive effects of *Rhizobium*, molybdenum (Mo) and lime (L) on seed yield (kg.ha^{-1}). L0= control, L1= 2 t lime.ha^{-1}, L2= 3 t lime.ha^{-1}; M0=control; M1=6 g M0.kg^{-1} seed; M2=12 g Mo.kg^{-1} seed. Bars followed by similar letter are significantly different at $P \leq 0.05$

CHAPTER 3

EFFECTS OF *RHIZOBIUM* INOCULATION, LIME AND MOLYBDENUM ON PHOTOSYNTHESIS AND CHLOROPHYLL CONTENT OF *P. VULGARIS* L.

3.1 Introduction

It is well established that inadequate levels of any mineral nutrient in the growth media may limit growth, Chl synthesis and photosynthesis process due to their involvement in carbohydrate synthesis (Lambers *et al*., 1998). Calcium (Ca^{2+}) and Molybdenum (Mo) are essential plant nutrients whose roles in plant physiology have been well documented (Marschner, 1995; White and Broadley, 2003). They are involved in several biochemical and physiological processes in plants (Hepler and Wayne, 1985; Bauchot *et al*., 1999). *Rhizobium* inoculation in legumes is accredited for stimulating growth and is an alternative to the expensive inorganic nitrogen fertilizers (Ndakidemi and Dakora, 2007). The use of appropriate strains of inoculants in nitrogen deficient soils may offer an excellent opportunity for improving legume growth and development.

Calcium may function directly in several aspects of photosynthesis. It appears to modulate activity of the phosphatase enzymes in the carbon reduction cycle in the synthesis of different sugar components (Haupt and Weisenseel, 1976; Brand and Becker, 1984). Some research evidence also supports Ca^{2+} function in the water-splitting complex, suggesting its role in the photosystem II (Brand and Becker, 1984). As light is absorbed, this catalytic centre drives the photosynthesis process (Marschner, 1995). In the chlorophyll molecule embedded in a protein, there is a catalytic centre of photosynthetic water oxidation, which is composed of Mn^4Ca cluster.

In practice, Ca^{2+} deficiency is corrected by supplying agricultural lime or other sources of Ca^{2+}. For instance, XiaoJun *et al.* (2004) showed that the photosynthesis and photosynthetic efficiency of the leaves of rice were significantly enhanced with the supply of Ca^{2+}. In this study, levels of net photosynthetic rate, stomatal conductance, chlorophyll, and soluble sugar of the leaves increased significantly compared to the control treatment without Ca^{2+}. Furthermore, addition of Ca^{2+} decreased the content of malondialdehyde and the permeability of cell membrane, but increased the superoxide dismutase activity (XiaoJun *et al*., 2004) hence affecting photosynthesis. It is therefore obvious that the supply of Ca^{2+} through lime application may have a significant influence on the photosynthesis process at cellular level in *P. vulgaris* L. plants.

Molybdenum is an essential micronutrient for plants. It plays an important key role in Chl synthesis. In plants, it is absorbed as MoO_4^{2-}. In Southern Africa, several cases of Mo deficiency have been identified in variety of crops (Pienaar and Bartel, 1968; Tanner, 1978; Rhodes and Kpaka, 1982; Tanner, 1982; Kang and Osiname, 1985; Thibaund, 2005). Molybdenum deficiency in soil is a widespread agricultural problem that induces yield and quality losses in many crop species worldwide (Liu, 1991, 2001, 2002). Molybdenum deficient plants exhibit poor growth and low contents of Chl,

ascorbic acid and show reduced leaf blade formation, interveinal mottling and chlorosis around the edges and tips of older leafs as well as reduced photosynthesis (Marschner, 1995; Liu, 2002).

It is generally accepted that legumes need more Mo than most other plants (Mcbride, 2005). Various studies have reported that the application of Mo enhances the yield in crops that grow in deficient soil (Liu, 2001; Min *et al*., 2005; Xue-Cheng *et al*., 2006). Thus, the objective of this investigation was to establish and quantify the influence of *Rhizobium* inoculation and Mo and Ca^{2+} supplied as lime on Chl formation and different photosynthetic activities in *P. vulgaris* L. grown in a selected area in South Africa.

3.2 Materials and Methods

3.2.1 Site location and description

The experiments were conducted in the glasshouse of the Cape Peninsula University of Technology (CPUT), Cape Town Campus, Keizersgracht from October 2008 to December 2008. Field experimentation was also conducted under irrigation at the Agricultural Research Council Nietvoorbij site (33º54'S, 18º14'E) in Stellenbosch, South Africa, during the summer seasons, from October 2008 to March 2009. The site lies in the winter rainfall region of South Africa at an elevation of 146 m above sea level. The mean annual rainfalls on the farm is 713.4 mm and mean annual temperatures range from 22.6ºC (day temperature) to 11ºC (night temperature).

According to the soil classification working group, the soil type was sandy loam classified as Glenrosa, Hutton form, (SCWG, 1991) equivalent to skeletic leptosol according to FAO classification system (FAO, 2001). Following land preparation, but prior to planting, soil sample was collected for nutrients analysis at a depth of 20 cm.

3.2.2 Experimental design

The experimental treatments consisted of 2 levels of *Rhizobium* inoculation (with rhizobia and without rhizobia), 3 levels of lime (0, 2 and 3 $t.ha^{-1}$) and 3 levels of Mo (0, 6 and 12 $g.kg^{-1}$ of seeds). The experimental design followed a spilt-split-plot design with 4 replications per treatment. The field plots measured 2.5 m x 4 m with 5 rows 0.5 m apart from one another. *P. vulgaris* L. was sown with inter-row planting distance of 20 cm. The plots were interspaced by small terraces of 1 m to prevent contamination. The plant population density was 200,000 $plants.ha^{-1}$

Planting was done after ploughing and harrowing. Lime application was done 2 weeks before planting. Twelve hours before planting, seeds were soaked into Mo solution. The zero Mo control was also soaked in a water solution containing no Mo. To avoid contamination, all *Rhizobium* uninoculated

treatments were sown first. *Rhizobium* inoculation was done manually by putting the inoculant (*Rhizobium leguminosarum* biovar phaseoli-bakteriee registrasienr. L1795 wet 36/1947) in the planting hole. The inoculants used were obtained from University of Pretoria, South Africa.
Determination of chlorophyll (Chl) contents in plant leaves

Extraction of Chl concentrations by dimethylsulphoxide (DMSO) was done as described in Hiscox and Israelstam, 1979. A third of the plants leaves from the tip were collected from each pot and/or plot. A hundred (100) mg of the middle portion of fresh leaf slices was placed in a 15 ml vial containing 7 ml DMSO and incubated at 4°C for 72 h. After the incubation, the extract was diluted to 10 ml with DMSO. The DMSO technique extracts Chl from shoot tissue without grinding or maceration (Hiscox and Israelstam, 1979). A 3 ml sample of Chl extract was then transferred into curvets for absorbance determination. A spectrophotometer (UV/Visible Spectrophotometer, Pharmacia LKB. Ultrospec II E) was used to determine absorbance values at 645 and 663 nm, which was then used to determine total leaf Chl as proposed by Arnon, (1949) and expressed as mg L^{-1} as follows:

$$\text{Chlorophyll total } (Chl_t = 20.2D_{645} + 8.02D_{663})$$

3.2.3 Measurement of photosynthesis in plant leaves

The photosynthesis measurements of intact leaves that were still attached to the main plant stem (flag leaf) was made at flowering stage with a portable infra-red gas chromatograph (LCpro+ 1.0 ADC, Bioscientific Ltd., 12 Spurling Works, Pinder Road, Hoddesdon, Hertfordshire, EN11 ODB, UK) in the glasshouse and in the field.

3.2.4 Statistical analysis

The data from this experiment was analyzed using the software of STATISTICA program 2008 (StatSoft Inc., Tulsa, OK, USA). Fisher's least significant difference was used to compare significant treatment means at $P \leq 0.05$ level of significance (Steel and Torrie, 1980).

3.3 Results

3.3.1 Effect of *Rhizobium* inoculation on the leaf chlorophyll content, the photosynthesis, the intercellular CO_2 concentration and the transpiration of *P. vulgaris* L.

The results in Table 1 clearly indicate that rhizobial inoculation had significant effects on leaf Chl content and all the other parameters assessed in this study. All parameters measured i.e. the leaf Chl content, A, Ci and E were significantly increased with rhizobial inoculation. For example, the leaf Chl

content for the glasshouse experiment increased significantly with rhizobial inoculation by 123% and 178% for the field experiment relative with uninoculated control (Table 1).

The photosynthesis similarly increased significantly with rhizobial inoculation by 140% for the glasshouse experiment and by 81% in the field experiment compared with control.

The intercellular CO_2 concentration was increased by 39% for the glasshouse experiment and by 17.4% for the field experiment in the treatment supplied with rhizobial inoculation compared with the control.

The transpiration rate (E) similarly increased significantly with rhizobial inoculation by 14.2% for the glasshouse experiment and 53% for the field experiment compared with the uninoculated treatment (Table 1).

3.3.2 Effect of molybdenum on the leaf chlorophyll content, the photosynthesis, the intercellular CO_2 concentration and the transpiration of *P. vulgaris* L.

In this experiment, the results in Table 1 indicate that Mo at 6 and 12 $g.kg^{-1}$ of seeds had significant response on leaf Chl content, A, Ci and E of *P. vulgaris* L. compared with the control (zero-level Mo). In the glasshouse experiment, compared with the control, supplying 6 or 12 g $Mo.kg^{-1}$ of seed increased the leaf Chl content by 26% and 72% respectively. In the field experiment, the leaf Chl content increased significantly in plant supplied with 6 or 12 g $Mo.kg^{-1}$ by 32.4% and 58% respectively compared with the control (Table 1).

The value of A increased significantly by 27% and 50% for the glasshouse experiment with the application of 6 or 12 g $Mo.kg^{-1}$ of seeds and by 25.4% and 59.3% in the field experiment respectively.

The Ci increased with the supply of Mo at 6 or 12 $g.kg^{-1}$ of seeds by 6.4% and 11% for the glasshouse experiment and 6% and 15.3% for the field experiment.

The transpiration of *P. vulgaris* L. increased significantly with Mo supply at 6 or 12 g $Mo.kg^{-1}$ of seeds by 23% and 23% for the glasshouse experiment and 12% and 24% respectively for the field experiment.

3.3.3 Effect of lime on the leaf chlorophyll content, the photosynthesis, the intercellular CO_2 concentration and the transpiration of *P. vulgaris* L.

The result in Table 1indicate that lime had significant effects on the leaf Chl content, A, Ci and E. Application of lime at the highest rate (3 t lime.ha^{-1}) was significantly superior to the control and 2 t lime.ha^{-1}. In the glasshouse experiment, compared with the control, supplying with 2 t lime.ha^{-1} or 3 t lime.ha^{-1} increased the leaf Chl content by 14% and 22.3% respectively. In the field experiment, the leaf Chl content was increased significantly in plots supplied by 2 or 3 t lime.ha^{-1} by 10% and 22% respectively.

The photosynthesis of *P. vulgaris* L. in the glasshouse experiment was increased significantly with the application of 2 or 3 t lime.ha^{-1} by 6% and 22% respectively. In the field experiment, the supply of 2 or 3 t lime.ha^{-1} increased significantly the values of A by 6% and 12% compared with the control.

In the glasshouse experiment, Ci increased significantly with lime application of 2 or 3 t lime.ha^{-1} by 7% and 9% compared with the control. In the field experiment, the value of Ci increased significantly with lime application of 2 or 3 t lime.ha^{-1} by 2.3% and 4.5% compared with the control.

The values of E for both glasshouse and field were significantly different especially for the treatment involving 2 t lime.ha^{-1} compared with 0 or 3 t lime.ha^{-1}. In the glasshouse experiment, the value of E increased by 7% when supplied with 2 t lime.ha^{-1} compared with 0 or 3 t lime.ha^{-1}. In the field experiment, E values increased by 6% when supplied with 2 t lime.ha^{-1} compared with 0 or 3 t lime.ha^{-1}.

3.3.4 Interactive effects of *Rhizobium*, molybdenum and lime.

The results in Figure 1 show that there was a significant interaction between *Rhizobium* and molybdenum on the leaf Chl content for both glasshouse and field experiment, A for the field experiment only, Ci for the field experiment and E for the glasshouse experiment. In all measurements, the lowest values were recorded in the control treatment, whereas the highest values were recorded in treatments supplied with *Rhizobium* and 12 g Mo.kg^{-1} of seeds (Figure 1).

The *Rhizobium* x lime interaction was significantly different for E in glasshouse experiment only. The values of E in the field experiment, the leaf Chl content in the glasshouse and field experiment, A and Ci in the glasshouse and field experiment were not significantly different (Figure 2).

The results in Figure 3 show that there was significant interaction between Mo and lime only in A, Ci and E of *P. vulgaris* L. in the glasshouse experiment, whereas the highest values were recorded: for A

in treatments supplied with lime at 3 t.ha^{-1} and 12 g Mo.kg^{-1} of seeds, Ci in treatment supplied with lime 0, 2 t lime.ha^{-1} with 0 level Mo and E in treatment supplied with lime at 0, 2 and 3 t.ha^{-1} with 6 and 12 g Mo.kg^{-1} of seeds.

The interaction between *Rhizobium*, Mo and lime in Figure 4 was significantly different only in the rates of A and Ci of *Phaseolus vulgaris* L. in glasshouse experiment, whereas the highest values were recorded for A in treatments supplied with rhizobia with 12 g Mo and 3 t lime.ha^{-1} compared with the treatments without rhizobia and Ci in treatments supplied without rhizobia with 0 level Mo and 0 level lime compared with the treatment with rhizobia.

3.4 Discussion

In our study, *Phaseolus vulgaris* L. plants inoculated with *Rhizobium leguminosarum* had leaves with the highest Chl content and recorded better measurements in A, Ci and E (Table 1) as compared with the uninoculated control. The leaf Chl content for the glasshouse experiment was increased by 123% and 178% for the field experiment relative to the uninoculated control. The photosynthesis increased by 140% for the glasshouse experiment and by 81% for the field experiment compared with the control. The intercellular CO_2 concentration was increased by 39% for the glasshouse experiment and by 17.4% for the field in the treatment supplied with rhizobial inoculation compared with the control (Table 1). The transpiration increased significantly by 14.2% for the glasshouse experiment and 53% for the field experiment compared with the uninoculated treatment (Table 1). The supplied treatments in this study were essential as they improved most of the parameters measured (Table 1; Figure 1-4). Similar to our study, it has been reported that rhizobial inoculation may influence the physiological growth conditions of leguminous plants (Volpin and Phillips, 1998; Lanier *et al*., 2005) by increasing leaf photosynthesis (DeJong and Phillips, 1981; Lippi *et al*., 1999; Zhou *et al*., 2006) and Chl contents in the leaves (Sekhon *et al*., 2002; Tajini *et al*., 2008). Results from this study suggest that the supplied *Rhizobium leguminosarum* promoted the plant growth through a mechanism which increased Chl synthesis and photosynthetic rate in *P. vulgaris* L. plants.

In this study, Mo played an important role in the nutrition of *P. vulgaris* L. plant by improving the leaf Chl content, A, Ci and E. Results in Table 1 and Figures 1, 3 and 4 clearly demonstrate that Mo at 6 and 12 g.kg^{-1} of seeds increased significantly the leaf Chl content, the value of A, Ci and E of *P. vulgaris* L. Molybdenum is an important micronutrient for nitrogen fixing legumes (Graham *et al*., 1982; Kucey and Hynes, 1989; Bottomley, 1992; Tu, 1992; Banath *et al.,* 1996; Andrade *et al*., 2002). Soils with a pH below 6.0, such as those used in this study, (Pienaar and Bartel, 1968; Tanner, 1978; Rhodes and Kpaka, 1982; Tanner, 1982; Kang and Osiname, 1985; Thibaund, 2005) usually have low Mo available to the plants and need to be supplied with this micronutrient (Brady, 2008). The remarkable increase of Chl, A, Ci and E in leaves supplied with Mo at 6 and 12 g Mo.kg^{-1} of seeds is

an indication that this nutrient was limiting as observed in the zero control treatment. Molybdenum in plants is known to be responsible in various redox reactions (Mendel and Hänsch, 2002; Williams and Frausto da Silva, 2002) such as those related to water relations and transpiration rates through stomatal control (Kaiser *et al*., 2005). It is worth mentioning that these parameters are closely related to the photosynthesis processes in plants. From this background, it is logical to appreciate its contribution in improving Chl synthesis, A, Ci and E as reported in our study. Similar to our study, Marschner (1995) and Liu (2002) also reported that Mo deficient plants exhibited low Chl contents and showed reduced A in their leaves. As indicated in this study and in a separate work by Mcbride (2005), our results suggest that Mo is one of the limiting factors to crop productivity in the study area.

Data collected in this experiment show that soil application of lime at 2 or 3 $t.ha^{-1}$, significantly increased the leaf Chl content for both glasshouse and field experiment, A for the field experiment only, Ci for the field experiment and E of *P. vulgaris* L. (Table 1, Figures 2, 3 and 4). Calcium is an important constituent of plant tissues and has a vital role in maintaining and modulating various cell functions such as stabilizing cell wall structures, regulating ion transport and selectivity, and controlling ion-exchange behaviour as well as cell wall enzyme activities (Conway, 1982; Conway and Sams, 1987; Elad and Kirshner, 1992; Rengel, 1992; Marschner, 1995). The reduced Ca^{2+} availability as observed in our study site (which was acidic in nature), could have impaired these functions as they are closely related to Chl synthesis and photosynthesis process.

In the present study, the addition of Ca^{2+} in form of lime increased the Chl content in *P. vulgaris* L. In a study involving cucumber, the supply of Ca^{2+} stabilized the apoproteins of the light-harvesting Chl a/b-protein complex of photosystem II and finally improved the Chl contents in the leaves (Tanaka *et al*., 1995), results which were similar to our study.

Physiologically, Ca^{2+} plays a key role in water oxidation during the process of photosynthesis (Yocum, 1991). In their study, Barry *et al.* (2005) confirmed that low levels of Ca^{2+} were associated with reduced A in plants. Similar to our study, XiaoJun *et al.* (2004) showed that the A and photosynthetic efficiency of rice leaves were significantly enhanced with the supply of Ca^{2+}. The levels of net photosynthetic rate, stomatal conductance and the contents of Chl in the leaves increased more significantly than the control treatment without Ca^{2+}. Therefore, the addition of Ca^{2+} in the form of lime in deficient soils could offer an economical and simple solution to *P. vulgaris* L. production problems caused by high acidity.

Results from this study showed that there was a marked *Rhizobium* x Mo x lime interaction on the Chl content, A, Ci and E, with better results when *Rhizobium*, molybdenum and lime were supplied together than when either treatment was applied alone (Figure 1-4). In all measurements, lowest values were recorded in the control treatment, whereas highest values were recorded in treatments supplied in

combination. The result reported in this study suggests that *P. vulgaris* L. yields on these acidic soils cannot be maximized unless all limiting nutrients are supplied. In their studies, (Hepler and Wayne, 1985; Marschner, 1995; Lambers *et al*., 1998; Bauchot *et al*., 1999; White and Broadley, 2003) established that inadequate levels of any mineral nutrient in the growth media may limit A and plant growth due to their involvement in carbohydrate synthesis. Calcium and Mo are essential plant nutrients. They are involved in several biochemical and physiological processes in plants such as those involving photosynthesis.

In conclusion, *Rhizobium* inoculation and the supply of Mo and lime significantly improved the leaf Chl content, A, Ci and E in our study. The interactive effects were also observed in different combinations of the above treatments in which significant interactive effects were reported by inoculating the soil with *Rhizobium* and supplying Mo and lime at the highest levels, indicating the impact and importance of these inputs in the study area. The potential of improving Chl synthesis, A, Ci and E in *P. vulgaris* L. through *Rhizobium* inoculants and the addition of lime and Mo was clearly explored. Successful bean production in these acidic soils is unlikely unless attention is paid to supply these three important nutrients in the study area.

Table 3-1. Effects of *Rhizobium* inoculation, lime and molybdenum on photosynthesis and chlorophyll content of *P. vulgaris* L.

Treatments	Glasshouse experiment				Field experiment			
	Leaf Chl content (mgL^{-1})	Photosynthesis (A) (µ mol CO^2 $m^{-2}S^{-1}$)	Intercellular CO_2 concentration (Ci) (mmol CO_2 mol^{-1} air)	Transpiration (E) (mmol H_2O m^{-2} S^{-1})	Leaf Chl content (mgL^{-1})	Photosynthesis (A) (µ mol CO^2 $m^{-2}S^{-1}$)	Intercellular CO_2 concentration (Ci) (mmol CO_2 mol^{-1} air)	Transpiration (E) (mmol H_2O m^{-2} S^{-1})
Rhizobium								
-R	5.9±0.50b	4.4±0.29b	222.2±3.15b	1.4±0.04b	7.6±0.45b	5.4±0.36b	254.0±4.19b	1.5±0.06b
+R	13.2±0.44a	10.6±0.27a	309.6±3.8a	1.6±0.02a	21.2±0.64a	9.8±0.22a	298.4±2.81a	2.3±0.04a
Molybdenum ($g.kg^{-1}$)								
0	7.2±0.93c	6.1±0.76c	251.5±9.53c	1.3±0.05a	11.1±1.36c	5.9±0.55c	257.9±5.92c	1.7±0.09c
6	9.1±0.81b	7.6±0.65b	267.6±9.31b	1.6±0.01b	14.7±1.43b	7.4±0.61b	273.2±6.12b	1.9±0.10b
12	12.4±0.78a	9.0±0.65a	278.6±10.5a	1.6±0.02b	17.5±1.61a	9.4±0.33a	297.4±3.90a	2.1±0.09a
Lime ($t.ha^{-1}$)								
0	8.5±0.99a	6.9±0.74b	253.1±8.91a	1.5±0.05a	13.05±1.50c	7.2±0.63b	269.9±7.40b	1.8±0.12b
2	9.7±0.90b	7.3±0.73b	270.0±10.43b	1.6±0.03b	14.3±1.52b	7.6±0.57ab	276.0±5.52ab	1.9±0.10b
3	10.4±0.93b	8.4±0.69a	274.6±10.26b	1.5±0.04b	15.9±1.63a	8.1±0.55a	282.6±5.78c	1.8±0.12a
3-Way ANOVA (F-Statistic)								
R	319.7***	1199.1***	934.0***	54.8***	1334.9***	497.9***	225.7***	208.2***
M	56.7***	92.8***	30.1***	84.8***	98.9***	99.7***	60.8***	21.3***
L	7.1**	24.1***	20.8***	3.4*	20.1***	6.3**	6.2*	4.9*

Effects of lime and molybdenum on photosynthesis and chlorophyll content: -R) without rhizobia; +R) with rhizobia. Values followed by dissimilar letters in the same column differ significantly at $P \leq 0.05$

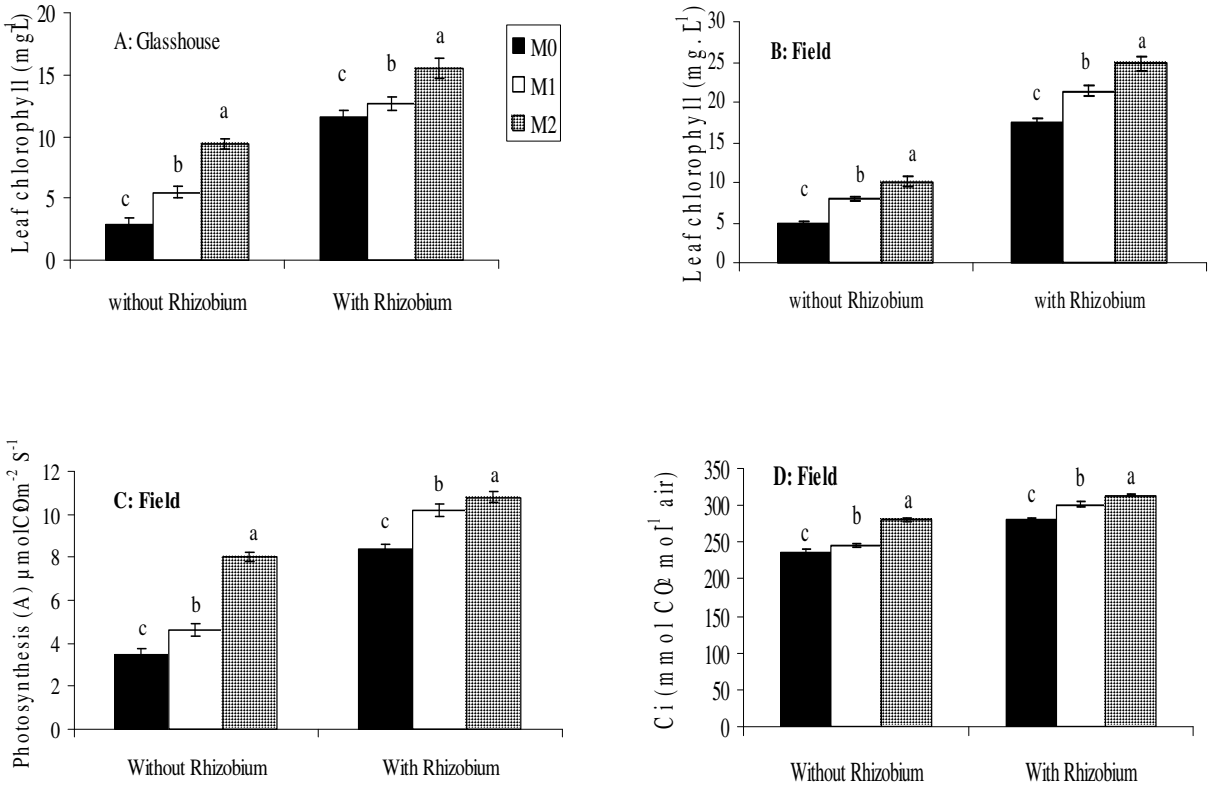

Figure 3-1. Interactive effect of *Rhizobium* inoculation and molybdenum (Mo) on: A) Leaf chlorophyll content (glasshouse); B) Leaf chlorophyll content (Field); C) Photosynthesis (Field); and D) Intercellular CO_2 concentration (Field). M0=control; M1=6 g Mo.kg^{-1} seed; M2=12 g Mo.kg^{-1} seed. Bars followed by similar letter are significantly different at $P \leq 0.05$.

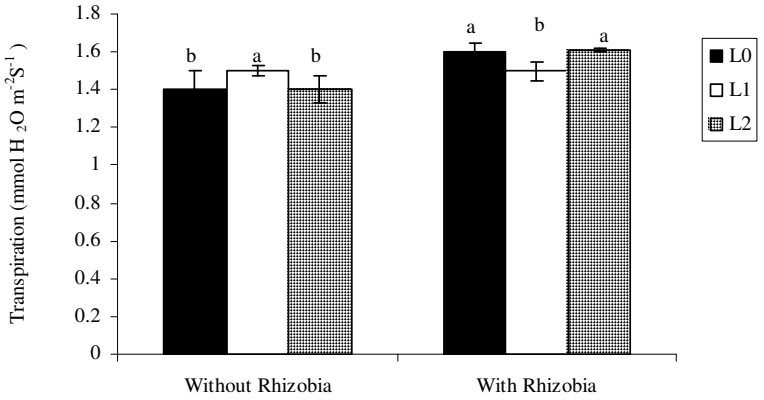

Figure 3-2. Interactive effect of *Rhizobium* inoculation and lime on the transpiration rate in the glasshouse. L0= control; L1= 2 t lime.ha^{-1}; L2= 3 t lime.ha^{-1}. Bars followed by similar letter are significantly different at $P \leq 0.05$.

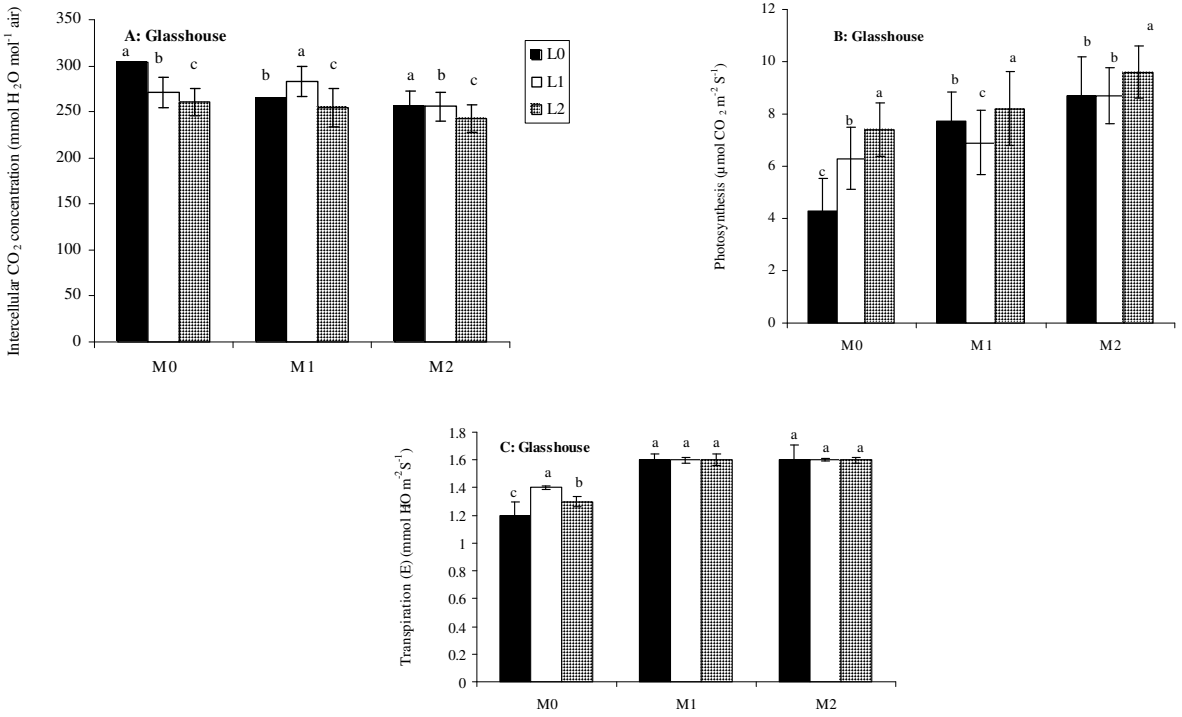

Figure 3-3. Interactive effects of molybdenum and lime on: A) Intercellular CO_2 concentration (glasshouse); B) photosynthesis (glasshouse) and C) transpiration (glasshouse). M0= control, M1= 6 g Mo.kg^{-1} seed, M2= 12 g Mo.kg^{-1} seed, L0= control, L1= 2 t lime.ha^{-1}, L2= 3 t lime.ha^{-1}. Bars followed by similar letter are significantly different at $P \leq 0.05$.

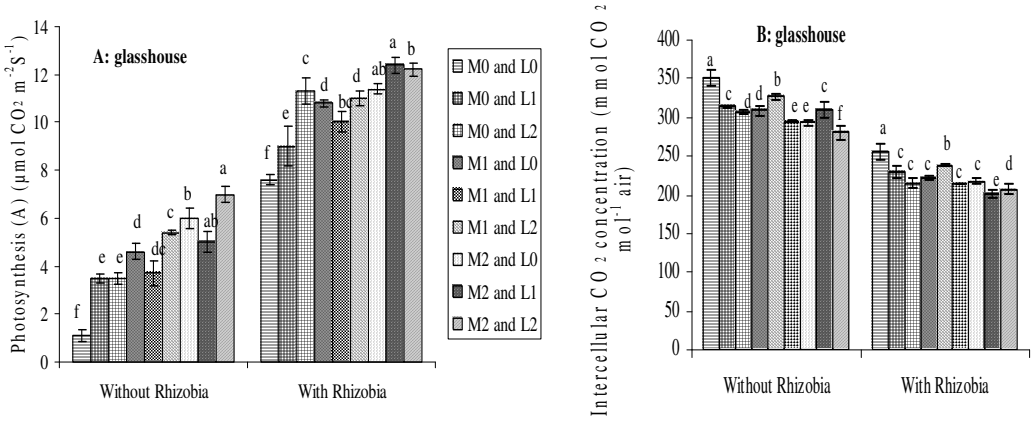

Figure 3-4. Interactive effect of rhizobia, molybdenum and lime on: A) photosynthesis (glasshouse); B) Intercellular CO_2 concentration (glasshouse). M0= control, M1= 6 g Mo.kg^{-1} seed, M2= 12 g Mo.kg^{-1} seed, L0= control, L1= 2 t lime.ha^{-1}, L2= 3 t lime.ha^{-1}. Bars followed by similar letter (s) are significantly different at $P \leq 0.05$.

CHAPTER 4

CHANGES IN SELECTED SOIL CHEMICAL PROPERTIES IN THE RHIZOSPHERE OF *P. VULGARIS* L. SUPPLIED WITH *RHIZOBIUM* INOCULANTS, MOLYBDENUM AND LIME.

4.1 Introduction

*Rhizobium*s are also known as bio-fertilizer (Alaa EL-Din *et al*., 1985) as they may increase the availability of soil nutrients to the plants and in the rhizosphere through processes such as biological N_2 fixation. They may also contribute to soil nutrition from their dead cells (McCulley, 2001) or making nutrients more available through solubilisation of phosphates and other minerals bound in unavailable forms such as Fe. Studies have reported the solubilisation of P in the rhizosphere by R*hizobium leguminosarum* bv. *Phaseoli* (Chabot *et al*., 1998). For instance, in a nodulated fixing pigeon pea plant, P availability was enhanced by the legume through release of piscidic acid in pigeon pea root exudates, which mobilised and increased P availability (Ae *et al*., 1990). Other mechanisms are related to siderophores production which helps facilitate the solubilisation of certain nutrients such as Fe from unavailable to more available form (Dakora and Phillips, 2002). There is research evidence that some rhizospheric bacteria produce siderophores which solubilise Fe (Bar-Ness *et al*., 1991; Wang *et al*., 1993; Dakora and Phillips, 2002).

Nitrogen fixed by the rhizobial in the host plant may be released into the rhizosphere through the root exudation (Eaglesham *et al*., 1981; Ndakidemi, 2006) and thus improving the N status of the soil. Plants fixing nitrogen may also manifest changes in soil pH (Nye, 1981; Bolan *et al*., 1991), and other physical and chemical characteristics such as the level of plant exudates (Uren and Reisenauer, 1988; Tavaria and Zuberer, 1998; Griffiths *et al*., 1999; Revsbech *et al*., 1999; Xu, 2000).

Research evidence on the effect of lime on soil chemical properties is widely available in the literature (Meiwes, 1995; Meda *et al*., 2002). For instance, lime is reported to play a key role in raising the soil pH and increasing the exchangeable Ca and Mg in the soil (Meiwes, 1995).

Besides the availability of adequate information on the effects of *Rhizobium* inoculation on N economy of soils, very few studies have assessed their impact on the availability of other nutrient elements in the rhizosphere of legumes. This study examines the effects of i) *Rhizobium* inoculation, ii) Mo supply, and iii) lime application on the concentrations of plant-available nutrients in the rhizosphere of *P. vulgaris* L.

4.2 Materials and Methods

4.2.1 Experimental site

The experiments were conducted in the glasshouse of the Cape Peninsula University of Technology (CPUT), Cape Town Campus, Keizersgracht from October 2008 to December 2008. Field experimentation was also conducted under irrigation at the Agricultural Research council Nietvoorbij site (33º54'S, 18º14'E) in Stellenbosch, South Africa, during the summer seasons, from October 2008 to March 2009. The site lies in the winter rainfall region of South Africa at an elevation of 146 m above sea level. The mean annual rainfalls on the farm is 713.4 mm and mean annual temperatures range from 22.6ºC (day temperature) to 11ºC (night temperature).

The soil type was sandy loam classified as Glenrosa, Hutton form (SCWG, 1991), equivalent to skeletic leptosol in the FAO soil classification system (FAO, 2001). Following land preparation, but prior to planting, soil samples were collected for nutrients analysis.

4.2.2 Experimental design

The experimental treatments consisted of 2 levels of *Rhizobium* inoculation (with rhizobia and without rhizobia), 3 levels of Mo (0, 6 and 12 $g.kg^{-1}$ of seeds) and 3 levels of lime (0, 2 and 3 $t.ha^{-1}$). The experimental design followed a spilt-split-plot design with 4 replications per treatment. The field plots measured 2.5 m x 4 m with 5 rows 0.5 m apart from one another. *P. vulgaris* was sown with inter-row planting distance of 20 cm. The plots were interspaced by small terraces of 1 m to prevent contamination. The plant population density was 200,000 $plants.ha^{-1}$.

Planting was done after ploughing and harrowing. Lime application was done 2 weeks before planting. Twelve hours before planting, seeds were soaked into Mo solution. The zero Mo control was also soaked in a water solution containing no Mo. To avoid contamination, all *Rhizobium* uninoculated treatments were sown first. *Rhizobium* inoculation was done manually by putting the inoculant (*Rhizobium leguminosarum* biovar phaseoli-bakteriee registrasienr. L1795 wet 36/1947) in the planting hole. The inoculants used were obtained from University of Pretoria, South Africa.

4.2.3 Collection and preparation of bulk soil

Soil samples were collected with auger (0 - 20 cm depth) from several locations within each replicate plot and mixed for determination of the initial nutrient concentrations in the soil. The soil samples were air-dried in the laboratory and sieved (2 mm) for analysis of nutrients and determination of pH and organic matter.

4.2.4 Collection and preparation of rhizosphere soil

At 60 days after planting (DAP), rhizosphere soil, defined as rich in roots and/or adhering to the roots and influenced by root activity, was collected from around *P. vulgaris* L. plants for nutrient analysis. To achieve this, soil was carefully excavated from around single plants or their pairs down to 30 cm or more (depending on root depth), and the island of soil around the plant dug up and removed, with the plant and its roots intact inside the lump of soil. Using one's hands, the volume of soil containing intact plant (s) was removed from the exterior down to a root-rich rhizosphere soil material of about 30 - 50 g. This sample was shaken into a labelled plastic bag and the process repeated for up to 72 samples. These rhizosphere soil samples were air-dried in the laboratory, and sieved (2 mm) for analysis of nutrients and the determination of pH and plant- available nutrients in rhizosphere soil.

4.2.5 Measurement of soil pH

The pH of soil was measured in 0.01 M $CaCl_2$ solution using a 1:2:5 soil-to-solution ratio.

4.2.6 Determination of plant-available nutrients in rhizosphere soil

The extractable P, K, Na, Ca and Mg were determined by citric acid method as developed by Dyer (1894) and modified by the Division of Chemical Services (DCS, 1956) and Du Plessis and Burger (1964). A 20 g air-dried soil sample was extracted in 200 mL of 1% (w/v) citric acid, heated to 80°C, shaken for 2 min at 10 min intervals over a total period of 1 h and filtered. A 50 mL aliquot was heated to dryness on a water bath, and 5 mL of concentration HCl and HNO_3 and 20 mL of deionised water added. The mixture was heated to dissolve the dry residue, and the sample filtered. Measurements of P, K, Na, Ca and Mg were done directly by direct aspiration on the calibrated simultaneous ICP.

The trace elements i.e. Cu, Zn, Mn and Fe were extracted from soil using di-ammonium ethylenediaminetetraacetic (EDTA) acid solution [Trierweiler and Lindsay (1969), as modified by Beyers and Coetzer (1971)]. The extractants were analysed for Cu, Zn, Mn, and Fe using the calibrated simultaneous ICP spectrophotometer.

4.2.6 Statistical analysis

The data from this experiment was analyzed using the software of STATISTICA program 2008 (StatSoft Inc., Tulsa, OK, USA). Fisher's least significant difference was used to compare significant treatment means at $P \leq 0.05$ level of significance (Steel and Torrie, 1980).

4.3 Results

4.3.1 Effects of *Rhizobium*, molybdenum and lime on pH of rhizosphere soil of *P. vulgaris* L.

Rhizobium inoculation, Mo and lime significantly increased the rhizosphere pH of *P. vulgaris* L. (Table 1). For instance, *Rhizobium* inoculation increased the rhizosphere pH by 3.2% compared with the uninoculated control. Molybdenum at 6 and 12 $g.kg^{-1}$ of seeds increased significantly the soil pH by 3.2% and 6.5% compared with the zero Mo control. Furthermore, lime at 2 and 3 $t.ha^{-1}$ increased significantly the rhizosphere pH by 3.2% and 4.9% respectively relative to zero lime control treatment (Table 1).

4.3.2 Effects of *Rhizobium*, molybdenum and lime on macronutrient concentrations in rhizosphere soil of *P. vulgaris* L.

The macronutrient concentrations (P, K and Mg) were not significantly affected by Rhizobium inoculation. However, Ca and Na were significantly increased by *Rhizobium* inoculation. *Rhizobium* inoculation increased Ca by 37% and Na by 78% compared with the uninoculated control (Table 1).

Molybdenum had no significant effect on any of the macronutrient concentrations in rhizosphere soil of *P. vulgaris* L.

Application of lime at 2 and 3 $t.ha^{-1}$ resulted into significant increase in Ca and Mg. In this study, the level of Ca and Mg in the rhizosphere of *P. vulgaris* L. increased by 29% and 71% for the Ca and 69% and 180% for Mg compared with the control (Table 1).

4.3.3 Effects of *Rhizobium*, molybdenum and lime on micronutrient concentrations in rhizosphere of *Phaseolus vulgaris* L.

The result in Table 2 indicates that *Rhizobium* inoculation significantly increased the concentrations of only Cu, Zn, Fe and Mn in the rhizosphere of *P. vulgaris* L. compared with the control. For example, the concentrations of Cu, Zn and Fe increased respectively by 20%, 67% and 28% with *Rhizobium* inoculation compared with the control (Figure 1).

Molybdenum and lime had no significant effect on all micronutrient concentrations in rhizosphere of *P. vulgaris* L. (Table 2).

4.3.4 Interactive effect of *Rhizobium*, molybdenum and lime on the concentration of nutrients in the rhizosphere of *Phaseolus vulgaris* L.

There was an interactive effect between *Rhizobium* and molybdenum only for pH values in the rhizosphere soil of *P. vulgaris* L. (Figure 2). *Rhizobium* inoculation combined with Mo gave significantly higher pH values compared with all other treatments (Figure 2).

4.4 Discussion

Rhizobium inoculation in this study significantly decreased the soil acidity by increasing the soil pH in the rhizosphere of *P. vulgaris* L. (Table 1). These are positive results especially in acidic soils where low pH is responsible for poor plant growth (Meiwes, 1995) which limits the uptake of some important nutrients such as phosphorous (Dakora and Phillips, 2002) and the decomposition of organic materials in the soil (Motavalli *et al*., 1995). Improved soil pH to optimum levels from different practices is advantageous as may improve the soil chemical properties and the availability of certain mineral nutrients in the soil (Condron *et al*., 1993; Bagayoko *et al*., 2000; Table 1) and hence the plant growth. The mechanism involved in *Rhizobium* inoculation improving pH of the soil is complex, but research evidence has shown

that plants that absorb nitrogen as NO_3^- tend to raise the pH in the rhizosphere (Nye, 1981; Dakora and Phillips, 2002) a phenomenon which was not proved in this study.

Rhizobium inoculation numerically but not significantly increased the concentrations of P and K. However, the rhizosphere concentrations of Ca, Na (Table 1), Fe, Cu, Zn, and Mn (Figure 1) were significantly increased with *Rhizobium* inoculation. It is not well established on how *Rhizobium* makes these mineral nutrients more available in the rhizosphere, but few possible options are proposed. Firstly, there is evidence that *Rhizobium* can increase the availability of nutrients such P and Fe through a mechanism involving their solubilisation from unavailable to available forms (Chabot *et al*., 1998; Dakora and Phillips, 2002). Secondly, certain rhizospheric bacteria may produce siderophores which solubilise Fe (Bar-Ness *et al*., 1991; Wang *et al*., 1993; Dakora and Phillips, 2002) and make it available into soil solution for plant uptake, a scenario which was supported by results from this study. Thirdly, the decaying rhizobial cells could also increase the nutrient availability in the rhizosphere, an argument supported by McCulley (2001). Lastly, it is possible that soil inoculated with *Rhizobium* may have increased their biological activity associated with roots of the host plant and the micro-organism, thus increasing the decomposition of organic matter in the soil, root-residue decomposition enhanced root exudation and hence increasing the amount of available nutrients in the soil (Lee and Pankhurst 1992; Murphy *et al*., 2004; Hauggaard-Nielsen and Jensen, 2005; Eskelinen *et al*., 2009; Sanon *et al*., 2009). Whatever mechanism employed, Rhizobium inoculation seemed to be very beneficial in increasing some mineral elements in the soil.

Molybdenum application at 6 and 12 $g.kg^{-1}$ of seeds significantly increased the soil pH relative to the control treatment (Table 1). Noticeable increases were reported by supplying Mo at 12 $g.kg^{-1}$ of seeds. The mechanisms involved are not clearly understood, but it is possible that Mo stimulated N-fixation and uptake and metabolism of nitrates which ultimately resulted into the soil pH increase (Nye, 1981).

As expected, application of lime at 2 and 3 $t.ha^{-1}$ resulted into significantly increased the soil pH and the available Ca and Mg relative to the control treatment. Liming is done in acidic soils to reduce acidity (Jessop and Mahoney, 1982; Okpara *et al*., 2007) and to supply important plant nutrients such as Ca or Mg (Pierce and Warncke, 2000; Steiner and Alderman 2003). As the used study site was previously reported to be deficient in Ca and Mg

(Ndakidemi, 2005), application of lime was justifiable as it improved the soil pH and the basic cations: Ca and Mg into the rhizosphere.

In conclusion, this study found that *Rhizobium* inoculation significantly increased the soil pH and the availability of Ca, Na, Fe, Cu, Zn and Mn in the rhizosphere. The increased available nutrients due *Rhizobium* inoculation could be due improved favourable pH conditions of near neutral values. Additions of lime resulted in increased soil pH and exchangeable Ca and Mg. The treatments involving the combination of *Rhizobium* and Mo at the highest supply rate resulted into significant interactions and gave the excellent pH changes near to neutral.

Table 4-1: Effect of *Rhizobium*, molybdenum and lime on macro-nutrients measured in field during 2008 - 2009 season.

Treatments	pH	P	K	Ca	Mg	Na
			mg kg^{-1}			
Rhizobium						
-R	6.2±0.0b	5.1±0.1a	105.5±2.7a	3.5±0.3b	2.4±0.2a	79.1±2.0b
+R	6.4±0.1a	5.8±0.5a	112.5±3.5a	4.8±0.3a	2.3±0.3a	85.4±2.0a
Molybdenum ($g.kg^{-1}$)						
0	6.1±0.0b	6.0±0.7a	107.9±4.0a	3.9±0.4a	2.3±0.3a	82.5±2.6a
6	6.3±0.0ab	5.3±0.3a	109.1±4.0a	4.0±0.4a	2.3±0.3a	80.8±2.6a
12	6.5±0.1a	5.2±0.3a	110.0±3.6a	4.5±0.4a	2.5±0.3a	83.4±2.4a
Lime ($t.ha^{-1}$)						
0	6.1±0.0b	5.1±0.4a	110.2±3.4a	3.1±0.3c	1.3±0.2c	84.4±2.4a
2	6.3±0.1ab	5.6±0.6a	109.1±4.5a	4.0±0.4b	2.2±0.2b	82.1±2.2a
3	6.4±0.1a	5.8±0.4a	107.6±3.7a	5.3±0.4a	3.6±0.2a	80.2±2.9a
3-Way ANOVA (F-Statistic)						
R	15.4***	1.28 NS	2.1 NS	10.6**	0.1 NS	4.7 *
Mo	43.1***	0.70 NS	0.06 NS	0.8 NS	0.2 NS	0.275
L	20.3***	0.46 NS	0.10 NS	9.7***	25.4***	0.678 NS

-R: without *Rhizobium*; +R: with *Rhizobium*. Values presented are means ± SE. **, *** = significant at $P \leq 0.01$, $P \leq 0.001$ respectively, NS = not significant. Means followed by similar letter (s) in a column are not significantly different from each other at $P \leq 0.05$.

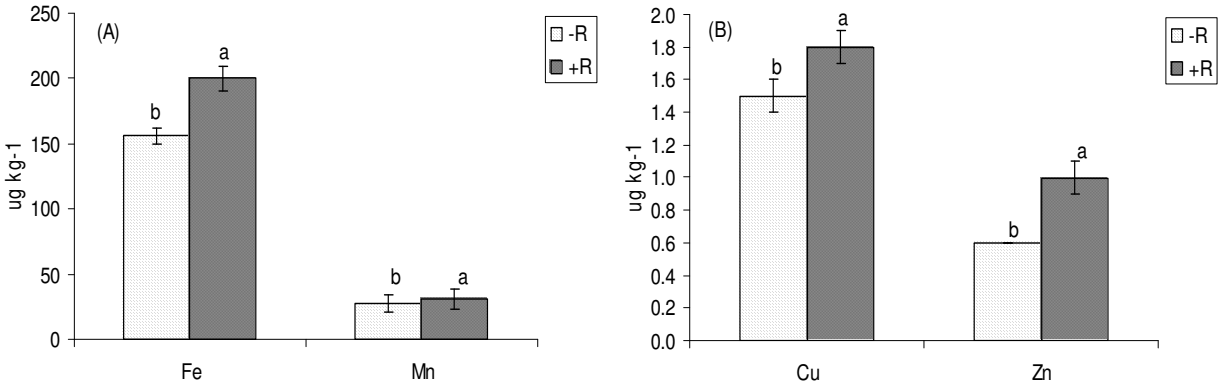

Figure 4-1. Effect of *Rhizobium* on soil micronutrients: A) Fe and Mn, B) Cu and Zn

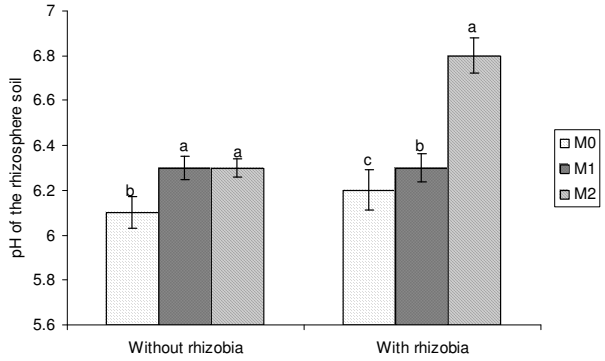

Figure 4-2. Interaction effect of *Rhizobium* and molybdenum on the pH of rhizosphere soil on field experiment in 2009.

CHAPTER 5

EFFECTS OF *RHIZOBIUM* INNOCULATION, LIME AND MOLYBDENUM ON NITROGEN FIXATION OF NODULATED *P. VULGARIS* L.

5.1 Introduction

Nitrogen is amongst the most limiting nutrient for plant growth. It is a constituent of all proteins, nucleic acids and many other biomolecules and it is essential in all living organisms (McCammon and Harvey, 1987; Marschner, 1995)**.** The availability of this important nutrient in the soil is constrained by many biotic and abiotic factors (Zahran, 1999). Leguminous plants such as *P. vulgaris* L. in association with *Rhizobium* bacteria have the ability to convert nitrogen from the air into the soil and transform it into ammonium (NH_4), which can be used directly by the host plant (Shanmugam *et al*., 1978). However, several reports have highlighted on low fixation capability of *P. vulgaris* L. especially if symbiotic association is constrained by various factors including inefficient strains capable of initiating the N-fixation process (Martínez *et al*., 1985; Isoi and Yoshida, 1991; Horta de Sa *et al*., 1993). This constraint could be alleviated through seed and/or soil inoculation with the proper *Rhizobium* bacteria before or at planting to facilitate N-fixation (Duque *et al*., 1985; Hardarson, 1993; Popescu, 1998**;** Ndakidemi *et al*., 2006). Studies in *P. vulgaris* L. have shown that N-fixation from *Rhizobium* inoculation contributed an N equivalence of 20 - 60 kg $N.ha^{-1}$ in Brazil (Da Silva *et al*., 1993). This is a significant amount which could otherwise only be supplemented through the use of artificial N fertilizers. Research reports have indicated significant achievements in legume growth and yield in many parts of the world following the inoculation with the appropriate inoculants (Ciafardini and Barbieri, 1987; Karanja and Wood, 1988; Hardarson, 1993; Carter *et al*., 1994; Brockwell, *et al*., 1995; Wani *et al*., 1995; Dakora, and Keya, 1997; Popescu, 1998; Zahran, 1999; Vargas *et al*., 2000; Ndakidemi *et al*., 2006).

However, nitrogen fixation involving symbiotic association between rhizobia in legumes is influenced by several factors including the availability of adequate amounts of Ca^{2+} and Mo for plant nutrition (Graham *et al*., 1982; Bell *et al*., 1989; Kucey and Hynes, 1989; Alva *et al*., 1990; Bottomley, 1992; Tu, 1992; Banath *et al.,* 1996; Andrade *et al*., 2002). According to established guidelines, some areas in the Southern Africa have been reported to be deficient in Ca^{2+} and Mo (Ndakidemi, 2005; Thibaund, 2005) and these may have N_2 fixation limitations.

Molybdenum has a notable influence on N-fixation and metabolism in N_2 fixing legumes (Parker and Harris, 1977; Franco and Munns, 1981; Marschner, 1995; Vieira *et al*., 1998). In nodulated legumes, Mo is necessary for the reduction of atmospheric nitrogen (N_2) to ammonia by nitrogenase enzyme. It has been established that the symbiotic bacteria require more Mo for N_2 fixation than does the host plant (O'hara *et al*., 1988). Molybdenum is also essential nutrient for nitrate reductase and nitrogenase enzyme activity (Westermann, 2005). The symbiotic bacterial enzyme nitrogenase is comprised of MoFe protein which is directly involved in the reduction of N_2 to NH_3 (Lambers *et al*., 1998) during fixation process. Supply of Mo to bacteroids is therefore an important process and most likely a key

regulatory component in the maintenance of nitrogen fixation in legumes that may influence plant growth (Kaiser *et al*., 2005).

When leguminous plants are grown under molybdenum deficiency conditions, phenotypes may develop with hindered or retarded plant growth characteristics due to reduced activity of molybdoenzymes and hence N-fixation (Agarwala and Hewitt, 1954; Spencer and Wood, 1954; Afridi and Hewitt, 1965; Randall, 1969; Jones *et al*., 1976; Agarwala *et al*., 1978). Generally speaking, we can conclude that, molybdenum deficiency is primarily associated with poor nitrogen health in plants and ultimately impaired growth.

Calcium supplied to plants through lime may perform multiple functions in plants. They are essential component in symbiotic N_2 fixation and nodule formation in legumes. Studies have indicated that Calcium deficiency in legumes depressed the calcium content of nodules, impairing nitrogen fixation due to inadequate calcium for nodule structure and/or metabolism (Graham, 1992; Banath *et al.,* 1996). In this context, Ca^{2+} deficiency in legume decreased the supply of fixed nitrogen from nodules to other organs, thus impairing plant growth. According to research by (Ndakidemi, 2005), the area used in our study has been reported to be deficient in Ca^{2+} and this may have N_2 fixation limitations to leguminous plants such as *P. vulgaris* L.

Despite the existence of substantial evidence on the influence of Ca^{2+}, *Rhizobium* and Mo on nitrogen fixation in pasture legumes and other related crops in Southern Africa, their effects on N_2 fixation in *P. vulgaris* L. in some parts of South Africa is not documented.

5.2 Materials and Methods

5.2.1 Site location and description

The experiments involving soils collected from the field were conducted in the glasshouse of the Cape Peninsula University of Technology, Cape Town Campus, Keizersgracht from October 2008 to December 2008. Soil material was collected from the same field experiment site described below. The field experiment was conducted under irrigation at the Agricultural Research Council Nietvoorbij site (33º54'S, 18º14'E) in Stellenbosch, South Africa, during the summer seasons from December 2008 to March 2009. The site lies in the winter rainfall region of South Africa at an elevation of 146 m above sea level. The mean annual rainfall on the farm is 713.4 mm and means annual temperatures range from 22.6ºC (day) to 11ºC (night).

The experimental site was under grass fallow for a period of 3 years. The soil type was sandy loam (Glenrosa, Hutton form), which according to the soil classification working group (SCWG, 1991) which is equivalent to skeletic leptosol according to FAO soil classification (FAO, 2001).

5.2.2 Experimental design and treatments

The experimental treatments consisted of 2 levels of *Rhizobium* inoculation (with rhizobia and without rhizobia), 3 levels of lime (0, 2, 3 $t.ha^{-1}$) and 3 levels of Mo (0, 6, 12 $g.kg^{-1}$ of seeds). The experimental design followed a spilt-split-plot design with 4 replications per treatment. The field plots measured 4 m x 4 m with 4 rows and 0.5 m apart from one another. *P. vulgaris* L. variety Provider – purchased from Rwanda was sown with inter-row planting distance of 20 cm. The plots were interspaced by small terraces of 1 m to prevent contamination. The plant population density was 200,000 $plants.ha^{-1}$.

Planting was done after ploughing and harrowing and Lime application was done 2 weeks before planting. Twelve hours before planting, seeds were soaked into Mo treatment solutions. The zero Mo (control) was also soaked in a water solution containing no Mo. To avoid contamination, all *Rhizobium* uninoculated treatments were sown first. *Rhizobium* inoculation was done manually by putting the inoculant (*Rhizobium leguminosarum* biovar phaseoli-bakteriee registrasienr. L1795 wet 36/1947 in the planting hole. The inoculants used were obtained from University of Pretoria, South Africa. In the glasshouse, 3seeds were sown in 2 kg soil carefully packed into 2 $kg.pot^{-1}$. These were thinned to two plants 10 days after sowing.

5.2.3 Plant harvest and sample preparation

At 60 d after planting, *P. vulgaris* L. plants were sampled for growth and Nitrogen analysis. About 10 plants were sampled respectively from the middle rows of each plot. The border plants within each row were excluded. The plants were carefully dug out with their entire root system, washed and divided into roots, shoots, pods. The plant organs were oven-dried at 60°C for 48 h weighed and ground into a fine powder for the analysis of Nitrogen.

5.2.4 Analysis of $\delta^{15}N$ and estimation of plant dependence on N_2 fixation

The ratio of $^{15}N/^{14}N$ and the concentrations of N in plant organs was measured using a Carlo Erba NA 1500 elemental analyser (Fisons Instruments SpA, Strada Rivoltana, Italy) coupled to a Finnigan MAT 252 mass spectrometer (Finnigan MAT Gmbh, Bremen, German) via a Conflo II open-split device. ^{15}N abundance is usually expressed in a relative, δ (delta) notation, which is the ‰ deviation of the ^{15}N natural abundance of the sample from atmospheric N_2 (= 0.36637 atom % ^{15}N) (Unkovich *et al.*, 1994):

$$\delta^{15}N = \frac{atom\%\ ^{15}N\ sample - atom\%\ ^{15}N\ air}{atom\%\ ^{15}N\ air} * 1000$$

Whole plant ^{15}N natural abundance was calculated as an average of $\delta^{15}N$ in all three plant parts used:

$$\delta^{15}N_{Whole\ plant} = \frac{\sum\left(\delta^{15}N_{root} + \delta^{15}N_{shoot} + \delta^{15}N_{pods}\right)}{3}$$

The ^{15}N natural abundance technique was used to quantify plant reliance upon N_2 fixation for growth (%Ndfa) as follows (Shearer and Kohl, 1986):

$$\%Ndfa = \left(\frac{\delta^{15}N_{RCr-}\delta^{15}N_{leg}}{\delta^{15}N_{RCr-}B_{va}}\right)\times 100$$

Where $\delta^{15}N_{RCr}$ is the ^{15}N natural abundance of the non - N_2 fixing reference plant, $\delta^{15}N_{leg}$ is the ^{15}N natural abundance of the N_2 fixing legume plant and B_{va} (B value) is the ^{15}N natural abundance of N_2 fixing plant relying on atmospheric N_2 as the sole N source. The B_{va} is included in the equation to account for ^{15}N discrimination during the N_2-fixing process in plant (Evans *et al*., 2001). Maize was used as reference plant for assessing the ^{15}N enrichment of soil. The $\delta^{15}N$ values (‰) of the reference plant material used were**:** 4.93 for roots and 4.43 for shoots and pods. B_{va} used for *P. vulgaris* L. in this study were -2.22‰ for shoots and pods and 0.95‰ for roots. (S.B.M. Chimphango and F.D. Dakora, unpublished data).

5.2.5 Statistical analysis

The data from this experiment was analyzed using the software of STATISTICA program 2008 (StatSoft Inc., Tulsa, OK, USA). When significant differences were detected by the analysis of variance (ANOVA), mean values of the $\delta^{15}N$, %N, %Ndfa, Total Nitrogen were used to compare treatment means at $P \leq 0.05$ level of significance (Steel and Torrie, 1980).

5.3 Results

5.3.1 Effects of *Rhizobium* inoculation, molybdenum and lime on dry matter yield, of nodulated *P. vulgaris* L.

Rhizobium inoculation significantly affected dry matter yield in shoots, pods and whole plant but had no effect on roots dry matter yield for glasshouse and field experiment compared with the uninoculated control treatment (Table 1). For example, *Rhizobium* inoculation increased significantly the dry matter yield for shoots by 45% for glasshouse experiment and 107% for field experiment,

whereas, with pods, the dry matter yield increased by 63% for glasshouse experiment and 104% for field experiment. At the whole plant level, *Rhizobium* inoculation increased the dry matter yield by 46% for glasshouse experiment and 88% for the field experiment.

Molybdenum application at the rate of 12 $g.kg^{-1}$ of seeds showed significant increases in dry matter yield for shoots, pods and whole plant in the glasshouse experiment compared with the control and Mo supplied at 6 $g.kg^{-1}$ of seeds (Table 1). However, highest dry matter yield was recorded in the treatment supplied with 12 g $Mo.kg^{-1}$ of seeds (Table 1).

In the field experiment, pods growth was the only parameter which was significantly increased with exogenous application at both 6 and 12 g $Mo.kg^{-1}$of seed relative to zero-Mo control treatment. Molybdenum at 6 g and 12 $g.kg^{-1}$ of seeds significantly increased the dry matter of pods by 15% to 23% respectively for field experiment compared with the control.

The supply of lime to *P. vulgaris* L. plants numerically, but not significantly, increased growth of all organs measured in this study (Table 1).

5.3.2 Effects of *Rhizobium* inoculation, molybdenum and lime on, N concentration in organs of nodulated *P. vulgaris* L.

The Rhizobial inoculation significantly increased %N of beans roots and shoots in the glasshouse, and shoots in the field study (Table 2). The exogenous supply of Mo and lime significantly increased the N concentration of roots in the glasshouse study. Shoot N concentration were significantly more at all levels of Mo and lime supply relative to zero control treatments (Table 2).

5.3.3 Effects of *Rhizobium* inoculation, molybdenum and lime on $\delta^{15}N$ of nodulated *P. vulgaris* L.

As shown in Table 2, *Rhizobium* inoculation significantly decreased $\delta^{15}N$ in shoots, roots, pods and whole plant for both glasshouse and field experiment relative to the uninoculated control treatment. Compared with the control, *Rhizobium* inoculation decreased $\delta^{15}N$ values in roots by 8% for glasshouse experiment and 37% for field experiment. *Rhizobium* Inoculation also decreased significantly the $\delta^{15}N$ values of shoots and pods thus, reflecting the observed lower $\delta^{15}N$ values at the whole-plant level (Table 3).

Molybdenum application also affected the $\delta^{15}N$ of roots, shoots, pods and whole-plant of both glasshouse and field experiment (Table 3). Relative to zero control treatment, Mo supplied at 6 and 12 $g.kg^{-1}$ of seed significantly decreased the $\delta^{15}N$ of their roots, shoots, pods, and whole plants of *P.*

vulgaris. The lowest $\delta^{15}N$ values were always recorded in the treatment supplied with 12 g Mo.kg^{-1} of seed (Table 3).

Applying lime to *P. vulgaris* L. in this study numerically, but not significantly, decreased the $\delta^{15}N$ values of roots in the field experiment. However, supplying lime at 2 and 3 t.ha^{-1} significantly decreased $\delta^{15}N$ values of roots, shoots, pods and whole plants in the glass house and those of shoots, pod and whole plant in the field experiment relative to the control treatment (Table 3). Pronounced decreases were recorded in plants supplied with lime at 3 t.ha^{-1} (Table 3).

5.3.4 Effects of *Rhizobium* inoculation, molybdenum and lime on percentage nitrogen derived from atmosphere (%Ndfa) in the nodulated *P. vulgaris*.

The percentage of nitrogen derived from the atmosphere (%Ndfa) was increased significantly with *Rhizobium* inoculation in all organs (roots, shoots, pods and whole plant) both in the glass house and field experiments compared with the uninoculated control treatment (Table 4).

Molybdenum and lime supply similarly increased the %Ndfa in all organs of *P. vulgaris* L. reported in this study (Table 4). In both glasshouse and field experiment, significantly more N was derived from fixation in treatments supplied with Mo (6 and 12 g.kg^{-1} of seed) and lime (2 and 3 t.ha^{-1}) as compared with the zero control treatments, with the highest rates of these inputs showing to facilitate the symbiotic fixation in *P. vulgaris* L. (Table 4).

5.3.5 Effects of *Rhizobium* inoculation, molybdenum and lime on total N (mg.plant^{-1}) in a nodulated *P. vulgaris* L.

Rhizobium inoculation treatment was the most influential one in increasing the plant total N content in different organs and whole plants of *P. vulgaris* L. (Table 5). Generally, inoculation significantly resulted into elevated N content of roots, shoots, pods and whole plant in the glasshouse (Table 5). Similarly, inoculation significantly improved N content of shoots, pods and whole plant in the field (Table 5).

With Mo application, there was no significant effects on total N of organs in most parameters measured except at the whole plant level whereby N content was markedly increased by the application Mo at 6 and 12 g.kg^{-1} of seed (Table 5).

The application of lime had no effect on total N of all bean organs (Table 5).

5.3.6 Effects of *Rhizobium* inoculation, molybdenum and lime on N-fixed in a nodulated *P. vulgaris* L.

Rhizobium inoculation of *P. vulgaris* L. had a significant influence on the amount of N-fixed (mg.plant^{-1}). Relative to the uninoculated treatments, inoculation significantly increased the N-fixed of roots, shoots, pods and whole plants of *P. vulgaris* L. grown in the glasshouse and in the field (Table 6). Similarly, when the contribution of N fixed was assessed on per ha basis, it clearly showed that the amount of N from bean residues of the whole plant level could account for 33 kg N.ha^{-1} in the *Rhizobium* inoculation as compared with 8.6 kg N.ha^{-1} in un-inoculation control.

As indicated in Table 6, supplying Mo to *P. vulgaris* L. grown in the glasshouse significantly increased the amount of N-fixed on roots, shoots, pods and ultimately in whole plants. Applying Mo to *P. vulgaris* L. in the field numerically, but not significantly, increased the amount of N-fixed values of roots (Table 6). However, application of Mo in the field significantly increased N-fixed in other parameters (shoots, pods and whole plants). Molybdenum application at 6 and 12 g.kg^{-1} of seed produced plants with higher N from fixation (kg N.ha^{-1}) relative to the zero Mo control treatments (Table 6).

5.3.7 Interactive effects of *Rhizobium*, molybdenum and lime on nodulated *P. vulgaris* L.

The interaction between *Rhizobium* and molybdenum was significant on the dry matter yield in roots and pods for glasshouse and field experiment and for only glasshouse experiment in shoots and whole plant dry matter yield. In most cases, measurements with the highest values were recorded in the treatments supplied with *Rhizobium* and Mo at different rates (Figures 1-1A, B, C, D; Figure 1-2A, B).

Results from the glasshouse showed that there was interactive effect between *Rhizobium* inoculation and Mo on $\delta^{15}N$ values in shoot in the glasshouse (Figure 2-1), and whole plants and pods in the field (Figure 2-2A, B). Reduced $\delta^{15}N$ values were mostly recorded in inoculated treatments supplied with Mo at 6 and 12 g.kg^{-1} of seed. Furthermore, fertilizing with lime at 2 and 3 t.ha^{-1} in combination with rhizobial inoculation also resulted into significant interactions. Plants receiving lime and rhizobial inoculants had significantly more reduced $\delta^{15}N$ values in pods harvested from field study as compared with their counterparts in the un-inoculated treatments (Figure 2-2C). In this study, the interaction between *Rhizobium* x molybdenum x lime influenced the $\delta^{15}N$ values of pods significantly in the field. *Rhizobium* inoculation together with Mo and lime gave the lowest values of $\delta^{15}N$ in *P. vulgaris* L. pods as compared with un-inoculated supplied with Mo and lime (Figure 2-2D).

Under glasshouse and field conditions, the inoculation with *Rhizobium* in combination with molybdenum significantly increased %Ndfa on shoots in the glasshouse and pods in the field (Figure

3A and B). The combination of these supplies increased the amount of N which was derived from atmosphere in *P.vulgaris* L. as compared with un-inoculated treatments. The effect of Mo and lime also interacted significantly at different levels of their application, with higher %Ndfa being recorded in pods collected from field experiment and supplied with higher levels of Mo and lime (Figure 3C). Overall, the combinations of *Rhizobium*, Mo and lime significantly stimulated the %Ndfa in pods grown in the field experiment (Figure 3D).

The results in Figure 4 show the interactive effects of *Rhizobium* inoculation and molybdenum and lime on total N (mg.plant^{-1}). They clearly demonstrate that molybdenum applied in combination with lime and rhizobial significantly improved the total N (mg.plant^{-1}) in the whole plant (Figure 4A) as compared with un-inoculated treatments.

In this study, a significant *Rhizobium* x Mo; *Rhizobium* x lime; and *Rhizobium* x Mo x lime were obtained on N fixed in pods of *P. vulgaris*. Supplying these inputs resulted into more N fixed per pod (Figure 5-1A, B and C) in the field experiment. Significant interactions between *Rhizobium* x Mo x lime were also observed on N fixed per plant (Figure 5-2A and B) at the whole plant level both in the field and in the glasshouse. The results generally indicated that lime combined with application of Mo and in presence of *Rhizobium* inoculation resulted into significantly increased N-fixed in the whole plant relative to the un-inoculated counterparts.

5.4 Discussion

In this study, nodulated *P. vulgaris* L. plants inoculated with *Rhizobium leguminosarum* showed positive effects on plant growth (i.e. roots, shoots, pods and whole plant) for glasshouse and field experiment compared with the un-inoculated control. These positive results are encouraging as N nutrition which finally improved plant growth was significantly achieved through the simple symbiotic relationship between *P. vulgaris* L. and the rhizobial bacteria. Nitrogen is one of the most limiting nutrients to plant growth. Its supply to plants is mostly done through the application of mineral fertilizers. This practice is not only expensive, but also unsustainable to small scale poor farmers' such as those found in Africa who cannot afford to purchase. Alleviation of N problem through *Rhizobium* inoculants is the best alternative in promoting legume productivity in Africa. Similar to our results, Ndakidemi *et al*. (2006) also reported significant improvements in *P. vulgaris* L. and soybean growth with the application of bean inoculants in Tanzania.

Mo application also significantly increased dry matter yield of some organs of beans compared with the zero control treatments. Noticeable significant results were reported in the glasshouse experiment. Highest significant whole plant dry weights in the glasshouse (8.66 g.plant^{-1}) and pods in the field (2.92 g.plant^{-1}) were obtained with Mo at the highest rate 12 g.kg^{-1} of seed. Similar to our results,

(Agarwala and Hewitt, 1954; Spencer and Wood, 1954; Afridi and Hewitt, 1965; Randall, 1969; Jones *et al*., 1976; Agarwala *et al*., 1978) also reported retarded plant growth in leguminous plants grown under molybdenum deficiency conditions such as those observed in the control treatment in our study (Table 1).

The $\delta^{15}N$ values of *P. vulgaris* L. in all organs measured in the glasshouse and field experiments were significantly decreased with *Rhizobium* inoculation and the supply of Mo and at 6 and 12 $g.kg^{-1}$ of seed and lime at 2 and 3 $t.ha^{-1}$ (Table 2). It is generally accepted that the lower $\delta^{15}N$ values in the organ of a legume, the greater will be the amount of N that will be derived from atmospheric fixation (Shearer and Kohl, 1986; Unkovich *et al*., 1994; Peoples *et al*., 1996; Gathumbi *et al*., 2002; Chikowo *et al*., 2004; Ndakidemi, 2005; Makoi *et al*., 2009) a phenomenon which was also reflected in our study (Table 4). The significant increases in %Ndfa with *Rhizobium* inoculation alone as compared with the inoculated control by 88% (Table 4) in the whole plants harvested from the field experiment represents a very significant contribution to N nutrition in *P. vulgaris* L. and a cheaper option for supplying N as compared with the expensive inorganic fertilizers. Molybdenum application at the rate of 6 and 12 $g.kg^{-1}$ of seed significantly increased %Ndfa by 22 and 52% respectively, whereas lime at 2 and 3 t per ha increased the %Ndfa from 8 to 18% respectively (Table 4). The increment in %Ndfa with Mo and lime supply indicates that the symbiotic functioning in *P. vulgaris* was enhanced by these three important inputs in the study area. Similarly, in their research, (Bhaskaran, 1936; Warington, 1950; Banath *et al*., 1966; Gurley and Giddens, 1969; Franco and Munns, 1981; Ishizuka, 1982; Brodrick and Giller, 1991; Graham, 1992) showed that Mo and Ca^{+} supply in legumes improved N_2 fixation in legumes. However, to quantify the use of these inputs in small scale holdings, an economic analysis is recommended.

The N-fixed in all organs of *P. vulgaris* L. assessed in glasshouse and field study ($mg.plant^{-1}$) were significantly increased by *Rhizobium* inoculation when compared with the un-inoculated control (Table 6). When computed on a per hectare basis, the estimated fixed rate with *Rhizobium* inoculation amounted to 32.7 kg $N.ha^{-1}$ which was an increase of 280% compared with the control treatment. This value is within the reported amounts (20 - 60 kg $N.ha^{-1}$) of N-fixation in *P. vulgaris* L. from *Rhizobium* inoculation in Brazil (Da Silva *et al*., 1993).

In the glasshouse and field study, Mo also played a crucial role on N- fixed in *P. vulgaris* L. For example, relative to the zero control in the field study, the application of Mo at 6 and 12 $g.kg^{-1}$ of seeds increased significantly the N fixed (kg $N.ha^{-1}$) by 45% and 71% respectively (Table 6). Molybdenum is known to be responsible in N fixation process by improving nodule functioning through improved nitrogenase enzyme activity (Westermann, 2005) and finally the N_2 fixation in legumes (Agarwala and Hewitt, 1954; Spencer and Wood, 1954; Afridi and Hewitt, 1965; Randall, 1969; Jones *et al*., 1976; Parker and Harris 1977; Agarwala *et al*., 1978; Franco and Munns, 1981;

Sharma *et al*., 1988; Marschner, 1995; Lambers *et al*., 1998; Vieira *et al*., 1998). Similar to our work (Table 6), experiments with a variety of other related legumes have shown that molybdenum fertilization enhanced nitrogen-fixing symbiosis (Parker and Harris, 1977; Rhodes and Kpaka, 1982; Adams, 1997; Nautiyal and Chatterjee, 2004).

The interactive effects between *Rhizobium*, Mo and lime application (Figures 1 and 5-2) were reported in our study. The maximum dry matter yield occurred in the treatments involving *Rhizobium* inoculation and highest rates of Mo (Figures 1-1A; B and C; 1-2A and B). The interactive effect between *Rhizobium* and Mo were also recorded on $\delta^{15}N$ values with significantly reduced $\delta^{15}N$ values appearing in inoculated treatments and combined with Mo at 6 and 12 $g.kg^{-1}$ of seed (Figures 2-1; 2-2 A and B) and lime at 2 and 3 $t.ha^{-1}$ (Figure 2-2C, D) thus resulting into significant interactions in %Ndfa (Figures 3 A, B, C and D) and N fixed per plant (Figures 1-1A, B and C; 5-2A and B). The combined application of *Rhizobium* inoculant along with the supply of Mo and lime proved to be the suitable combination of inputs for the cultivation of *P. vulgaris* L. in the study area.

In conclusion, N nutrition of *P. vulgaris* L. was improved by *Rhizobium* inoculation, Mo and Lime application both in the glasshouse and field experiment. *Rhizobium* inoculation alone significantly contributed 32.7 kg $N.ha^{-1}$ relative to un-inoculated control. This option seems simple and low-cost technology which could be adopted by farmers of all categories. Mo application at the rate of 6 and 12 $g.kg^{-1}$ of seed and lime at 2 and 3 $t.ha^{-1}$ significantly increased some of the symbiotic N fixation parameters compared with zero control treatments. Furthermore, the interactive effects were found between *Rhizobium* x Mo x lime application implying that supply of these inputs in the study area is important if higher yields of *P. vulgaris* L. have to be realized.

Table 5-1: Effect of *Rhizobium*, molybdenum and lime on dry matter yield (g.plant^{-1}) measured in glasshouse and in field during 2008 and 2009 seasons.

Treatment	Glasshouse				Field			
	Nodulated roots	Shoots	Pods	Whole plant	Nodulated roots	Shoots	Pods	Whole plant
Rhizobium								
-R	1.03±0.03a	2.66±0.21b	2.50±0.13b	6.19±0.31b	12.83±0.74a	47.97±4.46b	1.76±0.10b	62.56±4.63b
+R	1.09±0.02a	3.86±0.14a	4.08±0.13a	9.03±0.25a	14.52±0.67a	99.19±8.76a	3.59±0.10a	117.30±8.66a
Molybdenum (g.kg^{-1})								
0	1.01±0.04a	2.80±0.23b	3.00±0.25b	6.81±0.49b	13.94±1.09a	58.07±7.40a	2.38±0.25b	74.40±7.95a
6	1.08±0.03a	3.13±0.29b	3.15±0.22b	7.36±0.48b	13.60±0.76a	77.56±10.65a	2.73±0.25a	93.89±10.87a
12	1.08±0.03a	3.85±0.15a	3.72±0.18a	8.66±0.28a	13.48±0.76a	85.10±11.00a	2.92±0.16a	101.50±10.94a
Lime (t.ha^{-1})								
0	1.01±0.05a	3.18±0.26a	3.06±0.23a	7.25±0.46a	12.12±0.78a	75.88±10.80a	2.50±0.24a	90.50±11.11a
2	1.07±0.02a	3.38±0.25a	3.40±0.23a	7.85±0.45a	14.46±0.97a	72.11±9.06a	2.69±0.23a	89.26±9.34a
3	1.09±0.03a	3.22±0.24a	3.42±0.23a	7.73±0.46a	14.44±0.81a	72.75±10.34a	2.84±0.21a	90.03±10.36a
3-Way ANOVA (F-Statistic)								
R	3.168 NS	26.9449**	91.537**	67.395***	3.3712 NS	23.7888***	194.080***	27.4597***
Mo	1.889 NS	7.2506**	7.112**	10.026***	0.0916 NS	2.3523 NS	5.602**	2.3867 NS
L	1.814 NS	0.2975 NS	2.058 NS	1.133 NS	2.8438 NS	0.0492 NS	2.326 NS	0.0047 NS

-R: without *Rhizobium*; +R: with *Rhizobium*. Values presented are means ± SE. **, *** = significant at $P \leq 0.01$, $P \leq 0.001$ respectively; NS = not significant. Means followed by similar letter in a column are not significantly different from each other at $P \leq 0.05$.

Table 5-2: Effect of *Rhizobium*, Mo and Lime on $\delta^{15}N$ measured in glasshouse and in field during 2008 and 2009 seasons.

Treatment	Glasshouse				Field			
	Nodulated roots	Shoots	Pods	Whole plant	Nodulated roots	Shoots	Pods	Whole plant
Rhizobium								
-R	3.50±0.11a	1.56±0.17a	2.52±0.24a	2.53±0.15a	3.34±0.11a	2.63±0.12a	1.79±0.25a	2.59±0.14a
+R	3.22±0.07b	-0.32±0.11b	0.51±0.15b	1.14±0.09b	2.11±0.13b	0.60±0.14b	-0.79±0.03b	0.64±0.08b
Molybdenum ($g.kg^{-1}$)								
0	3.75±0.11a	1.50±0.27a	2.67±0.32a	2.64±0.10a	3.19±0.17a	2.36±0.22a	1.18±0.43a	2.24±0.26a
6	3.20±0.11a	0.50±0.21b	1.42±0.25b	1.70±0.16b	2.79±0.16b	1.65±0.22b	0.71±0.32b	1.71±0.22b
12	3.14±0.11b	-0.14±0.18c	0.45±0.21c	1.15±0.13b	2.19±0.19c	0.84±0.25c	-0.38±0.13c	0.88±0.17c
Lime ($t.ha^{-1}$)								
0	3.58±0.14a	0.83±0.29a	1.86±0.34a	2.09±0.23a	2.91±0.18a	1.88±0.25a	0.85±0.37a	1.88±0.25a
2	3.34±0.11ab	0.70±0.26b	1.57±0.32a	1.87±0.21ab	2.74±0.19a	1.65±0.24ab	0.52±0.36b	1.64±0.24b
3	3.17±0.11b	0.34±0.22b	1.11±0.29b	1.54±0.18b	2.52±0.21a	1.32±0.29b	0.13±0.30c	1.33±0.24c
3-way ANOVA (F-Statistic)								
R	4.90*	317.25***	129.71***	113.10***	72.93***	397.50***	4071.977***	908.483***
Mo	9.87***	82.02***	52.31***	44.12***	16.37***	74.13***	523.151***	150.331***
L	3.71*	7.72**	6.02**	5.96***	2.455 NS	10.04***	105.932***	24.756***

-R: without *Rhizobium*; +R: with *Rhizobium*. Values presented are means ± SE. *, **, *** = significant at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ respectively, NS = not significant. Means followed by similar letter (s) in a column are not significantly different from each other at $P \leq 0.05$.

Table 5-3: Effect of *Rhizobium*, molybdenum and lime on the %N in different parts of *P. vulgaris* L. measured in glasshouse and in field during 2008 and 2009 seasons.

Treatment	Glasshouse				Field			
	Nodulated roots	Shoot	Pod	Whole plant	Nodulated roots	Shoot	Pod	Whole plant
Rhizobium								
-R	1.01±0.04b	1.89±0.12b	5.43±0.44a	2.78±0.15a	0.98±0.05a	1.58±0.06b	2.87±0.06a	1.81±0.04b
+R	1.21±0.11a	3.15±0.25a	5.10±0.34a	3.15±0.13a	1.06±0.05a	1.83±0.08a	2.81±0.09a	1.90±0.05a
Molybdenum ($g.kg^{-1}$)								
0	1.26±0.15a	2.46±0.30a	5.44±0.58a	3.05±0.20a	1.08±0.07a	1.61±0.07a	2.58±0.04a	1.76±0.03c
6	1.11±0.05b	2.58±0.30a	5.10±0.44a	2.93±0.16a	1.00±0.05a	1.78±0.12a	3.08±0.12a	1.95±0.07a
12	0.96±0.06c	2.53±0.23a	5.25±0.42a	2.91±0.16a	0.98±0.05a	1.73±0.08a	2.87±0.07a	1.86±0.04b
Lime ($t.ha^{-1}$)								
0	1.30±0.15a	2.77±0.30a	5.55±0.58a	3.20±0.20a	0.98±0.06a	1.79±0.09a	2.93±0.08a	1.90±0.05a
2	1.04±0.05b	2.69±0.29a	5.20±0.44a	2.98±0.17a	1.05±0.06a	1.69±0.07a	2.78±0.07a	1.84±0.04a
3	1.00±0.06c	2.10±0.21a	5.05±0.41a	2.72±0.14a	1.03±0.05a	1.64±0.12a	2.81±0.12a	1.83±0.07a
3-Way ANOVA (F-Statistics)								
R	3.96*	20.4527*	0.30 NS	3.34NS	1.32 NS	5.90*	7.57NS	4.92*
Mo	3.21*	0.06 NS	0.10 NS	0.19NS	0.97 NS	0.92NS	23.43NS	8.44**
L	3.81*	2.28 NS	0.23 NS	1.917NS	0.41 NS	0.72 NS	3.01NS	1.38NS

-R: without *Rhizobium*; +R: with *Rhizobium*. Values presented are means ± SE. *, ** = significant at $P \leq 0.05$, $P \leq 0.01$ respectively, NS = not significant. Means followed by similar letter in a column are not significantly different from each other at $P \leq 0.05$.

Table 5-4: Effect of *Rhizobium*, molybdenum and lime on %Ndfa measured in glasshouse and in field during 2008 and 2009 seasons.

Treatments	Glasshouse					Field				
	Nodulated roots	Shoots	Pods	Whole plant	% Increase[h]	Nodulated roots	Shoots	Pods	Whole plant	% Increase[h]
Rhizobium										
-R	36.0±2.9b	47.1±2.4b	30.1±3.3b	37.7±2.9b	-	45.7±3.0b	27.1±1.8b	39.7±3.8b	37.5±2.9b	-
+R	42.9±1.9a	73.4±1.6a	59.0±2.2a	58.4±1.9a	55	75.5±3.5a	57.6±2.2a	78.5±0.5a	70.5±2.1a	88
Molybdenum ($g.kg^{-1}$)										
0	29.7±2.8b	48.0±3.8c	28.7±4.2c	35.5±3.6c	-	49.9±4.8b	31.1±3.4c	48.9±6.5c	43.3±4.9c	-
6	43.6±2.7a	61.9±2.9b	45.2±3.7b	50.2±3.1b	41	60.6±4.8ab	41.9±3.3b	56.0±4.8b	52.8±4.3b	22
12	45.0±2.7a	71.0±2.5a	59.8±3.2a	58.6±2.8a	65	71.3±4.8a	53.9±3.8a	72.4±1.9a	65.9±3.5a	52
Lime ($t.ha^{-1}$)										
0	34.0±3.5b	57.4±4.1b	40.1±4.6b	43.83±4.1b	-	57.1±5.1a	38.4±3.8b	53.8±5.6c	49.8±4.8b	-
2	40.0±2.8ab	59.2±3.7b	43.8±4.5ab	47.67±3.7ab	9	60.8±5.0a	41.8±3.6b	58.8±5.3b	53.8±4.6ab	8
3	44.3±2.5a	64.2±3.1a	49.9±4.4a	52.80±3.3a	21	63.9±5.1a	46.8±4.4a	64.7±4.5a	58.5±4.7a	18
3-Way ANOVA (F-Statistic)										
R	4.9**	317.2***	127.6***	149.9***	-	40.8***	397.5***	4072.0***	1503.4***	-
Mo	9.9***	82.0***	49.4***	47.1***	-	7.0**	74.1***	523.2***	201.4***	-
L	3.7*	7.7**	5.0*	5.5*	-	0.7 NS	10.0***	105.9***	38.9***	-

-R: without *Rhizobium*; +R: with *Rhizobium*. Values presented are means ± SE. *, **, *** = significant at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ respectively, NS = not significant. Means followed by similar letter in a column are not significantly different from each other at $P \leq 0.05$. ([h]: % increase was obtained by subtracting control from the treatment, divided by the control and multiplied by 100).

Table 5-5: Effect of *Rhizobium*, molybdenum and lime on total N (mg.plant^{-1}) measured in glasshouse and in field during 2008 and 2009 seasons.

Treatment	Glasshouse				Field			
	Nodulated roots	Shoots	Pods	Whole plant	Nodulated roots	Shoots	Pods	Whole plant
Rhizobium								
-R	10.38±0.50b	49.33±4.47b	140.10±13.80b	199.81±15.59b	12.83±0.74a	47.97±4.46b	53.58±1.66b	114.38±5.34b
+R	13.25±1.39a	118.89±9.00a	208.01±15.93a	340.16±16.56a	14.52±0.67a	99.19±8.76a	118.28±5.89a	231.99±11.10a
0	13.20±2.07a	70.36±10.61a	169.66±22.69a	253.21±27.49a	13.94±1.09a	58.07±7.40a	74.79±6.01a	146.81±12.69b
6	11.96±0.56a	87.96±14.16a	160.35±18.57a	260.27±26.18a	13.60±0.76a	77.56±10.65a	94.36±12.07a	185.52±19.74a
12	10.28±0.66a	94.01±7.88a	192.17±16.80a	296.47±18.31a	13.48±0.76a	85.10±11.00a	88.65±5.61a	187.23±14.20a
Lime (t.ha^{-1})								
0	13.63±2.07a	87.17±11.39a	171.94±22.25a	272.74±25.95a	12.12±0.78a	75.88±10.80a	87.19±9.88a	175.19±18.45a
2	10.99±0.51a	95.11±12.34a	180.29±20.13a	286.39±26.72a	14.46±0.97a	72.11±9.06a	89.82±8.85a	176.39±16.11a
3	10.82±0.70a	70.05±9.61a	169.94±16.19a	250.81±20.19a	14.44±0.81a	72.75±10.34a	80.79±6.66a	167.97±13.99a
3-Way ANOVA (F-Statistic)								
R	4.90*	52.10***	8.95**	36.38***	3.37 NS	23.79***	49.10***	127.89***
Mo	1.70 NS	2.16 NS	0.69 NS	1.32 NS	0.09 NS	2.35 NS	1.30 NS	6.44**
L	1.95 NS	2.35 NS	0.08 NS	0.79 NS	2.84 NS	0.05 NS	0.12 NS	0.26 NS

-R: without *Rhizobium*; +R: with *Rhizobium*. Values presented are means ± SE. *, **, *** = significant at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ respectively, NS = not significant. Means followed by similar letter in a column are not significantly different from each other at $P \leq 0.05$.

Table 5-6: Effect of *Rhizobium*, molybdenum and lime on N-fixed measured in glasshouse and in field during 2008 and 2009 seasons

Treatment	Glasshouse					Field					
	Nodulated roots	Shoots	Pods	Whole plant		Nodulated roots	Shoots	Pods	Whole plant		
	mg plant^{-1}				% increase	mg plant^{-1}				kg ha^{-1}	% increase
Rhizobium											
-R	3.73±0.35b	19.26±2.72b	46.45±6.31b	69.44±8.13b	-	5.89±0.55b	14.12±2.05b	22.8±2.6b	42.8±4.5b	8.6±0.9b	-
+R	5.53±0.48a	50.26±4.48a	124.32±10.88a	180.10±11.51a	159	10.85±0.63a	59.99±6.84a	92.6±4.5a	163.5±8.2a	32.7±1.6a	280
Molybdenum (g.kg^{-1})											
0	3.99±0.67a	21.99±3.77b	61.49±13.08b	87.47±15.36c	-	7.27±0.99a	22.15±4.55b	45.2±7.7c	74.7±12.2c	14.9±2.4c	-
6	5.40±0.53a	39.73±7.16a	77.33±11.14b	122.47±16.14b	40	8.30±0.86a	37.76±7.85ab	61.9±11.0b	108.0±17.0b	21.6±3.4b	45
12	4.49±0.37a	42.56±4.23a	117.33±13.84a	164.38±15.08a	88	9.54±0.75a	51.27±9.18a	65.9±5.4a	126.8±13.2a	25.4±2.6a	71
Lime (t.ha^{-1})											
0	4.54±0.69a	32.17±5.37a	74.58±11.52a	111.29±14.93a	-	7.18±0.91a	36.00±7.80a	57.0±9.5b	100.1±15.9a	20.0±3.2a	-
2	4.39±0.42a	40.77±6.74a	88.26±14.80a	133.41±18.66a	-	8.76±0.91a	35.46±7.12a	59.6±8.9a	103.8±15.0a	20.8±3.0a	-
3	4.96±0.51a	31.34±4.19a	93.31±14.10a	129.62±16.41a	-	9.17±0.81a	39.72±8.55a	56.5±7.1b	105.4±14.1a	21.1±2.8a	-
3-Way ANOVA (F-Statistic)											
R	9.32**	42.12***	43.05***	79.60***	-	36.83***	40.37***	14545.9***	335.8***	335.8***	-
Mo	1.97 NS	7.27**	7.84**	12.85***	-	2.59 NS	5.43**	479.2***	21.4**	21.4***	-
L	0.34 NS	1.59 NS	0.89 NS	1.21 NS	-	2.20 NS	0.14 NS	10.9***	0.2NS	0.2NS	-

-R: without *Rhizobium*; +R: with *Rhizobium*. Values presented are means ± SE. **, *** = significant at $P \leq 0.01$, $P \leq 0.001$ respectively, NS = not significant. Means followed by similar letter in a column are not significantly different from each other at $P \leq 0.05$.

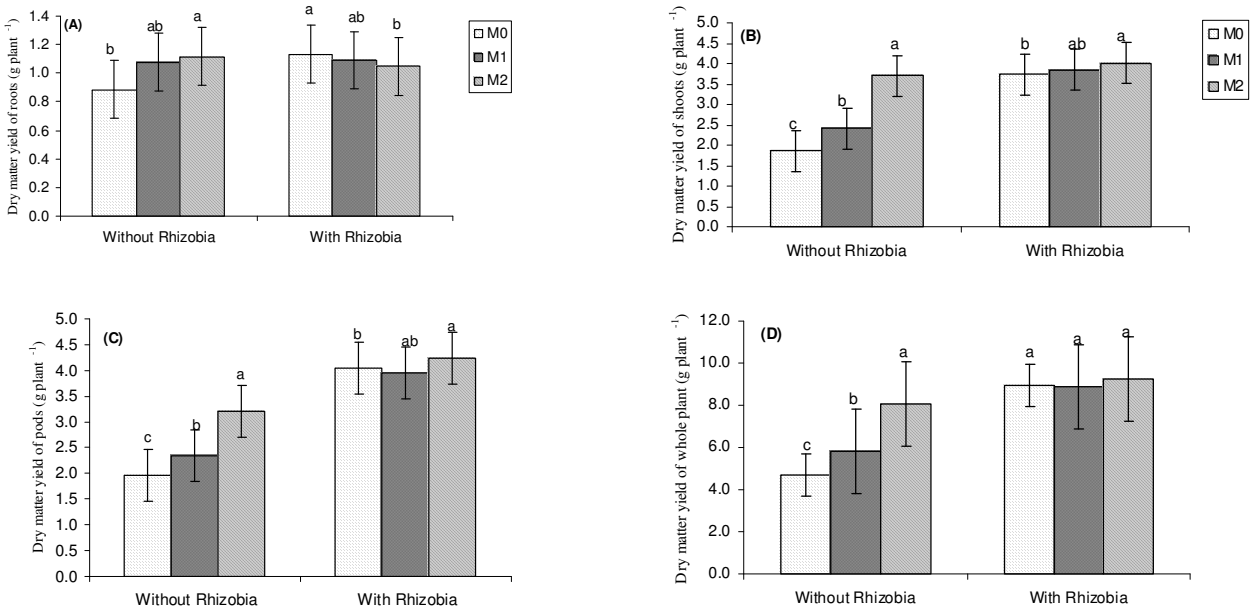

Fig.1-1: Interactive effect of *Rhizobium* and molybdenum in dry matter yield measured in the glasshouse experiment in 2008 (A): Roots, (B) Shoots, (C) Pods, (D) Whole plant.

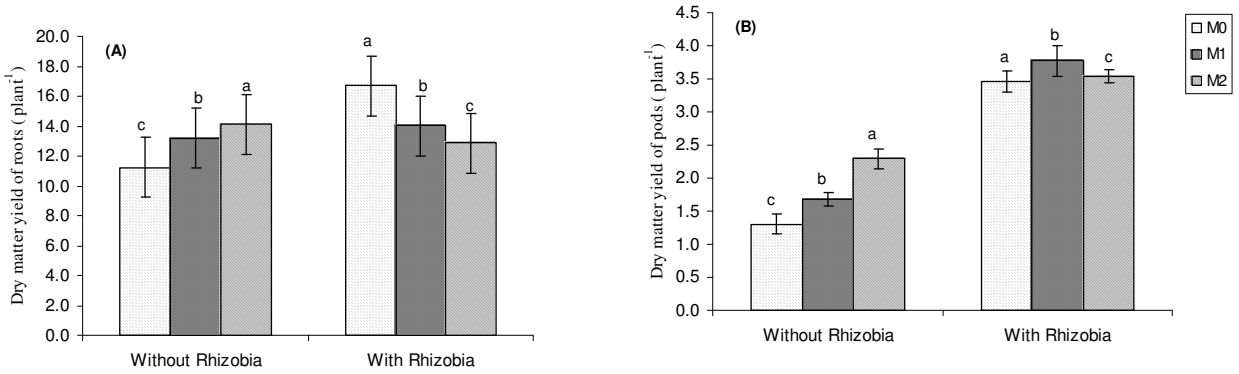

Fig.1-2: Interactive effect of *Rhizobium* and molybdenum in dry matter yield measured in the field experiment in 2009 (A): Roots, (B) Pods.

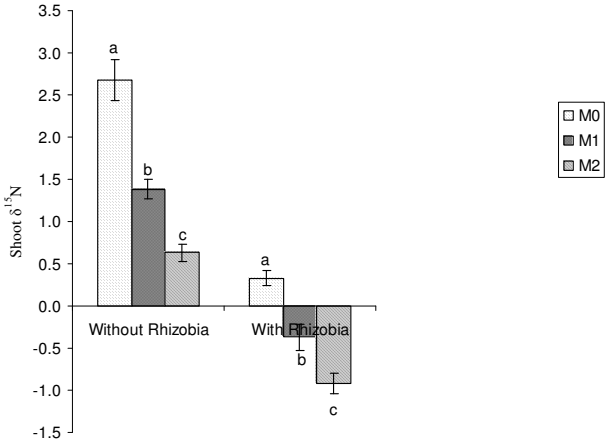

Fig.2-1: Interactive effect of *Rhizobium* and molybdenum on $\delta^{15}N$ in shoot as measured in the glasshouse experiment in 2008.

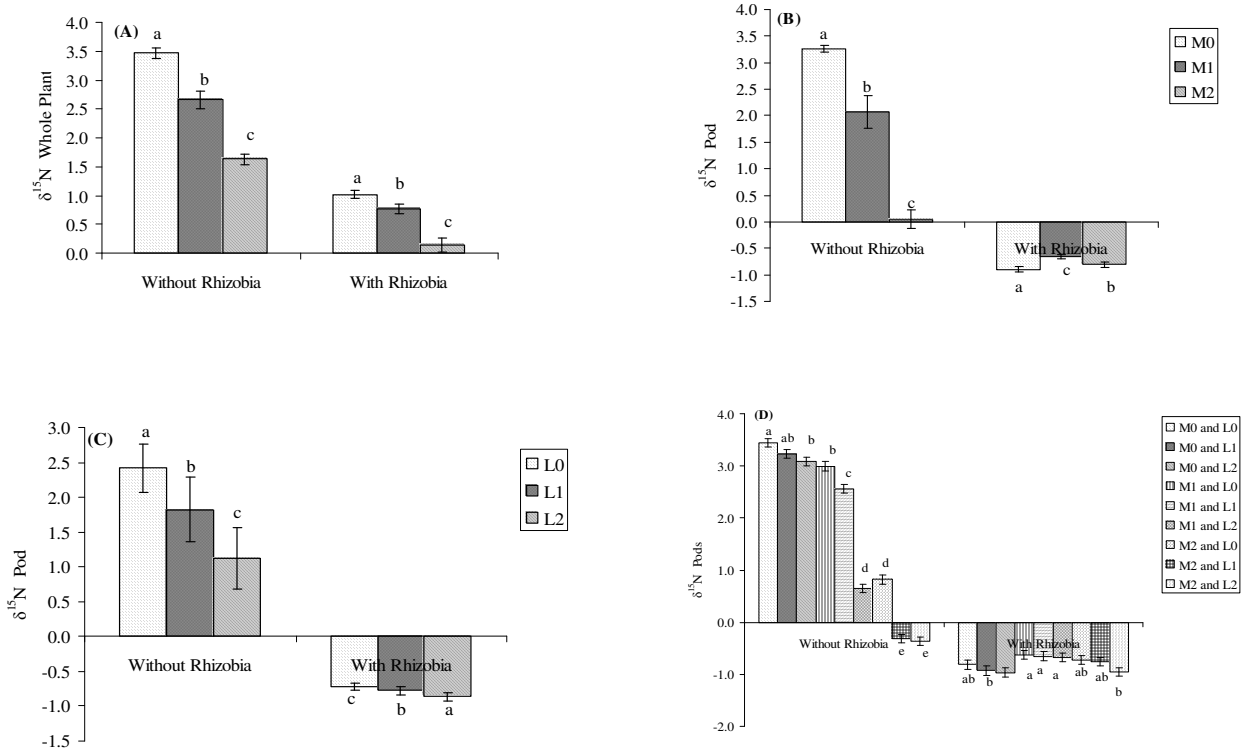

Fig. 2-2: Interactive effect of (A) *Rhizobium* and molybdenum on whole plant $\delta^{15}N$ in field experiment and (B) *Rhizobium* and molybdenum, (C) *Rhizobium* and Lime, (D) *Rhizobium,* molybdenum and lime on $\delta^{15}N$ pod in field experiment in 2009.

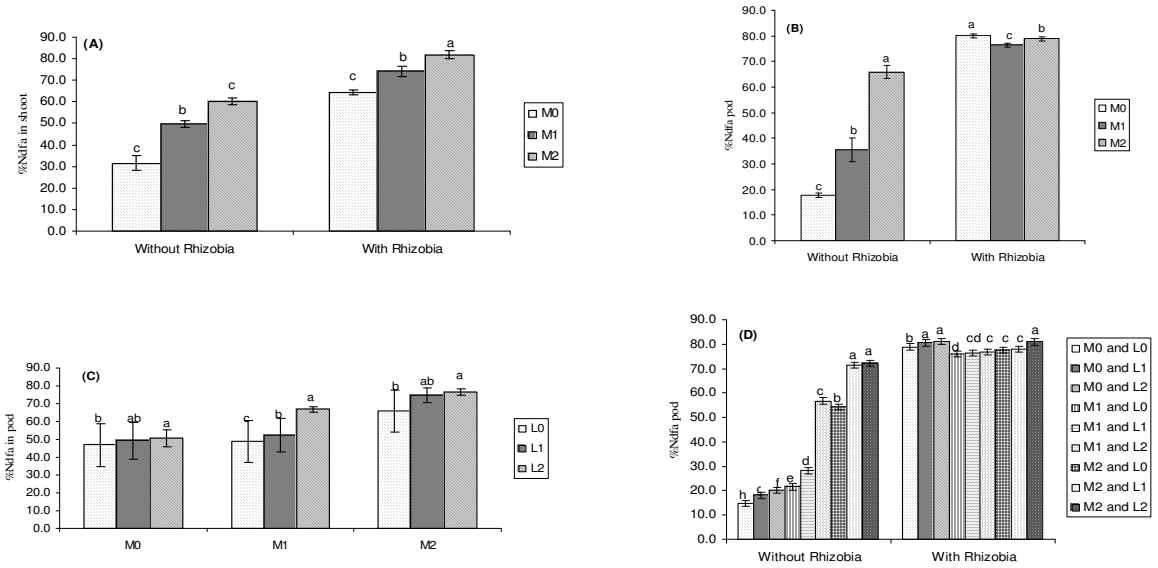

Fig.3: Interactive effect of (A) *Rhizobium* and lime on shoots %Ndfa in glasshouse experiment, (B) *Rhizobium* and molybdenum on pods %Ndfa in field, (C) molybdenum and lime on pods %Ndfa in field experiment, (D) *Rhizobium,* molybdenum and lime on pods %Ndfa in field experiment in 2009.

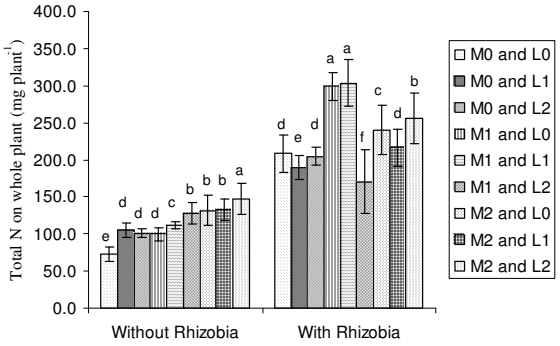

Fig.4: Interactive effect of *Rhizobium*, molybdenum and lime on whole plant total N in field experiment in 2009.

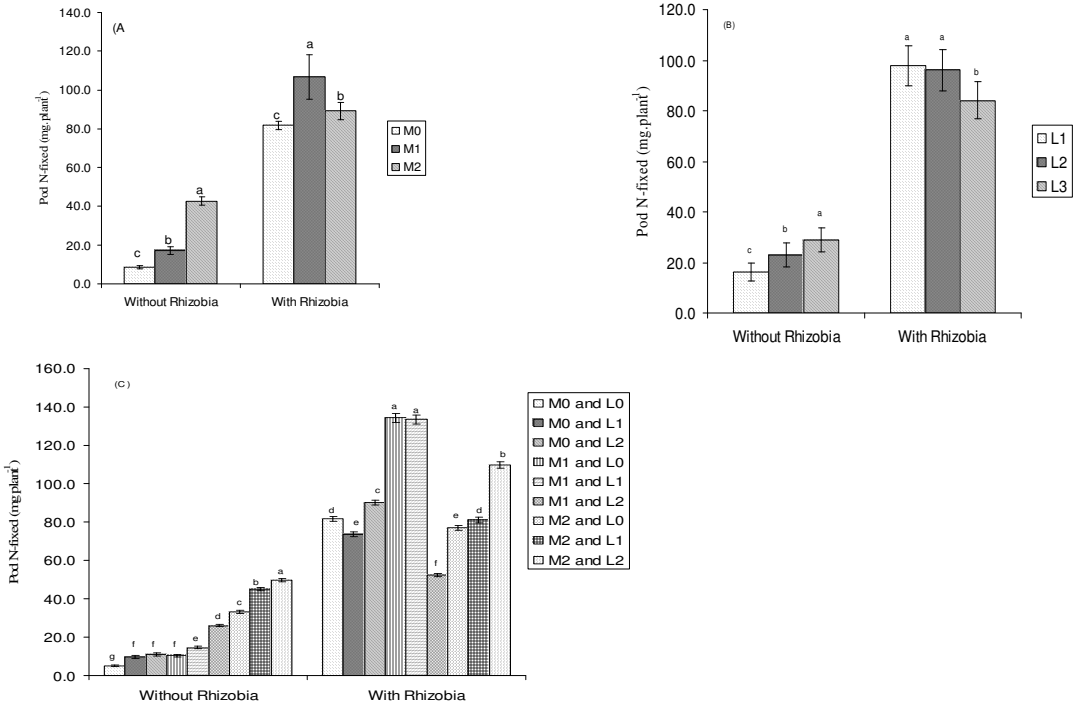

Fig.5-1: Interactive effect of (A) *Rhizobium* and molybdenum, (B) *Rhizobium* and lime, (C) molybdenum and lime, *Rhizobium,* molybdenum and lime on N-fixed in Pod in field experiment in 2009.

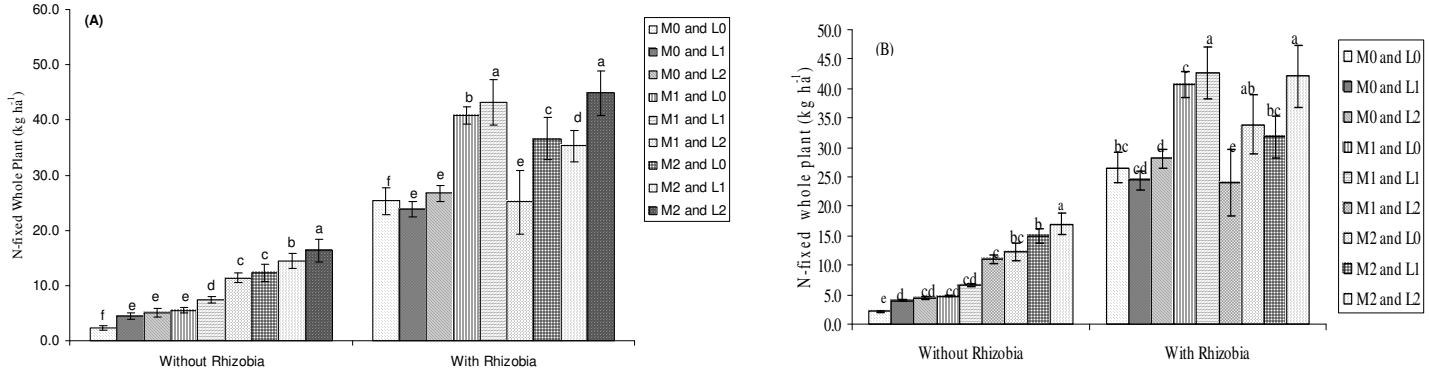

Fig.5-2: Interactive effect of *Rhizobium,* molybdenum and lime on N-fixed in whole plant in whole plant in glasshouse in 2008 (A) and field experiment in 2009 (B).

CHAPTER 6

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

Most African soils have acidic soil environments. Ca, Mg, and Mo have been reported as the main limiting nutrients in legume production. These acidic environments may also affect all stages of growth and specifically the legume-*Rhizobium* symbiosis and ultimately the nitrogen fixation (Munns, 1978; Keyser and Munns, 1979; Graham *et al*., 1982; Wood *et al*., 1984). Results from this study conducted in the glasshouse and verified in the field condition have shown that *Rhizobium* inoculation increased plant growth, N-fixation, Chlorophyll content in the leaves, the rate of photosynthesis and the grain yields of *P. vulgaris* L. Furthermore, rhizobial inoculation increased the rhizosphere concentration of mineral nutrients such as P, K, Ca, Mg, Na, Fe, Cu, Zn and Mn and the soil pH. In Africa, where farmers cannot afford the expensive inorganic N fertilizers, the use of this input may be a step towards doubling crop yields in farmers' fields. But care should be taken to ensure that the appropriate *Rhizobium* strains that are efficient under different environmental conditions are selected. If implemented properly, this may result into greater economic returns to farmers, as this technology is simple, cheap and sustainable.

As shown in this study, rhizobial inoculation resulted into significant elevated levels of mineral nutrients (P, K, Ca, Mg, Na Fe, Cu, Zn and Mn) in the soil. This implies that there is a synergistic effect from the micro organism. More studies to understand the mechanisms involved are recommended.

Furthermore, rhizobial inoculation as compared with uninoculated treatments improved the N content of *P. vulgaris* L. in different organs. For example, root, shoot and pod values of N were all elevated. These increases in N-content of *P. vulgaris* L. organs in this study could have implications in nutrient cycling into the ecosystem especially where the crop residues are all returned into the field after harvesting. At the whole plant level in the field, N-fixation of *P. vulgaris* L. from *Rhizobium* inoculation accounted for approximately 33 kg $N.ha^{-1}$. This is a huge contribution if farmers were to purchase equivalent of inorganic nitrogen fertilizers.

In areas where *P. vulgaris* L. organs are used as vegetables, the increased level of N in pods and leaves implies that the nutritional values were improved by rhizobial inoculation. *P. vulgaris* L. is known in Africa as poor mans meat (Deshphande *et al*., 1984). Any attempt to improve the nutritional value of this important grain legume (such as the use of *Rhizobium* inoculation) will reduce malnutrition in Africa.

Overall, lime also played a significant role in the growth of *P. vulgaris* L. Lime improved leaf chlorophyll content (Chl), the photosynthesis (A), the intercellular CO_2 concentration (Ci) and the transpiration rate (E), number of seeds per pod and the final seed yield. Highest lime rate of 3 $t.ha^{-1}$ was superior to the control and 2 t $lime.ha^{-1}$. Lime application also increased the soil pH and the amount of exchangeable Ca and Mg in the rhizosphere. In this study lime showed to increase the potential of *P. vulgaris* L. growth and reached its best capacity in a favourable pH environment best suited for its needs. These findings are useful as they can be used in the development of economically sound liming practices, an important step in the production of *P. vulgaris* L. in acidic environments.

Results from the studies have shown that the provision of Mo increased growth, N-fixation, and finally the N nutrition of *P. vulgaris* L. plants. Furthermore, supplying Mo significantly improved chlorophyll content and photosynthetic parameters. The Mo doses applied were as low as 6 - 12 $g.kg^{-1}$ of seeds. The increase in growth and productivity of *P. vulgaris* L. was due to enhanced N-fixation from Mo influence. Under acidic environment where Mo is unavailable in the soil solution, the use of this essential micronutrient is likely to boost of *P. vulgaris* L. growth and yields. But studies on different application rates and methods should be undertaken to ascertain the most appropriate optimums.

In conclusion the combination of *Rhizobium* inoculation x Mo x Lime were essential for better plant growth, N-fixation and yield of *P. vulgaris* L. Under such acidic soil conditions, the use of these resources is important if maximum yield potential in *P. vulgaris* L. is to be realized.

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