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Full Length Research Paper

Effects of *Rhizobium* innoculation, lime and molybdenum on nitrogen fixation of nodulated *Phaseolus vulgaris* L.

Sylvie Bambara and Patrick A. Ndakidemi*

Faculty of Applied Science, Cape Peninsula University of Technology, Cape Town Campus, Keizersgracht, P.O. Box 652, Cape Town 8000, South Africa.

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A field and glass house experiment was conducted with the aim of evaluating *Rhizobium* inoculation, molybdenum (Mo) and lime supply on growth and nitrogen fixation of nodulated *Phaseolus vulgaris*. The experiment was laid in a split-split plot 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}). *Rhizobium* inoculation showed significant increase in dry matter yield of different organs and decreased δ^{15}N values in all organs assessed, thus resulting to improved % nitrogen derived from atmosphere (%Ndfa) in all organs and the amount of N derived from fixation. Molybdenum and lime were significantly effective in promoting plant growth in most organs and decreased δ^{15}N values in all organs for glasshouse and field experiment. Lowest δ^{15}N values were recorded in Mo and lime supplied at the highest rates of 6 and 12 g kg^{-1} of seeds and 2 and 3 t lime per ha, respectively. Molybdenum and lime application also significantly improved %Ndfa in all organs and N derived from N-fixation in most organs. Significant responses in N nutrition were also reported in treatments involving the combination of *Rhizobium* x Mo x lime with better result being recorded in treatments involving *Rhizobium* inoculation and higher levels of Mo and lime.

Key words: Nodulated root, δ^{15}N, %Ndfa, total N.

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 *Phaseolus vulgaris*, in association with *Rhizobium* bacteria has 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 low fixation capability of *P. vulgaris* 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 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* have shown that N-fixation from *Rhizobium* inoculation contributed an N equivalence of 20 - 60 kg N ha^{-1} in Brazil (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).

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*Corresponding author. E-mail: NdakidemiP@cput.ac.za or ndakidemi56@yahoo.co.uk. Tel: +27214603196. Fax: +27214603193.

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 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 legu-minous plants such as *P. vulgaris*.

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* in some parts of South Africa is not documented.

MATERIALS AND METHODS

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 (33°54'S, 18°14'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 22.6°C (day) to 11°C (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), is equivalent to skeletic leptosol according to FAO soil classification (FAO, 2001).

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 x 4 m with 4 rows and 0.5 m apart from one another. *P. vulgaris* 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 per hectare.

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, 3 seeds were sown in 2 kg soil carefully packed into 2 kg pot. These were thinned to two plants 10 days after sowing.

Plant harvest and sample preparation

At 60 days after planting, *P. vulgaris* 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 and 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.

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* in this study were -2.22‰ for shoots and pods and 0.95‰ for roots (Chimphango and Dakora, unpublished data).

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 and total nitrogen were used to compare treatment means at $P \leq 0.05$ level of significance (Steel and Torrie, 1980).

RESULTS

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

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 per kg 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 per kg of seeds (Table 1). However, highest dry matter yield was recorded in the treatment supplied with 12 g Mo per kg 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 per kg of seed relative to zero-Mo control treatment. Molybdenum at 6 and 12 g per kg 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* plants numerically, but not significantly, increased growth of all organs measured in this study (Table 1).

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

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).

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

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 per kg 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 per kg of seed (Table 3).

Applying lime to *P. vulgaris* 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 3t per ha significantly decreased $\delta^{15}N$ values of roots, shoot 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 per ha (Table 3).

Table 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 $P \leq 0.05$.

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* 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* (Table 4).

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

Rhizobium inoculation treatment was the most influential one in increasing the plant total N content in different organs and whole plants of *P. vulgaris* (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 appli-cation of Mo at 6 and 12 g per kg of seed (Table 5). The application of lime had no effect on total N of all bean organs (Table 5).

Table 2. Effect of *Rhizobium*, Mo and Lime on δ^{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 ≤ 0.05, P ≤ 0.01, P ≤ 0.001 respectively, NS = not significant. Means followed by similar letter (s) in a column are not significantly different from each other at P ≤ 0.05.

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

Rhizobium inoculation of *P. vulgaris* 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* 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* grown in the glasshouse significantly increased the amount of N-fixed on roots, shoots, pods and ultimately their whole plants.

Applying Mo to *P. vulgaris* 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).

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

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

Table 3. Effect of *Rhizobium*, molybdenum and lime on the %N in different parts of *Phaseolus 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 $P \leq 0.05$.

with *Rhizobium* and Mo at different rates (Figures 1A, B, C, D, 2A and 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 3), and whole plants and pods in the field (Figure 4 A and 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 4C). 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* pods as compared with un-inoculated supplied with Mo and lime (Figure 4D).

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 (Figures 5A and B). The combination of these supplies increased the amount of N which was derived from atmosphere in *P. vulgaris* 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 5C). Overall, the combinations of *Rhizobium*, Mo and lime significantly stimulated the %Ndfa in pods grown in the field experiment (Figure 5D).

Table 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).

The results in Figure 6 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 6A) as compared with un-inoculated treatments.

In this study, a significant *Rhizobium* x Mo; *Rhizobium* x lime; *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 7A, B and C) in the field experiment. Significant interactions between *Rhizobium* x Mo x lime were also observed on N fixed per plant (Figure 8A 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.

DISCUSSION

In this study, nodulated *P. vulgaris* plants inoculated with *R. leguminosarum* showed positive effects on plant growth (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* 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

Table 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
Molybdenum (g.kg^{-1})								
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 $P \le 0.05$, $P \le 0.01$, $P \le 0.001$ respectively, NS = not significant. Means followed by similar letter in a column are not significantly different from each other at $P \le 0.05$.

Africa who cannot afford to purchase them. 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* 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 per kg 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* 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 the $\delta^{15}N$ 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 phenolmenon 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* and a cheaper option for

Table 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 ≤ 0.01, P ≤ 0.001 respectively, NS = not significant. Means followed by similar letter in a column are not significantly different from each other at P ≤ 0.05.

supplying N as compared with the expensive inorganic fertilizers.

Molybdenum application at the rate of 6 and 12 g per kg 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* 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* 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*. For example,relative to the zero control in the field study, the application of Mo at 6 and 12 g per kg of seeds increased significantly the N fixed (kg N

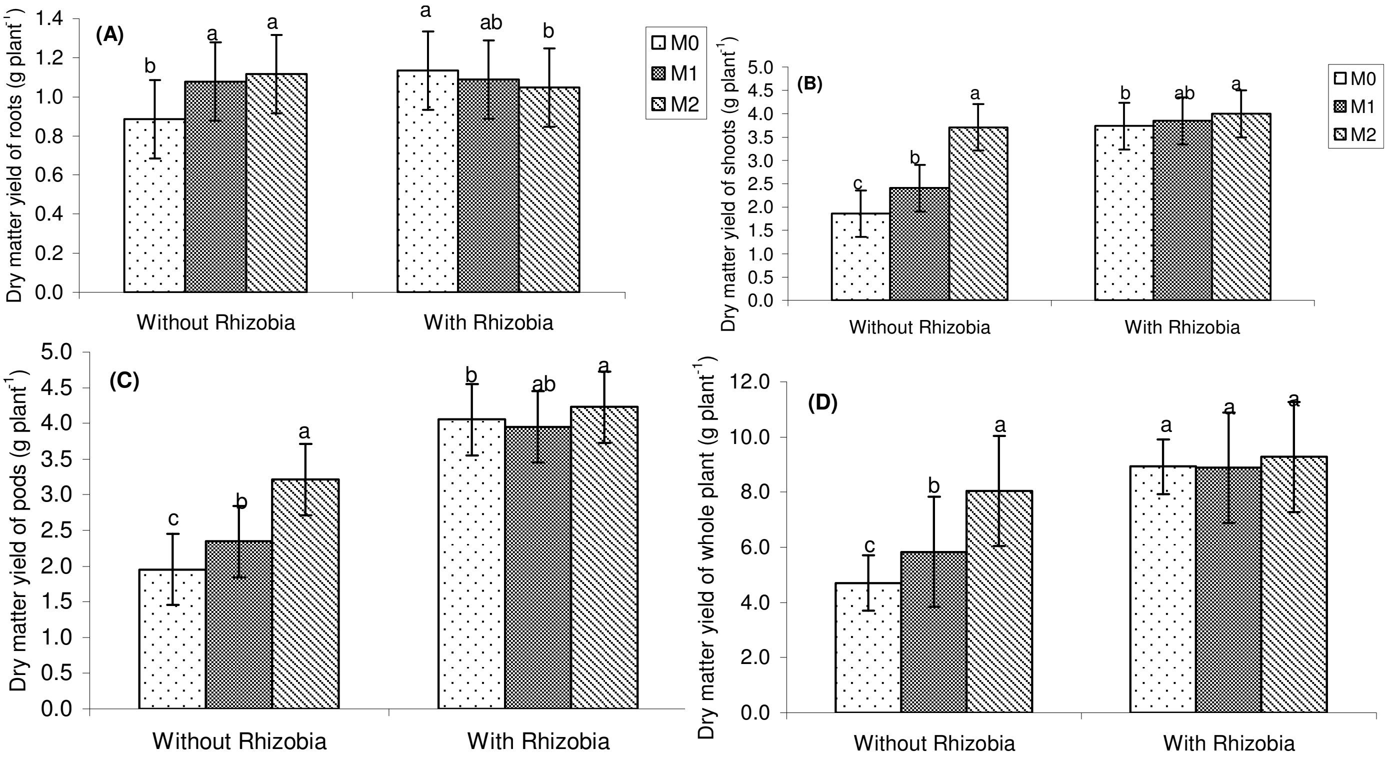

Figure 1. Interactive effect of *Rhizobium* and molybdenum in dry matter yield measured in the glasshouse experiment in 2008 (A): Roots, (B) Shoots, (C) Pods and (D) Whole plant.

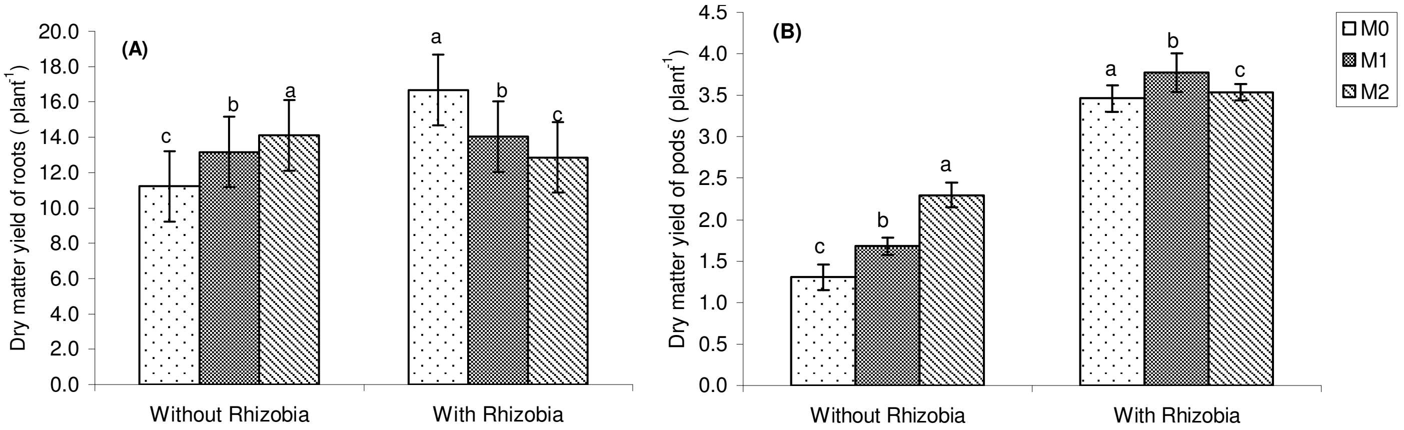

Figure 2. Interactive effect of *Rhizobium* and molybdenum in dry matter yield measured in the field experiment in 2009 (A): Roots and (B) Pods.

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 8) were reported in our study. The maximum dry matter yield occurred in the

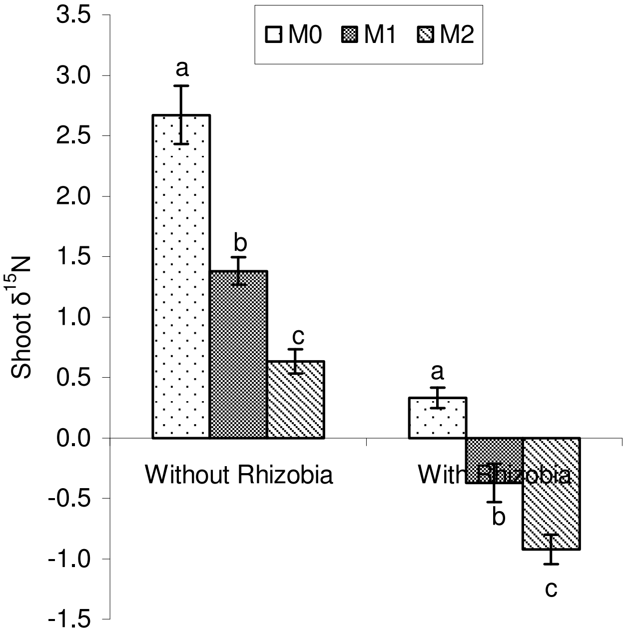

Figure 3. Interactive effect of *Rhizobium* and molybdenum on δ15N in shoot as measured in the glasshouse experiment in 2008.

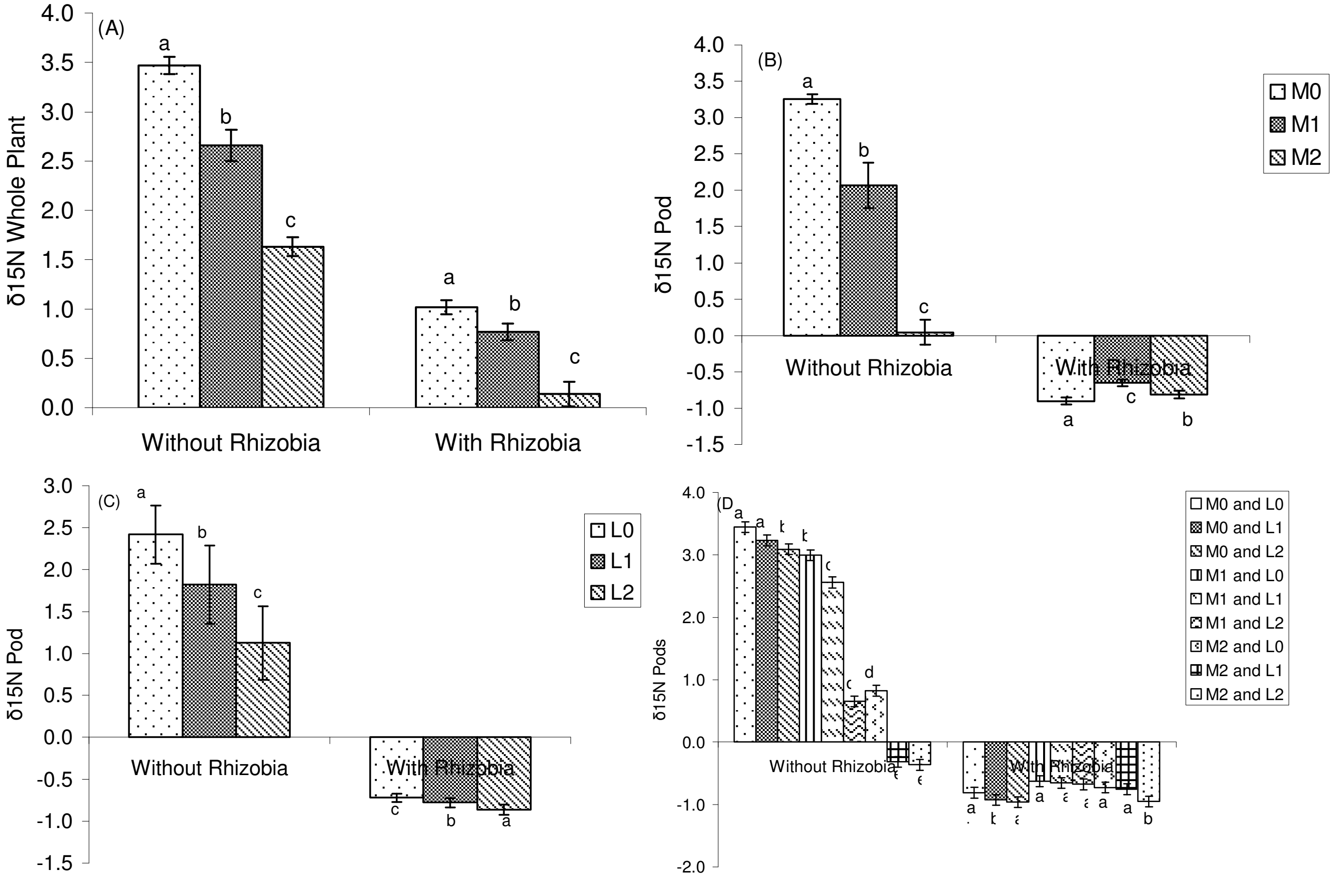

Figure 4. Interactive effect of (A) *Rhizobium* and molybdenum on whole plant δ15N in field experiment, (B) *Rhizobium* and molybdenum, (C) *Rhizobium* and Lime and (D) *Rhizobium,* molybdenum and lime on δ15N pod in field experiment in 2009.

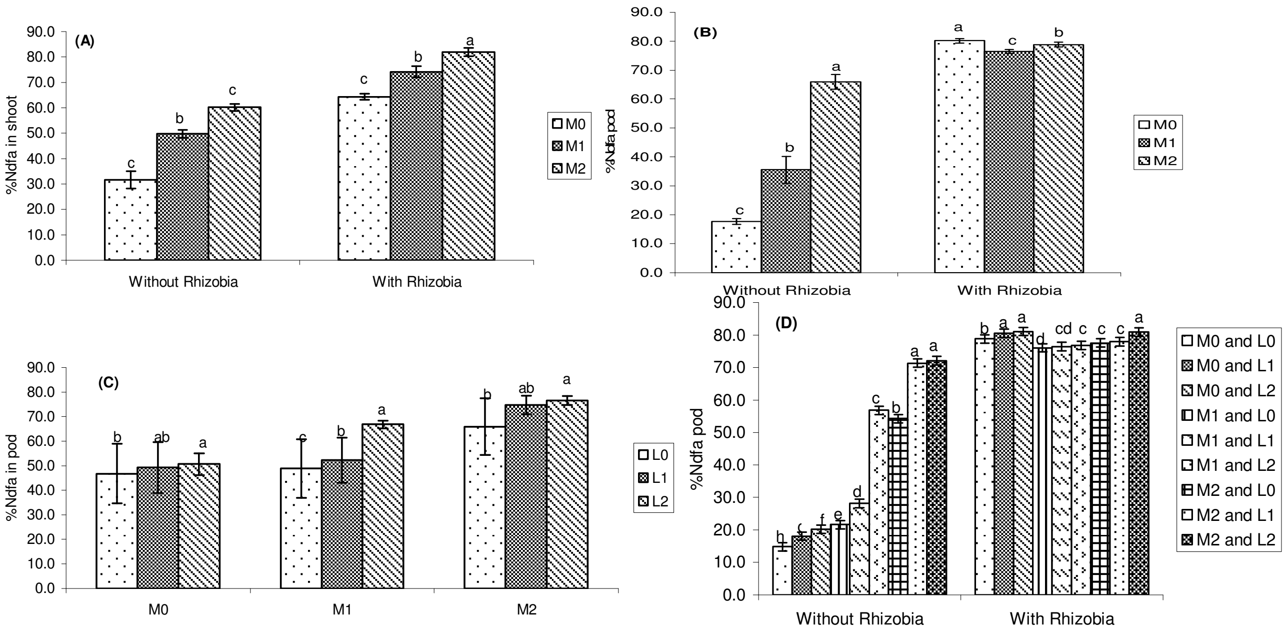

Figure 5. Interactive effect of (A) *Rhizobium* and lime on shoots % Ndfa in glasshouse experiment, (B) *Rhizobium* and molybdenum on pods % Ndfa in field, (C) molybdenum and lime on pods % Ndfa in field experiment and (D) *Rhizobium,* molybdenum and lime on pods % Ndfa in field experiment in 2009.

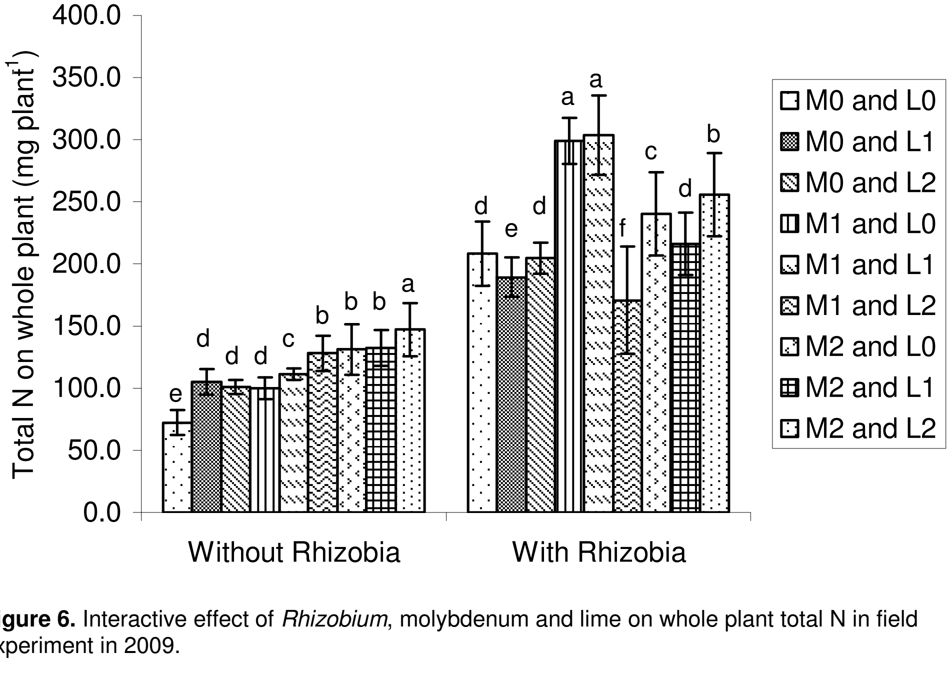

Figure 6. Interactive effect of *Rhizobium*, molybdenum and lime on whole plant total N in field experiment in 2009.

treatments involving *Rhizobium* inoculation and highest rates of Mo (Figures 1 A, B, C, 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 3, 4A and B) and lime at 2 and 3 t ha^{-1} (Figure 4C and D) thus resulting to significant interactions in % Ndfa (Figures 5 A, B, C and D) and N fixed per plant (Figures 7A, B and C, 8A 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* in the study area.

In conclusion, N nutrition of *P. vulgaris* was improved

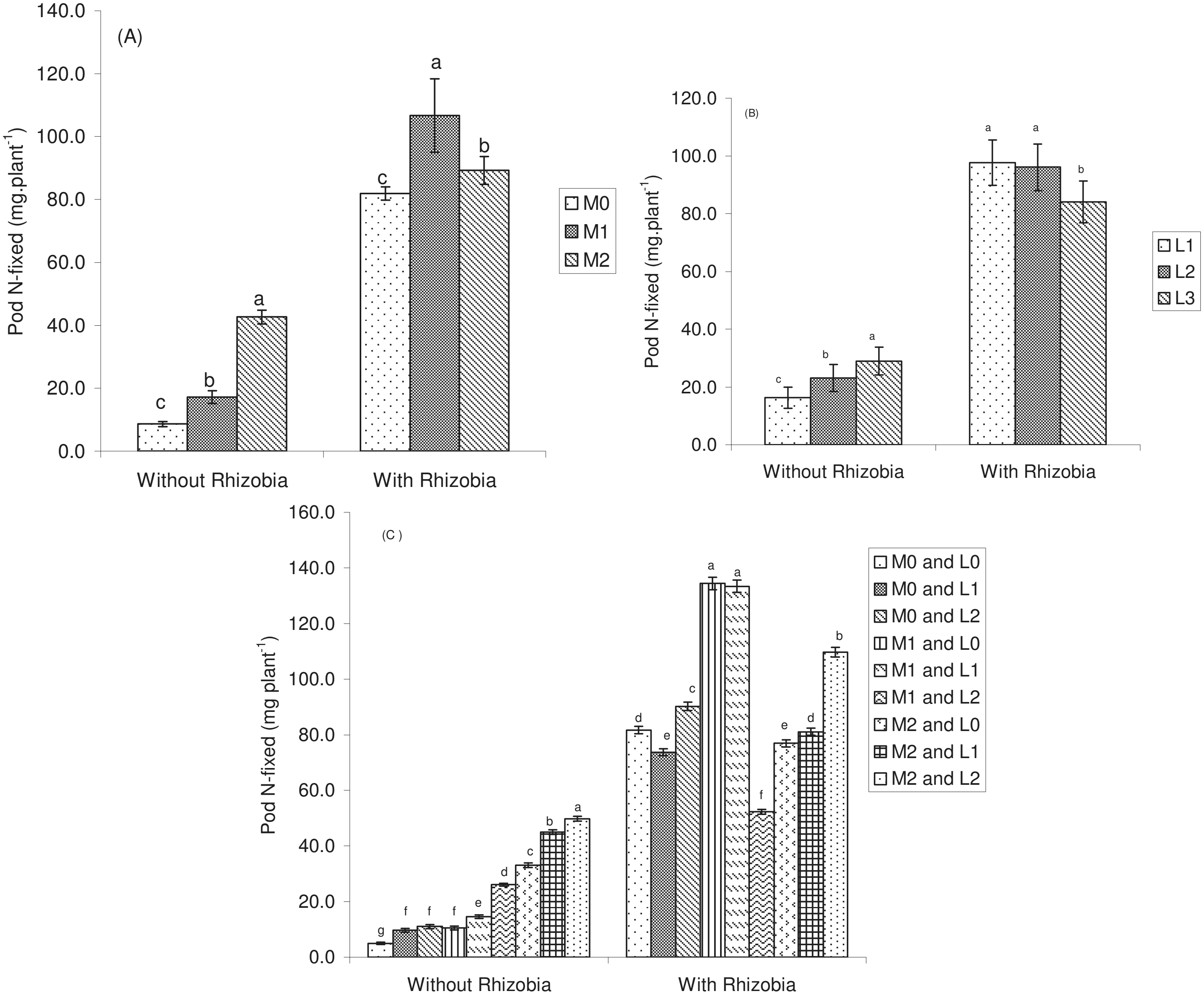

Figure 7. Interactive effect of (A*) Rhizobium* and molybdenum, (B) *Rhizobium* and lime and (C) *Rhizobium,* molybdenum and lime on N-fixed in Pod in field experiment in 2009.

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 per kg of seed and lime at 2 and 3 t per ha 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* have to be realized.

ACKNOWLEDGEMENTS

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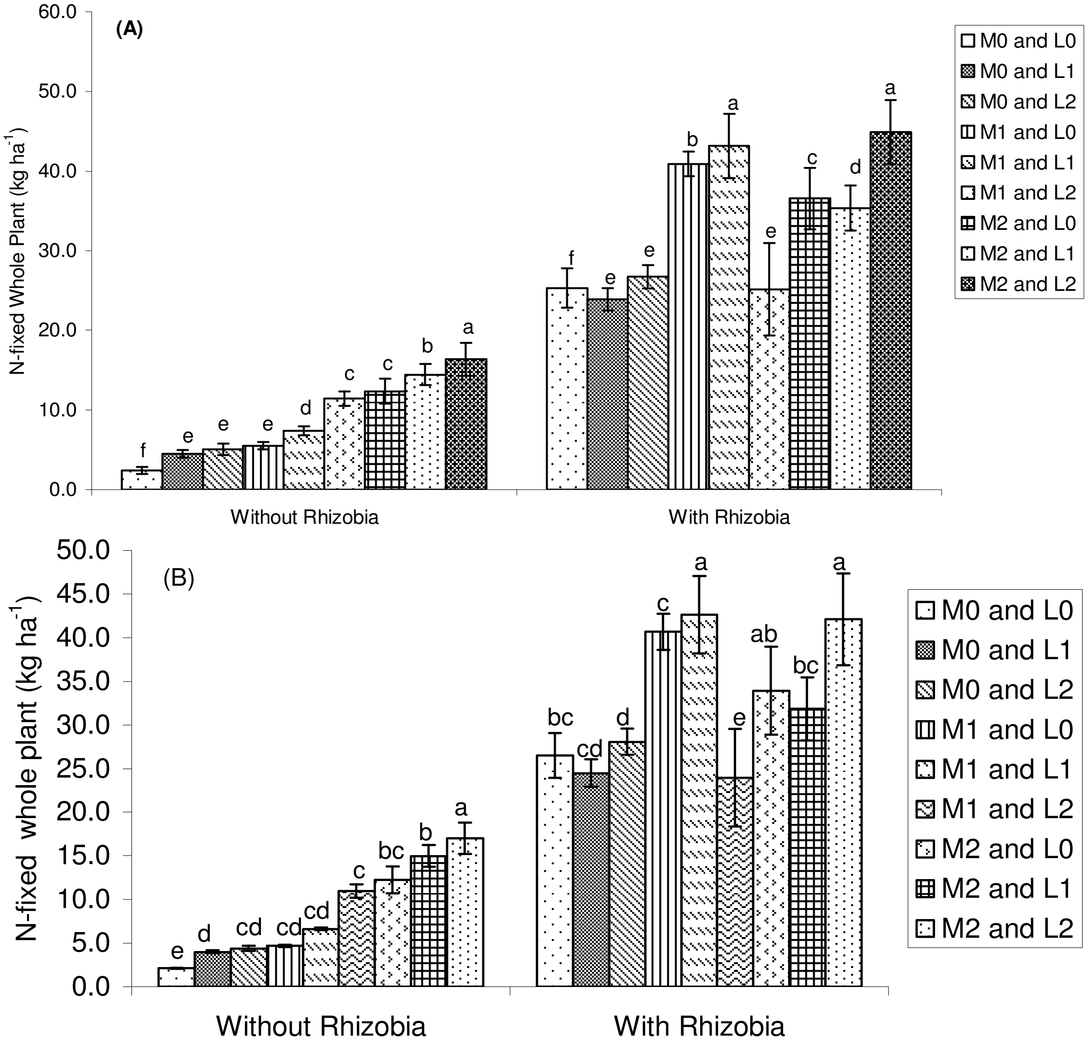

Figure 8. Interactive effect of *Rhizobium,* molybdenum and lime on N-fixed in whole plant in glasshouse in 2008 (A), and field experiment in 2009 (B).

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