Effect of molybdenum enzyme activity of green gram (*Vigna radiata*) grown in low land acid soils of Pudukottai region of Tamil Nadu

ABSTRACT

The role of molybdenum (Mo) in greengram in regulating the activities of enzymes such as nitrate reductase (NR) and glutamine synthetase (GS) in particular to nitrogen assimilation was investigated in acid soils of Semi-arid Tropics (SAT) of Tamil Nadu. Incubation studies were taken up with two sources of molybdenum namely sodium molybdate (Na_2MoO_4) and ammonium molybdate ($(NH_4)_6Mo_7O_{24}$) in which sodium molybdate proved to be a better performer and recommended as Mo fertiliser in SAT acid soils. Application of Mo increases root, shoot length and biomass. It also increases the number of nodules and rhizobium population in green gram. In particular to microbial aspect the rhizobium population count serial dilution technique was followed. The anatomy of nodule was imaged using high-resolution imaging Scanning Electron Microscope (FEI SEM Quanta 250 , Netherlands).

Keywords *– Nitrate reductase, Glutamine synthetase, Greengram, Molybdenum*

1. INTRODUCTION

Pulses are the important protein source for human diet. In India pulses can be cultivated with limiting resources which is less expensive than animal protein. Pulses are less expensive when compared to other vegetables which can be grown in rainfed conditions as intercrop as well as a mixed crop in particular of giving better returns. Apart from consumption pulses crop cultivation improves soil fertility, physical structure of soil and also act as nutrition fodder for cattle. The pulse availability per adult per day at present is only 36 which is against the minimum requirement of 50g/adult/day. The recommended dietary allowances (RDA) for adult male and female is 60 g and 55g per day. Greengram in Indian soils considered an an excellent source of high quality and digestible proteins but deficient in important micronutrients viz., Mo, Zn, Fe. Pulses are very exact in their requirement like neutral soil pH (liming acid soils), phosphate and sulphur manuring, use of Rhizobium culture and molybdenum for seed treatment for better nodule activity to achieve maximum yield.Pulses are cultivated in two seasons viz., Kharif and Rabi season with a production area of 143.41 lakh hectare, the productivity of 91.16 lakh tonnes and yield

of 636 Kg per hectare(Agricultural Statistics at a glance, 2017). Even India increased output of pulse production to 22.95 million tonnes from 16.35 million tonnes, need arises to import 50.8 lakh tonnes from China, Canada, Australia and Myanmar which costs about Rs 17 280 crores (Economic Times, 2018). Pulses contribute 11 percent of total gross cropped area. Among all pulses, the area under gram is 4% arhar 2% and other pulses in about 5% of the gross cropped area. The net irrigated area in the country is 47% while the remaining falls under rainfed conditions. The pulses under irrigation cultivated about 37% of the area while 63% of pulses are grown under rainfed conditions. India's outstanding contribution towards total global acreage and production of pulses at 35% & 25 % respectively

The biological importance of Mo in plants is due to its highly beneficial action in the fixation of nitrogen from the atmosphere by the nitrogen-fixing bacterium (*Azotobacter chroococum*). Some of the crops considered most sensitive to Mo deficiency are clover, cauliflower, broccoli, rape, beet, spinach, lettuce and alfalfa. Among the micronutrients, Mo is readily translocated and its deficiency symptoms generally appear on the whole plant. The deficiency for other micronutrients appears on the young leaves at the top of the plant because of their inability to translocate within the plant. Mo deficiency emerges as general yellowing and stunting of the plant, interveinal mottling and cupping of the older leaves followed by necrotic spots at leaf tips and margins. Mo deficiency reduces the availability of Fe and P and the utilisation of N, increases the contents of S, Cu and Mn in plants (Chatterjee *et al*.,1992). During the growth of pulses, atmospheric nitrogen get fixed in their root nodules by Rhizobium. Mo helps in localisation and regulation of nitrogen assimilation and amino acid accumulation via regulating enzymes (Hristozkova et al. 2007), and also is a co-factor of nitrate reductase (NR) enzyme. NR is required for the reduction of nitrates for protein synthesis. Mo also stimulates the activity of glutamine synthetase (Kevresan et al. 1998). Glutamine synthetase (GS) converts ammonia into protein via GS-GOGAT cycle. Thus Mo deficiency reduces NR and GS activities. Mo deficiency occurs mainly in acidic soils of temperate climatic conditions or in hilly acidic soils where the soil is usually acidic. In contrast, in some of the laterite low land acidic soils of Pudukottai and Sivagangai Districts of Tamil Nadu of southern India under SAT (semi-arid tropical) climatic conditions, Mo deficiency is very common. This study was undertaken to investigate the physiological response of greengram on the application of Mo fertiliser in acidic low land soil and the pathways of N-accumulation and utilisation for the same.

2. MATERIALS AND METHODS

Pudukottai District covers a geographical area of 4,56,449 ha receiving rainfall of 685mm from northeast monsoon season falls under the temperature range of 32.8^{0}C to 17.3^{0}C.Pudukottai extends across the north latitude of 8^0 30' to 10^0 40' Eastern latitude of 78^{0}24' to 79^{0}40'. Pudukottai District consists mainly of Cretaceous type of geological distribution which consists of sand, clay, sandy-clay lying over the crystalline rocks of Archean age in parts of Pudukkottai district. The soil is classified taxonomically as Paralithic Haplustalf, In particle size analysis as per international pipette method the percentage of sand(56%), silt(15%) and clay(28%).The pH pH (5.61) is acidic in nature ,organic carbon content(0.36) is low and calcium carbonate (3.22%) is non-calcareous

2.1 Incubation Experiment

A laboratory experiment was conducted to study the solubility of molybdenum fertilisers namely sodium molybdate and ammonium molybdate at varying doses. There were five levels of Mo (0, 1, 2, 3, 4 and 5 kg Mo ha^{-1}) replicated four times in completely randomised design (CRBD). The factors used for the incubation study were:

i) Sources of Mo : sodium molybdate and ammonium molybdate

ii) Levels of molybdenum (Six) : 0, 1, 2, 3, 4 and 5 kg Mo ha^{-1}

Sodium molybdate of 0.64 gram was dissolved in 1000 ml double distilled water which gives 640 mg L^{-1}. From the stock solution of 640 mg L^{-1}, 1, 2, 3, 4, 5 ml was pipetted out and made up the volume to 500 ml in order to mix the solution with the soil sample taken for incubation study. 0.55 gram of ammonium molybdate was dissolved in 1000ml double distilled water which gives 550 mg L^{-1}. From the stock solution of 550 mg L^{-1} 1, 2, 3, 4, 5 ml was pipetted out and made up the volume to 500 ml to mix the solution with the soil sample taken for incubation study (Table 1).

Table 1. Different dose levels of sodium molybdate and ammonium molybdate

Treatments	Sodium molybdate kg^{-1}soil	Ammonium molybdate kg^{-1} soil
1 kg Mo ha^{-1}	1.25 mg (L_1)	0.925 mg (M_1)
2 kg Mo ha^{-1}	2.50 mg (L_2)	1.85 mg (M_2)

3 kg Mo ha^{-1}	3.75 mg (L_3)	2.775 mg (M_3)
4 kg Mo ha^{-1}	5.0 mg (L_4)	3.700 mg (M_4)
5 kg Mo ha^{-1}	6.25 mg (L_5)	4.625 mg (M_5)

2.1.1. Incubation containers

Clear white plastic jars (14.0 cm height, 12.0 cm diameter and 1.0 kg capacity) fitted with tight lids were selected for conducting the experiment.

2.1.2. Incubation study

The incubation process was done physically by taking 1 kg of soil into plastic cups and two molybdenum sources such as sodium molybdate and ammonium molybdate applied at the rate of 1, 2, 3, 4 and 5 kg Mo ha^{-1}. Sodium and ammonium molybdate were thoroughly mixed and brought to the required moisture level with deionised water to get the moist friable condition of the soil. The plastic cups having fertiliser were covered with tight lids and kept in the laboratory at room temperature for thirty days.

To facilitate the normal chemical process, the soil – molybdenum fertilizer sources were stirred with a glass rod once a day. To maintain sufficient moisture level in each cup throughout the incubation period, the plastic cups were weighed every three days and the weight was recorded and the difference between the last and current weight was compensated by adding deionised water.

Soil samples were drawn at the beginning, 1^{st}, 2^{nd}, 3^{rd} and 4^{th} week of incubation for analysis of molybdenum. After sampling, the cups were weighed in order to add water to keep the moisture level constant in each cup.

2.2. Basic Studies

A pot culture experiment was conducted at the Department of Soil Science and Agricultural Chemistry in order to study the N-fixation process in greengram plants. Soil samples drawn from NPRC, Vamban was used for the study. Treatments composed of 5 levels of molybdenum (0, 200, 400, 600, 800 and 1000 g Mo ha^{-1}) replicated 5 times in a completely randomised design. During the experimentation, nodules count, Rhizobium population, N-fixation process and high-resolution imaging of nodules were taken up.

2.2.1. Nodulation number

At 40 DAS, plants were uprooted, washed thoroughly and the total nodule count was taken up. The data were presented as a number of nodules $plant^{-1}$.

2.2.2. Rhizobium population (Serial Dilution technique)

One gram of the soil sample was transferred aseptically to a sterile water blank of 9 ml to get 10^{-1}dilution. One ml of this dilution was transferred to 9 ml sterile blank to get 10^{-2} dilution. Similarly, the sample was serially diluted up to 10^{-9} dilution and one ml of this dilution was used for plating.

2.3. Nitrate reduction activity

Nitrate reductase enzyme was determined after 30th day of the vegetative stage by following the method developed by Hageman and Reed (1980). 0.5 ml phosphate buffer (pH 7.5) was pipetted out in a test tube. Then 0.2 ml of potassium nitrate solution, 0.4 ml NADH solution and 0.7 ml of water was added. The reaction was initiated by the addition of 0.2 ml enzyme extract, similarly, a control was set up in the same way but it should be done with water instead of enzyme extract. Incubation was done at 30^{0}C for 15 minutes. The reaction was terminated by the rapid addition of 1 ml of sulphanilamide followed by 1ml of naphthyl ethylenediamine reagent. It should be kept for 90 minutes. The absorbance was measured at 540 nm. A standard graph was prepared with potassium nitrite. Different known aliquots of potassium nitrite standard solution were pipette into a series of test tubes and the volume was made up in each tube to 2 ml by adding water.

2.4. Glutamine Synthetase:

Glutamine synthetase enzyme was determined after 30th day of vegetative stage by following the method developed by Pateman (1969). The reagent (ml) was pipetted out as mentioned in the order below : Glutamine 2.0 ml, Sodium arsenate 0.5 ml, $MnCl_2$ 0.3 ml, Hydrxylamine 0.5 ml, ADP 0.5 ml, enzyme extract 0.2 ml.

In order to set a blank 2 ml of 20 mM Tris –HCl was added instead of Glutamine. The reaction mixture was incubated for 30 minutes at 37^{0}C. The reaction was stopped by adding 1 ml of ferric chloride reagent. The brown colour was measured at 540 nm. A range of standards containing 100-500 µg γ–glutamyl hydroxamate in 4 ml buffer solution was prepared and the colour was developed by adding 1 ml of ferric chloride.

2.5. Scanning Electron Microscope (SEM)

The scanning electron microscope (SEM) is a type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. Here, a wide range of magnifications is possible, from about 10 times (about equivalent to that of a powerful hand-lens) to more than 500,000 times.

Nodules were removed from the roots of green gram plant. The nodules were washed with distilled water for 4 to 5 times to remove the soil particles. Then the moisture was removed from the nodule with the help of tissue paper. Then the cross section of the nodule was taken and loaded on the sample stage and images were taken in a Scanning Electron Microscope (FEI SEM Quanta 250 , Netherlands).

2.6. Statistical analysis

The data collected were subjected to statistical analysis in ANOVA (Ranganathan, 1990). Whenever the treatment difference was found significant (F test), critical difference was worked out at 5 per cent probability level and the values were furnished. If there is no significant difference between treatments, it was denoted by the symbol NS.

3. RESULTS AND DISCUSSION

3.1 Mo release pattern from two sources of Mo fertilization

In order to study the Mo release pattern from two sources of Mo fertilisers sodium molybdate and ammonium molybdate and release of Mo were periodically monitored for a duration of 4 weeks. An incubation experiment was conducted for a period of 4 weeks. The results showed that the AB-DTPA extractable Mo showed an increasing trend with the progression of the incubation period (Table 2 & Fig 1). The values increased progressively with an advancement incubation period regardless of sources of Mo fertiliser. However, the values were 10 to 15 per cent higher for sodium molybdate than ammonium molybdate at all stages of observations. Mo fertilisation at the rate of 1000 g ha^{-1} registered highest values 4 to 5 times higher than control and such response continued in the incubation period. At the last week of incubation, the available Mo status of M5 applied at the rate of 1000 g Mo ha^{-1} registered 79.3 and 62.3µg g^{-1} for sodium and ammonium molybdate, respectively. The available Mo value was nearly doubled in fertilised soils in comparison to an initial value in the experimental site. The basic solubility had shown that ammonium Mo solubility is enhanced when the medium gets heated up. The sodium molybdate is soluble under room temperature and therefore it is ideal as a recommended source of Mo fertiliser under field conditions. Performance of sodium molybdate (Na_2MoO_4) was better than ammonium molybdate [$(NH_4)_2MoO_4$] in terms of available Mo. Na_2MoO_4 is readily soluble at room temperature because its mineralisation occurs at a faster rate than $(NH_4)_2MoO_4$. So, Na_2MoO_4 is recommended as Mo fertiliser source.

Fig 1. Overall view of the Incubation Experiment

3.2.Effect of Molybdenum on Nitrate reductase and Glutamine synthetase

The key enzymes involved in N-assimilation namely nitrate reductase and glutamine synthetase. The nitrate reductase activity increased correspondingly with incremental levels of Mo. Molybdenum fertilisation with graded levels increased the nitrate reductase and glutamine synthetase activities (Table 3).Nitrate reductase is a substrate inducible enzyme which closely coincides with the availability of nitrate nitrogen in the soil. Mo fertilization through soil, seed and foliar spray favoured the availability of Mo in the soil vis-a vis availability of nitrogen. The highest nitrate reductase activity of 123.7 µg NO_2 g^{-1} of leaf $hour^{-1}$ registered in M5 which is 24.2 per cent higher than control. Since Mo fertilised greengram plants are adequately nourished with Mo and nitrogen it is understandable that N-assimilation has improved. Similar trend of response was seen for glutamine synthetase also. The glutamine synthetase activity registered the highest in M5 which is 18.8 per cent higher than control. Further, another N-assimilating enzyme glutamine synthetase facilitates assimilation of ammonium into aminoacid glutamine. It is quite evident that N-assimilatory pathway has modified or improved due to Mo fertilisation. Our data are in agreement with the findings of Viera *et al*. (1998). The correlation study indicated that available Mo positively correlated with nitrate reductase (r = 0.95) and glutamine synthetase (r = 0.85).

3.3.Effect of Mo fertilisation on growth parameters of green gram

Plant parameters such as plant height, shoot dry matter product and root drymatter product characteristics were measured at 40 DAS Molybdenum fertilisation done through seed, foliar sprays and soil had significantly increased the plant height irrespective of doses (Table 4). The highest values were recorded

in foliar spray treatments followed by soil and seed treatment. The lowest value was registered in control. In this experiment, Mo applied by foliar application of T4 (0.025 per cent) followed by T5 (0.050 per cent) favourably enhanced shoot and root characteristics. The correlation study indicated that available Mo positively correlated with shoot mass (r = 0.958) and root mass (r = 0.901). Our data are in agreement with the findings of (Adkine *et al.* 2011;Kovacs *et al.* 2015 and Qin *et al*. 2017).

3.4 Effect of Molybdenum on dry matter production (DMP) on shoot and root

Application of Mo through seed, foliar and soil had increased the individual plant dry matter significantly, the lowest value was registered in control 12.52 g. Among the method of Mo fertilization, soil application of Mo applied at the rate of 500 or 1000 g ha^{-1} increased the dry matter production nearly 1.5 to 1.7 times in comparison to control. The dry matter Mo produced in soil treatments was comparable to foliar spray treatments. These values were significantly higher than seed treatment. Root measurements namely length and dry matter were measured at the harvest stage of green gram. The control had the lowest values for all the three parameters measured. The foliar spray of Mo at the rate of 0.025 per cent or 0.050 per cent had increased all the 3 root parameters followed by soil application of Mo and seed treatment. Foliar spray of Mo had increased the dry matter by 3 to 4 times, root length by 2.5 times and volume by 2 times indicating the importance of foliar spray of Mo in root proliferation and dry matter product.This data is in accordance with the findings of Steiner *et al*. (2018).

3.5. Effect of Mo fertilization on nodulation and Rhizobium population

Application of Mo at incremental levels increased the number of nodules linearly and the highest number registered in M5 where Mo was applied at the rate of 1000 g ha^{-1} (25 nodules $plant^{-1}$) (Table5 & Fig2).The number of nodules registered in the best treatment was 2.5 times than control (10 nodules $plant^{-1}$).The increase in nodule numbers was higher with the higher level of Mo application. The rhizobium population of Mo fertilised treatments at the rate of 1000 g ha^{-1} had the highest value of 8 CFU ml^{-1} while control registered the lowest value of 2 CFU ml^{-1}. Molybdenum fertilisation with graded levels increased the Rhizobium population which in turn increases the number nodulation which resulted in the effective N-fixation process. The highest Rhizobium population (8 CFU ml^{-1}) was registered in M5 (1000 g Mo ha^{-1}) and the modulation number of 25. Our data are in agreement with the findings of Tripathi and Edward (1978), Dey and Ghosh (1986) and Bhuiyan *et al.* (2008). The correlation study indicated that available Mo positively correlated with nodulation number (r = 0.901). Our data have unequivocally demonstrated Mo fertilisation assist in nodulation and rhizobium population that promote N-fixation in greengram plants

Fig 2. Comparison of nodulation in greengram plant fertilised with (1000 g Mo ha^{-1}) and without Mo application (Control)

Roots showing nodules	Roots fertilized with molybdenum
	
Control –Mo 0 g ha^{-1} & Fertilised- Mo 1000 g ha^{-1}	Fertilized- Mo 1000 g ha^{-1}

3.6. SEM view of effective nodules

An experiment was conducted to undertake basic anatomical features of nodules developed in greengram at 45 DAS. The SEM view clearly indicated that the treatments that received Mo fertilisation were found to contain a large number of Bacteroides in comparison to control (Fig3a & Fig3b). In addition to the number of bacteroids, the peri bacterial membrane shown to be thicker and widespread as a result of Mo fertilisation. The best treatment was soil applied at the rate of 1000 g ha^{-1} had higher number of active bacteroids involved in N-fixation process. On the other hand, the control had less number of Bacteroides besides many of them appear to be non-functional. On the other hand, the control had less number of Bacteroides besides many of them appear to be non-functional. Our data are in agreement with the findings of Roth and Stacey (1989) and Benson *et al*. (2005).

SEM IMAGE OF GREENGRAM NODULES

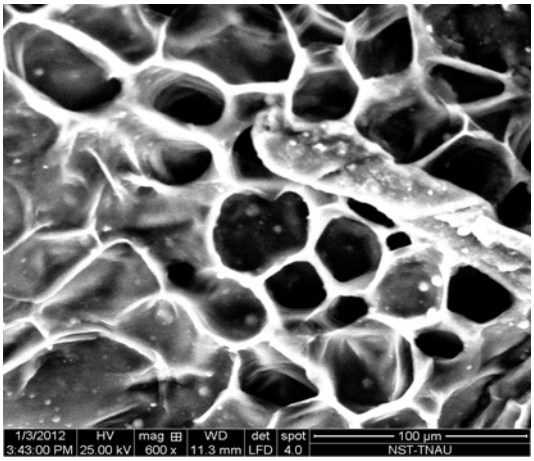

Fig 3a. Without Mo application-Control 100µm Magnification

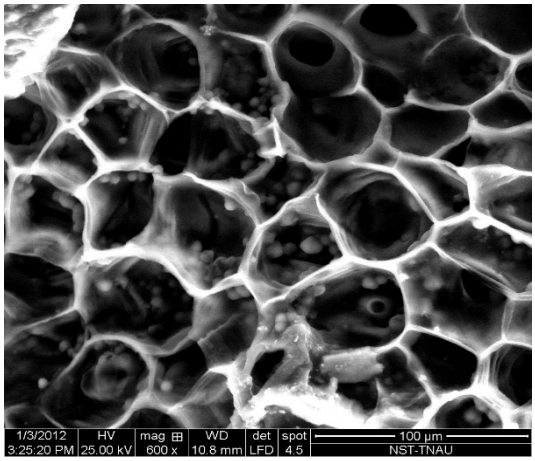

Fig 3b. With Mo application- 1000 g ha^{-1} 100µm Magnification

4.CONCLUSION

Overall, the data clearly indicated that the molybdenum deficiency is closely associated with acidic soil condition. Molybdenum application @ 1000 g ha^{-1} in the form of sodium molybdate or foliar spray of Mo @ 0.05 per cent at pre-flowering and 15 days later found to enhance the yield of green gram to the tune of 30-35 per cent. Besides, Mo improves the nodulation in green gram which resulted in enhanced available N content of the soil.

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Table 2. Molybdenum release pattern from two sources of Mo fertilizers

Treatments	0 DAS		1st week		2nd week		3rd week		4th week	
	$NaMoO_4$	NH_4MoO_4	$NaMoO_4$	NH_4MoO_4	$NaMoO_4$	NH_4MoO_4	$NaMoO_4$	NH_4MoO_4	$NaMoO_4$	NH_4MoO_4
Mo (0 g Mo ha^{-1})	0.04	0.03	0.06	0.04	0.08	0.06	0.1	0.08	0.13	0.09
M1 (200 g Mo ha^{-1})	10.8	9.2	14.3	12.3	19.0	16.2	24.7	22.3	28.7	26.3
M2 (400 g Mo ha^{-1})	15.1	13.3	21.3	19.5	25.3	23.4	30.3	28.1	34.5	32.7
M3 (600 g Mo ha^{-1})	19.2	17.2	26.3	24.8	32.4	30.3	37.6	34.6	40.2	37.3
M4 (800 g Mo ha^{-1})	35.5	28.5	42.6	33.4	46.7	37.5	50.1	43.7	53.7	47.3
M5 (1000 g Mo ha^{-1})	44.8	37.6	48.5	41.3	59.3	44.5	65.3	58.7	79.3	62.3
Average	**20.95**	**17.66**	**25.54**	**21.92**	**31.69**	**25.36**	**35.93**	**31.27**	**40.41**	**34.37**
SEd	**0.17**	**0.02**	**0.02**	**0.01**	**0.02**	**0.01**	**0.01**	**0.01**	**0.02**	**0.01**
CD(0.05)	**0.36**	**0.05**	**0.04**	**0.03**	**0.03**	**0.02**	**0.03**	**0.03**	**0.04**	**0.03**

Table 3. Effect of Mo fertilization on Nitrate Reductase and Glutamine Synthetase (GS) activities in greengram (*Vigna radiata*)

Treatments	**Nitrate reductase activity (µg of NO_2 g^{-1} of leaf $hour^{-1}$)**	**Gluatmine synthetase (mol min^{-1} mg^{-1} protein)**
Mo (0 g Mo ha^{-1})	93.8	45.8
M1 (200 g Mo ha^{-1})	98.7	49.7
M2 (400 g Mo ha^{-1})	100.9	51.2
M3 (600 g Mo ha^{-1})	106.7	53.7
M4 (800 g Mo ha^{-1})	110.4	55.8
M5 (1000 g Mo ha^{-1})	123.7	56.3
SEd	0.24	0.02
CD(0.05)	0.5	0.04

Table 4 . Effect of different methods of Mo fertilizer application on growth parameters in greengram (*Vigna radiata*)

Treatments	Shoot		Root	
	Plant Height (cm)	Drymatter Product (DMP) (g plant^{-1})	Drymatter Product (DMP) g plant^{-1})	Length (cm)
T1 (control)	38	12.52	0.65	13.5
Seed Treatment				
T2 (2 g Mo kg-1)	41.8	17.3	1.63	28.2
T3 (4 g Mo kg-1)	44.5	17.66	1.73	29.3
Foliar spray				
T4 (0.025 %)	53.4	19.86	1.94	32.3
T5 (0.050 %)	57.1	20.28	2.3	34.3
Soil application				
T6 (500 g Mo ha-1)	48.2	21.04	1.87	27.3
T7 (1000 g Mo ha-1)	49.7	21.88	1.94	29.3
SEd	1.02	1.3	0.04	0.8
CD (0.05)	2.09	2.7	0.07	1.7

Table 5. Effect of Mo fertilization on nodulation and *Rhizobium* population in greengram (*Vigna radiata*)

reatments	No of nodules plant^{-1}	Rhizobium population (CFU/ ml)
Mo (0 g Mo ha^{-1})	10	2
M1 (200 g Mo ha^{-1})	13	3
M2 (400 g Mo ha^{-1})	18	4
M3 (600 g Mo ha^{-1})	20	5
M4 (800 g Mo ha^{-1})	22	6
M5 (1000 g Mo ha^{-1})	25	8
SEd	0.61	0.45
CD (0.05)	1.25	0.92