Effect of molybdenum on nitrate reductase and glutamine synthetase in greengram (*Vigna radiata*) grown in acidic low land soils of Pudukottai region in 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), involved in primary nitrogen assimilation, was investigated in acid soils of SAT environment of Tamil Nadu. Incubation studies taken with two sources of molybdenum namely sodium and ammonium molybdate in which sodium molybdate proved to be a better performer and recommended as Mo fertilizer in SAT acid soils. Application of Mo increases root, shoot length and biomass. It also increases the number of nodules and rhizobium population in greengram. In particular to 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).

Key words – Nitrate reductase, Glutamine synthetase, Greengram, Molybdenum

1.INTRODUCTION

Low productivity of pulses is attributed to the extensive rainfed cultivation, intense pests and diseases incidence and multi-micronutrient deficiencies. Among the factors, micronutrient deficiencies are the most critical (Singh and Saha 2005). Molybdenum

(Mo) is recognized as one of the essential micronutrients for plant growth and development particularly for pulses, resulting in improved pulse production.

Molybdenum is known to improve the crop yield particularly pulses detailed study has not been undertaken due to the belief that molybdenum deficiency occurs primarily in temperate conditions or hilly soils where the soil pH is usually acidic.

In Tamil Nadu, some of the low land laterite soils particularly pudukottai and sivagangai districts where the soils are acidic to highly acidic causing molybdenum deficiencies in pulses grown there.

To address the problem of molybdenum deficiency, the scientific worker conference 2010 of the Tamil Nadu Agricultural university suggested to take up a detailed study on molybdenum application in pulses (greengram) to derive suitable recommendation to pulse growing farmers of the region.

In field experiment sowing was conducted both in rabi and kharif seasons.

In pot culture experiment completely randomized block design (CRBD) was followed.

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'.

During the growth of pulses atmospheric nitrogen get fixed in their root nodules by rhizobium. Mo helps in localiza 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 application of Mo fertilizer in acidic low land soil and the pathways of N-accumulation and utilization for the same.

2.MATERIALS AND METHODS

2.1 ~~Mo-use efficacy study~~

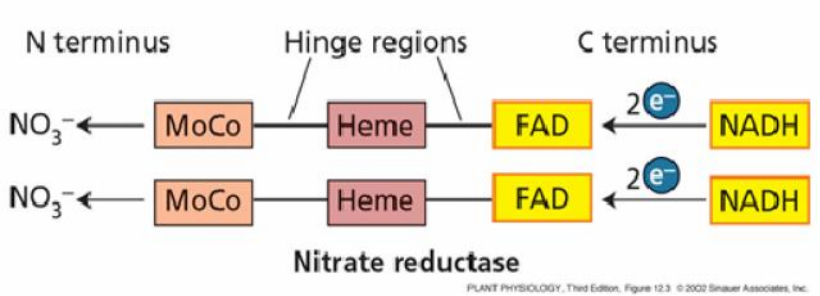
Ten clear white plastic jars (14 cm height, 12 cm diameter) of 1 kg capacity with tight lid were taken for the study. Each jar was filled with 1 kg soil taken from a field at National Pulses Research Centre (NPRC), Tamil Nadu, India (10°20'N, 78°54'E). The soil was acidic red lateritic (pH 5.6) type. The water holding capacity of the soil was 19.5% at 15 bar. Standard solutions of ammonium molybdate (52% Mo) and sodium molybdate (39% Mo) were added in plastic jars in five different treatments [M_1 (200 g Mo ha^{-1}), M_2 (400 g Mo ha^{-1}), M_3 (600 g Mo ha^{-1}), M_4 (800 g Mo ha^{-1}), M_5 (1000 g Mo ha^{-1})]. After addition of fertilizer solution, soil was mixed thoroughly and taken for estimating the available Mo. The jars with tight lid were kept in laboratory at room temperature for 1 month. The soil was stirred daily with a glass rod. Deionized water was also added on necessity basis to maintain the moisture level. Soil samples were taken from each jar at the interval of 1^{st}, 2^{nd}, 3^{rd} and 4^{th} week to measure available Mo by ammonium bicarbonate-DTPA method (Soltanpour and Schwab 1977).

2.2 Effect of Mo in N-fixation in greengram

The same soils as described above were used for pot culture experiment to study the effect of Mo in N-fixation process in greengram (variety Vamban 2) of short duration (60 to 65 days). Particle size distribution in soil was 56% sand, 16% silt and 28% clay. Fertilizer scheduling was followed as per the soil test based recommendation and 5 treatments of Mo [control, M_1 (200 g Mo ha^{-1}), M_2 (400 g Mo ha^{-1}), M_3 (600 g Mo ha^{-1}), M_4 (800 g Mo ha^{-1}), M_5 (1000 g Mo ha^{-1})] with three replicates of each were added as basal dose. Sodium molybdate was used as molybdenum fertilizer source. At 40 days, greengram were uprooted and washed thoroughly with water to take total nodule count and the anatomic features of nodules were studied under scanning electron microscope (FEI SEM Quanta 250, Netherland). Rhizobium population was also measured by serial plate dilution method in yeast extract mannitol agar medium (Allen 1953).

2.3 Determination of NR enzyme activity

Crude NR was extracted from fresh leaf tissue and its activity was determined by the method of Hageman and Reed (1980). The enzyme extract consisted of 1 g fresh tissue, 1mML^{-1} EDTA, 25-mML^{-1} cysteine and 25mML^{-1} potassium phosphate buffer (pH 8.8). In a test tube, 0.5 ml phosphate buffer (pH 7.5), 0.2 ml potassium nitrate, 0.4 ml NADH, 0.7 ml water and 0.2 ml enzyme extract were taken and incubated at 30°C for 15 min. Similarly blank was set without adding the enzyme extract. Nitrite in the solution was determined at 540 nm after 90 min of addition of 1 ml sulfanilamide [1% (w/v) in 1 N HCl] and 1 ml naphthylethylene-diamine dihydrochloride [0.02% (w/v)] by spectrophotometer.

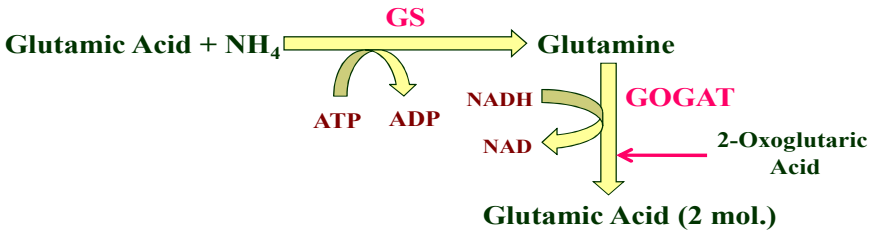


2.4 Determination of GS activity

1 g of fresh leaf sample from greengram was pre-chilled in a mortar pestle with 5 ml of 50 mM imidazole-acetate buffer (pH 7.8) containing 0.5 mM EDTA, 1mM dithiothreitol, 2 mM $MnCl_2$ and 20% glycerol as described by Pateman (1969). The enzyme extract was centrifuged at 10,000 r.p.m for 30 min. 2 ml 0.2 M L-glutamine; 0.5 ml 20 mM sodium arsenate; 0.3 ml 3 mM MnCl2; 0.5 ml 50 mM hydroxylamine; 0.5 ml 1 mM ADP and 0.2 ml enzyme extract were pipette out to make reaction mixture. The reaction mixture was incubated for 30 min at 37°C. To set blank 2 ml of Tris-HCl buffer was used in place of glutamine. GS activity was measured by colorimetric method at 540 nm after adding 1ml of $FeCl_3$ solution to reaction mixture.

GS – GOGAT Pathway

1. **Glutamine Synthetase (GS)**
2. **Glutamate synthase or GOGAT**



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 greengram 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 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 are no significant difference between treatments, it was denoted by the symbol NS

3. RESULTS AND DISCUSSION

Mo-use efficacy study shows that the availability of Mo (µg g^{-1}) increased day by day (Table.1 & Fig 1). Highest availability (79.3 µg g^{-1}) was found on 4^{th} week and lowest (0.03 µg g^{-1}) was on 1^{st} day of experiment in case of both molybdate fertilizers. 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 mineralization occurs at faster rate than $(NH_4)_2MoO_4$. So, Na_2MoO_4 is recommended as Mo fertilizer source.

Fig 1. Overall view of the Incubation Experiment

Mo fertilizer did not alter plant height though significant increase in root length was observed in greengram (Table 2). Without Mo application, root length was 13.8 cm whereas with 1kg ha^{-1} Mo application the increase of root length was 27.8 cm. There was a sharp increase in dry matter in both roots and shoots of greengram with Mo application. Highest number of nodule (25) was observed in greengram @1 kg ha^{-1} Mo application whereas it was only 10 in the control. In Fig.3(b), SEM images of greengram root cross section show more number of nodules rather than in control [Fig 3 (a)]. Similarly rhizobium population was the highest in M_5 (@1kg ha^{-1}) treatment. It was 8 CFU/ml whereas it was only 2 CFU/ml in the control (Table 3& Fig 2a, 2b). These results are in good agreement with earlier observations (Tripathi and Edward 1978; Dey and Ghosh 1986; Bhuiyan et al. 2008).

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

Control –Mo 0 g ha^{-1} **Fertilized- Mo 1000 g ha^{-1}**

A positive correlation between the activities of nitrate reductase (µg of NO_2 g^{-1} of leaf $hour^{-1}$) and Mo application (r^2 = 0.92) implies that the activities of nitrate reductase generally increase with increasing amount of Mo (Table 4). The highest NR activity (123.7 µg NO_2^- per gram leaf $hour^{-1}$) was found with the highest Mo application (1 kg ha^{-1}). These results are in good agreement with earlier observations (Viera et al. 1998). Mo application prominently enhanced NR and GS activities, indicating that Mo becomes biologically active when it make complex with molybdoprotein. The higher biological activity of molybdoprotein may lead to higher activity of NR and N assimilation. The correlation (r^2 = 0.95) between glutamine synthetase (GS) activities and Mo application is comparable to that of NR activity (fig. 3). However, the increase in GS activity with Mo concentration is not sharp as NR activity.

SEM IMAGE OF GREENGRAM NODULES

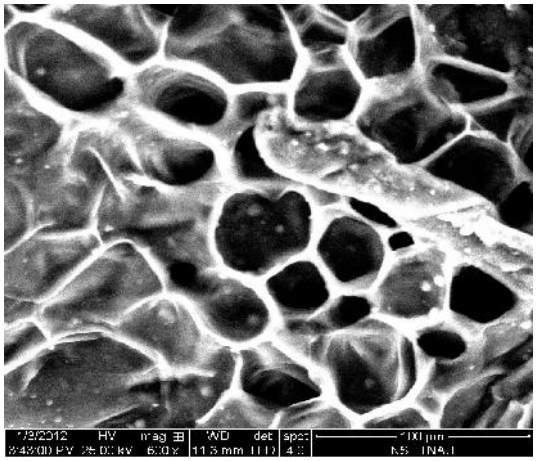


Fig 3a. Without Mo application-Control

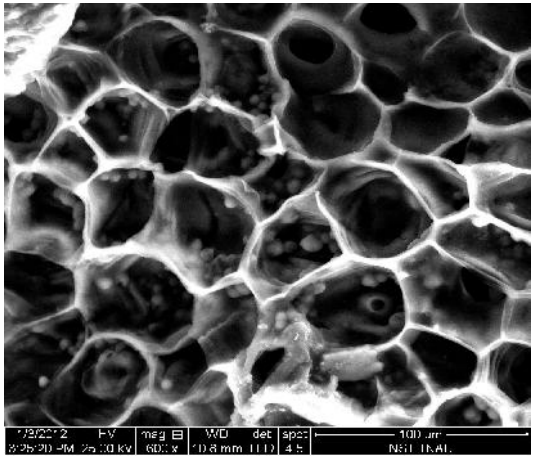


Fig 3b. With Mo application- 1000 g ha^{-1}

4.CONCLUSION

Application of Mo is also needed for Mo-deficient acid soils of SAT environments like in temperate acid soils to enhance rhizobium population and nodulation in greengram. Mo supplies also boost NR activity and N-assimilation. Higher Mo status resulted in higher accumulation of GS. This indicates the usefulness of Mo fertilizers for pulses in agriculture practices for acid soils of both SAT and temperate environments.

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Table 1. 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 2 . Effect of different methods of Mo fertilizer application on growth parameters in greengram (*Vigna radiata*)

Treatments	Shoots Height (cm)	Roots DMP (g plant^{-1})	Roots DMP (g plant^{-1})	Roots 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 3. 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

Table 4. 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