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Research Article
Role of Molybdenum in Relationship with *Rhizobium* sp. and its Effect on Physiological Characteristics and Soybean Production

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Abstract
Background and Objective: Molybdenum (Mo) is a micro nutrient that plays a role in activating the nitrogenase enzyme needed by *Rhizobium* bacteria to form root nodules in legume plants and every bacteria that fixes nitrogen requires molybdenum during the fixation process. The objective of the research was to evaluate the role of Mo in increasing the effectiveness of N_2 fixation and determine the response to soybean seed production and quality. Furthermore, it focused on collecting physiological and soybean production data. **Materials and Methods:** The study was conducted at Medan Selayang, Medan, Sumatera Utara from June to August, 2022. This research was conducted in an experimental field, using a factorial randomized block design with 2 factors and 3 replications. The first variable was Mo treatment at 0, 200, 400 and 600 g ha^{-1}. The second was the application of *Rhizobium* sp., inoculant at 0, 5 and 10 mL/polybag. The variables observed include chlorophyll, cuticle thickness, number of pods, number of filled pods, seed dry weight/sample, 100 dry seed weight and protein content. **Results:** The combination of *Rhizobium* sp., inoculation at 5 mL/plant and 200 g ha^{-1} Mo fertilizer produced the highest chlorophyll content and total chlorophyll. However, the highest cuticle thickness was produced without inoculation at 5 and 10 mL/plant. *Rhizobium* sp., inoculation at 5 mL/plant and 600 g ha^{-1} Mo increased the number of filled pods per plant to 92.73 pods, seed dry weight per plant and seed dry weight per plot. **Conclusion:** Inoculation of *Rhizobium* sp., 5 mL/plant and 600 g ha^{-1} molybdenum increased the number of filled pods per plant, seed dry weight per plant and seed dry weight per plot respectively 18.82, 29.13 and 13.89%.

Key words: Soybean, molybdenum, *Rhizobium* sp., nitrogenase enzyme, N_2 fixation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Soybean (*Glycine max* L. Merrill) is a legume that serves as the main source of food and vegetable protein for public consumption and the food industry. It is a source of a functional food because it contains highly efficacious isoflavones as the main secondary metabolites[1]. Isoflavones prevent osteoporosis, heart damage and degenerative diseases such as atherosclerosis, prostate cancer, colon cancer and diabetes mellitus, reduce menopausal syndrome and have anti-tumor properties[2-4].

Soybean is a leguminous plant that establishes a mutualistic symbiosis with *Rhizobium* in Nitrogen (N_2) fixation. In this symbiosis, *Rhizobium* resides in root nodules, while soybean plants obtain N from the belay by bacteria. This process produces root nodules containing *Rhizobium* which fix N_2 from the atmosphere[5-7]. The inoculation is expected to meet N_2 requirements in soybean plant, reducing the use of inorganic N_2 fertilizers. However, biological N_2 fixation as N_2 for plant growth has decreased due to the intensification of inorganic fertilization[8].

Nitrogen fixation in legume-rhizobia symbiosis is catalyzed by nitrogenase. This enzyme complex consists of nitrogenase (Mo-Fe protein) and nitrogenase reductase (Fe- protein). The Mo-Fe protein is encoded by the *nifD* and *nifK* genes, while the Fe protein is encoded by the *nifH* gene. Furthermore, nifBEN is a cofactor of Fe-Mo biosynthesis and nifA is a positive regulator of other nif genes[9]. The nitrogenase activity correlates with the expression of nif gene levels[7,10].

The micronutrient molybdenum (Mo) is needed to enhance the effectiveness of N_2 fixation by rhizobia because the nitrate reductase enzyme uses the nutrient for NO_3^- assimilation. When Mo concentration in the soil is low or around 0.7 ppm, it inhibits the formation of root nodules and interferes with N_2 fixation by rhizobia[11]. In addition, obstructed fixation of *rhizobia* reduces the availability of N_2 for plants, disrupting soybean plant growth and production[12].

The Mo in plants is a component of the nitrate reductase enzyme, reducing nitrate to nitrite ions and activating the enzymes nitrogenase, nitrate reductase and xanthine oxidase. Also, it is a part of the meta-protein nitrogenase that contributes to the N_2 fixation process and is required for bacterial N_2 metabolism[13]. The micronutrient acts as a cofactor for the enzymes nitrogenase and nitrate reductase, involved in the biological fixation and assimilation of N_2 in legumes. Furthermore, Mo plays a vital role in N_2 metabolism and protein synthesis and it facilitates various physiological and biochemical processes including photosynthesis and carbohydrate and sulfur metabolism. Research showed the significance of Mo in the growth, physiology, yield potential and nutritional quality of various legume grains including soybean, peanut and cowpea[14]. However, legume that depends on N_2 fixation are prone to Mo-deficiency-induced N_2 deficiency, specifically in acidic mineral soils in subhumid and humid tropics. The field studies showed that the application of Mo through legume leaves increases nodule mass levels, N_2 fixation, increased seed yield and N_2 uptake[15].

The effect of Mg on the physiological characteristics of soybeans in dry land[16] and N_2 uptake of two varieties inoculated with *Bradyrhizobium japonicum* have been reported[17]. There is limited research on the effect of Mo in its relationship with *Rhizobium* sp. Therefore, the role of Mo in relationship with *Rhizobium* sp., on physiological characteristics and soybean production was evaluated.

MATERIALS AND METHODS

Place and time of research: This research was conducted on dry land in Tanjung Sari, Medan, North Sumatra, Indonesia from June to August 2022. The soil analysis was carried out at the Socfind Laboratory before the research, while the analysis of chlorophyll content and stomatal density was done at the Plant Physiology Laboratory, Faculty of Mathematics and Natural Science, Universitas Sumatera Utara. Table 1 shows the soil analysis at the research location.

Materials and tools: The materials used included soybeans of the Devon variety from the Research Centre for Various Nuts and Tubers (Malang-Indonesia), manure and compost, *Rhizobium* sp., isolate, urea, SP-36 and KCl, Mo, organic insecticides and fungicides. The tools included hoes, scales, scratchers, research nameplates, Spectrophotometer Thermo scientific Multiskan Go USA, microscopes Microscope Carl Zeiss Primo Star Built with AxioCam ERc5S, software zen lite 2012 USA, analytical balances Precisa type HGS (from Switzerland) ovens and several tools for laboratory analysis.

Devon 1 is a superior variety with an average yield of 2.75 ton ha^{-1}, has a large seed size (14.3 g/100 seeds), resistant to leaf rust disease, light-resistant to pod-sucking pests and contains higher isoflavones (2200 µg g^{-1}).

Research methods: This research focused on collecting data on morphological, physiological and biochemical characteristics, as well as soybean production through the role of Mo and *Rhizobium* sp., in enhancing biological N_2 fixation

Table 1: Results of soil analysis before the research was carried out

Parameter	Results	Analytical method
pH H_2O	4.900	H_2O (1:5)-electrometry
C-organic (%)	5.14	Walkley and black with a spectrophotometer
N Kjeldahl (%)	1.24891	Kjeldahl with a spectrophotometer
P (%)	2.3511	Dry ashing HNO_3 with a spectrophotometer
K-total (%)	0.13240	HNO_3 with AAS

and soybean productivity. This factorial randomized block design with 2 factors and 3 replications was used to conduct this research in an experimental field. The first factor was Mo treatment at 0, 200, 400 and 600 g ha^{-1}, while the second factor involved the application of *Rhizobium* sp., inoculant at 0, 5 and 10 mL/polybag. *Rhizobium* sp., used was obtained from the Soil Biology Laboratory, Faculty of Agriculture, Universitas Sumatera Utara, Medan, Indonesia.

The soybean seeds were planted with a spacing of 40×20 cm, 2 seeds per hole. Furthermore, thinning was carried out at 7 WAP, leaving 1 plant per hole. *Rhizobium* sp., was applied with a population density of 10^8 sel. mL^{-1} based on the treatment carried out when the plants were 1 WAP. Similarly, Mo was applied in the form of ammonium molybdate according to the treatment at 1 WAP and watering was conducted based on the land condition. The fertilizer used was urea 25 kg ha^{-1}, with half a dose (12.5 kg ha^{-1}) given at planting and the rest (12.5 kg ha^{-1}) at 4 WAP. Additionally, SP-36 (100 kg ha^{-1}) and KCl fertilizers (75 kg ha^{-1}) were given at the start of planting. Manual weeding of plants around the soybean was carried out using a small hoe. Also, harvesting was conducted by cutting the stem above the base once it had exhibited the harvest criteria and reached physiological maturity, characterized by brownish-yellow pods. The variables include chlorophyll a, chlorophyll b, total chlorophyll, cuticle thickness, number of pods, number of filled pods, seed/sample dry weight, 100 dry seed weight and protein content.

Measurement of chlorophyll a content, chlorophyll b and total chlorophyll according to the method described by Hendry and Grimes[18]. The chlorophyll content was determined by taking a sample of soybean leaves (0.1 g), then macerated with 10 mL acetone using a mortar. Determination of chlorophyll a, chlorophyll b and total content chlorophyll is done by using the following Equation:

$$\text{Chlorophyl a} = \frac{(12.7\times \text{A663}) - (2.69\times \text{A645})}{10}$$

$$\text{Chlorophyl b} = \frac{(22.9\times \text{A645}) - (4.68\times \text{A663})}{10}$$

$$\text{Chlorophyl total} = \frac{(22.9\times \text{A649}) + (20.2\times \text{A645})}{10}$$

The chlorophyll content is expressed in mg g^{-1} fresh leaves.

A variable observed in cross-leaf incisions is the thickness lower cuticle layer (abaxial) of soybean leaves. The cuticle thickness unit used is μm.

Statistical analysis: The data was subjected to two way Analysis of Variance (ANOVA) procedures and comparison of means was tested for significance using Duncan's Multiple Range Test (DMRT) p = 0.05.

RESULTS

Chlorophyll a content: The interaction between Mo fertilizer and *Rhizobium* sp., inoculation had a significant effect on chlorophyll a. The combination of inoculation treatment at 5 mL/plant and 200 g ha^{-1} Mo fertilizer produced the highest chlorophyll a content, while 0 mL/plant and 400 g ha^{-1} fertilizer produced the lowest (Table 1).

Chlorophyll b content: The Mo treatment significantly affected chlorophyll b content. Meanwhile, the interaction between the Mo fertilizer application and *Rhizobium* sp., inoculation had no significant effect on the content. Table 2 showed that the 400 g ha^{-1} Mo treatment produced the highest chlorophyll content.

Total chlorophyll: The interaction between Mo and *Rhizobium* sp., had a significant effect on total chlorophyll. Furthermore, the inoculation of *Rhizobium* sp., at 10 mL/plant produced the highest content compared to the treatment at 5 mL/plant and without inoculation (Table 2).

This showed the role of *Rhizobium* sp., in enhancing total chlorophyll. In plants, N_2 acts as a constituent of chlorophyll molecules, protoplasm, nucleic acids and amino acids. The symbiotic relationship between *Rhizobium* sp. and soybean roots increased the availability of N, hence increasing soybean total chlorophyll.

Cuticle thickness: The interaction between Mo and *Rhizobium* sp., had a significant effect on cuticle thickness. Without inoculation, the cuticle thickness was higher compared to the treatment with inoculation at 5 and 10 mL/plant (Table 3), indicating that *Rhizobium* sp., inoculation decreased cuticle thickness. The treatment without Mo (0 g ha^{-1}) increased the thickness compared to others. Additionally, *Rhizobium* sp., inoculation at 5 mL/plant and 400 g ha^{-1} Mo produced the highest stomatal density.

Table 2: Chlorophyll a, chlorophyll b and total chlorophyll content of soybean leaves with the application of *Rhizobium* sp. and Mo

		Rhizobium sp. (mL/plant)			
Variables observed	Molybdenum (g ha^{-1})	R_0 (0)	R_1 (5)	R_2 (10)	Mean
Fresh weight (mg g^{-1})					
Chlorophyll a	M_0 (0)	2.81^{abc}	2.49^{bcd}	2.87^{ab}	2.72
	M_1 (200)	2.47^{cd}	2.98^{a}	2.84^{abc}	2.77
	M_2 (400)	2.19^{d}	2.71^{abc}	2.75^{abc}	2.55
	M_3 (600)	2.77^{abc}	2.56^{bc}	2.87^{ab}	2.74
	Mean	2.56^{b}	2.68^{ab}	2.83^{a}	
Chlorophyll b	M_0 (0)	4.43	4.53	4.41	4.45^{ab}
	M_1 (200)	4.55	4.30	4.40	4.42^{ab}
	M_2 (400)	4.66	4.40	4.47	4.51^{a}
	M_3 (600)	4.48	4.20	4.37	4.35^{b}
	Mean	4.53	4.36	4.41	
Total chlorophyll	M_0 (0)	7.23^{a}	7.02^{abc}	7.28^{a}	7.17
	M_1 (200)	7.02^{abc}	7.28^{a}	7.25^{a}	7.19
	M_2 (400)	6.85^{bc}	7.11^{ab}	7.22^{ab}	7.06
	M_3 (600)	7.26^{a}	6.76^{c}	7.24^{a}	7.09
	Mean	7.09^{b}	7.04^{b}	7.25^{a}	

Numbers followed by the same letter are not significantly different according to Duncan’s Multiple Range Test at level $\alpha = 5\%$

Table 3: Cuticle thickness of soybean leaves with the application of *Rhizobium* sp. and Mo

	Rhizobium sp. (mL/plant)			
Molybdenum (g ha^{-1})	R_0 (0)	R_1 (5)	R_2 (10)	Mean
µm				
M_0 (0)	29.54^{cd}	21.19^{d}	70.12^{a}	40.28
M_1 (200)	29.07^{cd}	46.41^{bc}	20.65^{d}	32.04
M_2 (400)	31.67^{cd}	54.13^{ab}	31.86^{cd}	39.22
M_3 (600)	55.05^{ab}	21.90^{d}	14.97^{d}	30.64
Mean	36.33	35.91	34.40	

Numbers followed by the same letter are not significantly different according to Duncan's Multiple Range Test at level $\alpha = 5\%$

Table 4: Number of fleshy pods, number of unripe pods, dry weight of 100 seeds, seed dry weight /plant, seed dry weight per plot of soybean with the application of *Rhizobium* sp. and Mo

		Rhizobium sp. (mL/plant)			
Variables observed	Molybdenum (g ha^{-1})	R_0 (0)	R_1 (5)	R_2 (10)	Mean
Number of the fleshy (pod)	M_0 (0)	75.27^{bc}	79.27^{abc}	68.53^{c}	74.36
	M_1 (200)	78.80^{abc}	76.67^{abc}	87.73^{ab}	81.07
	M_2 (400)	84.53^{abc}	70.47^{c}	74.07^{bc}	76.36
	M_3 (600)	72.67^{bc}	92.73^{a}	76.67^{abc}	80.67
	Mean	77.82	79.78	76.73	
Number of the unripe (pod)	M_0 (0)	19.13	21.10	19.07	19.77
	M_1 (200)	19.67	19.30	20.17	19.71
	M_2 (400)	19.20	19.13	20.23	19.52
	M_3 (600)	20.50	20.03	19.57	20.03
	Mean	19.63	19.89	19.76	
Dry weight of 100 seeds (g)	M_0 (0)	19.13	21.10	19.07	19.77
	M_1 (200)	19.67	19.30	20.17	19.71
	M_2 (400)	19.20	19.13	20.23	19.52
	M_3 (600)	20.50	20.03	19.57	20.03
	Mean	19.63	19.89	19.76	
Seed dry weight/plant (g)	M_0 (0)	20.48^{cd}	21.91^{bcd}	21.35^{bcd}	21.25
	M_1 (200)	23.07^{abcd}	23.89^{abcd}	25.99^{ab}	24.32
	M_2 (400)	25.39^{abc}	19.76^{d}	24.25^{abcd}	23.14
	M_3 (600)	21.05^{bcd}	28.9^{a}	22.79^{bcd}	24.25
	Mean	22.50	23.62	23.60	
Seed dry weight/plot (g m^{-2})	M_0 (0)	503.8^{ab}	490.77^{ab}	403.37^{b}	465.98
	M_1 (200)	577.30^{a}	565.67^{a}	505.80^{ab}	549.59
	M_2 (400)	579.43^{a}	472.57^{ab}	490.93^{ab}	514.31
	M_3 (600)	560.83^{a}	585.10^{a}	476.73^{ab}	540.89
	Mean	555.34^{a}	528.53^{ab}	469.21^{b}	

Numbers followed by the same letter are not significantly different according to Duncan's Multiple Range Test at level $\alpha = 5\%$

Table 5: Protein content of soybean with the application of *Rhizobium* sp. and Mo

	Rhizobium sp. (mL/plant)			
Molybdenum (g ha^{-1})	R_0 (0)	R_1 (5)	R_2 (10)	Mean
(%)				
M_0 (0)	36.32^{abc}	36.5^{abc}	35.67^{cd}	36.16
M_1 (200)	34.02^{e}	37.44^{ab}	35.32^{cd}	35.59
M_2 (400)	34.11^{e}	37.56^{a}	34.56^{de}	35.41
M_3 (600)	35.72^{cd}	35.82^{bcd}	35.11^{cde}	35.55
Mean	35.04^{b}	36.83^{a}	35.17^{ab}	

Numbers followed by the same letter are not significantly different according to Duncan's Multiple Range Test at level $\alpha = 5\%$

Soybean production: The production variables include the number of filled pods and empty pods, the weight of 100 dry seeds, dry seeds per plant and dry seeds/plot (Table 4). Based on the results, inoculation at 5 mL/plant and 600 g ha^{-1} Mo increased the number of filled pods per plant to 92.73. Meanwhile, the lowest number of filled pods, 68.53, were produced without Mo and inoculation at 10 mL/plant.

Rhizobium sp., inoculation and Mo treatments had no significant effect on the number of empty pods. However, inoculation at 10 mL/plant without Mo reduced the number of empty pods per plant to 19.07. Inoculation and Mo application had no significant effect on the dry weight of 100 seeds. The highest dry weight, 21.10, was observed at 5 mL/plant without Mo.

Inoculation of *Rhizobium sp.* and application of Mo significantly influenced seed/plant dry weight. The treatment with 5 mL/plant and 600 g ha^{-1} Mo resulted in the highest seed dry weight of 28.90 g. Similarly, highest yields of the dry seed weight per plot were obtained at 5 mL/plant and 600 g ha^{-1}, resulting in 580.10 g/plot. The seed weight per plot produced the highest yield of 555.34 g/plot without *Rhizobium* sp.

Protein content: The results showed that *Rhizobium* sp., inoculation and the interaction between inoculation and Mo application had a significant effect on protein content (Table 5). The soy protein increased with inoculation at 5 mL/plant, making up 36.83% and decreased without inoculation to 35.04%. Inoculating *Rhizobium* sp., at 5 mL/plant and applying 400 g/plant Mo yielded the highest soybean seed protein of 37.56%. However, applying 200 and 400 g ha^{-1} Mo without inoculation produced the lowest, 34.02 and 34.11%, respectively.

DISCUSSION

Rhizobium sp. plays a vital role in increasing the total chlorophyll, as well as chlorophyll a and b (Table 2). This is because N_2 is the main factor in the formation of chlorophyll, hence, increasing the availability in plants during growth will increase the chlorophyll content. According to Tairo and Ndakidemi[19] and Sara *et al.*[20], N_2 is a constituent of enzymes, proteins, chlorophyll and growth regulators and N_2 deficiency leads to stunted growth and yellowing of leaves. The improved vegetative growth in plants inoculated with *Rhizobium* strains was due to access to an N_2 source, which stimulated other growth characteristics such as total leaf chlorophyll content.

In legume plants, Mo plays an important role as a cofactor for the enzymes nitrogenase, nitrate reductase, xanthine oxidase and plays a role in N_2 metabolism and protein synthesis. Various physiological and biochemical processes including photosynthesis and carbohydrate and sulfur metabolism are also facilitated by Mo. The role of Mo is very crucial for legumes because it is a component of metal nitrogenase. The growth, physiology, yield potential and seed quality of legumes such as soybeans, peanuts and cowpeas are greatly influenced by Mo[14,21].

Table 2 showed that there was an increase in chlorophyll a with higher *Rhizobium* sp., inoculation. According to Nyoki and Ndakidemi[22], *Rhizobium* binds to free N_2 in the air to form ammonia (NH_3), which is converted into amino acids and then into N_2 compounds required by plants for growth and development. *Rhizobium* sp., obtains carbohydrates from soybean plants as a source of energy.

Table 4 shows that the application of *Rhizobium* sp. and Mo inoculants significantly affected seed yield. The highest soybean seed yield was obtained with *Rhizobium* sp., inoculation at 5 mL/plant and 600 g ha^{-1} Mo. This indicates the synergistic role of *Rhizobium* sp. and Mo in increasing soybean production. The bacteria fix atmospheric N_2 in the root nodules of legume plants. Rhizobia is a group of microbes that can bind free N_2 in the air to form ammonia (NH_3^-), which is converted into amino acids and then into nitrogen compounds needed for plant growth and development. Rhizobia obtain carbohydrates from host plants as a source of energy.

Meanwhile, Mo acts as a cofactor of the nitrate reductase enzyme which reduces nitrate ions to nitrite and activates the enzymes nitrogenase, nitrate reductase and xanthine oxidase. These enzymes aid in N_2 fixation and are essential components for bacterial N_2 metabolism[12,23].

The nitrogen requirements of legume plants are largely contributed by fixation through mutual symbiosis of bacteria that have nitrogenase enzymes with legumes which are capable of reducing dinitrogen into organic forms. In this symbiosis, rhizobia strains have different effectiveness between one strain and another. Activation of nitrogenase enzyme to function properly requires the role of Mo. The results of this research show that the addition of Mo up to 600 g ha^{-1} and the application of *Rhizobium* sp., 5 mL/plant increases the production of soybean seeds.

CONCLUSION

This research has proven the need for Mo in the mutualistic symbiosis between *Rhizobium* sp. and soybeans, related to the activation of the nitrogenase enzyme by Mo. Inoculation of *Rhizobium* sp., 5 mL/plant and 600 g ha^{-1} molybdenum increased the number of filled pods per plant, seed dry weight per plant and seed dry weight per plot respectively 18.82, 29.13 and 13.89%.

SIGNIFICANCE STATEMENT

This research resulted in the finding that Mo plays an important role in the mutualistic symbiosis between *Rhizobium* sp. and legume plants, because of Mo's role in activating the nitrogenase enzyme. The research helps researchers and farmers in obtaining the most appropriate Mo dose and *Rhizobium* sp., inoculant concentration to increase soybean seed production. Based on this research, a new theory was obtained that the use of inoculation of 5 mL/plant and 600 g ha^{-1} Mo, respectively increased the number of filled pods per plant, dry weight of seeds per plant and dry weight of seeds per plot 18.82, 29.13 and 13.89%.

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