INFLUENCE OF MOLYBDENUM DOSES IN INOCULATION AND MINERAL FERTILIZATION IN COWPEA BEANS

INFLUÊNCIA DE DOSES DE MOLIBDÊNIO NA INOCULAÇÃO E NA FERTILIZAÇÃO NITROGENADA EM FEIJÃO CAUPI

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ABSTRACT: In some leguminous plants, associations with nitrogen-fixing microorganisms allow their nutrition with nitrogen (N) from the atmosphere. This process is known as Biological Nitrogen Fixation (BNF), where through nitrogenase enzymes, N_2 is converted to an available form. This process can replace in part, or in total, nitrogen fertilizers. Cowpea bean is a legume species that is recognized for its high capacity to carry out BNF. In the last decades, studies have encouraged small farmers from north and northeast Brazil to use inoculants with rhizobia species since the results of researches have demonstrated that inoculation is an interesting strategy to improve cowpea production. Considering the specific function of molybdenum (Mo) in the N assimilation, different doses of Mo were tested in this study in order to find doses that could improve and enhance BNF. Therefore, this study aimed to compare nitrogen fertilization and BNF in the N assimilation by plants with different Mo doses. Inoculation was performed with the strains UFLA 03-84 and INPA 03-11B. Doses of Mo were applied in seeds and each pot contained five seeds. Thirty-five days after germination, the plants were analyzed for shoot dry matter and fresh matter, N contents and accumulation, as well as the Soil-Plant Analysis Development (SPAD) Index and nodulation in inoculated plants. The different doses of Mo and also the nodulation treatments did not show significant differences in the contents of N. Plants with N fertilization had significant higher shoot dry matter and root dry matter production, in addition to higher N foliar contents and N accumulation. Therefore, BNF was not as efficient as nitrogen fertilization in the evaluated experimental conditions using cowpea beans.

KEYWORDS: *Vigna unguiculata* L.. Micronutrient. *Bradyrhizobium.*

INTRODUCTION

In some Brazilian areas, part of the daily food production is based on cowpea beans (*Vigna unguiculata* L). This leguminous plant has a production potential in the order of 6000 kg ha^{-1}. However, it is important to emphasize that as any [illegible] beans are extremely efficient in performing symbiosis with nitrogen-fixing bacteria (ZILLI et al., 2006).

Nitrogen (N) is a mineral with a complex cycle, being highly demanded by plants as the most limiting nutrient in plant growth. Low N contents in soils, plants with a short cycle and roots growth in the surface layer can increase the demand for N, especially for leguminous plants (SOARES et al., 2016). To supply the need for N, some farmers have decreased the use of mineral fertilization, replacing it with atmospheric N-fixing bacteria. In the process of mutual symbiosis between bacteria and leguminous plants (in this case, *Bradyrhizobium*), the biological nitrogen fixation (BNF) is considered a key technique to provide N to plants (COOPER; SCHERER, 2012), especially regarding tropical [illegible] 2017; COSTA et al., [illegible] et al., 2013; LIMA; PEREIRA; MOREIRA, 2005; RUFINI et al., 2016).

The contribution of BNF in soybean (*Glycine max*) and common bean is already well stated and represents a significant economy regarding the use of nitrogen fertilizers. However, this technology has not been widely used since cowpea beans are mostly produced by small farmers. Therefore, considering the potential of BNF to be exploited and the intrinsic characteristics of this culture in interacting with N-fixing

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microorganisms (RUMJANEK et al., 2005; VIEIRA et al., 2010), some researchers have invested efforts in this direction (CASTRO et al., 2017; COSTA et al., 2014a, 2014b; FARIAS et al., 2016; SOARES et al., 2006), in order to make this technology accessible to any producer.

Nutrients such as cobalt (Co), iron (Fe), and molybdenum (Mo) in soils are essential for nitrogen-fixing microorganisms. For example, due to its role as a cofactor of nitrogenase and nitrate reductase, Mo is essential for the functioning of the enzymes in the N metabolism (BROADLEY et al., 2012). The form that N is supplied to plants and its metabolism is highly connected to the requirement of Mo and its functions in some leguminous plants (BROADLEY et al., 2012).

Several studies have demonstrated that Mo application in soils shows positive responses in some plants (GUPTA, 1981; KUBOTA, 2006), since this micronutrient is generally found in low levels in soils. According to some authors (FREIRE FILHO et al., 2005; JACOB-NETO; THOMAS; FRANCO, 1988), the greater availability of Mo occurs either by the application of limestone that promotes the reduction of the adsorption effect or by the application of this nutrient, which promotes significant increases of BNF.

Therefore, this study aimed to compare the efficiency of different doses of Mo (0, 0.05, 0.1, 0.2, and 0.4 mg pot^{-1}) in the BNF process, using lime to increase soil pH, and consequently increasing Mo availability. The fixation of N_2 was provided by the following strains: UFLA 03-84 and INPA 03-11B. Treatments using N fertilization were also tested in order to measure the efficient the nitrogen fixation. The doses of Mo were tested in the treatments with and without N addition. The strains were added the treatments without the addition of N, since this nutrient inhibit the activity of N-fixing bacteria.

This study aimed to find an adequate Mo dose in order to allow a better biological nitrogen fertilization performance.

MATERIAL AND METHODS

Soil sampling and preparation

The experiment was conducted under greenhouse conditions, using a clayey Red-Yellow Latosol, which was collected in a region that presents a transition Cwa-Cwb climate (according to the Köppen classification) (ANTUNES, 1986), with annual average temperature of 19.6 °C, annual average precipitation of 1511 mm, annual air humidity average of 76.2%, and annual evaporation of 901.1 mm (ALVARES et al., 2013). The soil was collected from the 0-0.2-m layer, in a mountainous area with the following geographic coordinates: 21º17'16" S and 44º48'07" W.

The soil was air-dried and passed through a 2-mm sieve to obtain air-dried fine earth (ADFE). The soil samples were sterilized twice with 24 h intervals between each sterilization process in order to remove soil microorganisms, especially native bacteria strains that could fix atmospheric N and compete with the inoculated bacteria. After sterilization, the soil was analyzed for its physical and chemical attributes (Table 1). Limestone (2.13 g pot^{-1}) and fertilizers (MALAVOLTA, 1980) were applied in order to allow plant growth. The fertilization was divided into three stages: five, 12 and 19 days after cultivation. In the first stage, P, K, Mg, S, and N (according to the treatments that receive it) were applied. In the second stage: N, K, and S were applied and the third stage, N, K, B, Zn, Mn, Fe, and Cu were added. The cowpea bean cultivar Cauamé was used in this study.

Inoculation preparation

The experiment followed a randomized complete block design, with three replications. The factorial scheme consisted of five doses of Mo (0, 0.05, 0.1, 0.2, and 0.4 mg L^{-1}) and two types of N application: fertilization with ammonium nitrate and inoculation with *Bradyrhizobium* bacteria strains. The seeds were treated with a molybdenum acid solution, leaving them soaked in the solution for 30 min. Then, the seeds were air-dried prior to cultivation.

The inocula were prepared with strains from the *Bradyrhizobium* genus, specifically UFLA 03-84 (COSTA et al., 2019) and INPA 03-11B (GUIMARÃES et al., 2015), which are recommended as a leguminous inoculant for cowpea. The inocula were grown in Petri plates, in YMA (VINCENT, 1970), from five to seven days and, as they showed enough growth, they were transferred to Erlenmeyer glasses filled with the same medium but without Agar (YM). After five days, 1 mL of the inoculum was applied in cowpea beans seeds, consisting of a mix of both strains.

Pots of 4 dm^3 were used, with 5 seeds in each pot. Twenty-five days after germination the plants were harvested, leaving three plants per pot. After germination, plants were cultivated for 35 days, when it was expected a maximum nodule activity.

Table 1. Soil chemical and physical attributes after sterilization.

Layer		0-20 cm
pH (H_2O)		4.9
K	mg dm^{-3}	26
P		0.28
Zn		1.37
Fe		12
Mn		1.26
Cu		0.61
B		0.24
S		7.03
Ca	cmol$_c$ dm^{-3}	0.3
Mg		0.1
Al		0.3
H+AL		2.01
*CEC (pH 7.0)		2.55
M	%	39
V		18.3
**O. M.	dag kg^{-1}	0.54
Sand		65
Clay		32
Silt		3

*CEC: Cation Exchange capacity, **O. M.: organic matter.

Experiment Evaluation

The analyzed variables were: number of nodules, nodules dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), N foliar content and SPAD index. Thirty-five days after the emergence of plants, the SPAD index was measured using the portable equipment SPAD502, in the leaves located in the upper third part of each plant. Studies have shown that the SPAD Index is an important predictor of the N contents in leaves (SIMIL et al., 2019) as well as for the chlorophyll contents (BOYDSTON et al., 2018). After that, the plants were removed from the pots and the root nodules were quantified.

The material was separated into shoot, root, and nodules. They were conditioned in properly identified paper bags and oven dried at 70±5 ^{o}C for five days until reaching constant weight, when they were weighed for dry matter quantification.

The semi-micro Kjeldahl methodology was used to evaluate the N foliar contents. The shoots were grounded and 1 g of each sample was used in the procedure.

Statistical Analysis

The data were analyzed using the SISVAR software, version 5.6 (FERREIRA, 2011). The Shapiro Wilk test was conducted to verify data normality and, whenever necessary, the data were transformed according to the formula (X+0.5)0.5 in order to meet the assumptions of the analysis. The data was also submitted to analysis of variance using the Scot Knott test with 5% probability.

RESULTS AND DISCUSSION

The initial objective of the study was to identify one or more doses of Mo that could optimize and increase the N contents in the plants from the BNF, considering the application of limestone as a facilitating process, since it promotes the increase of soil pH. At high pH conditions, Mo becomes more available. This hypothesis was initially reinforced by other studies, such as Ma et al. (2018), who obtained results indicating increased N uptake by Mo application and even noticed that it may be beneficial for subsequent crops, although there are few studies on availability for subsequent crops. However, it was observed that in the different interactions between the statistical variables (SDM, RDM, N accumulation, number and dry weight of nodules, N foliar content and SPAD index) (Table 2), there were no significant effects of different doses of Mo. The interaction between Mo doses and nitrogen fertilization/bacteria inoculation also did not reveal significant differences in any evaluated characteristics.

Table 2. Analysis of variance for the number of nodules, SDM, RDM, NDM, N content and SPAD index.

Variation	FD	Number of nodules	SDM	RDM	NDM	N content	SPAD Index
Block	2	7.217	3.610	0.011	0.002	1.602	36.888
Mo doses	4	1.174	0.585	0.009	0.0006	0.265	15.673
N fertilization	1	-	70.605*	0.090*	-	65.069*	48.794
Interaction	4	-	0.585	0.312	-	0.151	37.948
Residual	18	2.375	1.461	0.011	0.0004	0.403	31.718
Mean	-	3.777	2.241	0.956	0.073	7.32	40.582
VC%	-	40.80	53.94	11.26	2.99	8.67	13.88

SDM: Shoot dry matter; RDM: Root dry matter; NDM: nodules dry matter

Regarding the lack of significant difference in the Mo doses, similar results can be found in the literature for common beans (MATOSO; KUSDRA, 2014), soybeans (*Glycine max*) (GRIS; CASTRO; OLIVEIRA, 2005), peanuts (CAIRES, 2005), and cowpea beans (LEITE et al., 2009). Several aspects might be involved in the unexpected results found in the present study, such as the micronutrient application through seeds, the survival of the strains when inoculated in the seeds after the Mo application, the chemical and intrinsic characteristics of the soil and even the studied cultivar. In addition, Castro et al. (2017) reported that temperature might negatively influence the nodulation process, especially temperatures higher than 40 °C, which may reflect lower plant growth, as observed in the present study. Considering that the experiment was conducted during summer months in a tropical country under greenhouse condition, this temperature could have been reached. Studies have also shown that the efficiency of biological nitrogen fixation can be affected by varying edaphic conditions, such as nutrient levels, soil type, and management practices (ALAM et al., 2015; DABESSA; ABEBE; BEKELE, 2018).

Studies have shown that the application of Mo through seed or leaf may influence the fixation process. Fullin et al. (1999) found that providing the micronutrient through seed by pelletizing was not efficient. Some studies have revealed that foliar application may show more efficient responses for micronutrient application (FULLIN et al., 1999; YANNI, 1990). In addition, Amara and Nasr (1995) reinforced that better results were obtained when Mo was applied by spraying the leaves, and this higher efficiency of foliar application was corroborated by Lopes et al. (2014, 2016), after finding higher contents of Mo in bean leaves and seeds. However, for soybean, it is currently recommended the application of Mo with the inoculant via seed where *Bradyrhizobium* strains are inoculated (ALBINO; CAMPO, 2001). Thus, it can be deduced that for cowpea, better answers could have been obtained if foliar fertilization was applied, thus, benefiting the BNF. Other researchers concluded that the seed reserve could provide enough Mo contents to meet the plant's requirement for at least one generation, without compromising the productivity (BRODRICK; GILLER, 1991).

It is known that cowpea beans are able to nodulate with different bacteria species, from different genus such as *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium* (RUMJANEK et al., 2005). Studies previously reported that the species *B. elkanii* (INPA 03 11B), also tested in this study, has shown high efficiency in BNF. However, variations among strains show different values for the N contents (SOARES et al., 2006; ZILLI et al., 2006). These variations can occur due to the intrinsic characteristics of the organism or by external factors (temperature, for example) and also due to some chemical-molecular aspects involved in the rhizobia-leguminous interaction.

Studies indicate that the application of Mo may cause deleterious effects to the inoculated strains in the seeds of the inoculated culture (CAMPO; ARAÚJO; HUNGRIA, 2009). For example, high doses of Mo may decrease the number of viable cells of *Bradyrhizobium* strains on the root surface, impairing the biological nitrogen fixation process (TONG; SADOWSKY, 1994). In addition, the survival of *Bradyrhizobium* may be reduced in liquid inoculants when compared with peat moss (ALBINO; CAMPO, 2001). Therefore, it can be suggested that the inoculants survival can be affected by the application of Mo, when it was applied to seeds.

Tong and Sadowsky (1994) suggested that high doses of Mo affect *Bradyrhizobium*, and may decrease the number of viable cells on the seed surface, impairing nodulation and biological nitrogen fixation. Albino and Campo (2001) also stated that the application of Mo to soybean via seeds reduced the number of *Bradyrhizobium* cells due to its contact with the inoculant and, consequently, the nodulation and biological nitrogen fixation.

The specificity between the host plant and the symbiotic organisms is controlled by the production of bacterial and root exudates (PERRET; STAEHELIN; BROUGHTON, 2000) and by genetic factors. Plant exudates, known as flavonoids, are recognized by bacteria as they are released (FERNANDES JUNIOR; REIS, 2008). On the other hand, microsymbionts produce nod factors as a response, which are important molecular signals recognized by high-affinity receptors in plants that induce responses in the host plant, such as the deformation of the root hairs (DÉNARIÉ; DEBELLÉ; PROMÉ, 1996). Therefore, according to the low fixed N content through the nodules, the flavonoids could not have been recognized by the inoculated strains.

The establishment of interactions of legumes and rhizobia is coordinated by many other interactions between them. Initially, the nod factors receive the plant flavonoids signals (RIVAS et al., 2009) and, consequently, the nod factors of rhizobia act inducing nodules formation of plants. In this way, all processes involved in BNF, as well as the type and quantity of interactions are important to determine the leguminous-rhizobia specificity (LAGUERRE et al., 2001). Therefore, some of these characteristics might have interfered in the perfect symbiosis and, consequently, on the N fixation efficiency.

The absence of a response from Mo fertilization can be due to the natural Mo content in the soil, which could be enough to meet the requirements of the plant (FERREIRA et al., 2001). According to Jacob Neto and Rosseto (1998), regarding micronutrients, the seeds' internal reservoir is sufficient to allow plant growth without external dependence. Barbosa et al. (2010) also stated that Mo reservoir in seeds could be enough for a good plant development when they are well supplied with other nutrients. The soil used in this experiment did not show signs of previous cultivation, a fact that could be responsible for Mo exportation and availability decrease. This fact corroborates the hypothesis that the soil may present enough natural Mo contents.

The application of limestone to raise soil pH is responsible to increase Mo availability, especially Mo ions adsorbed on Fe and Al oxides (MARCONDES; CAIRES, 2005). Although Mo is a plant nutrient, especially for the ones that biologically fix atmospheric N, the required content of the nutrient is considered to be low (LEITE et al., 2007). The pH adjustment in soils could be enough to increase the Mo availability and supply according the need of the plant, masking the response for different Mo doses. Lantmann et al. (1989) reinforced this idea by stating that Mo addition is closely related to soil pH, with better efficiencies in soils with low pH.

Doses of Mo can be responsible for different responses in plants according to the tested cultivars of a given species. By analyzing the behavior of 17 evaluated beans, Amane et al. (1994) observed that the intensity of their responses to Mo was conflicting. Some cultivars show different behaviors regarding the application of Mo, and a given cultivar may have the same efficiency with the application of Mo both through seeds or leaves. Berger et al. (1995) observed that in the bean cultivar *Ouro*, there was no differentiated response regarding the form of application. In contrast, in the other tested cultivar, *Ouro Negro*, the foliar application was more effective than via seed. Alam et al. (2015) conducted a study in which several doses of Mo were applied to the soil to evaluate its effect on nodulation and hairy vetch (*Vicia villosa* Roth) biomatter production. The application of Mo increased the number and size of nodules and the activity of nitrogenase and nitrate reductase enzymes in plants, leading to increased N uptake and increased biomatter; in plants grown in soil receiving 0.5 mg of Mo per kg, there was increased N fixation in hairy vetch (*Vicia villosa* Roth). Thus, it is important to consider such events when choosing a cultivar to be studied.

Nitrogen fertilization, when analyzed lonely, promoted statistically higher SDM, RDM and N contents when compared with the treatments with bacteria inoculation (Table 3). Nitrogen accumulations in shoots were also significantly higher in treatments with nitrogen fertilization when compared with treatments with bacteria inoculation, as shown in Figure 1. Broadley et al. (2000) suggested a negative effect in plants with N deficiency and plants with normal N availability, and this effect can be noticed by the reduction of fresh leaves matter, in limiting conditions for N.

Regarding the highest production of dry matter in treatments with N fertilization when compared with bacteria inoculation, it can be considered that it is metabolically less expensive for plants to use N that is readily available in soil than to establish an association with diazotrophic bacteria (GYANESHWAR et al., 2002).

Table 3. Mean tests for treatments with nitrogen addition and treatments with bacteria inoculation.

Treatments	Means		
	SDM	RDM	N content
Nitrogen fertilization	2.62 a	1.01 a	8.79 a
Bacteria inoculation	1.61 b	0.90 b	5.85 b

Means followed by the same letters do not differ from each other by the Scott Knott Test with 5% probability. SDM: Shoot dry matter, RDM: root dry matter and N content: N foliar content.

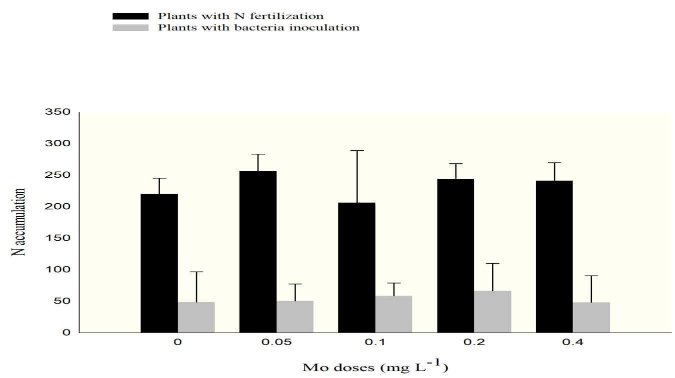

Figure 1. Nitrogen accumulation with different Mo doses in treatments with N fertilization compared with treatments with bacteria inoculation

Nitrogen is one of the responsible nutrients for plants growth and for production of new tissues and cells, influencing IAA (indolyl acetic acid) synthesis, which is the plant growth hormone (FAQUIN; ANDRADE, 2004). That is why, in this study, higher contents of N can result in higher matter production.

According to Malavolta, Vitti and Oliveira (1997), reference values for foliar N content in beans ranges from 3 to 5 dag kg^{-1} (30 to 50 g kg^{-1}). Considering that the average N contents in the inoculated treatments and in the fertilized treatments were 34.4 and 77 g kg^{-1}, respectively, the BNF was sufficient to reach this reference range.

In general, one can state that, in some way, such factors may have influenced the biological nitrogen fixation process, which contributes to the effects on the application of different doses of Mo in the soils that have their pH adjusted. All treatments that presented nodules were the ones inoculated with bacteria. No nodule was observed in treatments with nitrogen fertilization, confirming that there was not contamination among treatments.

CONCLUSIONS

No significant results were found for Mo application for each tested dose. Therefore, it was not possible to determine the adequate dose to improve and benefit BNF.

Many factors, such as the form of Mo application, the strains, the soil characteristics, as well as the studied cultivar might have contributed to low BFN and the no effect found after Mo application.

This study was the first step in understanding how Mo can be applied to improve the biological nitrogen fixation.

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RESUMO: Em algumas plantas de leguminosas, as associações com microorganismos de fixação permitem a sua nutrição com N (nitrogênio) da atmosfera. Este processo é conhecido como Fixação Biológica de Nitrogênio (FBN), onde através de enzimas nitrogenase N_2 é convertido na forma disponível. Este processo pode substituir em parte, ou no total, fertilizantes nitrogenados. O feijão-caupi é uma espécie de leguminosa que é reconhecida pela sua alta capacidade de FBN. Nas últimas décadas, estudos encorajaram os pequenos agricultores do norte e nordeste do Brasil a usar inoculantes com espécies de rizobio, uma vez que os resultados das pesquisas têm demonstrado que a inoculação é uma estratégia interessante para melhorar a produção dessa cultura. Considerando a função específica do molibdênio (Mo) na assimilação do N, diferentes doses de Mo foram testadas neste estudo, a fim de encontrar doses que possam melhorar e potencializar a FBN. A inoculação foi feita com as estirpes UFLA 03-84 e INPA 03-11B. As diferentes doses de Mo foram aplicadas na semente, cinco sementes por vaso. Trinta e cinco dias após a germinação, as plantas foram analisadas com relação à matéria seca aérea e radicular, teor de N e acúmulo, índice de análise de desenvolvimento solo-planta (SPAD) e nodulação nas plantas inoculadas. As diferentes doses de Mo não mostraram diferenças significativas no conteúdo de N, nem no processo de nodulação. As plantas com adubação nitrogenada tiveram mattera seca da parte aérea e de raízes superior as inoculadas, além de maiores teores foliares de N. Portanto, para feijão-caupi, a FBN não foi tão eficiente quanto o fertilizante nitrogenado nas condições experimentais avaliadas.

PALAVRAS CHAVE: *Vigna unguiculata* L.. Micronutriente. *Bradyrhizobium*.

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