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Research Article

Efficiency and assimilation of nitrogen in bean plants through foliar application of zinc and molybdenum nano fertilizer

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Abstract

Fertilization with nanoparticles offers alternatives to improve the efficiency of nutrient absorption in a sustainable way in crops. The objective of this work was to study the influence of foliar application of zinc-molybdenum nanofertilizer (Nano ZnMo) on nitrogen (N) use efficiency (NUE), as well as on yield parameters and N assimilation in green bean plants cv. "Strike". Three doses of Nano ZnMo (0, 4 and 8 ppm) were applied foliar in combination with four doses of N in the form of NH_4NO_3 in nutrient solution (0, 3, 6 and 12 mM). The treatment that obtained the greatest increase in total biomass was the combination of 6 mM N + 4 ppm Nano ZnMo, with an increase of 11.6% compared to the treatment without application of Nano ZnMo. The treatment that registered the highest yield was the combination of 3 mM of N + 4 ppm of Nano ZnMo with an increase of 39.3% compared to the treatment without foliar application of Nano ZnMo (3 mM of N). The utilization coefficients (NUtE) and NUE were favored with the foliar application of Nano ZnMo at 4 ppm in combination with 3 and 6 mM of N in nutrient solution. These treatments made it possible to reduce the dose of N fertilization without compromising production. Therefore, the foliar adhesion of Nano ZnMo makes it possible to improve the efficiency of nutrient absorption and sustainably increase crop productivity.

Keywords: absorption; nitrogen use efficiency; nitrogen metabolism; nanofertilizer Mo-Zn; *Phaseolus vulgaris* L.

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Introduction

The bean is a crop distributed throughout the world and is the most important legume for the diet in Latin America and Southeast Africa, due to its content of proteins, vitamins, minerals and fiber (Siddiq and Uebersax, 2012; Celmeli *et al.*, 2018). Among the different existing species with potential for human consumption, the common bean (*Phaseolus vulgaris* L.) is the most cultivated worldwide (Singh, 2013). According to FAO estimates, production in 2019 reached 28.9 million tons, with a global harvested area of 33.1 million hectares (Nadeem *et al.*, 2021). Despite this, the diversity of conditions under which beans are grown complicates obtaining high yields, so proper fertilization management plays a very important role in the improvement of food production. However, the progressive use of inputs versus the soil fertility loss has generated a contamination problem (Zhang *et al.*, 2015; Massawe, 2016). About 30% of agricultural inputs are under the effect of leaching, volatilization, among other loss factors (Pereira *et al.*, 2017). One of the most limiting elements in the development and production of crops is nitrogen (N), which presents losses in its application of up to 70% depending on the conditions of the crop (Mitra, 2015). Therefore, there is a need to improve conventional fertilization practices and change them to advanced technologies that allow sustainable agriculture (Chhipa, 2017).

Nanotechnology makes available the development of fertilizers based on nanocomposites such as Cu, Ag, Mn, Mo, Zn, Fe, Si and Ti oxides that have shown promising results in terms of optimal concentration in seeds, growth and production of plants with which could improve the N use efficiency (NUE) (Delfani *et al.*, 2014; Rameshaiah *et al.*, 2015). Nanofertilizers provide specific and controlled delivery sites for active ingredients to plants and reduce losses, which could provide ecological balance and mitigate climate change. (Mishra *et al.*, 2014; Solanki *et al.*, 2015).

Molybdenum (Mo) is an essential microelement for the plant and its absence could restrict its development. Different specific plant enzymes, such as nitrate reductase (NR) in N metabolism use Mo, so deficient plants exhibit reduced growth and low chlorophyll concentration (Hille *et al.*, 2011; Mendel, 2011; Rana *et al.*, 2020). Regarding the role of Zinc (Zn), its most important functions include enzymatic reactions and oxidation-reduction reactions, in addition to maintaining the structural and functional integrity of biological membranes, facilitating protein synthesis and gene expression, energy production and participation in the Krebs cycle (El Habbasha *et al.*, 2013; Hafeez *et al.*, 2013; Marshner, 2015). However, its absorption is hampered by antagonisms with other elements, such as phosphorus and the pH of the soil solution (Manuel *et al.*, 2018; Rana *et al.*, 2020). Despite this, research indicates that it is possible to increase the efficiency and accumulation of these microelements by foliar application in quantities of 1 and 8 ppm in legumes (Taran *et al.*, 2014), but there is still little literature on of the effects of nanofertilizers of these micronutrients on physiological responses in plants. Therefore, the objective of the present study was to evaluate the physiological responses, assimilation and NUE in the cultivation of common bean (*Phaseolus vulgaris* L.) against the application of different doses of a commercial ZnMo nanofertilizer.

Materials and Methods

Crop management

The experiment was carried out in the facilities of the Center for Research in Food and Development in Delicias, Chihuahua, México, during the months of August to October 2020. The experiment was carried out under greenhouse conditions with an average temperature of 28.6 °C and average relative humidity of 45%. Green bean plants cv. 'Strike' developed for 60 days, which were planted in germination trays and later transplanted into 13 L pots with a 2:1 mixture of vermiculite and perlite, placing 2 plants per pot. A standard nutrient solution was applied, without N, with pH 6±0.1, composed of 1.6 mM K_2HPO_4, 0.3 mM K_2SO_4, 4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5 µM Fe-EDDHA, 2 µM $MnSO_4$, 0.25 µM $CuSO_4$ and 0.5 µM H_3BO_3. 500 mL of the nutrient solution were applied per pot every third day. The foliar application of the treatments was carried out from the appearance of the true leaves (15 days after germination), every 10 days for a total of 5 applications during the crop cycle.

Experimental design and treatments

A completely randomized experimental design was used, with a 4x3 factorial arrangement with four repetitions, with the doses of N in the form of NH_4NO_3 applied in the nutrient solution as the factor A (0, 3, 6 and 12 mM of N) and factor B the doses of the commercial product of ZnMo nanofertilizer (Nano ZnMo) Broadacre® in a foliar way (0, 4 and 8 ppm) (Table 1).

Table 1. Description of the treatments

N doses in the solution	Doses of foliar nanofertilizer of ZnMo (ppm)	Code
0 mM N	0	Control
0 mM N	4	0 N + 4 ZnMo
0 mM N	8	0 N + 8 ZnMo
3 mM N	0	3 N + 0 ZnMo
3 mM N	4	3 N + 4 ZnMo
3 mM N	8	3 N + 8 ZnMo
6 mM N	0	6 N + 0 ZnMo
6 mM N	4	6 N + 4 ZnMo
6 mM N	8	6 N + 8 ZnMo
12 mM N	0	12 N + 0 ZnMo
12 mM N	4	12 N + 4 ZnMo
12 mM N	8	12 N + 8 ZnMo

Plant sampling

Once the plants reached the state of physiological maturity at 60 days of crop development, the plant material was harvested. The collected material was washed with distilled water to remove residues and finally separated into organs (root, stem, leaves and fruit). The samples were divided into fresh and dry material. The fresh material was used for the quantification of fruit yield, total fresh biomass, photosynthetic pigments and analysis of NR enzymatic activity. Part of the fresh material was placed in a drying oven (Shel Lab Forced Air Laboratory Oven SMO14-2, Baltimore, USA) at 70 °C for 48 h until it lost moisture. This material was weighed, ground and used for the quantification of Zn, Mo, N and total protein.

Plant analysis

Biomass

The total biomass of the different plant organs was evaluated separately from the dry weight of the plant. To quantify the weight, an analytical balance (AND HR-120, San José, California, USA) was used; the weights were expressed as grams per plant based on dry weight (g d.w. plant^{-1}).

Yield

The total fresh weight of fruits per plant was quantified. The results were expressed as grams of fresh weight per plant (g f.w. plant^{-1}).

Assay of the enzymatic activity of nitrate reductase (EC 1.6.6.1)

For the extraction and quantification of the "*in vivo*" NR activity, the methodology proposed by Sánchez *et al.* (2004) was used. From the fresh plant material (leaves), specifically from the leaf blade, taleolae of 7 mm were obtained up to an approximate weight of 0.1 g. The taleolae were placed in a test tube, to which 100 mM potassium phosphate buffer, pH 7.5, and 1% (v/v) propanol (P-Buffer) were added as infiltration medium, according to the NR determined: For endogenous NR, 100 mM P-buffer was used, for NO_3^- induced NR 100 mM P-buffer with 50 mM KNO_3, for Mo-induced NR, 100 mM P-buffer was used with 50 mM Mo, and finally for NR induced with NO_3^- + Mo, 100 mM P buffer with KNO_3 and 50 mM Mo was used. Once the plant material was submerged in the infiltration medium, the samples were subjected to pressure for 10 min in the dark at 0.8 bar. The samples were then incubated for 30 min at 30 °C in the dark. After this time, the samples were placed in a water bath at 100°C for 15 min to achieve enzymatic inactivation. Subsequently, 1 mL of the sample aliquot was placed in a test tube, 2 mL of 1% (p/v) sulfanilamide in 1.5 M HCl and 2 mL of 0 N-(Dihydrochloride) were added. 02% (w/v). 1-naphthyl)-ethylenediamine in 0.2 M HCl. Finally, the tubes were shaken and after 20 min of rest the sample was read in a Genesis 10S UV-VIS spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) at an absorbance of 540 nm against a standard curve of NO_2^-. Nitrate Reductase activity "*in vivo*" was expressed in μmol of NO_2 formed per g^{-1} per h^{-1} of fresh weight.

Quantification of photosynthetic pigments

For the quantification of photosynthetic pigments, the methodology described by Wellburn (1994) was followed. From the fresh leaf material, 7 mm taleolae were obtained up to an approximate weight of 0.125 g. Four replicates per treatment were obtained and placed in test tubes. 10 mL of pure methanol (99%) (CH_3COH) was added to each tube and sealed with parafilm tape. They were left to stand in the dark for 24 h. Once this time had elapsed, the samples were shaken and the reading was carried out in a Genesis 10S UV-VIS spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) at three wavelengths: 666, 653 and 470 nm. Photosynthetic pigment concentrations were expressed as mg g^{-1} fresh weight and were calculated using the following formulas:

$$\text{Chl a} = [15.65(A666) - 7.34(A653)]$$
$$\text{Chl b} = [27.05(A653) - 11.21(A666)]$$
$$\text{Carotenoids} = [(1000{*}A470) - 2.86(\text{Chl a}) - 129.2(\text{Chl b})]/\ 221$$

SPAD values

For the quantification of the chlorophyll index, a Minolta SPAD 502 chlorophyll reader (Konica Minolta Sensing, Inc., Osaka, Japan) was used, which measures "*in situ*" without causing damage to the leaf. Reading is achieved by projecting light through a sheet. Five measurements per plant were made at 30 and 40 days after sowing (das). The measurement was made on parts free of ribs. The results were expressed as SPAD units (Shrestha *et al.*, 2012).

Quantification of amino acids and soluble proteins

For the quantification of amino acids and soluble proteins, 0.5 g of fresh plant material were weighed and homogenized in cold 50 mM KH_2PO_4 buffer at pH 7. The sample was centrifuged at 12,000 x *g* for 15 min. The supernatant obtained was used for the determination of total amino acids by the ninhydrin method with slight modifications (Sánchez *et al.*, 2004). Total free amino acids were expressed as mg glycine g^{-1} fresh weight. The soluble protein content was measured with the Bradford reagent (Kruger, 2009) and expressed as mg g^{-1} of fresh weight, using bovine serum albumin as a standard.

Nitrogen Use Efficiency Parameters (NUE)

For the determination of the NUE parameters, the terms and formulas proposed by Moll *et al.* (1982), who define NUE as the grain yield per unit of N available in the soil, were used. Likewise, the processes that accompany the term were taken into account to complement it, N uptake efficiency (NUpE) and N utilization efficiency (NUtE). Additionally, the total nitrogen concentration (TNC) term was taken as the basis. The following formulas were used:

1. TNC. It represented the sum of organic N and the total concentration of NO_3^-.
2. TNA or cumulative total N. It represented the multiplication of the TNC by the total dry weight of leaves.
3. NUtE. Calculated by dividing the total dry weight of the leaf by the TNC.
4. NUpE. Calculated by dividing the TNA by the total dry weight of the root.
5. NUE. Calculated by dividing grams of fresh fruit weight by mM of N applied.

Mineral quantification

The concentration of Zn and Mo minerals were determined by inductively coupled plasma mass spectrophotometer (Agilent Technologies 700 Series ICP-OES, California, USA), following the method described by Karacan and Aslantas (2008). The mineral concentration was expressed as mg kg^{-1} of dry weight. For N, Organic Elemental Analysis (OEA) (Thermo Fisher Scientific™, Waltham, Massachusetts, USA) was used, following the methodology of Krotz and Giazzi (2014), adapted for plant material. Results were expressed as mg g^{-1} d.w.

Statistical analysis

The data obtained were subjected to an analysis of variance and a mean separation test using the Tukey technique using a significance level of α=0.05 ($P \leq 0.05$) through the SAS version 8 software (SAS, 2004).

Results and Discussion

Biomass and yield

The accumulation of biomass is one of the main indicators of the correct functioning of the plants and the effectiveness of the treatments used (Sánchez *et al.*, 2016). In this study, significant differences ($P \leq 0.05$) were found between treatments (Figure 1). The highest biomass accumulation was found in the 6 N + 4 ZnMo treatment, however, as there were no significant differences ($P \leq 0.05$) between the treatments with reductions to 50% and increase to 100% of N in solution, the combination of 3 N + 4 ZnMo could be the most efficient. The results obtained were similar to those reported by Kovacs *et al.* (2015), who observed a slight decrease in dry weight with the addition of Mo. Although some authors indicate that Mo toxicity in crops is rare (Mitra, 2015), in treatments with 0 mM N and 6 mM N a decrease in biomass production was observed, results similar to those obtained by Bambara and Ndakidemi (2010), who explain that it may be caused by an over-accumulation of Mo in legumes. Kanwar *et al.* (2019) indicate that the exposure of plants to nanoparticles can alter the absorption of nutrients and cause variations in growth and development.

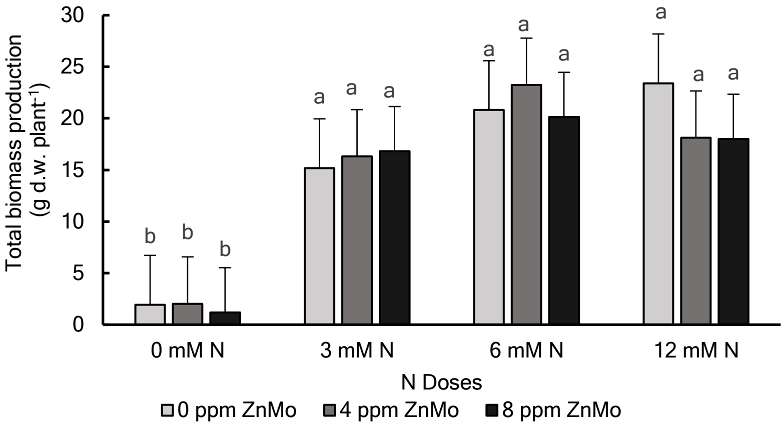

Figure 1. Effect of edaphic application of N (NH_4NO_3) and foliar application of ZnMo nanofertilizer on total biomass production in dry weight in green bean cv. 'Strike'.
*Different letters show statistically significant differences.

For its part, production or yield is an indicator component of the productivity of agrosystems (Martínez and Leyva, 2014). In the present study, the results obtained in production showed significant differences ($P \leq 0.05$) (Figure 2). The treatment that showed the highest fruit production was 3 N + 4 ZnMo with increases of 96% compared to the control without N, 29% in relation to the dose of 6 mM of N and 43% with the dose of 12 mM of N. These results support the theory that the addition of Mo and Zn nanofertilizers can help reduce the N doses applied to crops without compromising production, according to what was obtained by Gad and Kandil (2013), who managed to reduce the dose of N applied in 25% and obtain increases in yields of 39.8% with the addition of Mo in peas. Despite this, Frota *et al.* (2020) do not report significant differences in soybeans, in treatments with and without foliar application of Mo. As indicated by Preetha and Balakrishnan, (2017), Mo and Zn are linked to N cycles, and their metabolism is related, so adequate nutrition of these micronutrients can result in high yields.

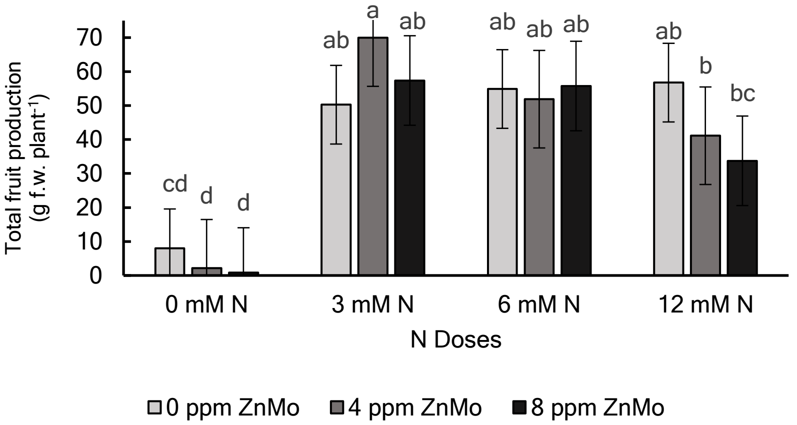

Figure 2. Effect of edaphic application of N (NH_4NO_3) and foliar application of ZnMo nanofertilizer on total fruit production in fresh weight in green bean cv. 'Strike'.
*Different letters show statistically significant differences.

In vivo NR activity

In the process of plant growth, after N uptake, either in the form of nitrate or ammonium, the nitrate is reduced to nitrite by the action of the enzyme NR, located in the cytosol. However, the activity of this enzyme is mainly dependent on the Mo co-factor (MoCo), as well as the presence of the substrate NO_3^- (Imran *et al.*, 2019). In the present work, significant differences ($P \leq 0.05$) were found in the activity of the NR enzyme (Table 2) with respect to the soil N application. The NR activity was stimulated from the treatments with application of 6 mM of N, being the treatments with the highest values 6 N + 4 ZnMo, 12 N + 0 ZnMo, 12 N + 4 ZnMo and 12 N + 8 ZnMo. These results, linked to those obtained in biomass production and yield, may indicate a possible overaccumulation of the nitrate substrate in the treatments under 12 mM N and also, a greater translocation efficiency by those with 3 mM N, in which the treatment with the highest yield and the lowest activation of the enzyme was found. When the treatments were induced with NO_3^-, the 6 N + 4 ZnMo treatment stood out, with increases of 98% compared to the control, 18% to the treatment with 6 mM of N and 472% compared to the treatment with the 12 mM dose of N. This could explain the decrease in biomass and production of treatments with high doses of N, when presenting NO_3^- toxicity, due to the low activity that these treatments presented when induced with this compound. Various studies have described decreases in biomass accumulation when there is an excess of NO_3^- in the plant (Li *et al.*, 2013; Hachiya and Sakakibara., 2017). When inducing the samples with Mo, the activity increased in the treatments with 6 mM and 12 mM of N, presenting the highest value in the 12 N + 0 ZnMo treatment, which agrees with what was explained by authors who highlight the participation of Mo in this first step of N assimilation (Kovács *et al.*, 2015; Hamlin, 2016).

In addition, an increase in NR activity was observed with the addition of Mo in the treatments without application of N, 3 mM of N and 6 mM of N in its three levels of ZnMo. These results were similar to those obtained by Alam *et al.* (2015) who reported a higher NR activity in the leaves and roots treated with Mo compared to the control in the pea crop.

Table 2. Effect of edaphic application of N and foliar application of ZnMo nanofertilizer on nitrate reductase activity in green bean cv. 'Strike'

Treatment	Endogenous	Induced with NO_3	Induced with Mo	Induced with NO_3+Mo
		Leaves		
0N - 0 ZnMo	0.0110 b	0.9619 bcd	0.0053 e	0.9052 cd*
0N - 4 ZnMo	0.0139 b	1.1781 abc	0.0140 de	0.7839 de
0N - 8 ZnMo	0.0072 b	1.3427 ab	0.0145 de	0.9042 cd
3N - 0 ZnMo	0.0140 b	1.7602 ab	0.0130 de	1.6271 ab
3N - 4 ZnMo	0.0092 b	1.6126 ab	0.0200 cde	1.3501 abc
3N - 8 ZnMo	0.0168 b	1.4562 ab	0.0187 cde	1.2342 abcd
6N - 0 ZnMo	0.1828 ab	1.6364 ab	0.1518 bcd	1.1757 bcd
6N - 4 ZnMo	0.2285 a	1.9107 a	0.1428 bcde	1.6773 a
6N - 8 ZnMo	0.1643 ab	1.3625 ab	0.1563 bc	1.0328 cd
12N - 0 ZnMo	0.3334 a	0.4073 cd	0.3628 a	0.4239 e
12N - 4 ZnMo	0.2535 a	0.3338 d	0.2465 ab	0.3251 e
12N - 8 ZnMo	0.3266 a	0.3871 cd	0.2520 ab	0.3384 e

*Different letters show statistically significant differences.

SPAD values

Chlorophyll content is an important variable to examine the physiological state of a plant, since it is the compound responsible for the green pigmentation in the leaves (Callejas *et al.*, 2013). In the present research work, significant differences ($P \leq 0.05$) were found in the chlorophyll indices measured in the treatments according to the application of soil N (Table 3). In both measurements, both the first at 30 das, and the second at 40 das, the highest value was found in the 12 N + 4 ZnMo treatment, which presented increases of 165 % compared to the treatment without application (0 N + 4 ZnMo) at 30 das and 172% at 40 das. A slight upward trend in SPAD values was observed when Mo was added to the treatments, a trend similar to that observed by Vieira *et al.* (2011), who obtained increases in SPAD values when applying foliar treatments with Mo in green beans, and by Wójcik (2020), who observed increases in all the treatments in which Mo was applied compared to its control in apple. The previous results suggest a higher content of chlorophyll in treatments with the addition of Mo, especially in the treatments with the highest rate of N, since this is necessary to maintain the structure of the chlorophyll molecule (Iqbal and Afzal, 2014). Despite the high chlorophyll index reflected in the SPAD values, the outstanding treatments did not present a significant difference compared to the treatments with lower N application rates, which may mean that sufficiency is reached at lower doses and that the foliar application of Mo, further complements this sufficiency (Treder *et al.*, 2016).

Table 3. Effect of edaphic application of N and foliar application of ZnMo nanofertilizer on the chlorophyll index (SPAD values) in green bean cv. 'Strike'

Treatment	SPAD values at 30 das	SPAD values at 40 das
0 N + 0 ZnMo	25.98 e	29.93 c*
0 N + 4 ZnMo	19.20 f	20.27 d
0 N + 8 ZnMo	19.28 f	30.16 c
3 N + 0 ZnMo	37.77 d	36.66 c
3 N + 4 ZnMo	40.55 d	35.88 c
3 N + 8 ZnMo	39.52 d	39.09 bc
6 N + 0 ZnMo	40.75 d	46.39 ab
6 N + 4 ZnMo	45.84 c	46.97 ab
6 N + 8 ZnMo	46.32 bc	46.92 ab
12 N + 0 ZnMo	47.22 abc	51.59 a
12 N + 4 ZnMo	50.90 a	55.21 a
12 N + 8 ZnMo	49.56 ab	54.49 a

*Different letters show statistically significant differences.

Photosynthetic pigments

Photosynthetic pigments are necessary for plants since they fulfill the function of harvesting light and are also reducing compounds (Münzbergová and Haisel, 2019). Regarding the present research work, significant differences were found ($P \leq 0.05$) regarding the soil application of nitrogen (Figure 3). The highest value was obtained in the 12 N + 0 ZnMo treatment, which had increases of 92% compared to the treatment without N, 71% to the treatment with 3 mM of N and 24% to the half dose, 6 mM of N. These results differ from those presented by Bambara and Ndakidemi (2009), who in their research work presented significant increases in the content of chlorophyll in leaves, in bean crops, with respect to their control with the application of Mo in a form foliar in doses of 6 and 12 g per kg of seeds. Newer works could explain the non-significant effect of the adhesion of Mo nanoparticles on the concentration of chlorophyll in leaves, despite the fact that this micronutrient is related to redox reaction processes and water relations through stomatal control. Mushinskiy and Aminova (2019) did not obtain significant differences in potato cultivation when adding Fe and Mo nanoparticles, possibly caused by a filtration by the plant to avoid the effects of nanoparticles. There is still a debate about the effect of nanoparticles on chlorophyll content, due to the contradictions between authors who show an increase and decrease in photosynthetic pigments (Korotkova *et al.*, 2017; Lebedev *et al.*, 2019).

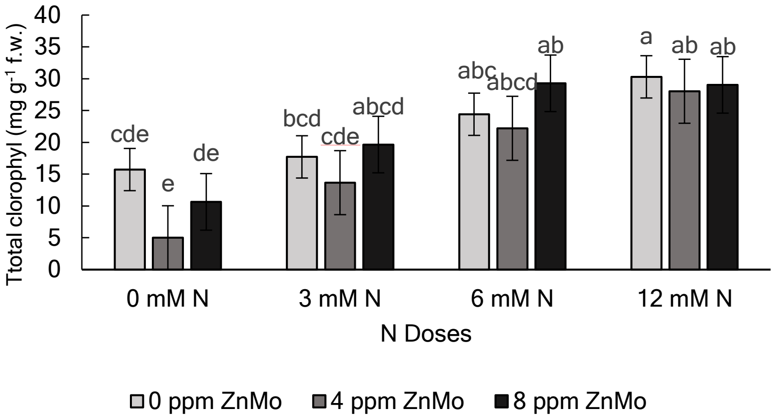

Figure 3. Effect of edaphic application of N (NH_4NO_3) and foliar application of ZnMo nanofertilizer on total chlorophyll content in green bean cv. 'Strike'
*Different letters show statistically significant differences.

Another of the physiological indicators of the photosynthetic process, along with chlorophyll a and b, are carotenoids. The data obtained in this research work indicate that the pigments known as carotenoids showed a significant difference ($P \leq 0.05$) compared to the foliar application of ZnMo (Figure 4). The concentration increased in the 6- and 12-mM N treatments, particularly in the high doses of foliar application. The treatment with the highest value was 12 N + 8 ZnMo, which presented an increase of 1.57 times with respect to the treatment without application of N, 52% with respect to the dose of 3 mM of N and 5.9 % with respect to the dose reduced to half of N. This trend was also reported by Mushinskiy and Aminova (2019) who indicate that after treatment with Cu and Mo, an increase in carotenoids was observed in the potato crop by 45%, which indicates an increased oxidative process or a nonspecific primary reaction to stress. The increase in the content of carotenoids in the treatments with high dose applications of both N and nano ZnMo could indicate the mobilization of the compensatory mechanisms of the plant to maintain normal functions in the assimilation process (Gupta and Gangwar, 2012).

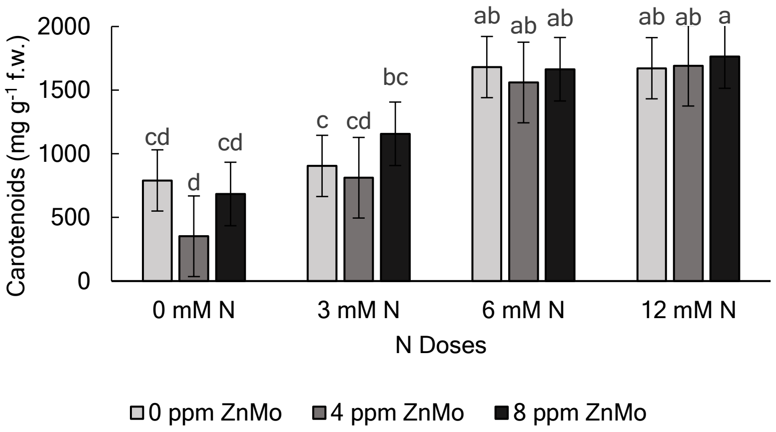

Figure 4. Effect of edaphic application of N (NH_4NO_3) and foliar application of ZnMo nanofertilizer on carotenoids content in green bean cv. 'Strike'
*Different letters show statistically significant differences.

Soluble proteins and amino acids

Amino acids perform numerous metabolic functions in addition to being constituents of proteins, such as control of intracellular pH, generation of metabolic energy or redox potential, and resistance to stress, so measuring the concentration of this biocompound can expose the physiological state of a plant (Pratelli and Pilot, 2014; Hildebrandt, 2015). The results obtained in the present study corresponding to proteins and soluble amino acids showed significant differences ($P \leq 0.05$) (Figure 5). An increase of these biomolecules was found in the treatments with edaphic application of 6 and 12 mM of N, the most notable treatment being the combination of 12 N + 8 ZnMo and the control treatment being the least favored. It is worth mentioning that the foliar addition of Mo was not significant, however, in 7 of the 8 treatments to which it was applied, the concentration of amino acids increased. These results show parity with those obtained by Ide *et al.* (2011), in which the levels of amino acids and other metabolites decreased significantly when there is Mo deficiency. On the other hand, the application of edaphic N presented a similar trend to that of the total chlorophyll content, with the 12 N treatment. and 8 ZnMo as the most favored, with increases of 110% compared to the control without N, 156% compared to the 3 mM dose and 83% compared to the 50% dose of N corresponding to 6 mM. As several authors have described, the assimilation of N ends in the synthesis of amino acids and proteins, so that high doses of N in the plant would mean an increase in the concentration of these biocompounds, in addition to the increase obtained with the addition of Mo and Zn as enzymatic reactions enhancers (Jabeen and Ahmad, 2017).

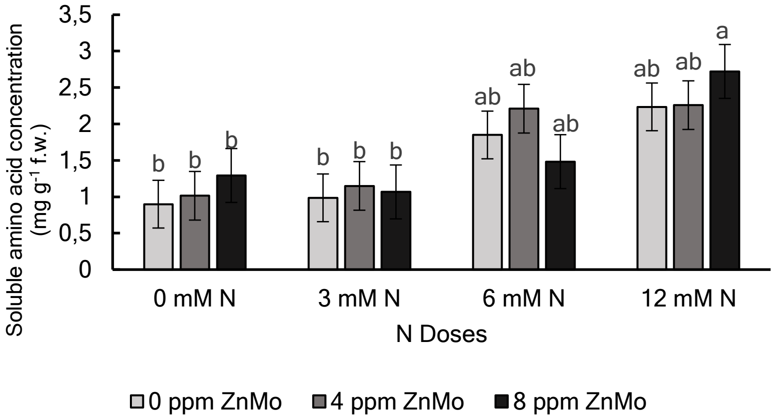

Figure 5. Effect of edaphic application of N (NH_4NO_3) and foliar application of ZnMo nanofertilizer on the concentration of soluble amino acids in green bean cv. 'Strike'

*Different letters show statistically significant differences.

With respect to protein content, the application of nano ZnMo presented significant differences in the treatments ($P \leq 0.05$) as a clear increase in protein concentration was observed with foliar application (Figure 6). Regarding the edaphic application of N, the treatment with 12 N + 8 ZnMo obtained the highest value, with a significant difference compared to the treatment without application of N increased up to 4 times its value and an increase of 31% compared to the dose 4 times lower (3 N and 8 ZnMo) and 16% compared to treatment with the 50% dose of N (6 N + 8 ZnMo). As authors have previously pointed out, adding Mo to the treatments increased the concentration of soluble proteins (Imran *et al.*, 2019; Al-Juthery and Al-Maamouri, 2020). Mo serves as an enhancer of enzymatic activity, along with nitrates for the process of protein synthesis. The rate of this synthesis will be increased if Mo is present, and therefore the absence of Mo reduces the total protein concentration in the plant (Symanowicz and Kalembasa, 2012; Nasar *et al.*, 2018).

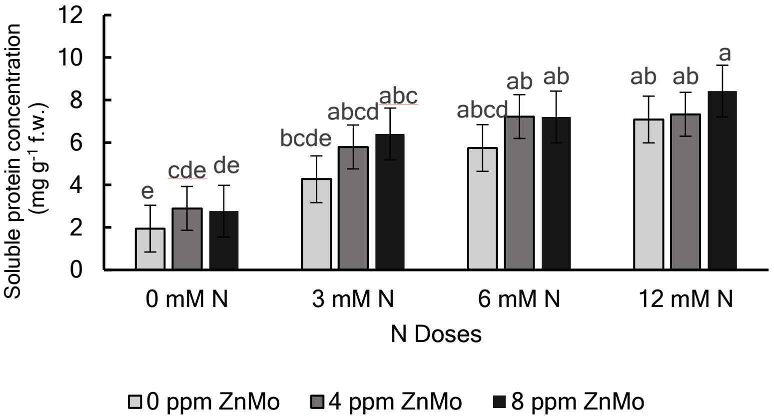

Figure 6. Effect of edaphic application of N (NH_4NO_3) and foliar application of ZnMo nanofertilizer on the concentration of soluble proteins in green bean cv. 'Strike'

*Different letters show statistically significant differences.

Organic N content

The final result of the N assimilation process in plants is organic N, amino acids and proteins (Moran-Zuloaga *et al.*, 2015). The results of this study showed significant differences ($P \leq 0.05$) when an increase in this element was observed with the dose of N applied (Table 4). The highest concentrations in the leaf were found in the treatments with 12 Mm of N and the treatment with 6 N + 8 ZnMo, as well as, the minimum concentrations were found in the treatments with 0 mM of N with 8 ZnMo + 3 N without foliar application (Table 4). In root, the highest concentration was presented in the treatment with 12 e N + 8 ZnMo, while the minimum concentration was obtained again by the 0 N + 8 ZnMo treatment. This behavior was similar for the fruit and stem organs, where the maximum concentrations were found in the highest doses of N application. In summary, it should be noted that, in general, the highest concentrations of organic N were found in leaves and fruits. Previously, an overaccumulation in the production of amino acids, proteins and organic N has been described in plants treated with high doses of N (Borgognone *et al.*, 2013; Horchani *et al.*, 2011), as well as a decrease in growth due to the toxicity caused by the accumulation of nitrate anion in the leaf tissue. The findings are consistent with the data obtained in leaves, the values that stood out were found in the treatments with the highest N dose, however, this was not reflected in biomass production and yield, since this group of treatments presented decreases, possibly toxicity consequences. The 3 N + 4 ZnMo treatment, despite the fact that it was not favored in the accumulation of organic N, was the treatment that stood out the most in terms of yield and that, according to Luque (2012), may be due to the fact that the photoassimilates were translocated from the leaves to the fruits.

Table 4. Effect of edaphic application of N and foliar application of ZnMo nanofertilizer on organic nitrogen content in green bean cv. 'Strike'

Treatment	Leaf	Root	Fruit	Stem
Organic nitrogen content (mg g^{-1} d.w.)				
0N - 0 ZnMo	2.67 b	2.41 cd	0.73 c	1.69 d*
0N - 4 ZnMo	2.13 bc	2.58 bcd	1.89 bc	1.78 d
0N - 8 ZnMo	0.80 c	1.90 d	**	1.68 d
3N - 0 ZnMo	0.88 c	2.72 abcd	3.64 ab	1.69 d
3N - 4 ZnMo	1.02 c	2.80 abcd	3.55 ab	1.77 d
3N - 8 ZnMo	1.44 bc	2.97 abcd	3.31 ab	1.72 d
6N - 0 ZnMo	1.05 bc	3.74 ab	4.68 a	2.48 cd
6N - 4 ZnMo	1.55 bc	3.69 ab	4.43 ab	2.83 bc
6N - 8 ZnMo	4.35 a	3.95 a	4.31 ab	2.58 cd
12N - 0 ZnMo	5.16 a	3.63 ab	5.31 a	4.39 a
12N - 4 ZnMo	4.97 a	3.50 abc	4.81 a	3.80 ab
12N - 8 ZnMo	5.34 a	3.23 abc	5.58 a	4.30 a

*Different letters show statistically significant differences.

**It was not possible to determine the concentration due to lack of plant material.

NUE parameters

The NUE can be defined as the production of plant biomass per unit of available N. The indices that complement this term are N absorption efficiency (NUpE) and N utilization efficiency (NUtE) (Kant *et al.*, 2011; Rosales *et al.*, 2020). In the present study, significant differences ($P \leq 0.05$) were found between treatments (Table 5). Regarding the total N content (TNC), the highest values were recorded in the 12 N + 0 ZnMo treatments, with an increase of 204.3% compared to the control treatment, an increase of 112.4% compared to 3 N + 0 ZnMo and an increase 59% relative to 6 N + 0 ZnMo; followed by the 12 N + 8 ZnMo treatment, with increases of 491% with respect to 0 N + 8 ZnMo, 95.3% increase with respect to 3 N + 0 ZnMo and 21.4% increase with respect to 6 N + 8 ZnMo. These results agree with what was found by Liu *et al.* (2017) in strawberry crops, who reported a significant increase in the N content in leaves compared to controls without spraying after application of Mo in foliar form at doses of 67.5 and 135 g Mo ha^{-1}. On the other hand, Silva *et al.* (2017) reported that the addition of Mo in green bean cultivars was not significant until the seeds were inoculated with N-fixing bacteria, so the application of Mo did not show significant increases. The treatments that presented the lowest content were 0 N + 8 ZnMo and the control.

To complement the meaning of NUE, the second parameter that was analyzed was that of total accumulated N (TNA), which contemplates not only the amount of N in the plant, but also its distribution and capacity to produce biomass. According to the results of this experiment, significant differences ($P \leq 0.05$) were found between treatments in the TNA with respect to the doses of edaphic N applied, with the most favored treatment being 12 N + 0 ZnMo, with increases of 158.3% with respect to the 3 N + 8 ZnMo treatment, the 25% N dose and an increase of 21.2% compared to the treatment with 50% N dose (6 N + 8 ZnMo). It is worth mentioning that both in the treatments without application of N, and in the treatments with 12 mM of edaphic N, there was a decrease in TNA when the foliar nano ZnMo was applied. This may be due to the slow release of the nanoparticles that caused Mo and Zn toxicity, a condition that, despite being uncommon, may include mild symptoms such as yellow pigmentation in leaves or reduced growth according to the results reported by Bittner (2014).

The N utilization efficiency (NUtE) parameter showed that the most favored treatments were 6 N + 4 ZnMo, 6 N + 0 ZnMo and 3 N + 0 ZnMo. These results agree with the biomass and yield results, in which one of the treatments with the highest production was 3 N + 4 ZnMo, to which a dose of N was applied 4 times lower than the treatments with the highest TNC indices and TNA, however, this decrease in the N rate application did not affect the production of pods, but on the contrary, it promoted a greater obtaining of these.

In addition, these results support what was reported by Shivay *et al.* (2015), who indicated that the Zn application made its absorption more efficient.

The N absorption efficiency (NUpE) data showed a higher absorption capacity when the plants were found in high doses of N, and as previous works indicate, the application of micronutrients in foliar form did cause an increase in this index, when N was present in the solution (Sánchez, 2006). An increase of 24.6% was obtained between the treatment 3 N + 8 ZnMo, of 63.6% between the treatment with 6 mM of N without application and with foliar application of nano ZnMo at 8 ppm and of 26.8% between the treatment with 12 mM of N without application and with foliar application of nano ZnMo at 8 ppm (Brown and Bassil., 2011; Wurzburger *et al.*, 2012; Ding *et al.*, 2018). Finally, the values obtained regarding the ratio coefficient of sheath production in grams per mM of N applied (Figure 7), showed the most efficient treatment as 3 N + 4 ZnMo, with increases of almost 10 times compared to the control without application of N (0 N + 4 ZnMo), 170% with respect to the treatment with a double dose of N (6 N + 4 ZnMo) and 582.1% with respect to the treatment with a 4 times greater dose of N (12 N + 4 ZnMo). The results obtained agree with what was found by Zewail *et al.*, (2021), who indicate that the application of micronutrients in the form of nanofertilizers can make their role in plant growth more efficient. Their results report an increase in growth and development parameters in stevia plants such as plant height, number of leaves and stem fresh and dry weight, after the application of a nanoformulation of microelements (Bo, Zn and Mo).

Table 5. Effect of edaphic application of N and foliar application of ZnMo nanofertilizer on parameters of N use efficiency (NUE), total N content (TNC), total accumulated N (TNA), efficiency in N utilization (NUtE) and efficiency in N uptake (NUpE) in green bean cv. Strike. TNC was expressed as mg g^{-1} d.w.; TNA was expressed in mg N; NUpE was expressed in mg N g^{-1} d.w.; NUtE was expressed in g^2 p.s. mg^{-1} N

Treatment	TNC	TNA	NUtE	NUpE	NUE
0N + 0 ZnMo	62.4 ef	47.4 e	0.012 de	66.9 fg	8.0 cd*
0N + 4 ZnMo	79.3 def	80.5 e	0.009 e	115.0 efg	2.1 c
0N + 8 ZnMo	31.2 f	17.6 e	0.017 cde	38.7 g	0.9 d
3N + 0 ZnMo	89.4 de	507.3 de	0.067 ab	211.9 defg	16.8 ab
3N + 4 ZnMo	91.4 de	517.8 de	0.061 ab	251.5 defg	23.3 a
3N + 8 ZnMo	94.4 de	549.7 cde	0.063 ab	273.6 def	19.1 ab
6N + 0 ZnMo	119.4 cd	945.9 bcd	0.067 ab	333.6 cde	9.1 ab
6N + 4 ZnMo	125.0 bcd	1179.0 ab	0.076 a	395.3 bcd	8.6 ab
6N + 8 ZnMo	151.9 abc	1171.7 abc	0.050 ab	545.3 abc	9.3 ab
12N + 0 ZnMo	184.9 a	1745.8 a	0.051 ab	596.1 bc	4.7 ab
12N + 4 ZnMo	170.8 ab	1178.3 ab	0.040 bcd	621.3 a	3.4 b
12N + 8 ZnMo	184.4 a	1420.2 ab	0.042 bc	712.3 a	2.8 bc

* Different letters show statistically significant differences.

Leaf content of Mo and Zn

Mo, like other micronutrients, plays an important role in plant development. Its role as a catalytically active metal during enzymatic catalysis classifies it as an essential micronutrient, since around 50 enzymes have been recognized that contain this element in the enzymatic systems of plants (Alloway, 2013; Rana *et al.*, 2020). In the present study, significant differences ($P \leq 0.05$) were found between treatments (Table 6), with the 12 N + 8 ZnMo, 0 N + 4 ZnMo and 3 N + 8 ZnMo treatments showing a greater accumulation of this micronutrient. The highest treatment was 12 N + 8 ZnMo, with increases of 25.3% with respect to the treatment with 3 mM of N and 42.3% with respect to the treatment with 6 mM of N, with no significant difference between them. Being the second most favored treatment and not presenting a significant difference with the first, the 0 N + 4 ZnMo treatment postulates the foliar dose of ZnMo of 4 ppm as the most efficient, because it allows an accumulation comparable to that obtained by doubling the dose, thus reducing the application to 50%. Various authors indicate that Mo nanocomposites could present improvements in development by having faster absorption and better interaction with the structure of the roots and bacterial symbiosis, however, high doses of Mo nanocomposites have shown toxicity, root necrosis and decrease in growth in chickpea cultivation (Azizi *et al.*, 2017; Thomas *et al.*, 2017; Yang *et al.*, 2020). In the present study, decreases in biomass accumulation (Figure 1) and production (Figure 2) were observed in treatments with high doses of foliar nano ZnMo, presenting similar results to those reported by Adhikari *et al.* (2013) in rice, and that explains, this decrease may be related to the ability of nanomaterials to cross the cell membrane due to their size. Another explanation for the high concentrations of Mo in the 0 N + 4 ZnMo treatment is the absence of N. In this treatment, Mo presented a greater accumulation because it was not used in the metabolism of N, which is evidence of the role of Mo in the N cycle (Tokasheva *et al.*, 2021).

Table 6. Effect of edaphic application of N and foliar application of ZnMo nanofertilizer on foliar Mo and Zn content in green bean cv. 'Strike'

Treatment	Foliar Zn (ppm)	Foliar Mo (ppm)
0N + 0 ZnMo	25.25 b	54.86 e*
0N + 4 ZnMo	365.00 a	360.47 ab
0N + 8 ZnMo	365.50 a	**
3N + 0 ZnMo	12.50 b	14.20 e
3N + 4 ZnMo	366.25 a	250.25 bcd
3N + 8 ZnMo	366.50 a	324.47 abc
6N + 0 ZnMo	52.50 b	9.00 e
6N + 4 ZnMo	363.75 a	139.72 de
6N + 8 ZnMo	368.75 a	299.17 abcd
12N + 0 ZnMo	41.25 b	6.72 e
12N + 4 ZnMo	360 a	158.25 cde
12N + 8 ZnMo	368.75 a	422.29 a

Data are means ± se. Different letters show statistically significant differences.

**It was not possible to determine the concentration due to lack of plant material.

Another of the so-called essential micronutrients for plants is Zn. The importance of the presence of Zn in plants ranges from the germination and development of seeds and generative tissues, to the maintenance of the integrity of biomembranes and energy processes (Sturikova *et al.*, 2018). In the present investigation, significant differences ($P \leq 0.05$) were obtained with respect to the application of nano ZnMo via foliar (Table 6). The results showed the treatments 12 N + 8 ZnMo and 6 N + 8 ZnMo as the treatments with the highest foliar Zn concentration value, however, no significant difference was found against the treatments 0 N + 4 ZnMo, 0 N + 8 ZnMo, 3 N + 4 ZnMo, 3 N + 8 ZnMo and 12 N + 4 ZnMo, so these results reinforce what was shown above and postulate the 4 ppm dose of nano ZnMo as the most viable due to the reduction of inputs without compromising the final concentration of the micronutrient in the plants. The results obtained are similar to those reported by Mahdieh *et al.* (2018), who found that vegetative growth parameters such as plant height, length of internodes and root, as well as the concentration of Zn in seeds, were favored with the foliar application of Zn nanoparticles.

Conclusions

The treatment with the combination of 3 mM of edaphic N + 4 ppm of nano ZnMo presented the highest values in terms of biomass accumulation, as well as the highest production values among all the treatments evaluated. On the other hand, the treatments fertilized with 12 mM of N showed the highest values regarding NR enzyme activity, chlorophyll index, pigment content and amino acid and soluble protein content where the foliar application at 8 ppm of ZnMo favored the following parameters. Regarding the accumulation of N and the NUE parameters, the treatment with the combination of 12 mM of edaphic N and 8 ppm of nano ZnMo obtained the highest values in terms of the presence of N in the tissues, however, the fertilized treatments with 3 mM of N tied with the treatments fertilized with 6 mM of N in the N utilization coefficient and the highest NUE coefficient was found in the treatment of 3 mM of edaphic N + 4 ppm of nano ZnMo, for which it is concluded that it was the combination that makes the N use more efficient, and that the foliar application of nano ZnMo at a dose of 4 ppm was the most favorable and efficient in the parameters evaluated. The application of Mo and Zn nanofertilizers in foliar form, at low doses, combined with the application of edaphic N at low doses, increases the efficiency of nitrogen use, by reducing the applied doses and obtaining a high biomass production and yield in snap bean plants cv. Strike. It is worth mentioning that more research is needed to explore the full scope of nanofertilizers and their impact on crop development and specifically on their interaction with biomolecules.

Authors' Contributions

E.S. and C.A.R.-E. designed the study. C.C.-M. and A.P.-M. analyzed the data. E.S. and C.A.R.-E. prepared the manuscript, while E.M.-M., M.A.F.-C. and K.H.-F. conducted the experiments. E.S., C.A.R.-E. and A.P.-M. organized the data and performed the statistical analysis. All authors read and approved the final manuscript

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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