University of São Paulo
"Luiz de Queiroz" College of Agriculture

Development, characterization and efficiency of nitrogen fertilizer associated with molybdenum nanoparticles

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Thesis presented to obtain the degree of Doctor in Science. Area: Soil and Plant Nutrition

Piracicaba
2022

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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DEDICATION

To my family, José Leonardo Moreira, Maria Margarida Moreira and Vívian do Rosário Moreira for their support and trust, my trues examples of dedication and character.

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"I am among those who think that science has great beauty.
A scientist in his laboratory is not only a technician:
he is also a child placed before natural phenomena
which impress him like a fairy tale."
Marie Curie

CONTENTS

RESUMO

Desenvolvimento, caracterização e eficiência de fertilizante nitrogenado associado a nanopartícula de molibdênio

A eficiência do uso do nitrogênio (N) está associada diretamente a agricultura sustentável. O uso de fontes renováveis de energia e o aumento de produtividade demandam cada vez mais a utilização de fertilizantes e estratégias para melhorar o uso dos fertilizantes. Dentre as estratégias para aumentar a eficiência dos fertilizantes nitrogenados temos o uso de inibidores de urease, inibidores de nitrificação, adição de micronutrientes, polímeros, dentre outros. Neste sentido a adição de molibdênio (Mo) é uma alternativa promissora devido seu potencial de influenciar o metabolismo e oaproveitamento do N pelas plantas, podendo ser aplicado na forma de fontes solúveis (molibdato de amônio) ou nanopartículas (trióxido de Mo). Foram conduzidos alguns estudos para avaliar a interação entre fontes de Mo, ureia e N-(n-butil) tiofosfórico triamida (NBPT) nas características físico-quimicas, volatilização de amônia (NH_3), recuperação do ^{15}N pela cana-de-açúcar e metabolismo e absorção de N pelo milho cultivado em solução nutritiva. As fontes de Mo foram granuladas com a urea, enquanto o NBPT foi aplicado como recobrimento. Após a caracterização dos fertilizantes verificou-se que o Mo reage com o NBPT, eliminando seu efeito como inibidor de urease. As perdas dessa mistura foram próximas a 44 % do N aplicado, enquanto apenas adição de NBPT à ureia resultou em perda de 33 % de N-NH_3. Em seguida foi selecionado dentre as misturas de ureia e Mo as que apresentaram as melhores características físico-químicas, utilizando-se a dose média de 600 g de Mo por 100 kg^{-1} de N, como a adequada para prosseguir com as avaliações do fertilizante em mudas de cana-de-açúcar. Neste momento foi avaliado a recuperação do ^{15}N-ureia e observou-se que não houve alteração em função da fonte e adição de Mo, com média de 80 % na planta. A produção de biomassa e o conteúdo de N também não foram influenciados, sendo respectivamente 408 g $vaso^{-1}$ e 3,8 g $vaso^{-1}$. Com o objetivo de avaliar as mudanças metabólicas e a preferência de absorção das formas de N em função da adição de Mo foi conduzido experimento em condições controladas com a cultura do milho. Os tratamentos consistiram da omissão de Mo e N e do suprimento destes nutrientes 28 dias após o transplante das mudas para solução nutritiva. Verificou-se que o Mo adicionado na solução nutritiva resultou na mesma produtividade do tratamento com N e com Mo, aproximadamente 215 g $vaso^{-1}$. Nesta condição observou-se também que na ausência de Mo e suprimento de N as plantas absorveram maior quantidade de amônio, comprovando que o Mo favorece absorção de nitrato. Portanto, a adição de Mo à ureia é viável como estratégia de distribuição uniforme do nutriente nas lavouras, porém sem afetar o metabolismo ou a recuperação do N pela planta.

Palavras-chave: Molibdato, Nitrato, *Zea mays*, *Saccharum* spp

ABSTRACT

Development, characterization and efficiency of nitrogen fertilizer associated with molybdenum nanoparticles

Nitrogen (N) use efficiency is directly associated with sustainable agriculture. The use of renewable energy sources and increase in productivity have increased the amountof fertilizers used and strategies to improve their use are required. Among the strategies to increase the efficiency of N fertilizers are the use of urease inhibitors, nitrification inhibitors, addition of micronutrients, polymers, among others. Therefore, the molybdenum (Mo) addition is a promising alternative due to its potential to influence N metabolism in plants and its utilization, and Mo can be supplied as soluble sources (ammonium molybdate) or as nanoparticles (Mo trioxide). Studies were conducted to evaluate the interaction between Mo sources, urea and N-(n-butyl) thiophosphoric triamide (NBPT) on physical-chemical characteristics, ammonia (NH_3) volatilization, recovery of ^{15}N by sugarcane and metabolism and N uptake by corn in nutrient solution. Mo sources were granulated with urea, whereas NBPT was treated as a cover. After fertilizers characterization it was found that Mo reacts with NBPT, eliminating its effect as a urease inhibitor. The N-NH_3 losses of urea + NBPT were close to 44 % of the N applied, while only urea + NBPT reached 33 %.. Then, the fertilizer with best physical-chemical characteristics were selected among the mixtures of urea and Mo, using the average rate 600 g of Mo per 100 kg^{-1} of N, as the appropriate one to proceed with the evaluations that consisted of applying the fertilizer to sugarcane seedlings. At this point, the recovery of ^{15}N-urea by sugarcane was evaluated and it was observed that there was no change as a function of the source and addition of Mo, with an overal recovey of 80 % by the plant. The biomass production and N content were also not influenced, presenting the average of 408 g pot^{-1} and 3.8 g pot^{-1}. In order to evaluate the metabolic changes and the uptake preference of N forms as a function of Mo addition, an experiment was conducted with corn under controlled conditions. The treatments consisted of the omission of Mo and N and the supply of these nutrients 28 days after transplanting the seedlings into a nutrient solution. It was verified that the Mo added to the nutrient solution after 28 days resulted in similar productivity as the treatments with N and Mo, approximately 215 g pot^{-1}. In this condition it was also observed that in the absence of Mo and supply of N the plants absorbed a greater amount of ammonium, proving that Mo favors nitrate absorption. The finding is that the addition of Mo to urea is a feasible strategy of uniform distribution of the nutrient in crops, but with no potential in affecting the metabolsim of the recovery of N by the plant.

Keywords: Molybdate, Nitrate, *Zea mays*, *Saccharum* spp

1. GENERAL INTRODUCTION

In recent years the development of agriculture have increased yields and the use of fertilizers. Thus, nutrients that were less used before became to receive attention, because in many cases productivity was limited by them, such as boron, zinc, copper and molybdenum (Mellis et al., 2017). In other words, increasingly fine-tuning of the system became necessary to achieve the potential productive of the crops.

Molybdenum (Mo) is required in leaf tissues at contents close to 1 mg kg^{-1} (Polidoro et al., 2006), and in soil its quantification in routine soil analysis is not performed due to methodological difficulties. Micronutrients are often responsible for limiting the productivity of crops, such as Mo, because it is directly related to the metabolism of nitrogen (N) and, consequently, to its utilization by the plant. The Mo omission reduces the activity of nitrate reductase, urease, nitrate reductase, and detoxification of sulfides; besides participating in the process of biological N fixation (Sun et al., 2009; Xiong et al., 2001; De Carvalho et al., 2011; Wu et al., 2014).

The productive gain by molybdenum supply is evaluated in crops that take up nitrogen expressively due to its participation in the nitrogenase present in the nodules (Philippi et al., 2021). The gains for non-nitrogen-fixing crops, such as sugarcane and corn, are questionable due to the limited number of studies in the literature (Thapa et al., 2016; Mellis et al., 2017; Santos et al., 2018). Several factors contribute to this, among them the difficulty to quantifying the element in leaf tissues due to its low concentration, the variable responses to the application of Mo, and the difficulty in distributing small amounts of Mo in the field. But it is recognized that in the presence of Mo, most plants reduce the accumulation of free nitrate in the cells, in other words, more N is assimilated.

The interaction between Mo and N is reported in the literature as a better utilization of N in plant tissue. The N efficiency utilization by crops in the suply or omission of Mo is often not supported, emphasizing only the enzymatic activities of nitrate reductase. However, Mo can also influence the uptake of N forms, modifying the proportion of nitrate and ammonium in the plant. This condition is possible because the uptake of nitrate is by negative feedback control, i.e., if there is less free nitrate in the cells, the uptake of nitrate is higher.

Molybenum is a nutrient not commonly used by most growers in Brazil, due to the lack of official recommendation to most crops and the dificulty of applying small amounts of Mo homogeneously to the field. However, more recently, most official bulletins recommend Mo adition to most crops, but the large-scale adoption of Mo fertilization requires feasible strategis to

supply it to the crops. In this study, we propose the production of urea enriched with Mo sources, trough granulation or as a coating, in order to facilitate the Mo distribution in the field, supply of Mo to crops and improvement in the N use efficiency by crops. The hypothesis is that adding Mo to urea will not reduce the physical-chemical quality of the urea while increasing nitrate uptake and plant productivity. We aimed (i) to characterize laboratory produced urea enriched with Mo sources, treated or not with NBPT, to support future studies on formulation and its impact on fertilizer efficiency; (ii) to evaluate the potential of reducing NH_3 losses from urea treated with nanoparticulate molybdenum trioxide and ammonium molybdate, coated or incorporated into the urea granule; associated or not NBPT; (iii) to evaluate the utilization of N-urea with Mo coated and incorporated into the granule by sugarcane; (iv) to assess modifications in the N metabolism in maize plants, to provide a better understanding between the relationship of Mo supply and N metabolism, which may contribute to improve and optimize agricultural products.

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2. ADDITION OF MOLYBDENUM TO UREA CHANGES CHARACTERISTICS AND REACTS WITH N-(N-BUTYL) THIOPHOSPHORIC TRIAMIDE

Abstract

Development or blending of new products in order to improve the efficiency of already commercialized fertilizers is a constant practice. New formulations have different physicochemical characteristics from the raw materials. These characteristics directly influence the quality of the fertilizer because they can alter the storage and application requirements, which can influence the use of the new fertilizer. Our hypothesis is that Mo can be added to urea, but at a maximum dose of 600 g per 100 kg of N regardless of the source of urea used. In view of this, this study aimed to characterize the addition of molybdenum (Mo) sources and rates to urea and n-(n-butyl) thiophosphoric triamide (NBPT). Thirty fertilizers were formulated combining three factors: three types of urea (urea, urea+NBPT coated and urea+NBPT incorporated), two sources of Mo (ammonium molybdate and molybdenum trioxide) and 5 rates of Mo (0, 150, 300, 600 and 1200 g of Mo per 100 kg of N). Properties such as nutrient content in the granules, salinity index, hardness, hygroscopicity and angle of repose were evaluated. Our results showed that the addition of ammonium molybdate changes the characteristics of the granules less, especially at rates lower than 600 g Mo per 100 kg of N.

Keywords: Nitrogen; Nanoparticles; Hygroscopicity; Salinity; Fertilizers.

2.1. Introduction

Agriculture has grown and developed intensively in the last 80 years, part of this development is attributed to nitrogen fertilizers. Currently, the demand of N in the world is 118,763 thousand tonnes (FAO, 2020) and it is possible to supply this nutrient to plants through numerous sources. The variability of sources has led to numerous investigations to evaluate the efficiency of nitrogen sources, and the difference between materials (Mariano et al., 2015; Barth et al., 2020). The variability of sources and the need to increase the efficiency of fertilizers has required the industry to improve them (Trenkel, 2010), resulting in the development of various formulations and characteristics (Guelfi, 2017).

In Brazil today the most used and studied fertilizer is urea (IFA, 20018; ANDA 2021). The reason is because it is the cheapest source of N, but it is also the source with the greatest losses, mainly from volatilization (Trenkel, 2010; Silva et al. 2017). Given this, a variability of products has been studied, such as urea with the addition of inhibitors (Mira et al., 2017), polymer coating (Xu et al., 2013), the addition of micronutrients (Cancellier et al., 2016; Rech et al., 2017), among other methods (Kasim et al. 2009). The best results have been obtained for urea with NBPT, the product responsible for urease inhibition (Pan et al., 2016). However, the addition of Mo is an innovative strategy, not reported so far.

Mo is directly related to numerous physiological and biochemical processes in plants, and also acts directly on the assimilation of N, since it is a cofactor of nitrate reductase. It can also be responsible for the greater absorption of this nutrient by the plant. In addition, it was found that this transition metal has the potential to inhibit the activity of the urease enzyme (Tabatabai, 1977), but we don't know how this process occurred, i.e., what is the interaction between Mo and the enzyme, how does this interaction happen, and in which part of the enzymatic domain. Thus, the interaction of the products could have a double action for the plant, first to reduce the loss of N and increase the availability of N to be absorbed and second to increase the absorption by the best N metabolism.

In this context, the development of a new fertilizer combining the two strategies may be a measure to increase the efficiency of nitrogen fertilization and improve the fertilization process. However, it is known that product mixtures alter the chemical and physical properties of fertilizers, often reduce the critical moisture content and become impractical. However, these characteristics are often ignored in research, and only how the plants respond to the formulated mixtures is evaluated.

The uncertainties about the interaction between urea, NBPT, and molybdenum sources led to the search for answers about it and how these would reflect on the physicochemical characteristics of fertilizers. Therefore, our hypothesis is that Mo can be added to urea, but at a maximum dose of 600 g per 100 kg of N regardless of the source of ureua used. This study aims to characterize laboratory produced urea treated with NBPT and Mo sources to support future studies on formulation and its impact on fertilizer efficiency.

2.2. Materials and Methods

2.2.1. Experimental site and design

The study was performed in the Laboratory of Stable Isotopes at the Center for Nuclear Energy in Agriculture under controlled conditions. The 3x2x5 factorial experimental was evaluated an entirely randomized design with 3 repetitions. The factors studied where (i) three types of urea: common urea, urea with NBPT coated and urea with NBPT incorporated (granulated) into the granules; (ii) two sources of Mo: ammonium molybdate (soluble) and nanoparticulate molybdenum trioxide (low solubility); (iii) and five rates of Mo: 0, 150, 300, 600 and 1200 g of Mo per 100 kg^{-1} of N (Figure 1, Table 1). All sources used were p.a. reagents.

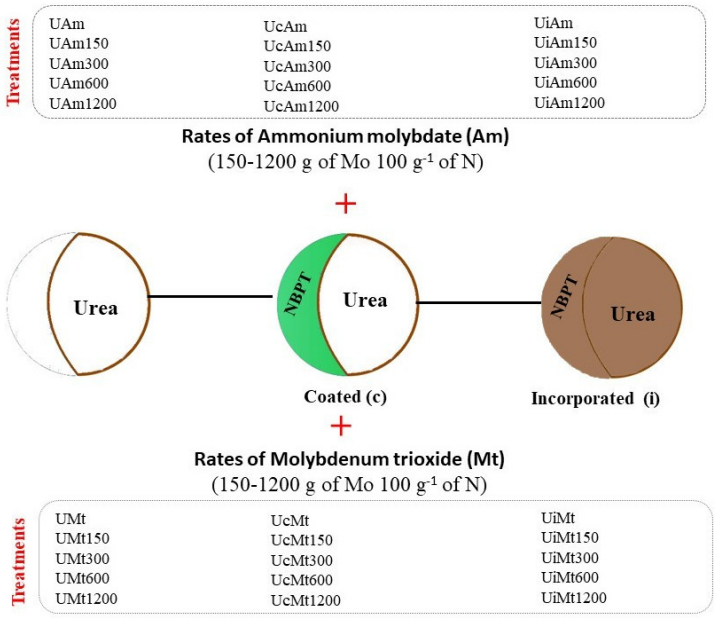

Figure 1. Fertilizers formulated with urea incorporated (Ui) and coated (Uc) with Mo sources (ammonium molybdate, Am; and molybdenum trioxide, Mt) and rates (0; 150; 300; 600; and 1200 g of Mo per 100 kg of N).

Table 1. Chemical properties of N fertilizers formulated with treated-urea (U, urea; Uc, coated; Ui, incorporated), and sources (Am, ammonium molybdate; and Mt, molybdenum trioxide) and rates of molybdenum (Mo, 0; 150; 300; 600; 1200 g of Mo per 100 kg of N)

Urea types	Mo rates	N (%)	Mo*(%)	Treat	N (%)	Mo*(%)	Treat
		Ammonium molybdate			Molybdenum trioxide		
Urea (U)	0	45.5	0.0	UAm	45.2	0.0	UMt
	150	44.9	96.3	UAm150	44.5	95.9	UMt150
	300	44.1	95.3	UAm300	44.3	95.0	UMt300
	600	44.2	100.0	UAm600	45.1	100.0	UMt600
	1200	44.5	100.0	UAm1200	44.7	96.4	UMt1200
Urea with NBPT coated (Uc)	0	46.0	0.0	UcAm	45.9	0.00	UcMt
	150	44.6	100.0	UcAm150	45.0	100.0	UcMt150
	300	44.7	100.0	UcAm300	44.6	100.0	UcMt300
	600	44.6	100.7	UcAm600	47.7	97.2	UcMt600
	1200	44.5	100.8	UcAm1200	44.1	100.0	UcMt1200
Urea with NBPT Incorporated (Ui)	0	44.4	0.0	UiAm	45.0	0.0	UiMt
	150	44.4	95.5	UiAm150	45.0	95.2	UiMt150
	300	44.4	101.3	UiAm300	44.0	101.4	UiMt300
	600	44.9	95.8	UiAm600	44.4	101.9	UiMt600
	1200	44.2	95.2	UiAm1200	44.3	101.5	UiMt1200

*percentage of Mo recovered in relation to that expected in the determination by ICP-OES. Molybdenum trioxide as nanoparticulate. Treat, treatments. Ammonium molybdate and Molybdenum trioxide present 81.0 and 99.5 % of MoO_3, respectively.

Mo was added using the same process as NBPT, i.e. for the first two types of urea Mo was coated and in the third type, it was incorporated into the granule during the granulation process. In the case of Mo incorporated two strategies were adopted to add Mo sources.

Ammonium molybdate was added in urea solution, later dried and granulated, while for Mo trioxide was added in powder form due to low solubility. To maintain uniformity, the trioxide was homogenized to urea maintaining the mass ratio (Suppl. Figure 1A). In other words, mixing was accomplished by progressive dilution of canstant mass.

The molybdenum was coated to the granule using the adherent Pearl Forti product. When the urea was also treated with Mo, it was applied after NBPT application (Suppl. Figure 1B, 1C, 1D). The NBPT used was provided by KochTM Agronomic Services, as a solution containing 135 mg NBPT kg^{-1}. The rate used for the coating of urea was 530 mg of NBPT per kg of urea, following commercial products.

2.2.2. Measurements

The first step was checking the size of the nanoparticles, measured using the dynamic light scattering technique according to Hackley and Clogston (2011). For the measurements, an aliquot of 1 mL of molybdenum trioxide suspension was transferred to a seven polystyrene cuvette and directly loaded into the equipment. The granules presented an average particle size of 64.10 nanometers, being therefore considered nano fertilizers (Figure 2).

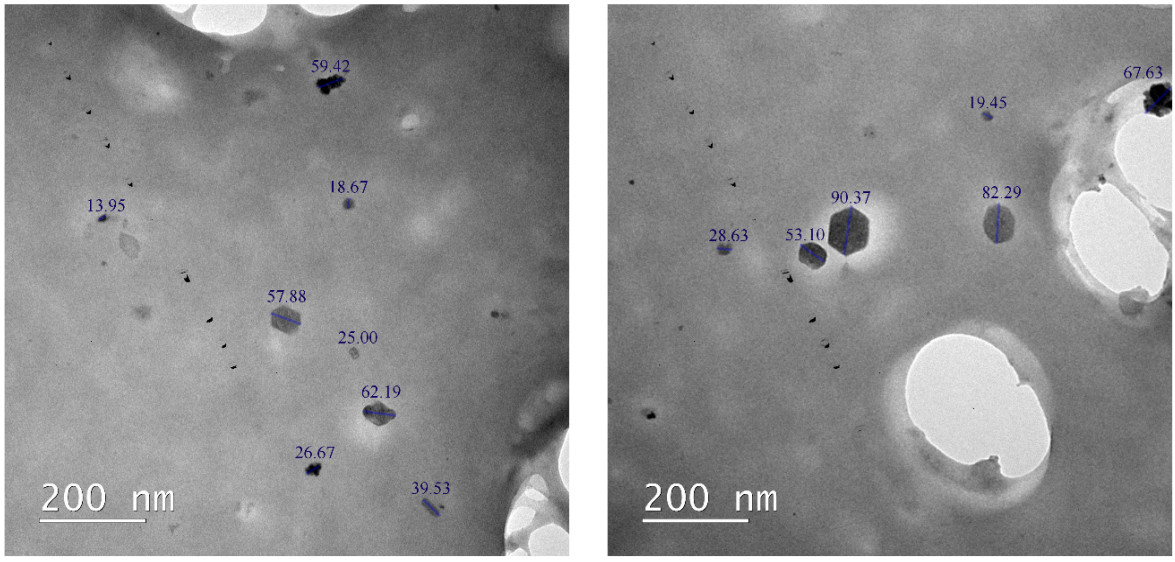

Figure 2. Electron microscope images of molybdenum trioxide for material characterization.

The total N content of the fertilizers was determined by wet digestion (Kjeldahl) using H_2SO_4, $CuSO_4.5H_2O$, NA_2SeO_3, K_2SO_4, and Raney's alloy, followed by distillation and titration with H_2SO_4 of the samples (Alcarde, 2009; MAPA, 2017). The total Mo content was determined by the atomic absorption spectrometric method (MAPA, 2017). The samples underwent acid

digestion using HCl, and the amount of Mo in the granule was expressed as a function of the amount added using equation 1. Where *% of Mo* is the percentage of molybdenum recovered in the determination, *Mou* is the amount of Mo determined after digestion and *Moe* is the amount of Mo added (Table 1; Equation 1).

$$\% \text{ of Mo} = \frac{Mo_u}{Mo_e} \times 100 \quad (1)$$

The salinity of the fertilizer was determined using the method (MAPA, 2017). 1 g of each fertilizer was weighed and added in a 100 mL volumetric flask, then the volume was completed with deionized and agitated water until the samples were dissolved and homogenized. After this process, the solution was transferred to a bequer and rested for 10 min to then determine the conductivity.

The determination of hygroscopicity followed the methodology proposed by Alcarde et al. (1992) and adapted by Faria et al. (2014) (Suppl. Figure 1F). 10 g of each fertilizer was added in a Petri dish approximately 4 cm in diameter and 1 cm high. The fertilizers were taken to the oven for 24 h at 50 ºC heavy to determine the dry mass and then added in desiccators with a relative air humidity of 38, 55, 76, 86, and 92 %. The humidity in each desiccator was obtained with the addition of sulfuric acid varying concentration. At each humidity, the samples were weighed 3, 6, 12, 24, and 48 h after being added to this condition. The amount of water absorbed was calculated in relation to the weight of the dry sample, i.e. after 24 hours in the oven.

Hardness evaluation of fertilizer granules was performed according to Manual for the Determination of Physical Properties of Fertilizers (IFDC, 1986) using Ethink Technology compression and traction meter, model 298 DGP (Suppl. Figure 1E). Each treatment had 21 granules analyzed, being 7 granules per repetition. To determine the resting angle, the fertilizer was spilled at constant speed forming a heap. The height and length of the heap were used to define the resting angle by trigonometric equations. The angle is equivalent to the inverse of the tangent, obtained by height divided by the length of the heap (MAPA, 2017).

After the preparation of the fertilizers, a reaction was observed with the formation of blue coloration between Mo and NBPT. To identify the change that occurred six fertilizers were submitted to Fourier-transform infrared (FTIR) analysis. The fertilizers analyzed were U, Uc, UcMa600, UiMa600, UcMt600, UiMt600. FTIR spectra of fertilizers were collected by an FTIR appliance (Jasco FTIR 4100). The fertilizers were pressed into pellets for analysis, and the infrared spectra were collected in the range of 4000–400 cm^{-1}.

2.2.3. Data processing and statistical analysis

The assumptions of homogeneity of variance and normality of residues were evaluated by the Bartlett-Test and the Shapiro-Wilk-Test, respectively. Outliers were removed when found by the Grubbs test. The outcomes of hardiness, saline index, angle of response, and the mean of hygroscopicity were analyzed in machine learning using the k-means clustering algorithm (unsupervised learning). The data were separated into 2 clusters (Cluster 1: 60 observations; Cluster 2: 30 observations) by the Silhouette method; which is based on the comparison of tightness and separation (Rousseeuw 1987). The distance between the observations and a cluster was calculated from the Euclidean distance. A total of 90 samples was tested in the k-means clustering algorithm using the treatments with treated-urea associated with rates and sources of molybdenum. The averages of hardiness, saline index, angle of repose, and hygroscopicity within clusters were compared by the t test (Student; $p \leq 0.05$), correlated by the Pearson correlation ($p \leq 0.05$).

The data of hardiness, saline index, angle of repose, and hygroscopicity (at 38, 55, and 76; 86, and 92% of humidity) were submitted to an analysis of variance (ANOVA) based on the F-test. When the F-Test was significant ($p \leq 0.05$), the treated-urea and Mo sources were tested by the LSD test ($p \leq 0.05$), and the Mo rates were compared by the Regression test using quadratic and linear models ($p \leq 0.05$). Statistical analysis was performed using the R Statistical Software (version 4.0.0; R Foundation for Statistical Computing); and the programming language in Python (version 3.8.3; Python Software Foundation).

Figure 3. Visual aspect of formulated N fertilizers formulated with treated-urea (U, urea; Uc, coated; Ui, incorporated), and sources (Am, ammonium molybdate; and Mt, molybdenum trioxide) and rates of molybdenum (Mo, 0; 150; 300; 600; 1200 g of Mo 100 kg of N).

2.3. Results

2.3.1. The visual aspect of formulated fertilizers

Three days after the end of the granulation and coating process part of the products suffered visual changes. The base of all formulated compounds was urea, product white and without impurities (UAm, UMt) (Figure 3). To add Mo to urea it was necessary to use a sticky, which was slightly yellowish, this process resulted in yellowish granules, regardless of the source and rate of Mo, UAm150-1200, and UMt150-1200 (Figure 3). This coloration is characteristic of adherent and was expected, since Ma has coloration like that of urea and Mt is slightly grayish. Mt amounts added were insufficient to change the fertilizers color, if no reaction occurs.

The urea that received along with an adherent, the NBPT and Am resulted in green products, with a slight yellowing with the increase of Mo rates, which was expected due to the increase of adherent in the granules. On the other hand, the urea, which underwent the same procedure, but with Mt, resulted in products with a bluish coloration, which was more intense as the Mo rate increased (Figure 3). The coloration change after the end of the process indicates chemical reactions, which in this case may be undesirable. The same reactions were observed in the urea with Mt and NBPT incorporated in the granules, but less intense, indicating that the process occurred between Mt and NBPT when in direct contact and a more concentrated manner, due to the location of the products around the urea granule.

In order to elucidate the modifications that occurred in the fertilizers, they were analyzed by infrared spectroscopy and the results are shown in figure 4. Here we observe that the transmittance of urea differs from the other fertilizers, and it is possible to indicate some possible bonds present in the fertilizers that had a color change.

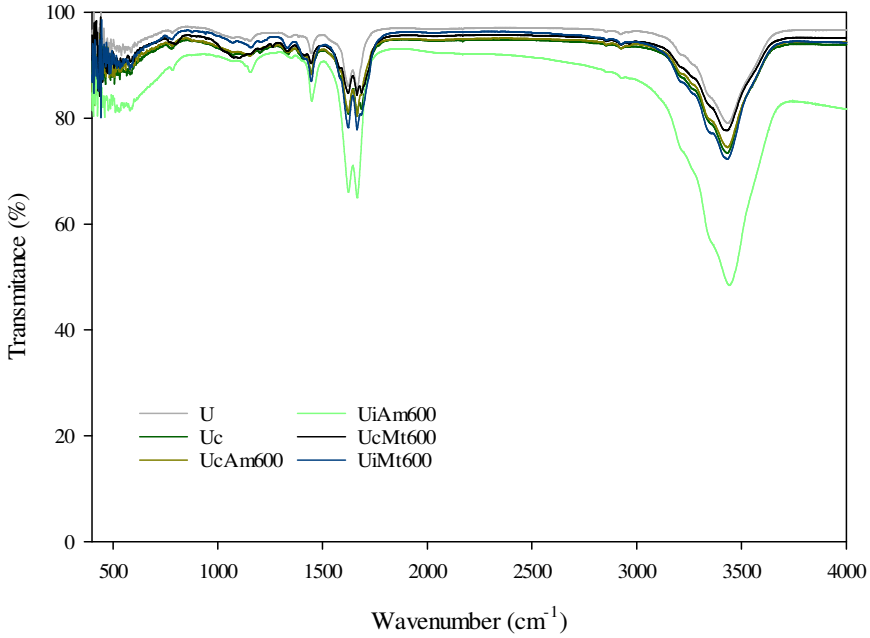

Figure 4. Spectra of the U, Uc, UcAm600, UiAm600, UcMt600, UiMt600 formulates fertilizers. 1650 ± 50 cm^{-1} = asymmetric (C=O) of amides; 1350-1250 = organic phosphates (P=O); 1420-1370 = organic sulfates(S=O).

2.3.2. Formulated fertilizer clusters

There was a formation of a one-dimensional plane with two Clusters accounting for a sum of squares of 63%, represented by the sum of squares within a cluster of 24328.4 and 19824.5, respectively in Cluster 1 and 2 (Table 2). The high values of the sum of squares indicated that there was a high variability within Cluster 1, which was composed of all treatments with treated-urea and sources of Mo at the rates of 150 and 300 g of Mo per 100 kg of N, and the control. While the treated-urea and molybdenum at the rates of 600 and 1200 g of Mo per 100 kg of N were associated with Cluster 2 (Figure 5).

In Cluster 2, there was a superior average of the saline index, angle of repose, and hygroscopicity with a general mean of 94.2 μS cm^{-1}; 33.2°; and 27.8; which were considered 64, 10; and 23'% higher than the respective averages in Cluster 1, respectively. There was no difference in hardiness between the clusters with a general average of 1.2 and 1.1 kgf in Cluster 1 and 2, respectively (Table 2).

In Cluster 1, there was a negative correlation of hardiness with saline index (r: -0.34; $p \leq 0.05$), and hygroscopicity (r: -0.41; $p \leq 0.05$), indicating that the increase of hardiness decreased the saline index and hygroscopicity. On the other hand, there was a positive correlation of angle of repose with saline index (r: 0.53; $p \leq 0.05$), but a negative correlation between the angle of repose and hygroscopicity (r: -0.34; $p \leq 0.05$). Therefore, the increase of angle of response plays

positively in the saline index, but it is negative in hygroscopicity. Interestingly, in Cluster 2, there was no correlation between hardiness, saline index, angle of repose, and hygroscopicity (Table 3).

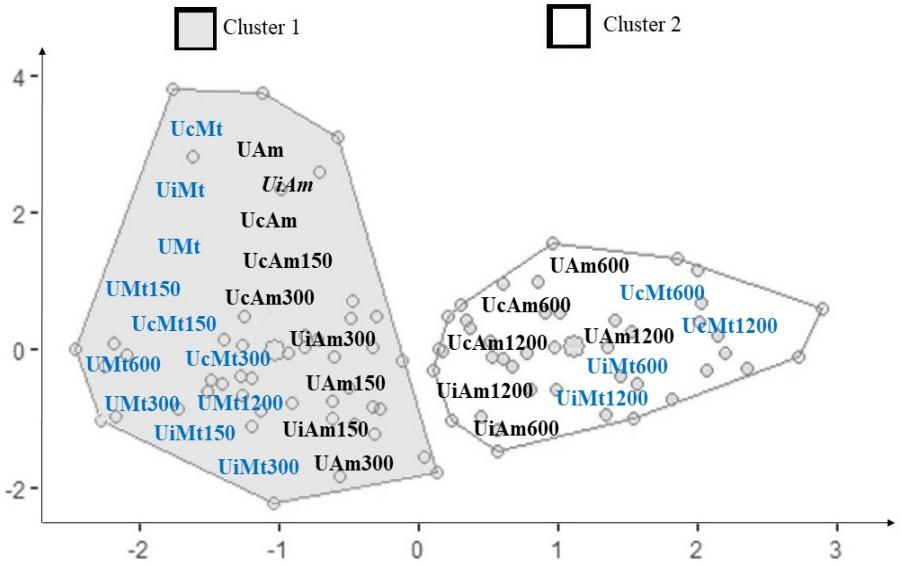

Figure 5. Distribution of N fertilizers formulated with treated-urea (U, urea; Uc, coated; Ui, incorporated), and sources (Am, ammonium molybdate; and Mt, molybdenum trioxide) and rates of molybdenum (Mo, 0; 150; 300; 600; 1200 g of Mo per 100 kg of N) in the Clusters 1 and 2. A total of 90 observations were separated into 60 and 30 observations in Cluster 1 and 2, respectively. Identification names in black and blue represent the ammonium molybdate and molybdenum trioxide, respectively.

Table 2. Values of hardiness, saline index, angle of repose, and hygroscopicity of N fertilizers formulated within clusters (Cluster 1 and 2)

Variables	Cluster 1	Cluster 2
Hardiness (kgf)	1.2±0.2 A	1.1±0.2 A
Saline index (µS cm^{-1})	33.5±9.9 B	94.2±12.9 A
Angle of repose (°)	30.1±2.8 B	33.2±1.6 A
Hygroscopicity	21.5±5.6 B	27.8±7.7 A
Sum of squares	24328.42	19824.5
Number of members	60	30

Sum of squares within the cluster. The averages of variables within clusters were compared by the t test (Student; $p \leq 0.05$). Hygroscopicity represents the mean of 38, 55, 76, 86, and 92% of humidity. Total of 90 observations.

Table 3. Correlations of hardiness, saline index, angle of repose, and hygroscopicity in Cluster 1 and 2

	Saline index	**Angle of repose**	**Hygroscopicity**
		---------- Cluster 1----------	
Hardiness	-0.34 (≤ 0.05)	-0.06 (0.60)	-0.41 (≤ 0.05)
Saline index	-	0.53 (≤ 0.05)	0.11 (0.39)
Angle of repose	-	-	-0.34 (≤ 0.05)
		---------- Cluster 2 ----------	
Hardiness	0.17(0.36)	-0.15(0.40)	-0.31(0.09)
Saline index	-	-0.26(0.15)	0.22(0.22)
Angle of repose	-		0.27(0.14)

Total of 90 observations. Significant correlations are highlighted in bold according to Pearson correlation ($p \leq 0.05$).

Considering that the N commercial urea, our quality standard, present a saline index lower than 30 μS cm^{-1}, and angle of repose higher than 25 °, and a hygroscopicity lower than 20; all attributes close to characteristic common urea. The Am associated with U at 150 g of Mo per 100 kg of N, and Mt associated with U at 150 and 300 g of Mo per 100 kg of N are presented as great fertilizers to supply both N and Mo to plants. If it is requested to increase the Mo rates, the data demonstrate that Mt is a better Mo source when associated with U, which positive adequate characteristics of the angle of repose and hygroscopicity were measured, but with a saline index higher than 30 μS cm^{-1} (Figure 6).

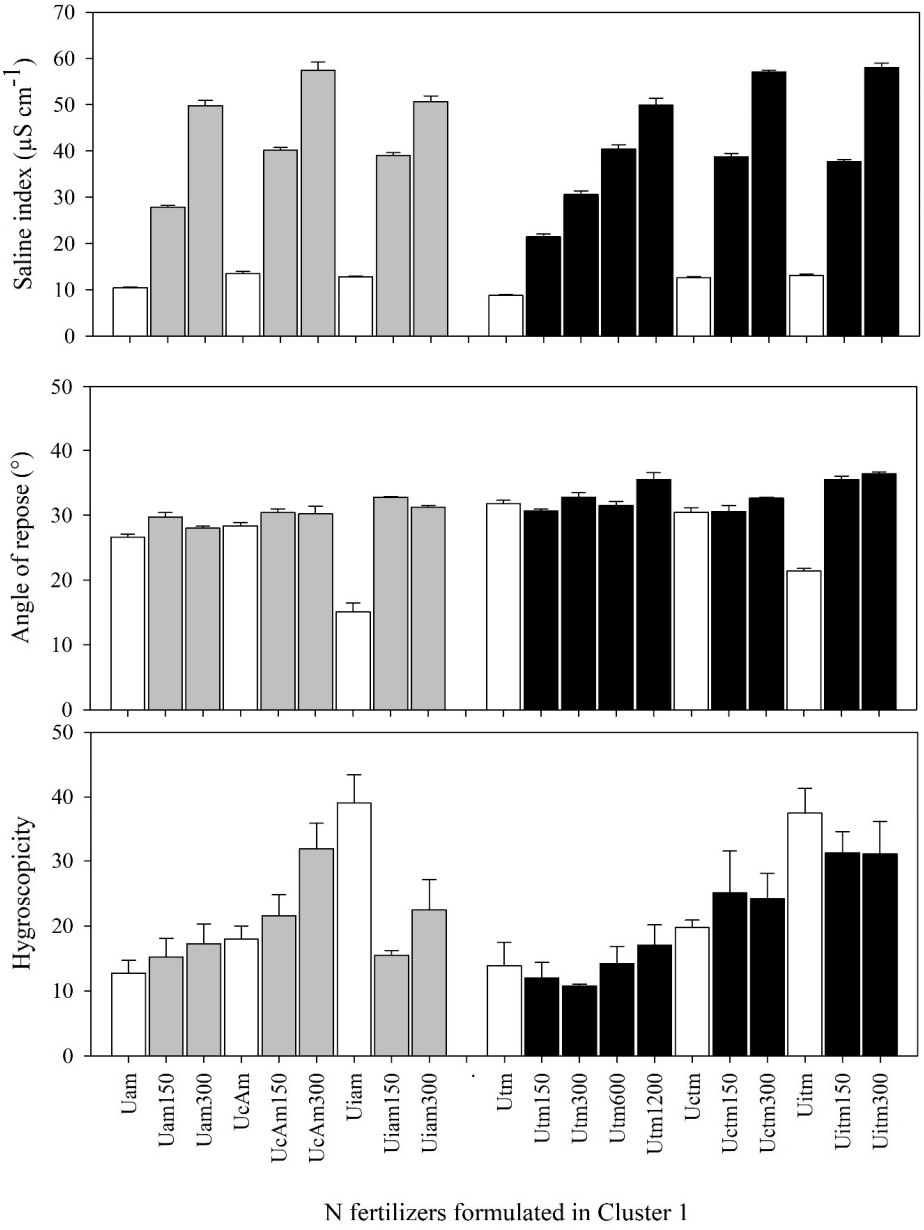

Figure 6.N fertilizers formulated with treated-urea (U, urea; Uc, coated; Ui, incorporated), and sources (Am, ammonium molybdate; and Mt, molybdenum trioxide) and rates of molybdenum (Mo, 0; 150; 300; 600; 1200 g of Mo 100 kg of N) in the Clusters 1.

2.3.3. Fertilizer characterization

The angle of repose presented three significant double-interactions between (i) treated-urea and Mo rates, (ii) source and rates of Mo, (iii) and treated-urea and Mo sources ($p \leq 0.05$); results are presented by the decompositions of interaction ($p \leq 0.05$), Figure 5. With both sources of Mo and treated-urea, the additions of Mo rates fitted linear responses in the angle of repose ($R^2 > 35\%$), with an increase of 11; 13; 25 and 22 % from the control to the rate of 1200 g of Mo

per 100 kg of N, respectively using the general average in U, Uc, Am, and Mt. While Ui fitted a quadratic response with an optimal rate of 700 g of Mo per 100 kg of N (Figure 7A-B). Among treated-urea, Ui presented a higher angle of repose at rates from 0 to 600 g of Mo per 100 kg of N. Interesting, there was no difference of treated-urea in the rate of 1200 g of Mo per 100 kg of N. Without exception, the Mt presented a higher angle of repose with a general average of 36 °, and a 13 % increase compared with Am (Figure 7A-B). The association of Mt with treated-urea promoted a greater angle of repose with an increase of 11; 5; and 15 % in U, Uc, and Ui, respectively compared with the associations of Am and treated-urea (Figure 7C).

The data of hardiness, saline index, and hygroscopicity at 38, 55, 76, 86 % of humidity presented a significant triple-interaction to the treated-urea, and sources and rates of Mo ($p \leq 0.05$). These results are presented by the decomposition at each level in the interaction (Figures 8 and 9).

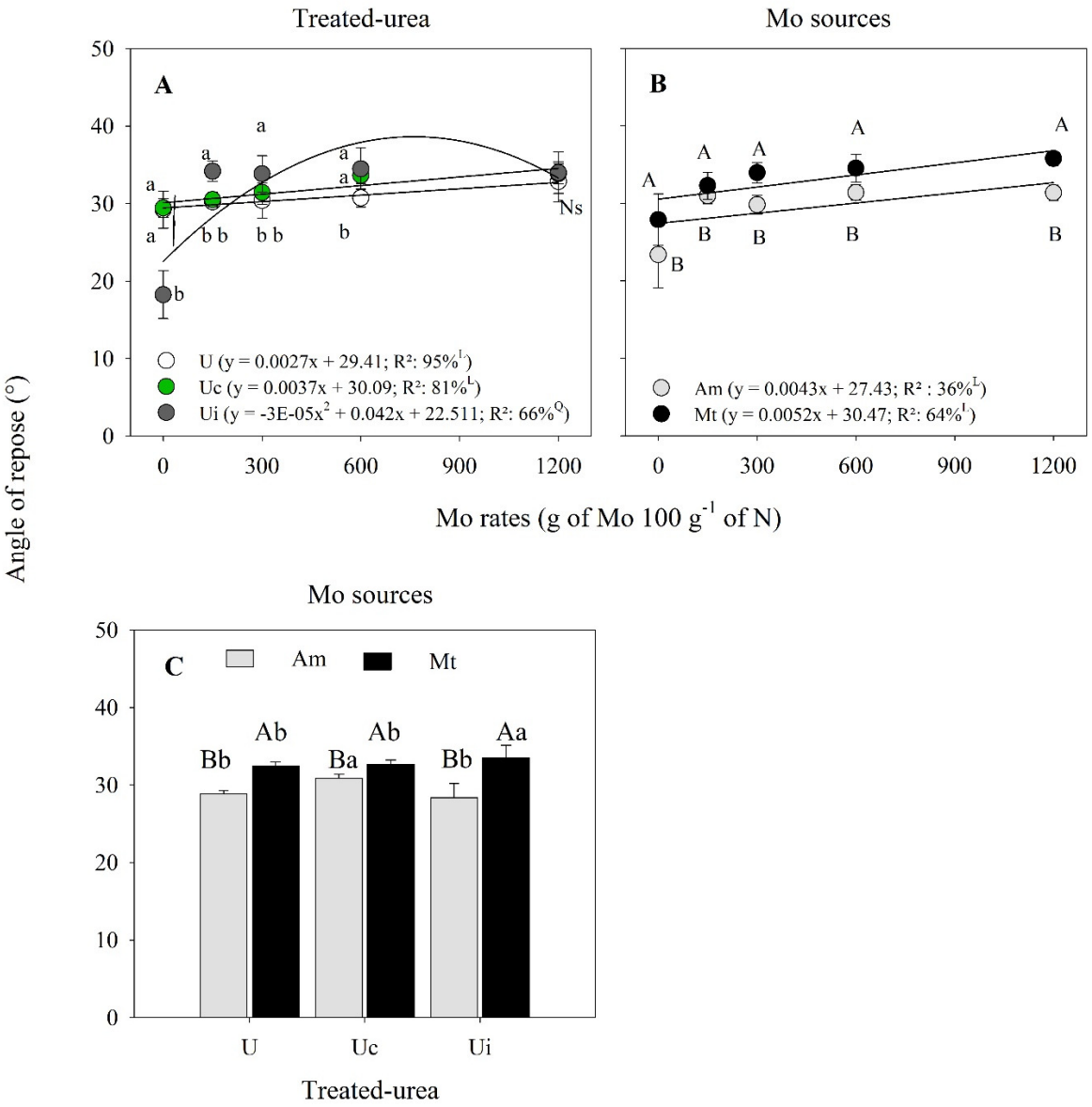

Figure 7. Angle of repose of N fertilizers formulated with treated-urea (U, urea; Uc, coated; Ui, incorporated), and sources (Am, ammonium molybdate; and Mt, molybdenum trioxide) and rates of molybdenum (Mo, 0; 150; 300; 600; 1200 g of Mo per 100 kg of N). There was three double-interactions between (i) treated-urea and Mo rates, (ii) source and rates of Mo, (iii) and treated-urea and Mo sources ($p \leq 0.05$), according to the Regression test ($p \leq 0.05$; Mo rates), and the LSD test ($p \leq 0.05$; Mo sources, uppercase letter; treated-urea, lower case). L, Q, and Ns represent linear and quadratic model, and no significant effect. Bar with standard error.

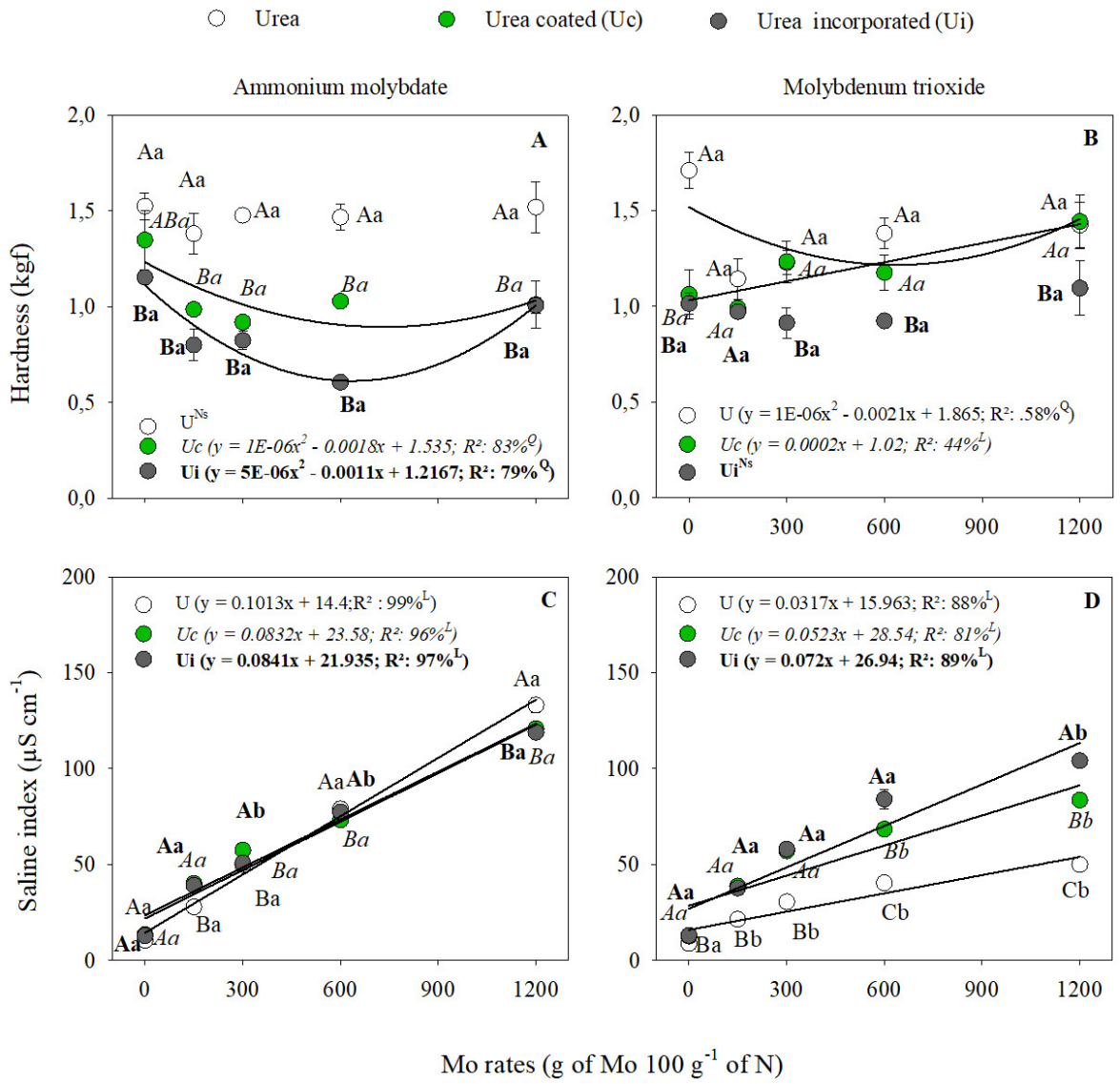

Figure 8. Hardiness and saline index of N fertilizers formulated with treated-urea (U, urea; Uc, coated; Ui, incorporated), and sources (Am, ammonium molybdate; and Mt, molybdenum trioxide) and rates of molybdenum (Mo, 0; 150; 300; 600; 1200 g of Mo per 100 kg of N). The effect of Mo rates was tested by the Regression test ($P \leq 0.05$), and results were explained by linear (^{L}) and quadratic model(^{Q}). Treated-urea and sources of Mo were tested by the LSD test ($p \leq 0.05$), and results were represented by uppercase and lowercase letters, respectively. ^{Ns}: no significant effect. Average with standard error.

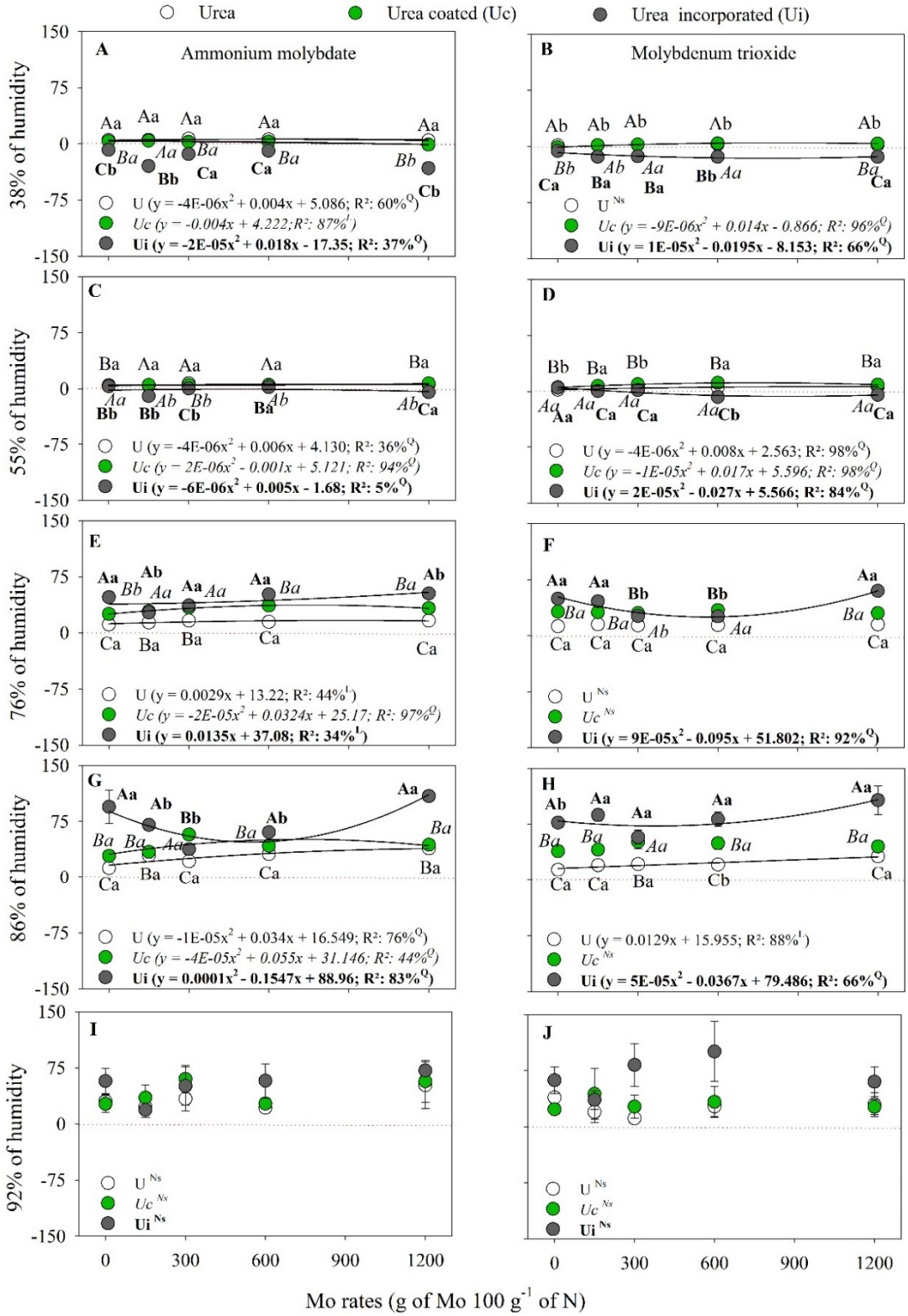

Figure 9. Hygroscopicity of N fertilizers formulated with treated-urea (U, urea; Uc, coated; Ui, incorporated), and sources (Am, ammonium molybdate; and Mt, molybdenum trioxide) and rates of molybdenum (Mo, 0; 150; 300; 600; 1200 g of Mo per 100 kg of N) at 38, 55, 76, 86 and 92% of humidity. The effect of Mo rates was tested by the Regression test ($p \leq 0.05$), and results were explained by linear (^{L}) and quadratic model(^{Q}). Treated-urea and sources of Mo were tested by the LSD test ($p \leq 0.05$), and results were represented by uppercase and lowercase letters, respectively. ^{Ns}: no significant effect. Average with standard error. Line red represents zero-point.

To hardiness of fertilizers, there was no difference between the Am and Mt with a general average of 1.1 and 1.2 kgf, respectively (Figure 6A-B). In Am, the U presented higher hardiness with an average of 1.5, which had significative difference from the Uc and Ui, which presented an average of 1.1 and 0.9 kgf, respectively. The Uc and Ui fitted quadratic responses with the highest reduction of hardiness at rates of 900 and 1100 g of Mo per 100 kg of N, and an R^2 of 83 and 79 %, respectively ($p \leq 0.05$), Figure 7A. In Tm, both U and Uc presented higher hardiness with averages of 1.4 and 1.2, which represented a mean increase of 23 % compared to Ui. The U fitted a quadratic response with the highest reduction of hardiness at rates of 1050 g of Mo per 100 kg of N (R^2: 58; $p \leq 0.05$); while, Uc fitted a linear response with an increase from 1.1 to 1.4 kgf (R^2: 44 %; $p \leq 0.05$), Figure 7B.

The saline index was increased by the Mo rates fitting linear responses and increments from 12.2 to 124.3; and 11.5 to 79.2 μS cm^{-1}, respectively in Am and Mt with all treated-urea (Figure 6C-D). In Tm, there was a clear difference in treated-urea with a higher saline index in Ui, followed by Uc and U with a general average of 59.4; 52.1; and 30.2 μS cm^{-1}, respectively. These differences were increased by the additions of Mo rates higher than 600 g of Mo per 100 kg of N (Figure 6D). In Am, there was a low variation of the saline index with a general average, ranging from 59 to 61 μS cm^{-1} (Figure 7C).

The hygroscopicity of fertilizers presented a consecutive increase and a general average of -3.1; 3.7; 29.3; 48.7; and 39.3, respectively in 38, 55, 76, 86 to 92 % of humidity (Figure 7). In the humidity of 38 %, there was a low and negative variation of hygroscopicity between Am and Mt with a general average of -3.6 and 3.1, respectively. Specifically, U presented a higher hygroscopicity in Am compared with Mt in all rates of Mo, as well in both sources of Mo the hygroscopicity in U was higher than Uc and Ui. The Mo rates fitted quadratic responses in the association of Am with U (R^2: 60 %) and Ui (R^2: 37 %), as well in the association of Mt with Uc (R^2: 96 %) and Ui (R^2: 66 %), with the plateau-rate at 500; 900; 777; and 975 g of Mo per 100 kg of N. An exception to the association of Uc and Am, when Mo rates fitted a linear response (R^2: 87 %), Figure 8A-B.

The humidity of 55 % played as an intermediate outcome between low and high humidity. There was a low variation between Am and Tm, and the rate of Mo fitted quadratic response in all associations of Am with U (R^2: 36 %), Uc (R^2: 94 %), and Ui (R^2: 5 %), as well as the associations of Mt with U (R^2: 98 %), Uc (R^2: 98 %), and (R^2: 84 %) with the plateau-rate at 750; 250; 416; 1000; 850; and 675 g of Mo per 100 kg of N (Figure 8C-D). With the increase of humidity from 76 to 86 %, the U presented the lower hygroscopicity in all associations with a general mean of 14.3 and 24.3, respectively, and a decrease of 55 and 44 % compared to Uc, and

66 and 69 % compared to Ui (Figure 8E-H). On the other hand, Ui presented the higher hygroscopicity in all rates and sources of Mo with a general average of 42.3 and 78.4, and an increase of 25 and 45 % compared to Uc, respectively in the humidity from 76 to 86 %. An exception, in Am and Tm, where Uc presented higher humidity in the Mo rates of 300 (86 % of humidity), and 300 and 600 (76 % of humidity), respectively, Figure 8E-H.

In 76 % of humidity, there were linear responses of Mo rates in the associations of U and Ui with Am (R^2: 44; 34 %); and the quadratic response of Ui and Mt (R^2: 92 %), and Uc and Am (R^2: 97 %) with optimal Mo rates at 810 g of Mo 100 g^{-1} of N (Figure 8E-F). In 86 % of humidity, quadratic responses were noticed in Am with U (R^2: 76 %), Ui (R^2: 44 %), and Uc (R^2: 83 %), as well as in Mt with Ui (R^2: 66 %) with optimal rates at 1350; 687; 773; 367 g of Mo per 100 kg of N, while there was a linear response in Ui (R^2: 88 %) (Figure 8E-F). There was no effect of treatments in the humidity of 92 % with a general average of 39.3 (Figure 8I-J).

2.4. Discussion

The fertilizer's visual appearance as well the color formation was associated with the nanoparticles and mode of addition, as identified by the micro-xrf result. The process of coating and incorporation of the products into urea highlights the distribution of S, P, and Mo in the urea granules with blue color. These elements are derived from NBPT (S and P) and Mt, in Figure 4. In the beads without blue coloring, we observe that there is no S and P, even in the presence of the adherent for molybdenum (Figure 4). In contrast, we noticed that the use of Am did not result in staining even in the presence of S and P, such condition is linked to the oxidation state of Mo in each compound (Nagul et al., 2015), making the reaction pH-dependent.

Nagul et al. (2015) describe many possible reactions between Mo and P and show this reaction as a function of the Mo oxidation state, pH and medium reaction wich is extremally reductor in our case. In addition, the Mo rates influenced the color formation. It happens due the reaction between Mo and P be intense above 12:1, being the rate of 600 g of Mo a relation between the elements of 8.6, while in the rate of 300 g of Mo the relation was only 4.3, making the reaction unfeasible (Veen et al., 1986).

Numerous projects have been developed to evaluate whether nanofertilizers are more efficient in delivering nutrients to plants, with the particles generally being evaluated after being absorbed or applied to the plants (Lin and Xing, 2007; Stampoulis et al. 2009; Lin and Xing, 2008; Shah and Belozerova, 2009; Taran et al. 2014; Thomas et al. 2017). In the colorimetric reaction formed we observed that the use of nanofertilizers can result in product interactions,

causing depreciation of the mixture formed, which did not always result in visual changes. Under favorable environmental conditions, nanoparticles will have high reactivity, as they have a large specific surface area (Dreizin, 2009; Mudunkotuwa et al. 2012; Faivre and Bennet, 2016), and are also widely used as catalysts for reactions. In this case, Mt nanoparticles were responsible for the reaction with NBPT, resulting in the infeasibility of the mixture. It was observed that the FTIR result did not differ between the treatments, different from that found by McCarty et al. (1989), thus the initial structure of NBPT was altered, specifically in the urease inactivation site, it is noted that the P-S peak is not observed (Figure 4).

Physicochemical changes can reduce the quality of the fertilizer, make transport and application more difficult and, of course, alter plant utilization depending on the stability of the compound formed (Faria et al. 2014). Rutland and Polo (1951) showed that the angle of repose is mainly influenced by the shape, size and coating of the granules, which was also observed in this experiment, since changes in the coating material and the method of adding NBPT to urea influenced the response to this variable. Furthermore, it is found that the flow of the granules made with Am will be classified as free flow while those with Mt will be classified as easy flow (Gaylord and Gaylord, 1984), indicating that physically the mixture of Am with urea brought the best results, which was also observed for the other parameters. The clusters show that rates higher than 600 g Mo resulted in lower hardness and higher angle of repose, which would make the transport and application of these products more difficult. In addition, higher salinity and hygroscopicity, i.e. lower critical moisture, is noted, indicating that these products have a higher capacity to absorb water from the environment and, if applied near the root, initially induce root dehydration (Mahmoud et al. 2020).

The process of incorporating of the products to urea showed a formation of fertilizer more susceptible to undesirable changes (Figure 6 and 7), such condition however is explained by the difference in the formulation process of the granules (Franco and Saraiva Neto 2007). The incorporated products received NBPT together with urea and due to it did not undergo high-temperature drying after granulation, because NBPT is sensitive to temperature. In other words, the difference between the formulation processes was expected, and solutions to this bottleneck in the formulation should be further sought for the application of NBPT incorporated in urea.

2.5. Conclusions

It is possible to mix Mo to urea without causing significant physicochemical alteration, regardless of the source of Mo used, as far as the rate of Mo does not exceed 600 g per 100 kg of

N. Under these conditions the formulated product is promising for use and commercialization. Adding rates of Mo higher than 600 g per 100 kg of N-urea reduced hardness, increased salinity, and increased water absorption from the environment, regardless of the Mo source. The formulations between urea with NBPT and molybdenum showed that the use of molybdenum trioxide reacted with NBPT, due to change in color after mixing; in rates below 600 g per 100 kg N this effect was reduced. This mixture should be avoided due to the degradation potential of NBPT, making it inefficient in inhibiting urease.

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3. MOLYBDENUM ADDITION O UREA DOESN'T CHANGE AMMONIA LOSSES IN THE N-(N-BUTYL) THIOPHOSPHORIC TRIAMIDE ABSENCE

Abstract

Urea is the most widely used source of N due to its low cost per N unit. However, this fertilizer, when added to the topsoil, undergoes hydrolysis and part of the N is lost through volatilization, reducing its agronomic efficiency. Strategies to reduce N-NH_3 losses are constantly being studied, and currently the main inhibitor of urea hydrolysis is N-(n-butyl) thiophosphoric triamide (NBPT). There is a demand for new strategies or molecules that make urea more efficient, resulting in a lower environmental impact on fertilizer use. In this context, the hypothesis is that adding coated Mo to urea with NBPT reduces ammonia losses. The aim was to evaluate N-NH_3 losses in urea treated with molybdenum (Mo) sources and NBPT. The experiment was conducted in the laboratory under controlled conditions, the factors were randomized block design with three repetitions. The factors were urea type (urea, urea+NBPT coated and urea+NBPT incorporated), Mo source (ammonium molybdate and molybdenum trioxide) and Mo rates (0, 150, 300, 600 and 1200 g of Mo per 100 kg of N). Fertilizers were granulated in laboratory using a disk granulator system. The experimental units were composed by a volatilization system that allowed the daily capture of ammonia losses individually for 30 days. Soil pH was also determined in each experimental unit. After measuring N-NH_3 losses, the data were fitted to a two-phase model, and it was observed that the addition of Mo sources to urea without NBPT does not cause changes in N-NH_3 losses and, therefore, can be a pratice incorporated to the industry. Molybdenum trioxide promotes higher N-NH_3 losses, equivalent to urea, when added to the urea coating with NBPT.

Keywords: Volatilization, Nanoparticle, Molybdenum trioxide, Fertilizers.

3.1. Introduction

High agricultural yields are related to adequate inputs, both in quantity and efficiency. The use of N in agriculture has contributed to the productivity increase and food availability. Even thought numerous sources of fertilizers could provide the N demand of plants, urea is the most widely used worldwide (IFA, 2018). The main advantage of urea is its physical-chemical characteristics and high N content, which can reach up to 46 %, which reduces costs with transportation, storage and application (Chien et al., 2009; Trenkel, 2010; Cantarella et al., 2018). However, this fertilizer also presents a disadvantage that compromises its efficiency, part of the applied N is lost by volatilization due to the hydrolysis of the urea molecule by urease that results in NH_4^+ production and pH increase near the granule region (Mikkelsen 2009; Tasca et al., 2011). The increase in pH, which reaches values close to 10, favors the conversion equilibrium of NH_4^+ into NH_3, a gas that is easily lost to the atmosphere. This process is favored when the fertilizer is applied in the soil surface without incorporation (Bremner 1995; Sommer et al., 2004). Ammonia losses are highly variable depending on environmental conditions, plant residues, soil type, and

irrigation, ranging from 50-70 % (Rochette et al., 2009; Pan et al., 2016; Drury et al., 2017; Silva et al. 2017). The result of this process is lower fertilizer utilization by plants, high energy expenditure and high environmental damage (Galloway et al., 2003; Behera et al., 2013; Otto et al., 2017; Sunderlage and Cook, 2018; Hill et al., 2019).

The addition of heavy metals, inhibitors, mixing sources, polymer coating, among other strategies (Kasim et al., 2009; Xu et al., 2013; Cancellier et al., 2016; Pan et al., 2016; Campos et al., 2017; Guelfi, 2017; Rech et al, 2017; Mira et al., 2017) can be used to reduce the speed of urea hydrolysis, delaying the volatilization peak which usually occurs between day 3-8 after urea application (Rochette et al., 2009; Mira et al., 2017; Silva et al., 2017). Nowadays the most promising strategy for inhibiting losses is the use of N-(n-butyl) thiophosphoric triamide (NBPT). This compound acts by occupying the place of urea in the binding site of the enzyme and consequently reduces the hydrolysis of urea favoring the permanence of hydrolyzed N in the NH_4^+ form and later a part will be transformed into NO_3^-. NBPT has the potential to reduce ammonia emissions to the atmosphere up to 52 % (Silva et al., 2017). Other strategies such as the use of micronutrients and metals like Cu, B, Zn, Cd, Hg either associated or not with NBPT (Tabaibai, 1967; Stafanato et al., 2013; Cancellier et al., 2016; Rech et al., 2017) have also been evaluated, though the results are diverse in the literature and the action mechanisms of these elements are not clear (Stafanato et al., 2013; Cancellier et al., 2016; Rech et al., 2017).

Tabaibai (1967) demonstrated the potential of several chemical elements to inhibit urease activity, one of them not reported so far by anyone else util present moment. Mo, was responsible for reducing on average 12 % of the urease enzyme activity. Mo is a transition metal required in small amounts by plants for full development. The application of Mo is done either by foliar or seed treatment using ammonium molybdate, sodium molybdate and recently molybdenum trioxide. Both ammonium and sodium molybdate are soluble products, whereas molybdenum trioxide has low solubility and therefore has been used in nanoparticulate form.

Nanofertilizers have shown potential in agriculture (Lin and Xing, 2007; Stampoulis et al. 2009; Lin and Xing, 2008; Shah and Belozerova, 2009; Taran et al. 2014; Thomas et al. 2017). A new strategy for applying this product associated with the possibility of reducing NH_3 losses would facilitate the adoption of molybdic fertilization in crops. The use of new technologies which can promote the mitigation of NH_3 losses and improve urea efficiency has great importance for a less environmental impact production. Many strategies have been adopted for the urea development with improved efficiency but research is still needed to evaluate the potential of new mixtures and products. Our hypothesis is that adding coated Mo to urea with NBPT reduce ammonia losses. The objective was to evaluate the NH_3 losses from urea treated

with nanoparticulate molybdenum trioxide and ammonium molybdate, coated or incorporated in the urea, associated or not with the addition of NBPT.

3.2. Material and Methods

3.2.1. Rationale for diffusion system development and experiment design

To achieve the aims of this study, a laboratory experiment was performed under controlled conditions at microscale (i.e. soil moisture, temperature, air circulation) while keeping the soil N dynamics as in the field scale. Thereby, we set below three parameters of sugarcane commercial field to bring them out proportionally in a microscale system (Figure 1): i) most of the sugarcane ratoon in Brazil are cropped under 1.5 m inter-row, ii) during sugarcane ratoon sprouting period, the straw-soil interface receives 100 kg ha^{-1} of N fertilizer commonly topdress applied as urea by machinery, iii) during the urea topdress application, the granules spread out about 0.1 m width over the soil-straw interface. From these fixed parameters we assumed a microsite located effect of N fertilizers on soil N dynamics as already been proposed in earlier studies (Black et al., 2004; Dawar et al., 2011; Sherlock et al., 1986). To access those effects on soil microsite, we developed a *quasi-hermetic* diffusion system constituted by two basic components: i) a glass jar (8.0 cm of inner diameter, 15.0 cm of height, ~0.754 L of inner volume) added of soil and sugarcane straw to represent in microscale the field parameters above mentioned, and, ii) a diffusion retaining apparatus attached to the jar lid for NH_3 volatilization measurements identical from that created by Khan et al. (1997). The diffusion systems were assembled by 160 g of dried soil enough to form a thin layer of ~2.5 cm depth in which the N fertilizer most respond (Black et al., 2004; Rachhpal-Singh and Nye, 1984; Sherlock et al., 1986) and 6 g of dried sugarcane straw gently disposed over the soil that is equivalent to 12 Mg ha^{-1} on average of sugarcane ratoons (Fortes et al., 2012; Leal et al., 2013) (Figure 2).

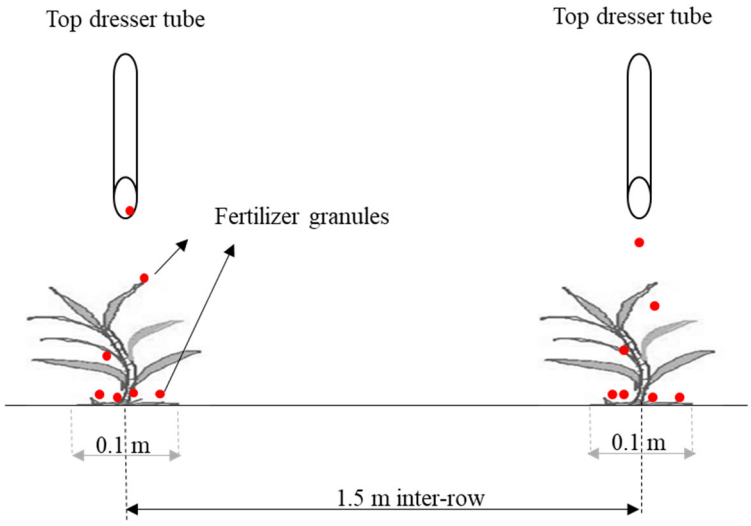

Figure 1. Representative scheme from sugarcane commercial fields during urea topdress application on ratoon sprouting period.

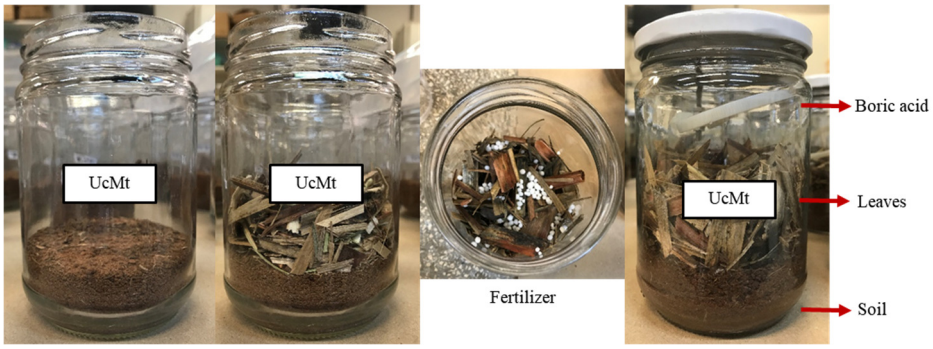

Figure 2. Illustration of the experimental units used to determine volatilized ammonia. Addition of soil for incubation for 21 d. B: Dry leaf addition on topsoil and incubation for 7 d. C. Fertilizer application in the experimental units after 28 d. D: Final composition of the experimental units.

The soil for this study was collected at 0-0.2 m layer thus sustaining the microbial community and activity commonly found on it (Paungfoo-Lonhienne et al., 2015, Yeoh et al., 2016). The soil was oven-dried at 40 ºC for 72 h (Haney et al., 2004), ground to pass through a 2.0 mm-sieve, homogenized and characterized for fertility and physical (**Erro! Fonte de referência não encontrada.**) (Raij et al., 1997; Gee and Or, 2002). The low result for soil BS evidenced the requirement of lime application to reach 60 % prior the onset of the experiment as recommended by van Raij et al. (1997) for sugarcane commercial fields. To compose the straw we collected dry leaves of sugarcane (variety RB96 6928) that were oven-dried at 65 °C for 96 h and cut manually down to 20 mm-size (Malavolta et al., 1997).

Table 1. Chemical and physical analysis of the soil experiment collect field at 0-0.2 m layer

pH	OC	N	P	S	K	Ca	Mg	BS	Als
$CaCl_2$	g dm^{-3}	---------- mg dm^{-3} ----------			-------- $mmol_c$ dm^{-3} --------			------ % ------	
5.1	21.0	819.0	12	<6.0	1.8	13	8	23	0
Al	**H+Al**	**CEC**	**SB**	**Zn**	**B**	**Cu**	**Fe**	**Mn**	**Mo**
---------- $mmol_c$ dm^{-3} ----------				---------- mg dm^{-3} ----------					
0	22	45	51	2.5	0.29	2.5	56.0	24.2	< dl
Sand	**Silt**	**Clay**							
---------- g kg^{-1} ----------									
740	34	226							

OC = organic carbonr, BS = base saturation, Als = Al saturation, CEC = cation-exchange capacity, SB = sum of bases, dl= detection limits.

Table 2. Table 1. Chemical analysis of sugarcane leaves disposed on the topsoil

N	P	K	Ca	Mg	S	Fe	Mn	Cu	Zn	B	Mo
---------- g kg^{-1} ----------						---------- mg kg^{-1} ----------					
5.5	0.9	4.3	5.8	0.2	1.1	510.0	41.5	3.0	12.0	17.1	<dl

dl= detection limits.

3.2.2. Experimental design and treatments

Under the microsite assembled into the diffusion system we experienced different N fertilizers associated to Mo. The fertilizers were formulated in laboratory through complete interaction of three main factors: i) urea type: standard (U), coated with NBPT (Uc) and incorporated to NBPT (Ui), ii) Mo source: ammonium molybdate (Am, soluble $(NH_4)_6Mo_7O_{24}$) and nanoparticulate molybdenum trioxide (Mt, low solubility MoO_3), and, iii) Mo rates: 0 (no Mo addition), 150, 300, 600 and 1200 g of Mo per 100 kg of N. The fertilizers formulation constituted a previous study of product physicochemical characterization of the products (Chapter I). Briefly, the NBPT content used was the same as commercially formulated by industry of 530 mg kg^{-1} of urea (Mira et al. 2017). The NBPT used was provided by Koch™ Agronomic Services. The mixtures were prepared one month before application and stored in an environment protected from light and humidity during this period. Mo was added using the same process as NBPT, i.e. for the first two types of urea the Mo was coated and in the third type it was incorporated into the granule during the granulation process. The experimental was carried out in factorial scheme (3x2x5) in randomized complete block desing, with three replications (n = 90) in which the diffusion systems constituted the experimental units (i.e. plots).

3.2.3. NH_3 volatilization and pH evaluation in short-term evolution essay

Prior the onset of the experiment, soil moisture was adjusted to 60 % of water-holding capacity (WHC) and left open in pre-incubation at 24 ± 1.5 °C for 21 d. After this period, the

sugarcane straw was gently accommodated on soil surface and the moisture was restored to 60 % of WHC (ISO. 2003). The jars were kept open for further 7th d and the moisture was restored whenever necessary to activate the microbial community and avoid initial release of NH_3 biased by soil disturbance (i.e. handling, liming) and straw addition(Suppl. Figure 2A, 2B). After the pre-incubation, the treatments were thoroughly and uniformly applied over sugarcane straw at 750 mg of N fertilizer (Figure 2). Immediately, a 40-mL vial containing boric acid solution was trapped in the diffusion retaining apparatus attached to the jar lid for NH_3 volatilization evaluation (Khan et al., 1997). The diffusion jars were kept in a room at 24 ± 1.5 °C for incubation. Inside the closed jars, the released NH_3 diffuses into the boric acid solution that accumulate as NH_4^+ during the incubation period. Following this period, the vial was removed and the consumed H^+ from acid boric solution was titrated to a pH end-point of 3.8 with 0.02 M H_2SO_4 standardized solution assisted by automated commercial instrument (848 Titrino plus, Metrohm, Switzerland) (Mulvaney, 1996) (Suppl. Figure 2D).

The soil pH was determined using a cylindrical potentiometer for semi-solids purpose with a conical-shaped glass bulb (5 mm-bulb of length and diameter, Hanna FC 240B model) (Suppl. Figure 2C). Soil pH readings were made by introducing the electrode bulb into ~5 mm soil depth always at the same micro-hole to reduce the disturbance and mobilization in the soil-straw system. The NH_3 emissions and pH readings were measured every day during 30 d. The volume and concentration of acid boric solution varied from 10 to 40 mL and 2 to 4 % (w/v) to optimize the balance between analysis sensibility and yield (i.e. time consuming) (Yuen and Pollard, 1953). The volume of H_2SO_4 dropped in the titration was used to calculate the amount of NH_3-N released during the incubation periods on oven-dry basis (g kg^{-1}).

The behavior of ammonia volatilization was explained by the association of two models, the first being the Hill-Langmuir function, a model used to describe enzymatic activity rates, in this case indirectly urease. Second is a constant rate function obtained by the inclusion of a linear term, necessary since volatilization continues after 20 d even at lower rates, but above nitrification. In the model used, we can observe the parameters of accumulated loss of NH_3 as a percentage of applied N (θ_a), the time after fertilization in days (t), θ_v which has three interpretations being the moment when 50 % of the maximum loss occurs, the when the maximum loss rate occurs, and the inflection point of the function, θ_k is the parameter associated with the maximum release rate, θ_b is the point at which the final second release starts and θ_x is the daily N lossed rate in the final stage of release (eq. 1,2 and 3).

$$y = y1 + y2 \quad (1)$$

$$y1 = \frac{\theta_a}{1 + (\theta_v | t)^{\theta_k}} \quad (2)$$

$$y2 = \begin{cases} 0 & t < \theta_b \\ \theta_x & \theta_b < t > \theta_{b+1} \\ \theta_x & t > \theta_{b+1} \end{cases} \quad (3)$$

3.2.4. Data processing and statistical analysis

Curves were fitted for each experimental unit, then the parameters obtained were found in the analysis of variance (ANOVA) based on the F test, when the F test was significant ($p \leq 0.05$), the treatemets were tested by the LSD test ($p \leq 0.05$). The variables were correlated using the Pearson-Correlation ($p \leq 0.05$).

3.3. Results

The N-NH_3 volatilization data were fitted to a model composed of two phases. The first phase was called ready release phase (y1) and the second slow-release phase (y2) because the volatilization rate was a constant determined by the daily rate of loss (θ_x). In the ready-release phase approximately 75 % of the released nitrogen was lost, so in this phase the maximum daily loss (R) and the day of maximum loss (θ_v) were determined.

In the ready-release phase of N (y1), it was observed that the average amount of N lost (θ_a) was 32.3% and there was an interaction between the type of urea and the Mo rates used (CV = 14.0%). The results show that Ui is the best option to reduce losses in the ready release phase but only for Ui, treatments without Mo. In this condition the losses were 21.3 %, while with the addition of Mo they were approximately 33 % (Table 3). The addition of Mo to urea with incorporated NBPT, or Ui products, was not efficient, and the contact between NBPT and Mo, independent of the source of Mo, led to the loss of inhibitor efficiency. The source of Mo used did not influence the responses when NBPT was not added, and the loss of the ready-release phase in this case was 34% of the N applied.

The NBPT incorporated into urea reduced volatilization by 30 % compared to not adding or covering the pellet. Moreover, the incorporation also led to a delay in the day of maximum loss (Ov), which for Ui occurred on the 12th day and for the others between the eighth and tenth day after application (CV = 13 %). However, the maximum loss was not influenced by the study factors, being on average 3.3 % of the N applied (Table 3) (CV = 11 %). The results

show that regardless of the source of Mo, volatilization occurs at the same rates, so in the ready-release phase there was no influence of the nanoparticulate material (Table 3). The only response obtained was in relation to the incorporation of NBPT in the urea, a result that was already expected.

In the second phase of the model, the daily N loss (θ_x) increased to 0.8 % regardless of the factors evaluated (CV = 22 %), but the transition point between the phases was influenced by the Mo source (CV = 20 %). The use of Mt advanced the transition by one day, in other words, the slow release of N was initiated earlier in the treatments with Mt (Table 4).

The parameters of the model indicate that the variation in function of the treatments was low, and these results were reflected in the predicted total N losses. It was observed that there was a response only for the interaction between the type of urea and source of Mo and also to the type of urea and the Mo rate (Table 5) (CV = 9%). The addition of Mo increased the losses of N in Ui, a fact that was not repeated for U and Uc, since the volatilization was the same within the evaluated rates. For Uc, the source of Mo influenced the loss of N, with Mt promoting greater losses, independent of the rate used. These results indicate that the trioxide has a greater potential of reaction with the inhibitor, as well as the greater the contact between products, the less efficient it is in reducing volatilization. This condition was expected due to the preparation of the fertilizers. The UcMt products had strong alteration in color and although too many physical-chemical characteristics were stable between treatments, the contact between NBPT and Mt resulted in indirect loss of quality, because the alteration in the inhibitor did not lead to great physical-chemical alterations of the granules, it only promoted inefficiency when in contact with the urease enzyme.

The correlation between total N lost and the quantified pH values were significant using the electrode for semisolids and pH in water, but not for calcium chloride (Table 6). This result indicates that total volatilized N can be predicted by soil pH values, but it is important that the pH value determined is close to the solubilization site of the urea granule, where the greatest changes in values occur. The day on which the maximum loss rate (θ_v) occurred is associated with the pH peak, the average value being 8.4. After this date the pH values rose to 8.8 on the 22nd d and after that present reduction, which corroborates with the second phase of the model, where the daily losses were lower (θ_x).

Table 3. Accumulated N-NH_3 loss (θa), day the maximum rate of loss occurred (θv), and maximum daily rate of loss (R) in the ready-release phase of moodle for urea treated with NBPT, molybdenum sources and rates for 30 d

	U			Uc			Ui			Rates
Rates	**Am**	**Mt**	$\overline{X}$	**Am**	**Mt**	$\overline{X}$	**Am**	**Mt**	$\overline{X}$	$\overline{X}$
g Mo (100 kg of N)$^{-1}$					θ_a (% of applied N)					
0	33.1	33.5	33.3 Ba	30.7	25.7	28.2 Ba	19.3	23.2	21.3 Aa	27.6
150	36.4	35.3	35.9 Aa	35.8	32.6	34.2 Aa	34.8	28.2	31.5 Ab	33.9
300	32.6	35.5	34.0 Aa	30.3	31.7	31.0 Aa	35.8	36.1	35.9 Ab	33.4
600	34.4	32.3	33.3 Aa	33.6	31.5	32.6 Aa	34.0	29.3	31.6 Ab	32.5
1200	35.7	31.5	33.6 Aa	33.6	34.7	32.2 Aa	35.3	33.1	34.2 Ab	34.0
$\overline{X}$	34.4	33.6	*34.0*	32.8	31.2	*32.0*	31.8	30.0	*30.9*	*32.3*
$\overline{X}$ **Mo sources**			**Am** 33.0						**Mt** 31.6	
g Mo (100 kg of N)$^{-1}$					θ_v (days)					
0	8.8	8.8	8.8 Ba	9.5	10.7	10.1 Ba	12.6	12.4	12.5 Aa	10.5
150	9.4	8.9	9.1 Aa	9.8	8.0	8.9 Aa	8.6	8.2	8.4 Ab	8.8
300	10.0	9.2	9.6 Aa	10.3	7.7	9.0 Aa	8.6	9.3	8.9 Ab	9.2
600	8.2	8.2	8.2 Aa	8.9	8.4	8.6 Aa	8.2	9.1	8.6 Ab	8.5
1200	8.7	8.2	8.5 Aa	9.1	8.1	8.6 Aa	8.9	8.2	8.6 Ab	8.5
$\overline{X}$	9.0	8.6	*8.8*	9.5	8.6	*9.0*	9.4	9.4	*9.4*	*9.1*
$\overline{X}$ **Mo sources**			**Am** 9.3						**Mt** 8.9	
g Mo (100 kg of N)$^{-1}$					R (% of applied N day^{-1})					
0	3.3	3.5	3.4	3.7	3.2	3.4	3.3	3.8	3.5	3.5
150	3.5	2.9	3.2	3.2	3.1	3.2	3.2	3.7	3.5	3.3
300	3.2	3.0	3.1	2.8	3.7	3.2	3.1	3.3	3.2	3.2
600	3.3	3.2	3.2	3.1	3.0	3.1	3.1	3.4	3.3	3.2
1200	3.1	3.7	3.4	3.3	3.3	3.3	3.2	3.4	3.3	3.3
$\overline{X}$	3.3	3.2	*3.3*	3.2	3.3	*3.2*	3.2	3.5	*3.4*	*3.3*
$\overline{X}$ **Mo sources**			**Am** 3.2						**Mt** 3.3	

U = urea, Uc = urea coated, Ui = incorporated, Am = ammonium molybdate, Mt = molybdenum trioxide. Capital letters in the same row indicate a difference between urea type and lower case letters in the same column indicate a difference between Mo rates.

Table 4. Point transition from the fast-release phase to the slow-release phase (θ_b) and daily rate of loss in the slow-release phase (θ_x) of moodle for urea treated with NBPT, molybdenum sources and rates for 30 d

	U			Uc			Ui			Rates
Rates	**Am**	**Mt**	$\overline{X}$	**Am**	**Mt**	$\overline{X}$	**Am**	**Mt**	$\overline{X}$	$\overline{X}$
g Mo (100 kg of N)$^{-1}$					θ_b (days)					
0	16.1	15.8	15.9	13.8	9.4	11.6	10.9	12.7	12.7	13.1
150	17.0	15.7	16.3	17.2	14.2	15.7	14.7	14.2	14.2	15.5
300	13.9	16.3	15.1	15.5	16.6	14.5	15.2	13.7	13.9	14.7
600	15.1	14.7	14.9	15.6	13.3	14.3	14.1	10.3	10.3	13.8
1200	15.0	14.6	14.8	16.2	14.0	15.1	15.6	14.6	14.6	15.0
$\overline{X}$	15.4	15.4	*15.4*	15.6	12.8	*14.2*	14.1	13.1	*13.6*	*14.4*
$\overline{X}$ **Mo sources**			**Am** 15.0 A					**Mt** 13.8 B		
g Mo (100 kg of N)$^{-1}$					θ_x (% of applied N)					
0	0.8	0.9	0.8	0.7	0.8	0.8	0.7	0.7	0.7	0.8
150	0.7	0.7	0.7	0.6	0.8	0.7	0.9	0.8	0.9	0.7
300	0.6	0.8	0.7	0.7	1.0	0.8	0.9	0.7	0.8	0.8
600	0.8	0.8	0.8	0.7	0.8	0.8	0.8	0.7	0.8	0.8
1200	0.8	0.7	0.8	0.7	0.8	0.8	0.8	0.8	0.8	0.8
$\overline{X}$	0.7	0.8	*0.7*	0.7	0.8	*0.8*	0.8	0.7	*0.8*	*0.8*
$\overline{X}$ **Mo sources**			**Am** 0.7					**Mt** 0.8		

U = urea, Uc = urea coated, Ui = incorporated, Am = ammonium molybdate, Mt = molybdenum trioxide. Capital letters in the same row indicate a difference between Mo sources.

Table 5. Total volatilized N-NH_3 predicted by the model after 30 days

	U			Uc			Ui			Rates
Rates	**Ma**	**Mt**	$\overline{X}$	**Ma**	**Mt**	$\overline{X}$	**Ma**	**Mt**	$\overline{X}$	$\overline{X}$
g Mo (100 kg of N)$^{-1}$					N-NH_3 (% of applied N)					
0	43.8	45.3	44.5 Ba	43.0	41.0	41.2 Ba	31.3	34.3	32.8 A a	39.8
150	44.5	43.9	44.2 Aa	42.8	44.6	43.7 Aa	47.6	41.1	44.4 Ab	44.1
300	40.7	45.0	43.9 Aa	38.5	47.3	42.9 Aa	47.9	45.7	46.8 Ab	44.2
600	45.1	43.6	44.3 Aa	43.6	45.4	44.5 Aa	46.3	43.3	44.8 Ab	44.5
1200	47.2	42.7	44.9 Aa	42.6	47.7	45.1 Aa	45.7	44.6	45.2 Ab	45.1
$\overline{X}$	44.3 *A*	44.1 *A*	*44.2*	42.1 *A*	45.2 *B*	*43.6*	43.8 *A*	41.8 *A*	*42.8*	*43.5*
$\overline{X}$ **Mo sources**			**Ma** 43.4					**Mt** 43.7		

U = urea, Uc = urea coated, Ui = incorporated, Ma = ammonium molybdate, Mt = molybdenum trioxide. Capital letters in the same row indicate a difference betwwen type of urea within the Mo rates and lower case letters in the column indicate a difference between Mo rates within the type of urea. Capital letters in italics in the same row indicate a difference between Mo sources within urea type.

Table 6. Pearson's correlation coefficient for variables subject to type of urea. sources and rates of molybdenum evaluated at the end of the experiment

	pH	**pHCaCl$_2$**	**pHwater**
Ntotal	0.38**	-0.17^{ns}	-0.18°
pH		-0.26*	-0.23*
pHCaCl$_2$			0.78**
pHwater			

^{ns}.$^{\circ}$.*.** = not significant and significant at 10. 5 and 1 % probability, respectively, by the F test.

3.4. Discussion

The volatilization process was evaluated under optimum conditions of urease activity. The average ambient temperature was kept at 25 ºC, the air humidity near 60%, soil moisture was kept at 60% of field capacity, sugarcane straw (enzyme source) was added, and nutrients were available for the proper enzyme functioning and reestablishment of the microbiota in the incubation period (Barth et al. 2020). Such condition favored N losses, this simulation was performed to select only the treatment that would withstand the environmental conditions most unfavorable to the application of this fertilizer. The favoring of urease activity culminated in N losses already in the first 24 hours after fertilizer application, reaching the highest rates near the eighth day after fertilizer application, and in the case of UiAm or UiMt only on the 12th d, like in the Christianson et al. (1990). Thought, some authors report that the maximum rate occurred earlier, near the 3th day after fertilizer application (Fillery and de Datta, 1986), this difference is associated with the experimental conditions and the rate of N applied (Silva et al. 2017).

Effect of Mo addition was expected to reduce N losses due to the results obteined in Tabatabi (1977), when Mo had an inhibitory effect on urease enzyme activity. The divergence between the results occurred because in the present work we evaluated the indirect activity of urease and the Mo sources were ammonium molybdate and molybdenum trioxide were used, while Tabatabi (1977) used molybdic acid and evaluated the activity in solution. In this condition there might have been an effect of acidity on enzyme activity, which was attributed to Mo. In addition the ratio between urea availability and the amount of urease was probably distinct, being a factor of great relevance to the final response.

θv was altered as a function of the treatments, and the efficiency of NBPT in reducing urease activity when incorporated into the granule was clear. It favored the distribution of the inhibitor in the soil and led to lower final N-NH_3 losses due to the higher proportion of occupied urease sites. The efficiency of NBPT in reducing volatilization losses was already expected, this effect was reported in literature by numerous papers, although it mostly occurs when the inhibitor is only added to the coating of the granule, which did not occur in our evaluations (Pan et al. 2016; Silva et al. 2017). This divergence from the data presented in the literature is possibly due to the favorable conditions for urease activity. In this case the addition of higher rates of NBPT could be efficient, as demonstrated by Mira et al. (2017) in the evaluation of six experiments in sugarcane fields.

The pattern of N loss evidence that under favorable conditions volatilization occurred constantly in the presence of urea in the system, as indicated by θx. After an average of 15 days

the rates were constant and close to 0.8 % of the N applied daily. This result is unsual in the evaluated field experiments, because urea application is done when there is a high probability of precipitation and consequently incorporation of urea into the soil (Cantarella et al., 2018).

The values of N lost were correlated with daily soil pH measurements by the semisolids electrode and also with pH values in water and calcium chloride on the last experimental day. The correlations indicated that soil pH monitoring using semisolids electrode is an alternative to predict the loss of N. This proposal had been previously performed in the literature, but with less efficient methodologies, such as laboratory pH determinations. In this case the sample preparation for determination can influence the result, this step being avoided with the sensor of semisolids.

Although the expected responses were not obtained, we have proven that the addition of Mo to urea without any other compound can be performed because this procedure does not influence the parameters of the model used as basis for comparison of the treatments, and does not influence the final N losses. That is, there will be no negative environmental impact from this formulation. The combination of NBPT and Mo as a search for a double strategy to reduce volatilization was inefficient, especially in the presence of Mt. The results indicated that the NBPT was in usable condition and had not been degraded, but the contact with Mo resulted in structure alteration of the inhibitor and loss of efficiency. The difference between molybdenum trioxide and ammonium molybdate is due to the difference in the oxidation state of Mo and also the specific surface area of the particles. The Mt, being nanoparticulated, presented a larger specific surface and therefore a greater reaction with the NBPT.

Many studies have evaluated potential urease inhibitors for addition to urea (Cantarella et al. 2018, Mariano et al. 2019), and in parts of the studies the results were opposite to what was expected or the molecules and chemical elements used were major environmental contaminants. Mo, as well as other micronutrients, B, Cu, and Zn (Cancellier et al., 2016; Rech et al., 2017; Adotey et al., 2017; Mariano et al., 2019) can be added to urea. Volatilization losses will not be minimized, but the logistics of using this nutrient as a fertilizer will be favored.

3.5. Conclusions

The addition of Mo to urea without NBPT, regardless of the source and rate, is a viable alternative because it doesn't increase the N loss by volatilization. This process would facilitate the distribution of Mo in the field. The addition of Mo to urea with NBPT is not feasible because there is a loss of efficiency of the inhibitor, indicating that there was degradation of the inhibitor;

the losses increase 10 % in the mixture of Mo trioxide and NBPT. The interaction between NBPT and Mo occurred differently depending on the addition method of the products to urea, because the addition method influences the particle contact. The direct contact between the products (Mo and NBPT), should not be used because it causes the N loss by volatilization in the same rate of urea.

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4. NITROGEN RECOVERY, GAS EXCHANGE AND ENZYME ACTIVITY IN SUGARCANE FERTILIZED WITH MOLYBDENUM ADDED TO UREA GRANULES

Abstract

Ethanol derived from sugarcane represents an important renewable energy source in the Brazilian economy. Nitrogen use efficiency (NUE) can contribute the sustainable replacement of petroleum by ethanol as energy source. Due to the molybdenum (Mo) contribution in the N metabolism, add Mo to urea can change N recovery by plantas and NUE. Our hyphotesis is that urea fertilizer enriched with Mo have the potential to improve sugarcane N-recovery (NRF). The experiment in factorial 2x2+1 was carried in blocks, with five repetitions. The treatments were composed by the combination of the method of Mo addition to urea, coated (Uc) or incorporated (Ui), molybdenum source, ammonium molybdate (Am) or nanoparticulated molybdenum trioxide (Mt), and the control treatment (- Mo, without addition of Mo). Fertilizers were granulated in laboratory using a disk granulator system. ^{15}N recovery by sugarcane, N and Mo content, photosynthetic rates and chlorophyll *a* fluorescence, and activity of nitrate reductase, glutamine synthetase, and urease were measured in sugarcane plant cultivated under controlled conditions. Mo addition to urea did'nt change NRF by sugarcane. Net photosynthesis was also non affected by Mo supply, but Mo supply altered the stomatal conductance in situation of increased temperatures, as well as influenced the activity of enzymes over the plant growth. Mo addition to urea is a feasible strategy to supply Mo to cultivated plants, but the effect of Mo in sugarcane growth wans't observed in this experiment.

Keywords: Molybdenum trioxide, Ammonium molybdate, Nitrate reductase, Photosynthesis, Fertilizers.

4.1. Introduction

Brazil is the world's largest producer of sugarcane, reaching production of 592,031 million tons, which are destined for the production of sugar and ethanol, that in 2021 will reach 25.8 billion liters (CONAB, 2021). Sugarcane ethanol have the potential to reduce greenhouse gas emissions (Zabed et al. 2017). However, the N use efficiency (NUE) of sugarcane is low and interferes with its performance as a sustainable energy source (Erisman et al. 2010). Nitrogen is widely used in agriculture and its inadequate management has contributed to the greenhouse effect due to the emission of N, N_2O and NO_2 into the atmosphere (Crutzen et al. 2007). Annually 107.74 million tons of N are used (FAO, 2019) in the world which may result in potencial 5.39 million tons of gases in the atmosphere. In this way, alternatives that aim to improve NUE are important, either by reducing losses or by increasing the utilization of the crop (Crutzen et al. 2007, Bernhard, 2010).

Numerous N sources are available on the market, but urea is responsible for approximately 60 % of N fertilizer used in agriculture (FAO, 2019). This source has the advantage of lower cost and higher concentration of N, but when misused the losses can reach

68 % of the N applied, causing inefficiency in the fertilizer (Cantarella et al. 2018, Mariano et al. 2019). There is a broad search for more efficient solutions, since the best alternative is currently N-(n-butyl) thiophosphoric triamide (NBPT) which reduces urea losses by 50 % (Silva et al, 2017) and only 30 % of the applied nitrogen is absorbed by the plants (Gava et al. 2003, Otto et al. 2016). Thus, there is a gap in nitrogen fertilizer development that needs to be addressed. New alternatives to improve the utilization of N-urea need to be evaluated in order to make agriculture more sustainable. One possible alternative is the use of micronutrients combined with urea and NBPT, because micronutrients besides contributing to reduce urease activity can be absorbed by plants and increase productivity. Elements such as Zn, Cu and B (Cancellier et al., 2016, Rech et al., 2017, Adotey et al. 2017, Mariano et al. 2019) are frequently studied in the literature but other micronutrients also have potential for use, one of them is Mo (Tabatabai, 1977).

Mo is responsible for acting in the first step of nitrate reduction, turning it into nitrite. The nitrate reduction process is critical, since only ammonium can be assimilated (Mendel 2011, Reyes et al. 2018). When nitrate to ammonium conversion is low the plant accumulates nitrate in the vacuole and reduces its nitrogen fertilizer utilization efficiency (Ide et al. 2011). In addition, high rates of nitrate are responsible for pest and disease attack as this is a signal that the sap content is rich in amino acid. Because it is a nutrient required in very small amounts, Mo does not always show an effect on biomass due to experimental variations, but we can observe effects on photosynthetic activity and biochemistry of plants that indicate better functioning of the structure linked to N metabolism (Polidoro, 2001, Hu et al. 2002, Yu et al. 2006, Mellis et al. 2017, Santos et al. 2018). Another important factor is related to the Mo ability to influence resistance to water deficit and heat stress in plants, due to its participation in the synthesis of abscisic acid and possible influence on stomatal regulation, favoring the maintenance of productivity under adverse conditions (Sun et al. 2009, Xiong et al. 2001).

Mo addition to the soil has the advantage that the nutrient is available throughout the entire cycle of the crop. Currently, the Mo source most used in agriculture is sodium molybdate, but this has a high pH, a point not presented by ammonium molybdate. Both sources are soluble and their availability to plants is immediate. Recent studies point to the use of nanoparticulate molybdenum trioxide in agriculture, a source of low solubility, moreover, molybdenum is in a form not absorbed by plants and needs to be transformed into molybdate to act in the metabolism. Nanoparticles show controversial results, especially regarding the mode of application (Huang et al. 2021, Sharma et al. 2021). The used source of micronutrient can directly interfere with the inhibition of urease activity and the utilization efficiency of the plant. Therefore, it is necessary to evaluate the effect of commercially available sources and their mode

of application. We hypothesized that the use of Mo combined with urea will increase the efficiency of N recovery by sugarcane plants. Our goal was to evaluate the recovery of N-fertilizer applied urea with Mo coated and incorporated into the granule.

4.2. Material and methods

4.2.1. Experimental conditions

The ^{15}N recovery was evaluated in a greenhouse experiment at ESALQ/USP. The incomplete factorial 2x2+1 was carried in randomized block design, with five repetitions, totaling 25 experimental units. The factors evaluated were addition mode of Mo urea (coated-Uc or incorporated-Ui), Mo sources (ammonium molybdate-Am and molybdenum trioxide-Mt) at a rate of 600 g per 100 kg of N, and the additional treatment without molybdenum (- Mo, control). The fertilizers granulated and prepared prior to application were labeled urea containing 2.666 % of ^{15}N.

The soil was collected in the superficial, 20 cm layer, in an area cultivated with sugarcane for 15 years, to preserve the microbiota. The soil was characterized according to Gee and Or. (2002) and Raij et al. (2001), presenting values of pH ($CaCl_2$), organic matter, phosphorus, sulfur, potassium, calcium, magnesium, hydrogen+aluminium, boron, copper, iron, manganese, zinc, molybdenum respectively of 4.9, 13.0 g dm^{-3}, 56.0 g dm^{-3}, 7.0 mg dm^{-3}, 1.2 $mmol_c$ dm^{-3}, 15.0 $mmol_c$ dm^{-3}, 5.0 $mmol_c$ dm^{-3}, $mmol_c$ dm^{-3}, 0.1 mg dm^{-3}, 4.5 mg dm^{-3}, 67 mg dm^{-3}, 3.3 mg dm^{-3}, 6.6 mg dm^{-3}, below the limit of quantification. The soil was classified as Entisols, sandy texture and the content of clay, silt and sandy were 50, 16 and 934 g kg^{-1}, respectively.

Sugar cane seedlings (variety CTC4) with approximately 30 cm were transplanted after some procedures (Suppl. Figure 3A). The seedlings were obtained from commercial nurseries, free of pests and diseases. Before to the transplanting, the seedlings were grown for 30 d in the greenhouse receiving only deionized water to minimize the effect of the germination substrate. During the planting, the seedlings were immersed in deionized water and the substrate completely removed and a sample of the seedlings was directed for characterization according to Malavolta et al. (1997) methodology. The seedlings presented values of nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, iron, manganese, copper, zinc, boron, nickel and molybdenum, respectively of 0.8 g kg^{-1}, 1.3 g kg^{-1}, 4.8 g kg^{-1}, 1.3 g kg^{-1}, 2.4 g kg^{-1}, 0.9 g kg^{-1}, 129.0 mg kg^{-1}, 34.8 mg kg^{-1}, 8.9 mg kg^{-1}, 28.8 mg kg^{-1}, 4.8 mg kg^{-1}, 10.3 mg kg^{-1} and 11.0 mg kg^{-1}.

The seedlings were transplanted into pots with 18 dm^{-3} of soil, with constant humidity of 60 % of the maximum water holding capacity. After transplanting, the seedlings were grown

for 50 days without the Mo and N addition (Suppl. Figure 3B). Fertilization with the remaining nutrients for the full development of the plants was applied three times, in the form of nutrient solution. The first application (50 % of the total rate) was performed 10 days after transplanting (DAT) and the two other times, 25 % of the total recommended, were applied respectively 40 and 65 d after transplanting. The nutrient solution was applied to the soil surface homogeneously in the pot. The total nutrients supplied were those adapted from Malavolta et al. (1980), containing P (200 mg dm^{-3}), K (150 mg dm^{-3}), Ca (75 mg dm^{-3}), Mg (50 mg dm^{-3}), S (60 mg dm^{-3}), B (1.5 mg dm^{-3}), Cu (1.5 mg dm^{-3}), Fe (5 mg dm^{-3}), Zn (5 mg dm^{-3}), Mn (4 mg dm^{-3}) (Figure 1). N was applied as urea at a rate of 150 mg dm^{-3} and Mo was added to urea at a rate of 600 g per 100 kg of N, 50 DAT.

At 65 DAT, the plants suffered from an infestation of aphis and mites, which required the application of abamectin twice until the end of the cycle. Figure 1 shows all the management and activities performed during the development of the plants, as well as the temperature and humidity.

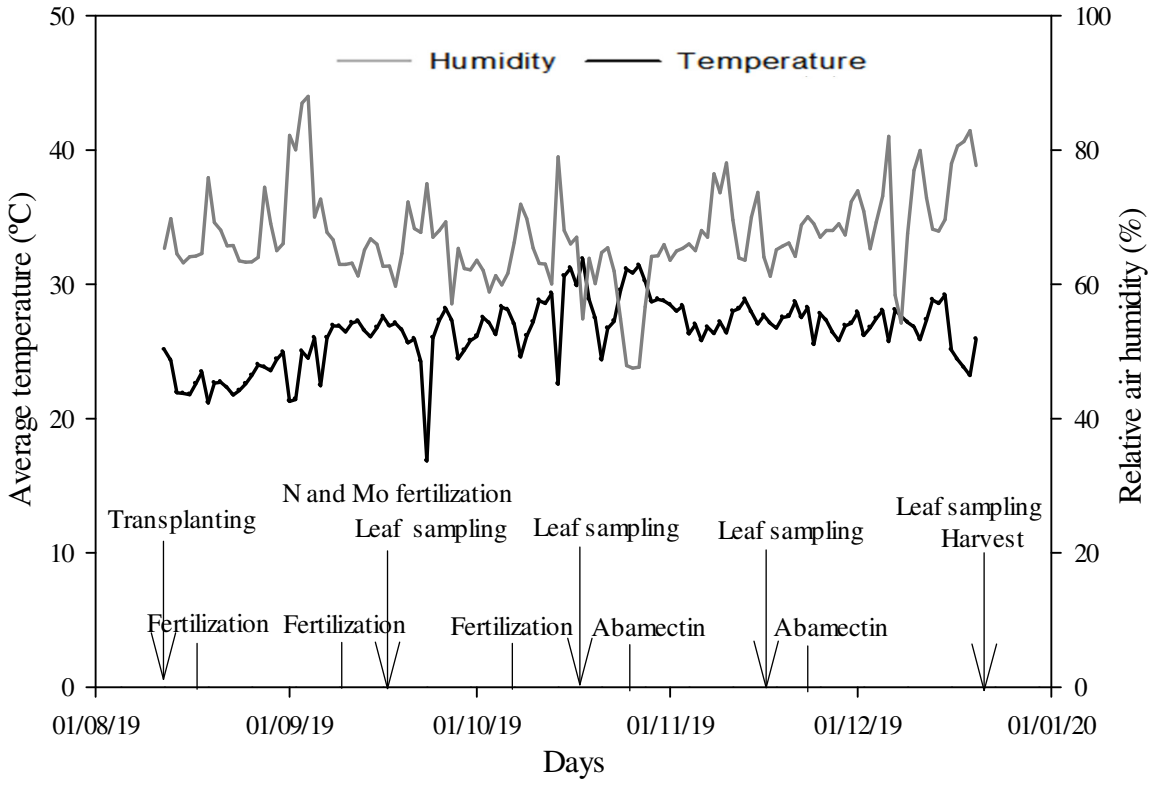

Figure 1. Management and leaf sampling performed throughout the experimental period. The leaf sampling were performed at 50, 80, 110 and 140 days after transplanting.

4.2.2. Plant measurements

Temporal evaluations were performed to determine N and Mo content, urease, nitrate reductase and glutamine synthetase activity, in addition to the gas exchange determination and chlorophyll a fluorescence (Chl *a*). At harvest, 140 DAT, the plants were compartmentalized into tillers+leaves, stalk, and roots for ^{15}N determination and Mo content (Suppl. Figure 3D). The compartments were oven-dried (65 °C for 72 h) and then the dry mass was determined.

In the leaf samples, at 50, 80, 110 and 140 DAT the isotopic abundance and total N content was determined, allowing the calculation of nitrogen derived from fertilizer (Ndff). Total N and ^{15}N abundance was determined with an automatic N analyzer connected to an isotope ratio mass spectrometer. At harvest, were calculated plant N from fertilizer (Ndff, %), and plant N-fertilizer recovery (Rnp, %) according to Trivelin et al. (1994) by equation 1, 2 and 3. In addition, N content in plant compartments was determined (g pot^{-1}). In the same leaves and compartments the Mo content was determined according to Malavolta et al. (1997). Mo content was used to calculate the absorption efficiency, as suggested by Swiader et al. (1994).

$$\text{Ndff (\%)} = (a/b) \times 100 \quad (1)$$

$$\text{Ndff (g/pot)} = (\text{Ndff(\%)}/100) \times \text{Total N} \quad (2)$$

$$^{15}\text{N recovery (\%)} = (\text{Ndff} / \text{NR}) \times 100 \quad (3)$$

where: Ndff is the nitrogen in the plant or soil derived from the fertilizer (% and g pot^{-1}), a and b are the ^{15}N abundance (atom % ^{15}N excess) in the plant and the fertilizer respectively, Total N is the nitrogen content of the plant (g pot^{-1}), and NR is the amount of N applied per pot (g pot^{-1}).

One day before leaf sampling for nutrient determination, photosynthetic parameters were determined using an infrared gas system (IRGA, model LI-6400) and Chl *a* with an attached fluorometer. At night, during the dark period (between 01:00 a.m and 03:30 a.m), the initial fluorescence and maximum fluorescence was obtained to calculate the potential quantum yield of photosystem II (FSII) (Genty et al., 1989). Associated with the variable fluorescence in light-adapted sample before the saturation pulse and maximum fluorescence in light-adapted sample (between 08:30 a.m and 12:30 p.m) (Suppl. Figure 3C), obtained with actinic light pulse, the forms of energy dissipation were calculated (Genty et al., 1989, Hendrickson et al., 2004) (Eq. 4, 5, 6). The effective quantum yield of photochemical energy conversion in FSII was used to estimate the apparent rate of electron transport (Bilger and Björkman, 1990) (Eq. 7). Daytime period, between 08:00 a.m and 12:00, were determined net assimilation rate (*A*) and stomatal conductance (*gs*). Measurements were performed using photosynthetically active radiation of 1500 μmol m^{-2} s^{-1}, predetermined in light curves for these experimental conditions, CO_2

concentration of about 400 μmol mol^{-1}, block temperature of 28 °C, air humidity in the block of 55 %, and flux of 500 μmol s^{-1}.

$$\hat{Y}_{II} = (Fm` - F)/Fm \quad (4)$$

$$\hat{Y}_{NPQ} = (F/Fm`) - (F/Fm) \quad (5)$$

$$\hat{Y}_{NO} = F/Fm \quad (6)$$

$$ETR = \hat{Y}_{II} \times PAR \times 0.84 \times 0.5 \quad (7)$$

where F is the fluorescence of a light-adapted sample before the saturation pulse, Fm' is the maximum fluorescence of a light-adapted sample, Fm is the maximum fluorescence, and PAR is the photosynthetically active radiation.

The leaves used in gas exchange were sampling for enzymatic analysis. After sampling, the material was immediately stored in liquid nitrogen to inactivate physiological degradation processes, and subsequently stored in a -80 ºC freezer. The activities of the enzymes nitrate reductase (NR), glutamine synthetase (GS) and urease (US) were determined. US activity was determined by method McCullough (1967) and Hogan et al. (1983), and NR was determined according to Cambraia et al. (1989), except a twice larger volume of extract was used for the reaction and the samples were incubated at 37 °C. The results were expressed as nmol NO_2^- h^{-1} g^{-1} FW (fresh weight). And GS activity was determined by the method of Elliott (1953) with modifications (Moreira et al. 2020), results expressed in μmol of γ-glutamylhydroxamate h^{-1} g^{-1} of FW (μmol GH h^{-1} $(g\ FW)^{-1}$).

4.2.3. Statistical Analysis

The data normality (Shapiro-Wilk-Test) and homogeneity of variance (Bartlett-Test) were evaluated, as well as, outliers by the Grubb-Test and, removed when present. Data was subject to analysis of variance (ANOVA) and when the F-Test was significant ($p \leq 0.05$), the means were compared by the LSD-Test, at the 0.05 confidence level.

4.3. Results

4.3.1. ^{15}N recovery, N content and biomass

The Ndff was not influenced by the factors in any of the sampling, averaging 0.368 % at 50 dDAT, a value that represents the natural abundance of 15N in the leaves. The isopotically labeled fertilizer was applied soon after the first leaf collection. At 80, 110 and 140 DAP the

plants leaves presented respectively Ndff of 54.0, 77.1 and 70.5 % (Figure 2). The biomass in the tiller+leaf and root compartments had average of 174.1 and 118.0 g pot^{-1}, respectively (Table 1). There was a response to the Mo source in the talk compartment and it was observed that the Mt addition increased 11.6 % mass. Although the stalk biomass was greater with the Mt addition, it was not repeated in the total plant biomass. Furthermore, in all compartments the response of the control (-Mo) was equivalent to the factorial mean (+Mo) (Table 1). The factorial average biomass was 393.3 g pot^{-1} while that of the control treatment was 407.8 g pot^{-1} (Table 2) (Suppl. Figure 3E).

Ntotal of the tiller+leaf and root compartments as well as the biomass was not influenced by the variation factors, neither by the addition of Mo. N content in this compartments averaged was 0.06 and 1.95 g pot^{-1}, respectively (Table 1). The stalks presented a response to the method of Mo adding to urea. The treatments where Mo was incorporated into the urea granule resulted in N accumulation 2.1 % higher than the addition of coated Mo, although the average of the Mo addition was the same as the control, 1.2 g pot^{-1} of N (Table 1). N accumulation in the plant was 1.3 % higher when Mo was incorporated into urea, following the same pattern as in the stalks, although the average addition of Mo did not differ from the control treatment, with 3.8 g pot^{-1} of N (Table 2).

Ndff, the N absorbed from fertilizer, was the same among the study factors for tiller+leaf and for the stalk, with an average of 32.3 and 70.7%, respectively, a value also obtained for the control average (Table 1). In the roots, the Ndff average of the combination of the urea type and Mo source was 4.8% higher than the control treatment (Table 1), indicating that in the Mo presence, the N absorption from fertilizer is greater regardless of the Mo source and the mode of addition to the fertilizer.

The RNP was not influenced by the experimental factors (Figure 3), as well as the N recovered by the soil (data not shown), average of 12.2 % of the N applied. The average N recovered by the plant was 81.0 %.

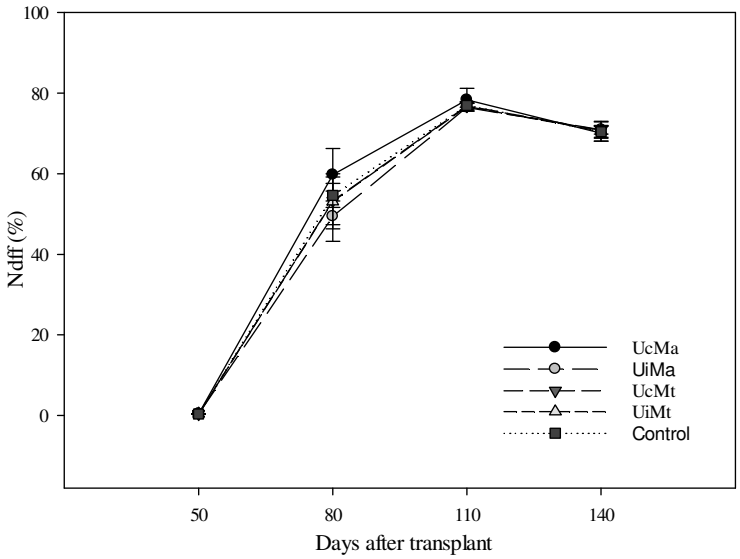

Figure 2. Nitrogen derived from fertilizer (Ndff) in the diagnostic leaf over 140 days after transplanting. Bar represent the standard error.

Table 1. Biomass, N content (Ntotal) and plant N from fertilizer (Ndff) compartments at 140 days after transplanting

	Mo sources			Mo sources			Mo sources		
	Am	**Mt**	**$\bar{X}$**	**Am**	**Mt**	**$\bar{X}$**	**Am**	**Mt**	**$\bar{X}$**
	Tiller+Leaves			**Stalk**			**Root**		
Type				**Biomass (g pot^{-1})**					
Uc	173.8±15.4	171.8±8.9	172.8±11.9	99.1±8.9	107.9±11.5	103.5±10.8	109.3±29.7	119.1±21.7	114.2±25.0
Ui	168.3±4.7	178.2±15.0	173.2±11.7	97.7±8.9	111.7±5.9	104.7±10.2	120.3±16.3	116.1±24.9	118.2±20.0
$\bar{X}$	171.0±11.1	175.0±12.1	*173.0±11.5*	98.4±8.4 B	109.8±8.8 A	*104.1±10.2*	114.8±23.3	117.6±22.1	*116.2±22.2*
Control			*175.2±5.8*			*107.2±7.5*			*125.4±5.6*
p_{urea}	0.91			0.77			0.44		
p_{Mo}	0.32			0.01			0.67		
$p_{Urea*Mo}$	0.14			0.53			0.77		
$p_{\bar{X}*Control}$	0.62			0.49			0.47		
CV (%)	5.00			8.68			18.1		
				Ntotal (g pot^{-1})					
Uc	1.9±0.1	1.9±0.1	1.9±0.1	1.2±0.1	1.2±0.1	1.2± 0.1 b	0.51± 0.1	0.52± 0.1	0.51± 0.1
Ui	2.0±0.1	2.0±0.1	2.0±0.1	1.3±0.1	1.3±0.1	1.3± 0.1 a	0.52± 0.1	0.51± 0.1	0.52± 0.0
$\bar{X}$	1.9±0.1	2.0±0.1	*2.0±0.1*	1.2±0.1	1.3±0.1	*1.2±0.1*	0.52± 0.1	0.52± 0.1	*0.52± 0.1*
Control			*2.0±0.2*			*1.3±0.0*			*0.53± 0.1*
p_{urea}	0.09			0.01			0.84		
p_{Mo}	0.16			0.54			0.95		
$p_{Urea*Mo}$	0.91			0.86			0.59		
$p_{\bar{X}*Control}$	0.70			0.23			0.63		
CV (%)	4.56			7.41			13.78		
				Ndff (%)					
Uc	30.9±1.4	34.5±2.7	32.7±.28	71.0±2.1	70.9±2.2	70.9±2.2	53.6±1.3	54.7±2.6	54.1±2.0
Ui	33.2±2.7	33.0±2.9	33.1±2.6	70.4±2.5	70.7±2.0	70.5±1.9	53.5±2.2	53.4±2.6	53.4±2.3
$\bar{X}$	33.0±2.3	33.7±2.8	*32.9±2.6*	70.7±2.2	70.6±2.0	*70.7±2.0*	53.5±1.7	54.0±2.5	*53.8 ±2.1A*
Control			*31.7±1.1*			*71.5±0.8*			*51.3 ±0.9 B*
p_{urea}	0.73			0.67			0.48		
p_{Mo}	0.13			0.97			0.63		
$p_{Urea*Mo}$	0.09			0.82			0.53		
$p_{\bar{X}*Control}$	0.32			0.50			0.04		
CV (%)	7.23			2.94			4.04		

Uc = urea coated, Ui = urea incorporated, Am = ammonium molybdate, Mt = molybdenum trioxide. Capital letter in the same row indicate difference between Mo source. Lower case letter in the same column indicate difference between urea type. Capital letters in italic indicate difference between the factorial mean and control by LSD test (p<0.05). Average followed by standard error.

Table 2. Biomass and N content (Ntotal) per pot 140 days after transplanting

	Mo sources			Mo sources		
	Am	**Mt**	$\overline{X}$	**Am**	**Mt**	$\overline{X}$
			Whole plant			
Type	**Biomass (g pot^{-1})**			**Ntotal (g pot^{-1})**		
Uc	382.2±37.7	398.8±23.0	390.5±30.8	3.6±0.1	3.8±0.2	3.6±0.2 a
Ui	386.3±24.7	406.0±28.4	396.1±27.2	3.8±0.2	3.6±0.1	3.8±0.2 b
$\overline{X}$	384.2±384.2	402.4±402.7	*393.3±28.4*	3.7±0.2	3.7±0.2	*3.7±0.2*
Control			*407.8±15.7*			*3.8±0.1*
p_{urea}	0.63			0.03		
p_{Mo}	0.13			0.57		
$p_{Urea*Mo}$	0.89			0.86		
$p_{\overline{X}*Control}$	0.27			0.31		
CV (%)	6.52			5.02		

Uc = urea coated, Ui = urea incorporated, Am = ammonium molybdate, Mt = molybdenum trioxide. Lower case letter in the same column indicate difference between urea type by LSD test (p<0.05). Average followed by standard error.

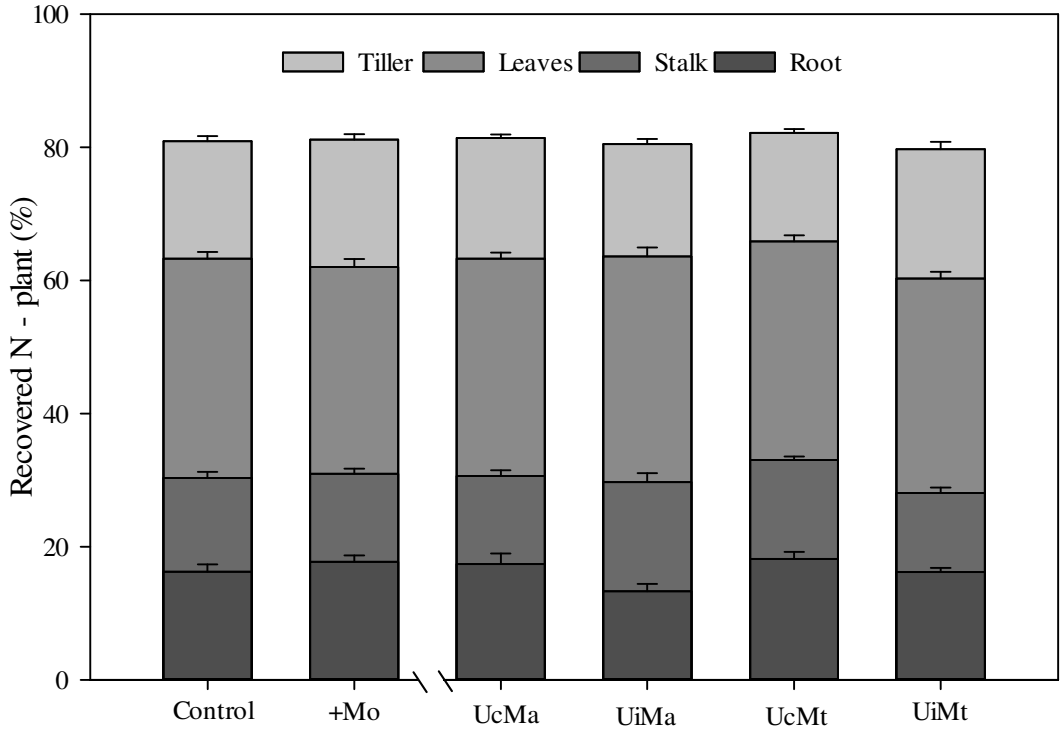

Figure 3. Recovery of N from fertilizer (RNF) in plant compartments at harvest (140 DAT). Uc = urea coated, Ui = urea incorporated, Am = ammonium molybdate, Mt = molybdenum trioxide. Average followed by standard error.

4.3.2. Mo concentration and accumulation

Mo concentration in the diagnostic leaf throughout time was influenced by the factors (Table 3). At 80 DAT the average of the factorial was approximately 9 times higher than the -Mo. In this period it is possible to observe that the performance of the Mo source was a function of the addition method to the urea. The UiAm and UcMt were the treatments with the highest leaf

contents. At 100 DAT the treatments had a reduction in Mo content, possibly due to a dilution effect, caused by plant growth. Even under this condition, the difference between the addition or non-addition of Mo was maintained. For the treatments - Mo, there was an inversion of the response, with the UcAm and UiMt treatments having the highest average Mo content in the plant tissue. In the last sampling, there was also a difference between the addition or not of Mo, being the -Mo with a average of 0.8 mg kg^{-1} and the +Mo of 6.7 mg kg^{-1}(Table 3). The Mo contents of the leaves in the control treatment over time are those commonly reported in literature, close to 1 mg kg^{-1}.

The UcMt showed a different behavior. In general, the treatments maintained high leaf Mo contents over time, while this one did not, presenting a reduction in the Mo content in the diagnostic leaf. This indicates that the plant did not absorb the Mo after 80 DAT, and or did not translocate the absorbed element. Based on the Mo content and the UE Mo was absorption, the reduction of the content being explained by the low translocation in the plant.

Mo content of the compartments was influenced by the Mo addition, as well as the leaves content. For tillers+leaves the behavior of the data was similar to the diagnostic leaf, the highest Mo contents were found for Mt and Ui, with no interaction between the factors. For the total Mo content in the plant, there was an interaction between the factors, indicating that the UiMt promoted the greatest accumulation of Mo, although there was no difference in the final biomass of the plants (Table 4). This response is due to the higher levels of Mo in the compartment of this treatment, since the final biomass was not influenced, being also the treatment with the highest Mo levels in the diagnostic leaf. As the biomass was the same, the UE responded to the Mo addition, demonstrating that regardless of the urea type and Mo source, the Mo absorption was the same.

Table 3. Molybdenum content in leaves throughout the experimental period and molybdenum content in the compartments after harvest (140 DAT)

	Mo sources			Mo sources			Mo sources		
	Am	**Mt**	**$\overline{X}$**	**Am**	**Mt**	**$\overline{X}$**	**Am**	**Mt**	**$\overline{X}$**
	80 d			**110 d**			**140 d**		
Type				**Mo concentration (mg kg^{-1})**					
Uc	8.6±1.7 Bb	14.0±1.2 Aa	11.3±3.2	9.1±1.4 Aa	4.3±0.3 Bb	6.7±2.7	8.0±1.3 Aa	2.2±0.3 Bb	5.1±3.1
Ui	14.0±2.9 Aa	11.4±1.1 Bb	12.7±2.5	6.0±1.2 Bb	11.8±2.7 Aa	9.0±3.7	6.0±0.7 Bb	10.4±1.6 Aa	8.2±4.02
$\overline{X}$	11.3±3.6	12.7±1.7	*12.0±2.8 A*	7.6±2.1	8.1±4.4	*7.7±3.3 A*	7.0±2.7	6.3±4.4	*6.7±3.6 A*
Control			*1.4±0.1 B*			*0.8±0.1 B*			*0.8±0.1 B*
p_{urea}	0.09			<0.01			<0.01		
p_{Mo}	0.09			0.46			0.04		
$p_{Urea*Mo}$	<0.01			<0.01			<0.01		
$p_{\overline{X}*Control}$	<0.01			<0.01			<0.01		
CV (%)	18.03			23.61			16.71		
	Tiller+Leaves			**Stalk**			**Root**		
				Mo content (mg pot^{-1})					
Uc	0.8±0.1	0.9±0.0	0.9±0.1 b	0.1±0.02 Aa	0.1±0.00 Aa	0.1± 0.01	0.5± 0.1	0.6± 0.1	0.6± 0.1
Ui	0.9±0.2	1.3±0.4	1.1±0.3 a	0.1±0.01 Bb	0.2±0.04 Aa	0.2± 0.07	0.5± 0.2	0.5± 0.1	0.5± 0.1
$\overline{X}$	0.9±0.1 B	1.1±0.3 A	*1.0± 0.3 A*	0.1±0.01	0.2±0.06	*0.13±0.1 A*	0.5± 0.1	0.6± 0.1	*0.6± 0.1 A*
Control			*0.3± 0.1 B*			*0.03±0.0 B*			*0.2± 0.0 B*
p_{urea}	<0.01			<0.01			0.10		
p_{Mo}	<0.01			<0.01			0.06		
$p_{Urea*Mo}$	0.06			<0.01			0.65		
$p_{\overline{X}*Control}$	<0.01			<0.01			<0.01		
CV (%)	22.47			20.06			21.12		

Uc = urea coated, Ui = urea incorporated, Am = ammonium molybdate, Mt = molybdenum trioxide. Capital letter in the same row indicate difference between Mo source. Lower letter in the same column indicate difference between urea type. Capital letters in italic indicate difference between the factorial mean and control by LSD test ($p<0.05$) Average followed by standard error.

Table 4. Molybdenum content (Mo content) and molybdenum uptake efficiency (UE) in sugarcane plants

	Mo sources			Mo sources		
	Am	**Mt**	$\overline{X}$	**Am**	**Mt**	$\overline{X}$
			Whole plant			
Type	**Mo content (mg pot^{-1})**			**UE (g kg^{-1})**		
Uc	1.4±0.2 Aa	1.5±0.1 Ab	1.5±0.2	13.4±2.1	13.3±0.2	13.4±2.7
Ui	1.6±0.2 Ba	2.2±0.4 Aa	1.9±0.4	12.9±2.4	17.3±0.1	15.1±4.1
$\overline{X}$	1.5±0.2	1.7±0.4	*1.7±0.4 A*	13.2±2.2	15.3±4.4	*14.2±3.5 A*
Control			*0.5±0.0 B*			*4.2±0.6 B*
p_{urea}	<0.01			0.03		
p_{Mo}	<0.01			0.57		
$p_{Urea*Mo}$	0.03			0.86		
$p_{\overline{X}*Control}$	<0.01			0.31		
CV (%)	16.46			5.02		

Uc = urea coated, Ui = urea incorporated, Am = ammonium molybdate, Mt = molybdenum trioxide. Capital letter in the same row indicate difference between Mo source. Lower letter in the same column indicate difference between urea type. Capital letters in italic indicate difference between the factorial mean and control by LSD test ($p<0.05$). Average followed by standard error.

4.3.1. Photosynthetic rates, Chl*a* fluorescence and enzyme activity

The net assimilation of CO_2 (*A*) was not influenced by the treatments, but the stomatal conductance (*gs*) was influenced at 140 DAT by the addition of molidenium and by the fators interaction (Figure 4A). In this period the +Mo and UcAm provided greater *gs* (Fgirua 4B). The stomatal conductance results at 140 DAT show similar behavior to the Mo content in the leaves, indicating a relationship between the two variables. The response only to this period may have been influenced by the temperature of the environment, after the 110 DAT, the temperature increased, reaching values of 45 ºC, evidencing the role of Mo in heat stress conditions. In other words, under stress conditions the plant reduced the *gs* to reduce water loss, this fact reduces the photosynthetic activity. The reduction in photosynthetic rate was not significant, indicating that despite controlling gas exchange, Mo did not reduce plant growth.

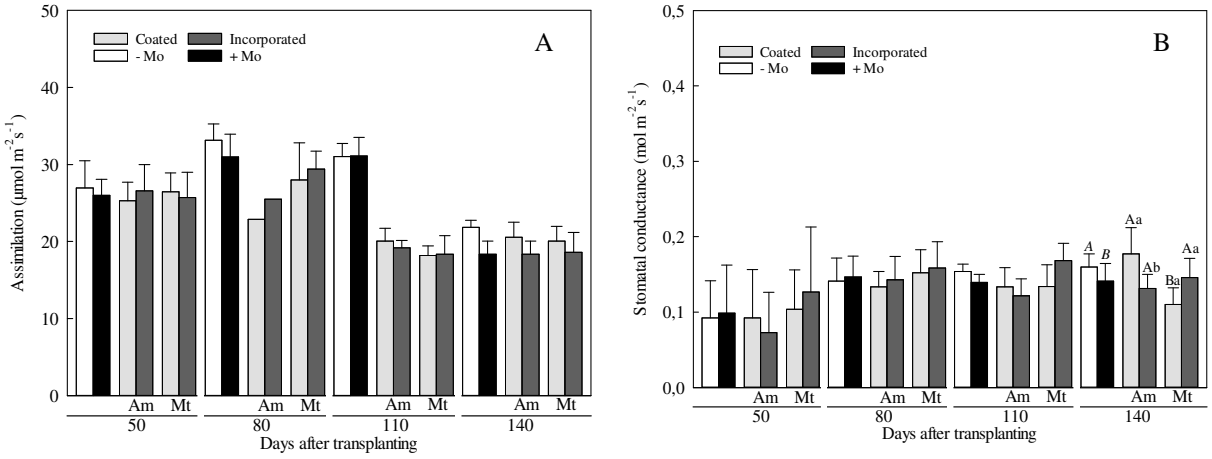

Figure 4. Response of photosynthetic parameters to molybdenum addition mode and source. CO_2 assimilation rate (A) and stomatal conductance (B). Capital letter indicate difference between urea type, lower letter indicate the difference between Mo source, capital letters in italics indicate the difference between Mo addition and no addition compared by LSD test ($p<0.05$). Bars indicate the standard error.

Mo application did not alter the apparent rate of electron transport and the amount of energy used in photosynthesis (Figure 5A, D), because the application of Mo and N was made after the measurement of 50 DAT. There were no changes in the energy dissipated in the form of fluorescence (Figure 5B) and in the modulated energy dissipation (Figure 5C), as in the previous cases, indicating that the availability of Mo does not influence the fluorescence of Chl *a* and the ability of photosytems to oxidize. The addition of Mo did not alter the electron transport and energy utilization performed by the plant even though it promoted alteration in stomatal conductance.

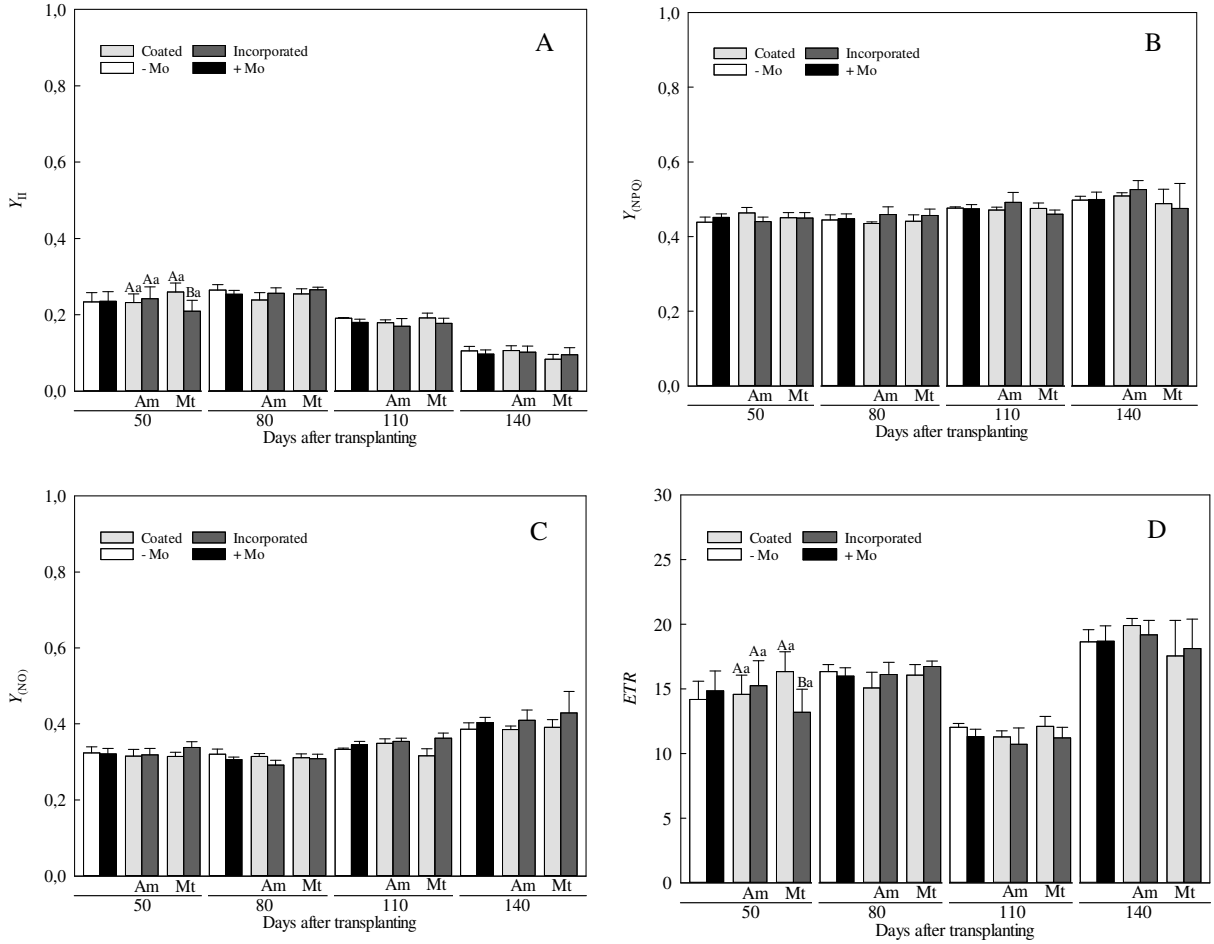

Figure 5. Effective quantum yield of linear electron flow through FSII (Y_{II}, A), regulated dissipative energy quantum yield (Y_{NPQ}, B), unregulated dissipative energy quantum yield (Y_{NO}, C) and apparent electron transport rate (*ETR*, D) in sugarcane plants as a function of molybdenum addition mode and source. Capital letter indicate difference between urea type, lower case letter indicate difference between Mo source compared by LSD test ($p<0.05$). Bars indicate the standard error.

Enzyme activity showed greater sensitivity to experimental factors and also to experimental conditions, as can be seen by the response of the data at 50 DAT (Figure 6A). The GS activity showed an effect in all periods the interaction of factors. At 80 DAT the GS activity was lower in the control (-Mo) compared to the factorial (+Mo), and at 140 DAT the opposite occurred. The UcAm showed the highest enzymatic values at 80 and 110 DAT, being on average 37 % and 27 % higher. At 140 DAT the Uc treatments were superior except for the Am, where the Ui was 40 % superior to the Uc. The Uc presented higher enzymatic rates until 110 DAT and later the treatments with incorporated Mo presented higher enzymatic rates.

NR activity was influenced by Mo addition only at 140 DAT, 12 % higher, due to lower N availability, since the UE was lower of Mo and led to lower Ndff of the roots (Table 1 and 6). That is, at lower N concentrations from fertilizer the plant increased NR activity. As well as for GS, treatments with coated Mo showed higher values at the beginning of plant development, but

at the end this condition changed. For NR the treatments with Mt presented the best performance (Figure 6B). For US activity it was observed that the factorial average did not differ from the control average control, but there was a response to interaction of the factors, which means that part of the treatments reduced the enzymatic activity (Figure 6C). At 80 and 140 DAT the UcMA was superior in relation to the other treatments by 19 and 3 %, on the average, but was equal to the UiMt.

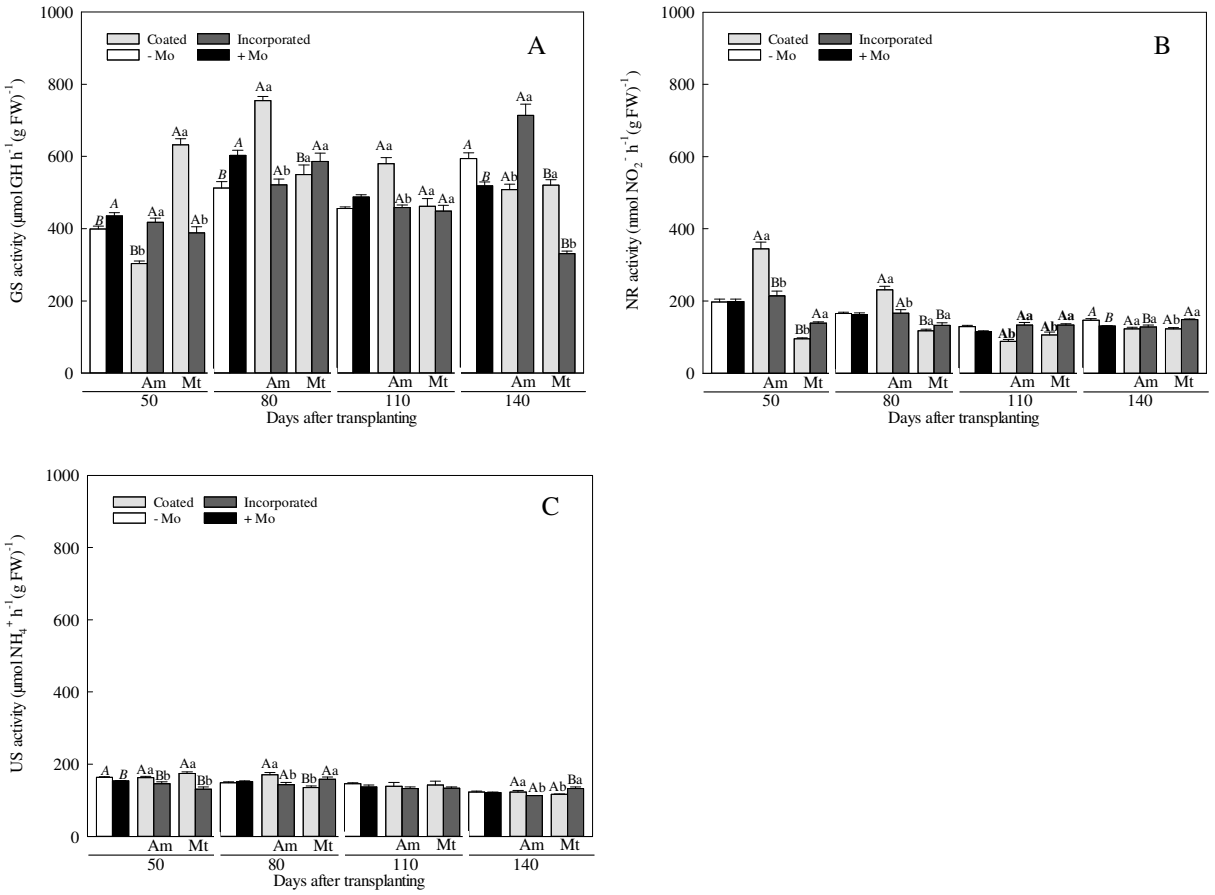

Figure 6. Glutamine synthetase (GS, A), nitrate reductase (NR, B) and urease (US, C) in sugarcane plants as a function of the mode of addition of molybdenum to urea and the source of molybdenum. Capital letters indicate difference between Mo source, lower case letters indicate difference between the Mo type urea, capital letters in italics indicate difference between Mo addition and the factorial average, bold capital letters indicate difference between source of Mo without interaction of factors, bold lower case letters indicate difference between the type of urea without interaction of factors by LSD test ($p<0.05$). Bars indicate the standard error.

4.4. Discussion

There is a scarcity of studies in the literature about the interaction of Mo and N in sugarcane plants (Santos et al. 2019, Santos et al. 2021), and the same occurs for the effect of Mo on productivity (Polidoro, 2001, Oliveira, 2012, Mellis et al. 2017). Associated with it, the results

obtained up to date are controversial. Oliveira (2012) found no effect of Mo application, whereas Polidoro (2001) reported a 25 % increase in yield with the application of 400 g ha^{-1} of Mo, and Mellis et al. (2017) showed that yield increased with the addition of 2 kg ha^{-1} of Mo in three of the eleven locations evaluated. The absence of response to Mo can be influenced by many factors. However, based on the data obtained so far, it is clear that some parameters commonly used in the literature, such as the critical leaf level, need to be reviewed.

Mo content in the control, without Mo application, was 1.0 mg kg^{-1}, higher than the literature, of 0.6 mg kg^{-1} (Polidoro et al. 2001, Santos et al. 2019.). Whereas for the treatments with Mo, the values ranged from 4.3 to 14 mg kg^{-1} depending on the sampling season and Mo source. Mellis et al. (2017) obtained values close to 6 mg kg^{-1} Mo in leaves. So, the values addressed in the literature of 0.6 mg kg^{-1} and the critical level of 1 mg kg^{-1} (Raij et al. 1997) are below the contents presented under conditions of nutrient response or productivity maintenance. In this study, mainly due to the location of the experiment and application of the nutrient the values were higher than Mellis et al. (2017). The restriction of the root system in the pot favors the nutrient uptake. Even with contents of 14 mg kg^{-1} no symptoms of toxicity were identified which highlights a wide tolerance range of the nutrient and supports the use of higher values for the critical level.

The restriction of the root system to a volume of soil favored not only Mo uptake, but also N-fertilizer recovery, averaging 81 % (Figure 3). In addition, urea application was incorporated to minimize volatilization losses, a factor that when present contributes to low efficiency of this fertilizer (Silva et al. 2017). Under field conditions, N-fertilizer utilization is close to 30 % in sugarcane (Otto et al. 2016). The same happened for quantified Ndff from leaf +1, with an increase until 110 DAT due to the dilution N effect in the plant throughout the crop by N uptake from the soil (Otto et al. 2016). Despite high N-fertilizer recovery and high Mo contents the biomass did not differ as a treatments function (Table 2). Anas et al. (2021) growing in similar conditions evaluating N rates found that biomass accumulation was variable with genotype and fertilizer availability. The same was reported by Santos et al. (2019) when evaluating the interaction of N and Mo under field conditions, and showed that yield and sugar content varied according to the variety evaluated when 200 g ha^{-1} of Mo as sodium molybdate was applied, i.e., the Mo response is attributed to the variety, as for N.

Gs variation with increasing temperature confirms the role of Mo under stress conditions in the plant. Stressed wheat plants showed reduced *gs* and reduced water loss in the presence of Mo (Sun et al. 2009, Wu et al. 2014). It is due to the participation of the LOS5/ABA3 gene in the encoding of a sulfurase that contains molybdenum cofactor present in

the abscisic acid (ABA) synthesis. Thus, Mo status is related to heat and water stress tolerance by inducing gene expression in the metabolic pathway (Xiong et al. 2001, Sun et al. 2009, Yue et al. 2011). However, *A* was not influenced by the change in *gs*, differing from Anas et al. (2021) where the gs and *A* variables increased until the development of ninth leaf of the plant. Marchiori et al. (2014) detected that there is a wide genotypic variety regarding the photosynthetic potential of sugarcane under the same evaluation conditions, with photosynthetically active radiation of 1500 μmol m^{-2} s^{-1}, CO_2 concentration of about 400 μmol mol^{-1}, temperature of 28 ºC. Results similar to that of Bassi (2018) with genotypes under high and low N (10 and 270 mg of N per kg^{-1} of sand) that received application in three times with an interval of 15 days, i.e., close to that performed in this study. Bassi (2018) found that *A* results varied from 3 and 13 μmol m^{-2} s^{-1} and stomatal conductance from 0.03 and 0.07 μmol m^{-2} s^{-1} as a function of N supply and genotype, factors that did not vary in this study.

Rhein and Silva (2016) evaluating nitrogen rates at field conditions found that the maximum photochemical efficiency of photosystem II is influenced by the availability of nitrogen and time of cultivation, thus the absence of response to energy dissipation is understood by the contents and accumulation of N in the plant that were the same. It can be seen that Mo has a greater influence on *gs* and only under ample stress other parameters linked to photosynthesis will be sensitive to this nutrient, because in the Mo absence the plant continues to assimilate N in the ammoniacal form, which may be in large quantities depending on soil humidity. On the other hand, this condition does not apply to enzymes linked to the N cycle.

Mo rapidly influences the activity of GS, NR, and US, but with variability throughout the crop (Figure 6). The average values observed for nitrate reductase of 0.2 μmol NO_2^- h^{-1} (g $FW)^{-1}$ are three times higher than determined by Santos et al. (2018) and lower than Boschieiro et al. (2019) where the evaluation of the proportion of nitrate and ammonium led the enzyme activity to mean values of 3 μmol NO_2^- h^{-1} (g $FW)^{-1}$. These results indicate that in addition to responding with high sensitivity to N and Mo the NR responds to growing conditions and, protected environments, with greater experimental control show higher activity. Possibly due to the faster growth of the plants. Furthermore, Santos et al. 2019 shows that NR activity is higher at the beginning of cultivation and decreases near 100 days after planting, confirming the results found. In this period there was a slight reduction of Mo terrors in the leaves showing the direct relationship between Mo content and enzyme activity as presented by Li-Ping et al. (2007).

The small Mo rates required by the plants often results in the non-application of the nutrient, foliar application or the need for adaptations, as done by Santos et al. (2019) where the use of sodium molybdate via soil was only possible after dilution of the salt in water for

distribution in the experimental area. Given the results and those presented so far in the literature, we suggest that the application of Mo be performed together with urea in the coating of the granules independing on the Mo source, the utilization of the fertilizer is favored and the absorption of Mo is also favored. Under these conditions more responsive varieties will have better productivity.

4.5. Conclusions

The Mo addition to urea did not change the amount of N in the plant derived from fertilizer and sugarcane biomass over 140 days of development. As expected, the Mo content increased with Mo supply, with the Mo trioxide incorporated into urea granules representing higher Mo contents in the plant, but not presenting higher absorption efficiency. Therefore, the sources are considered similar. This condition is supported by the variability of the enzymatic data. The supply of Mo increased enzyme activity and contributed to N metabolism in the plant independent from the source of Mo. The photosynthetic data indicate that Mo enables the plant to more rapidly adjust photosynthesis under heat stress.

The high N availability observed in the current study possibly reduced the effect of Mo in plant N metabolism. Addition of Mo to urea is a feasible strategy to supply low amounts of Mo to cultivated plants, whereas the Mo effect in biomass of sugarcane growth isn't observed due to the low Mo demand for sugarcane development.

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5. PHYSIOLOGICAL CHANGES IN NITROGEN METABOLISM OF MAIZE AS A RESULT OF MOLYBDENUM SUPLY

Abstract

Maize is a crop of worldwide importance and the efficiency of fertilizers in this plant becomes indispensable for sustainable agricultural development. Nitrogen (N) is widely used in maize production and the recovery of N by plant can be increased by the supply of Mo due to the role of Mo in the assimilation of nitrate, which first needs to be reduced to ammonium by nitrate reductase (NR). The NR is an enzyme constituted of Mo and the increase of this nutrient favors the enzymatic activity. The objective was to evaluate nitrate uptake and N metabolism in maize as a function of combined N and Mo omission and supply under controlled conditions. The plants were grown for 28 days in a nutrient solution without Mo and N, with Mo and N, and under omission of Mo or N. The treatments with omission received nutrients with foliar application or via nutrient solution after 28 days. Gas exchange, chlorophyll index and anthocyanins, N content were evaluated at 28, 35 and 45 days after transplanting (DAT). At 28 and 35 DAT amino acid profile in diagnostic leaves and roots were also evaluated. At 45 DAT the plant biomass was determined by harvest. The amino acid profile was altered as a function of Mo and N supply to the plants, as well as the uptake and accumulation of nitrate. The highest dry mass production was quantified in the positive control, in the Mo supplied by the nutritive solution and later by the absence of Mo, being the foliar application inferior to this treatment. Maize yield with omission of N and Mo was 31 % compared to the yield when both nutrients were supplied, demonstrating the role of N and Mo for maize production.

Keywords: Amino acids, Molybdate, Nitrate, Ammonium, Photosynthesis.

5.1. Introduction

Maize is the most produced crop in the world and according to FAO in 2019 approximately 1.1 billion Mg were produced worldwide. This agricultural product is one of the main responsible for world food safety, since it is used as food for several animals and as the base of processed food. In addition, maize can also be used as fuel (ethanol) and is capable of reducing greenhouse gas emissions by 20 %, since it is a renewable source (Börjesson, 2009).

Brazilian Maize yields in Brazil are very low compared to yields of other top producing countries around the world. According to the United States Department of Agriculture (USDA), in the 2020/2021 growing season the United States, which has the highest maize yield per area, reached more than 11 Mg ha^{-1} whereas Brazil had an average of 5.0 Mg ha^{-1} (USDA, 2021). These low yields in Brazil are the result of a series of inadequate management practices adopted in the different production systems. An alternative practice to improve maize productivity in Brazil is to improve the use of fertilizers, mainly nitrogen (N) (Noor, 2017; Andrea et al., 2018). Below (2013) considers N the second most important factor to obtain high yields, but considering that climate is the most important one, adequate N management is the most important factor that can

be influenced by farmers. Thus, the correct supply and management of N in many cases would result in a yield gain of 100 %, which shows the great importance of this macro nutrient in many crop systems.

Nitrogen is the element generally applied in great amounts in the maize crop during its growing period. According to Duarte et al. (2014) in order to reach high yields, approximately 200 kg ha^{-1} of N are necessary, because nitrogen is the most extracted and exported element by the crop. However, N fertilization practices are severely compromised by N-losses from soil, resulting in low N-fertilizer efficiency (Li et al., 2017; Noor, 2017). In this manner, new technologies that can contribute to better N utilization by plants are required. In maize, as well as in other crops, the nitrogen use efficiency (NUE) is determined by ^{15}N technique, which is very variable, presenting values between 30 and 55 % (Dobermann, 2005; Gava et al., 2010; Couto-Vázques and González-Prieto, 2016; Kumar et al., 2016), due to the dependence of several factors that can affect its utilization. Several genes are involved in the N utilization process and its expression can be affected by the supply of N and other nutrients. Given the complexity, an alternative for the direct measurement of NUE is to use morphological and physiological parameters (Gallais and Hirel, 2004; Yan et al., 2011).

Many variables in the process of N availability, absorption and metabolism allow the development of a wide range of strategies to increase N uptake. The current technologies are focused on the maintenance of N in the soil, which could result in a higher uptake by plants (Cantarella et al., 2008; Sanz-Cobena et al., 2008). In plant tissues, nitrate and ammonium are the major inorganic nitrogen sources. However, nitrate has to be reduced to ammonium in order to be incorporated into organic molecules. The reduction of nitrate is achieved by the two enzymes nitrate reductase (NR) in the cytosol and nitrite reductase in the plastids. NR is a homodimeric protein composed of three functional domains: N-terminal, central heme (cytochrome b557) and C-terminal flavine adenine dinucleotide (Mendel and Schwarz, 1999). The activity of NR is dependent on three co-factors, one of them being a molybdopterin, a molybdenum containing co-factor.

The use of molybdenum (Mo) in agriculture is recently not common and so far, only few studies have reported its effect on the physiological, biochemical and agronomical parameters (Calonego et al., 2010; Zoz et al., 2017). In addition, most of these studies are carried out in legume crops due to the role of Mo in the enzyme nitrogenase, which is responsible for N_2 fixation (Mengel and Kirkby, 1987). However, it is well known that this element affects directly the N metabolism of plants because Mo is a constituent of one of the prosthetic groups (MoCo) of several enzymes which are essential to N and C metabolism, such as nitrate reductase,

aldehyde oxidase, xanthine dehydrogenase and sulfite reductase (Campbell, 1999; Zimmer and Mendel, 1999; Mendel and Haensch, 2002; Zdunek-Zastocka and Lips, 2003). Therefore, plants with Mo-deficiency might show symptoms of nitrogen deficiency and the addition of Mo in crops may promote physiological and biochemical alterations, which may result in a greater root development and better utilization of N for biomass production (Wei et al., 2007; Škarpa et al., 2013).

Only few studies using Mo in plants were performedand most of them focued on the change of plant metabolism when Mo is added to the soil. Other recent studies provided Mo through foliar application or seed coating, even though it is still not clear how these practices interfere in nitrate uptake via roots (Wei et al., 2007; Calonego et al., 2010; de Albuquerque et al., 2012; Zoz et al., 2012; Sapucay et al., 2016; Silva et al., 2017). As a result of these experiments, researchers noted that N metabolism was unbalanced leading to higher total N and nitrate under Mo deficiency (Hu et al., 2002; Yu et al., 2006), even though it is not clear how Mo addition leads to biochemical changes. The goals were to assess modifications in the N metabolism with particular uptake nitrate, since it allows higher yields with the same amount of N applied in the system and guarantees greater food safety. Secondly, aims to provide a better understanding between the relationship of Mo supply and N metabolism, which may contribute to improve and optimize agricultural products.

5.2. Material and Methods

5.2.1. Growth conditions and experimental design

Maize seedlings, Likeit variety, were treated with 10 % sodium hypochorite solution for 3 min, then, seedlings were placed on germitest paper moistened with 1mM CaSO4. 2H2O solution for 7 days (Suppl. Figure 4A). Then, the plants were transplante to for nutrient solution in 5 L pots with 25 % strong and omission or presence Mo and N during 3 days (Suppl. Figure 4B). The nutrient solution was changed, and for another 3 days the plants grew in a half-strong solution, when the plants received nutrient solution with totaling strong for 28 days (Suppl. Figure 4C). It contained in a complet solution:2 mM K_2NO_3, 0.25 nM $(NH_4)_2HPO_4$, 0.75 $Ca(NO_3).4H_2O$, 0.125 mM $CaCl_2.2H_2O$, 1 mM $CaSO_4.2H_2O$, 1 mM $MgSO_4.7H_2O$, 0.025 mM $Ca(H_2PO_4)_2.H_2O$, 0.3 mM Fe-EDTA, 3 μM H_3BO_3, 2.5 μM $MnSO_4.H_2O$, 1 μM $ZnSO_4.7H_2O$, 1 μM $CuSO_4.5H_2O$ and 0.3 μM $Na_2MoO_4.2H_2O$. The nutriente solution was prepared with potassium nitrate 0.760 % $^{15}N\text{-}NO_3^-$ and the nitrification inhibitor dicyandiamide (DCD) was added, 7 μmol L^{-1}, to control the NH_4^+conversion in NO_3^- (Song et al. 2011). The DCD effect

was verified in a pre-test and its efficiency was at most 4 days, so it was the changeover time for the solutions that were prepared at pH 5.9.

A greenhouse experiment was conducted for 44 days ah the Institute of Applied Plant Nutrition with 14 hours light and 8 hours dark. The experimental was consisted of a completely randomized design using six treatments, four replications and two times, totaling 24 experimental untis. The treatments were complete nutrient solution (+ Control), low N and whitout Mo all the time (- Control, 5 % of N complet nutrient solution), low N and Mo (-Mo+NR, suppling N in solution after 3 weeks growth), without Mo (-MoR+N, suppling Mo in solution after 3 weeks growth), without Mo (-MoL+N, suppling Mo in leaf after 3 weeks growth) and without Mo (-Mo+N, all the time). The plants were grown for 28 days under the initial conditions and then for another 16 days with the combination of N and Mo supply or not. The foliar application was performed only once, while the root Mo supply was provided constantly.

5.2.2. Assessments throughout the growth

The dry matter (DM) production was determined 44 days after transplanting, two plants per pot were harvested and partitioned in roots, leaves and stems. The plantas compartment were washed, weighted and completed dryed at a 65ºC. At this moment DM was determined and material was ground. The same procedure was realizeted with leaf sampling at 28 and 35 DAT, for inorganic N according Tedesco et al. (1995).

For determination of Mo concentration 300 mg of DM were microwave digested in a 3 mL concentrated HNO_3 and 2 mL of 30 % H_2O_2 at 200 °C and 15 bar for 120 min (Tränkner et al., 2016) and the concentration were measured with an ICP-MS. N isotope abundance and total N content were determined an isotope ratio mass spectrometer, 6 mg of DM were placed in the titanium capsule, which this values em % ^{15}N atom and quantities of N accumulated in plant portioned was calculated, Ndff expressed in g pot^{-1}, and ^{15}N recovery were estimated using equation by Trivelin et al. (1994) (Eq. 1, 2). For the plant compartment the same procedure was adopted.

$$Ndff\ (\%) = (a/b) \times 100 \quad (1)$$

$$Ndff(g/pot) = (Nddf\ (\%)/100) \times Total\ N \quad (2)$$

where Ndff is the N in the plant derived from fertilizer (nitrate sources, % and g pot^{-1}), a and b are the ^{15}N abundance (atom % ^{15}N excess) in the plant (a) and in the fertilizer (b) ^{15}N-fertilizer, and Total N is the plant N content (g pot^{-1} of N).

The leaf and root sampling for ^{15}N had a part destined for amino acids quantification (Fountoulakis and Lahm, 1998). Total amino acid content was extracted using 6M HCl given to the dry samples for hydrolysis at 100 ºC for 24 h. Then, samples were dried in vacuo over NaOH for amino acids measurements. The depicted results were obtained by UHPLC-DAD on an Agilent 1290 system after derivatization of amino groups. Before leaf sampling for amino acid quantification, it was measured the plant chlorophyll index, anthocyanins index and NBI using the Dualex. Then, the leaves were also used to determine gas exchange using GFS-3000 (Heinz Walz GmbH, Germany) system on youngest fully expanded leaves. Chamber on 4×1 cm^2 size, temperature was set to 23 °C and relative humidity was 55 %, CO_2 concentration was kept constant at 400 ppm and flow was 1000 mmol $m^{-2}s^{-1}$. After *A* getting stabilized measurements were performed per 10 min for each experimental units. The measurements were performed between 9 a.m. and 4 p.m. (Suppl. Figure 4D).

5.2.3. Statistical Analysis

The data was analiyed using R software (R Core Team, 2019). Previously normality (Shapiro-Wilk-Test) and homogeneity of variance (Bartlett-Test) were evaluated. The treatments were subject to analysis of variance (ANOVA), if F-test was significant ($p \leq 0.05$), the means were compared by LSD-Test, at the 0.05 level of confidence.

5.3. Results

5.3.1. Leaf gas exchange and pigments

At 28 and 35 DAT the chlorophyll index was influenced by the -Mo-N and -Mo+NR treatments (Figure 1A). The absence of these nutrients representing a 47 % reduction in the chlorophyll index. The -Mo+NR showed a 45 % increase in chlorophyll index at 35 DAT compared to -Mo-1N (negative control). The -Mo-N was the treatment with the lowest chlorophyll index at all measurements (18, Figure 1A). 16 days after N supply, 44 DAT, the chlorophyll index of -Mo+NR was equal to +Mo+N (positive control) and Mo supplied. The opposite was observed with the anthocyanin index, an accessory and protective plant pigment. Only -Mo-N and -Mo+NR showed a response, with production 76 % higher than the others treatments at 28 DAT. The N supply reduced the production of anthocyanin over time and at the end was the same as the +N treatments throughout the cycle, this being the factor responsible

for the variation of pigments in the plant and visual coloration observed throughout the experiment.

Gas exchange presented the same behavior of the pigments and showed that despite not altering the *A*, Mo influences the *gs* in the short and long term. At 28 DAT the -Mo-N and -Mo+NR showed lower photosynthesis and *gs*, but at 35 DAT the photosynthetic rate increased when there was N supply, -Mo+NR, while the *gs* did not change in this treatment (Figure 1C and D). The N omission reduced the chlorophylls amount, probably the rubisco amount and consequently the *A*. Mo supply reduced conductance at 44 DAT. In other words, the higher chlorophyll indices are related to the increased photosynthetic rates and lower stomatal conductance, due to the C4 metabolism of the plant that reduces water loss and favors the maintenance of photosynthesis.

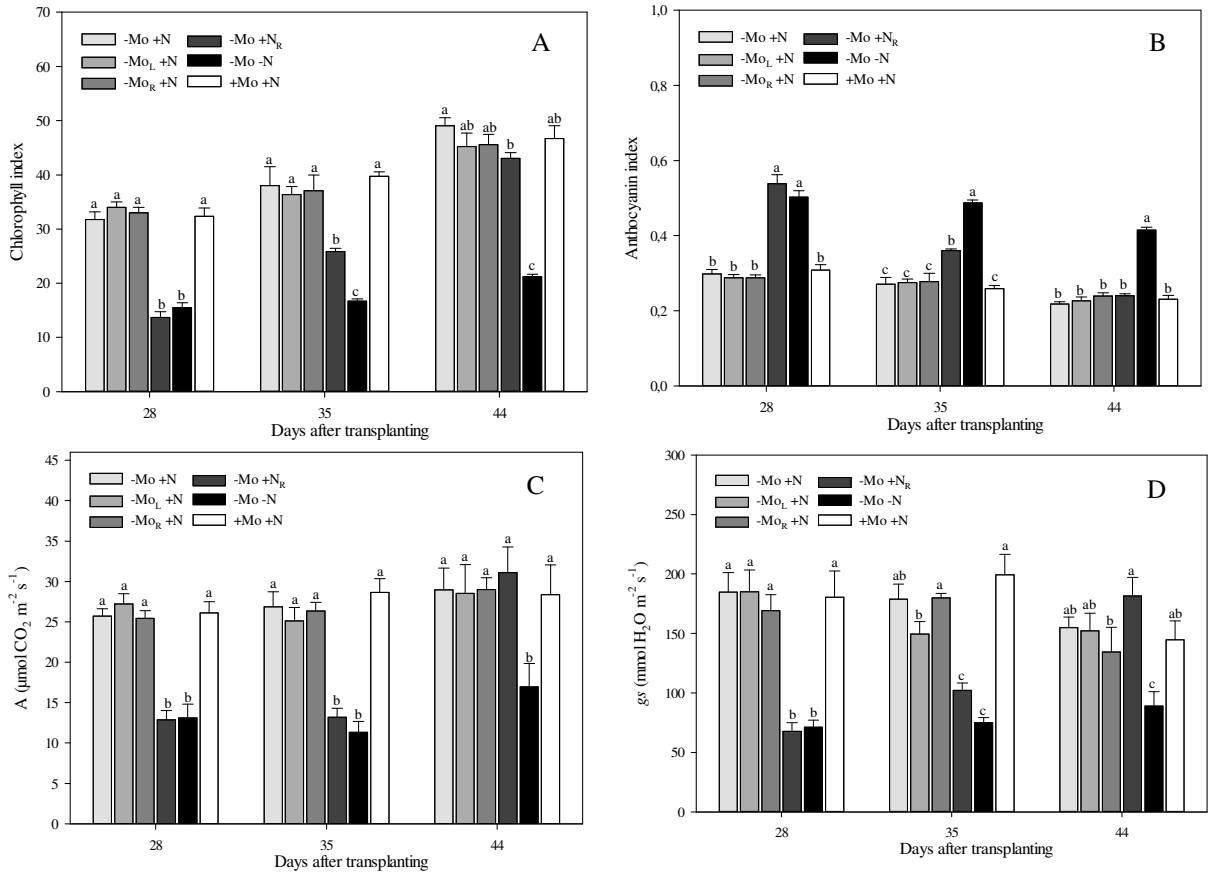

Figure 1. Chlorophyll index (A), anthocyabin index (B), CO_2 assimilation rate (*A*, C) and stomatal conductance (*gs*, B) in maize plants grown on nutrient omission up to 28 DAT and afterwards with resupply. -Mo+N: with out Mo, -Mo$_L$+N: Mo supply in leaves after 28 DAT, -Mo$_R$+N: Mo supply in roots after 28 DAT, -Mo+N$_R$: N supply in roots after 28 DAT, -Mo-N: control negative, and +Mo+N: control positive. Letters indicate difference between treatments in the same date by LSD test (p<0.05). Bars indicate the standard error.

5.3.2. Amino acids

All essential amino acids were determined, but in the root most were found below the detection limit, thus it was possible to quantify only Ala, Asp, Glu, Gly, Leu and Val. The N omission showed the lowest levels of amino acids, being that Asp, Glu and Gly, after the N supply were equivalent to the other treatments. The levels of Ala were lower in the treatments with Mo omission, but the supply of this nutrient increased the levels of this amino acid. The same occurred with Val, which was identified only at 35 DAT, being on average 7 times lower in the -Mo-N (Table 1). The presence of Leu, Val, and Ala are related to amino acid degradation and pyruvic acid degradation, indicating cellular photorespiration. Glu levels increased with the Mo addition, indicating that there was a major presence of N available, which was also verified with the N supply (Table 1).

The amino acids in the leaves were identified in larger quantities, the Mo omission resulted in an accumulation of amino acids in the leaves, higher than the positive control. The N omission reduced, on average, 3 times the content of amino acids in the leaves (Table 2). The Mo supply maintained the content of amino acids similar to the +Mo+N, while the N addition increased the amino acids content, indicating a rapid recovery of metabolic pathways. Thr content indicates that the transmethylation reactions were compromised when only N was supplied. Furthermore, the amino acids regulating growth and plant defense (Tyr and Phe) were found in higher amounts after Mo supply in leaves, indicating better functioning of the shikimate pathway. Glu contents increased after Mo supply, indicating increased N availability for amino acid formation. Pro, recognized for its osmoprotective role, showed higher levels with Mo application. The same occurred with Arg, which, despite not differing from the +Mo+N, showed an increase with the Mo supply, indicating a greater reserve of N assimilated by the plant. The levels of His were higher in the leaves that received Mo, probably due to the stress of the plant, since it is known as a chelator and transporter of metals and semi-metals.

Table 1. Amino acid concentration in maize roots grown for 28 DAT on Mo and N omission and one week after nutrient resupply (35 DAT)

	-Mo +N	**-Mo$_L$ +N**	**-Mo$_R$ +N**	**-Mo +N$_R$**	**-Mo -N**	**+Mo +N**
			28 DAT			
Ala	91.5±25.6 abc	78.5±12.0 bc	116.8±17.0 ab	51.8±3.3 c	59.7±4.8 bc	143.0±23.7 a
Asp	66.0±7.4 a	57.5±5.3 a	76.3±5.3 a	13.3±11.5 b	11.8±10.2 b	76.5±8.5 a
Glu	74.0±8.6 a	63.5±6.2 a	87.3±7.8 a	13.3±11.5 b	13.0±11.3 b	92.3±10.6 a
Gly	68.3±9.6 a	56.8±5.0 a	73.3±7.9 a	<DL	<DL	73.5±8.8 a
Leu	59.7±7.5 a	55.5±4.8 a	66.5±5.2 a	11.3±9.7 b	<DL	63.0±6.4 a
			35 DAT			
Ala	106.8±27.4 bc	119.25±8.8 ab	155.8±14.0 a	58.8±4.2 cd	45.0±1.5 d	80.0±13.7 bcd
Asp	57.0±17.0 a	65.8±5.4 a	58.0±2.2 a	47.7±4.9 a	<DL	58.8±7.7 a
Glu	63.3±19.7 a	70.5±6.8 a	72.3±6.6 a	47.1±5.0 a	<DL	61.3±8.9 a
Gly	56.8±18.5 a	52.0±15.2 a	43.0±12.9 a	45.4±4.5 a	<DL	63.3±6.5 a
Leu	60.3±4.3 a	58.5±0.6 ab	47.0±0.6 b	34.1±6.9 c	<DL	55.8±4.0 ab
Val	51.9±4.6 a	52.2±0.5 a	42.8±0.7 a	11.3±9.7 b	<DL	14.0±12.1 b

DL: detection limit, -Mo+N: with out Mo, -Mo$_L$+N: Mo supply in leaves after 28 DAT, -Mo$_R$+N: Mo supply in roots after 28 DAT, -Mo+N$_R$: N supply in roots after 28 DAT, -Mo-N: control negative, and +Mo+N: control positive. Letters in the same row indicate difference between treatments by LSD test (p<0.05). Average followed by standard error.

Table 2. Amino acid concentration in maize leaves grown for 28 DAT on Mo and N omission and one week after nutrient resupply (35 DAT)

	-Mo +N	-Mo$_L$ +N	-Mo$_R$ +N	-Mo +N$_R$	-Mo -N	+Mo +N
			28 DAT			
Ala	444.5±7.6 ab	482.0±35.1 a	453.5±11.9 ab	139.5±10.3 c	132.25±13.9 c	393.5±18.8 b
Arg	140.3±4.1 ab	193.6±18.2 a	171.0±6.4 ab	57.7±2.6 c	56.0±2.9 c	140.3±13.1 b
Asp	316.2±5.3 ab	373.3±33.2 a	325.8±12.7 ab	108.8±7.5 c	100.3±10.8 c	274.5±20.9 b
Glu	308.8±3.9 ab	364.8±32.5 a	321.0±8.8 ab	95.8±6.9 c	85.8±9.9 c	264.5±19.6 b
Gly	317.3±9.0 ab	368.0±34.4 a	324.0±12.1 ab	86.5±7.3 c	79.5±8.4 c	267.5±24.3 b
His	59.5±1.8 ab	71.3±7.0 a	61.25±2.9 ab	<DL	<DL	52.7±3.8 b
Ile	189.0±3.9 ab	218.0±21.2 a	192.5±7.2 ab	61.8±1.5 c	76.1±10.5 c	158.0±14.1 b
Leu	334.0±7.8 ab	388.3±37.6 a	341.8±13.5 ab	109.5±8.2 c	103.0±10.6 c	279.0±28.9 b
Lys	185.0±5.9 ab	212.3±19.8 a	187.3±8.1 ab	60.0±4.3 c	74.2±10.7 c	158.5±13.9 b
Phe	148.3±4.2 ab	175.0±17.4 a	151.8±6.4 ab	50.0±2.2 c	52.5±1.3 c	123.8±11.7 b
Pro	210.8±4.3 ab	242.5±23.7 a	215.5±.7.3 ab	77.5±5.5 c	73.4±8.0 c	178.3±16.1 b
Ser	176.5±5.7 b	215.0±20.1 a	186.75±5.8 ab	57.0±3.4 c	56.7±2.7 c	156.8±12.6 b
Thr	183.0±4.9 ab	218.0±20.9 a	190.0±6.5 ab	56.8±4.3 c	58.3± 2.7 c	155.5±13.3 b
Tyr	75.0±2.2 ab	89.3±8.9 a	77.8±4.1 ab	<DL	<DL	64.5±6.3 b
Val	264.8±4.1 ab	303.8±29.1 a	269.5±9.5 ab	86.8±6.5 c	81.8±8.7 c	222.3±20.1 b
			35 DAT			
Ala	496.5±25.7 a	524.3±55.0 a	417.3±55.4 a	399.0±25.6 a	149.5±11.0 b	421.3±38.4 a
Arg	184.8±10.2 a	195.3±19.2 a	159.3±19.0 a	150.0±11.7 a	58.5±4.2 b	153.3±15.9 a
Asp	364.5±15.8 a	386.0±38.8 a	316.5±37.4 a	302.5±20.8 a	118.5±8.5 b	310.0±30.2 a
Glu	388.8±20.1 a	403.3±42.6 a	317.5±38.6 a	309.3±19.7 a	111.3±9.0 b	320.8±32.9 a
Gly	386.8±21.7 a	408.8±44.1 a	329.0±39.0 a	317.3±23.9 a	106.8±8.5 b	317.3±34.0 a
His	69.3±4.0 ab	73.5±7.8 a	59.3±6.9 ab	54.3±3.9 b	<DL	62.3±2.7 ab
Ile	211.0±12.4 a	222.8±23.0 a	181.8±20.3 a	168.0±12.3 a	65.3±4.8 b	174.5±18.0 a
Leu	384.8±22.0 a	405.8±42.0 a	331.5±37.7 a	304.5±23.2 a	119.0±8.7 b	316.8±32.8 a
Lys	209.8±9.9 a	222.5±21.0 a	184.8±21.7 a	178.8±11.7 a	70.5±4.4 b	172.0±17.0 a
Phe	174.6±10.3 ab	186.3±20.2 a	150.8±17.1 ab	135.0±10.6 b	51.0±3.7 c	143.0±15.2 ab
Pro	240.8±13.2 a	253.5±25.1 a	207.5±24.8 a	192.0±15.0 a	82.3±5.5 b	199.6±20.2 a
Ser	208.0±13.2 a	222.0±22.4 a	176.5±22.2 a	169.0±12.9 a	59.5±4.5 b	175.3±18.7 a
Thr	212.8±11.0 ab	227.0±23.3 a	182.8±21.4 ab	169.5±12.9 b	61.8±5.0 c	178.8±19.0 ab
Tyr	86.8±3.8 ab	92.0±8.8 a	76.8±9.0 ab	68.8±5.5 b	<DL	71.0±7.4 ab
Val	292.0±17.1 a	308.5±31.3 a	250.5±28.2 a	235.8±17.1 a	91.8±6.5 b	243.0±24.7 a

DL: detection limit, -Mo+N: with out Mo, -Mo$_L$+N: Mo supply in leaves after 28 DAT, -Mo$_R$+N: Mo supply in roots after 28 DAT, -Mo+N$_R$: N supply in roots after 28 DAT, -Mo-N: control negative, and +Mo+N: control positive. Letters in the same row indicate difference between treatments by LSD test (p<0.05). Average followed by standard error.

5.3.3. Biomass, N and Mo content

The DM was influenced by N and Mo supply. In roots, the Mo omission caused a 13.5 % reduction, whereas Mo supply via solution increased DM by 19 % compared to omission or foliar application (Table 3). Furthermore, Mo supply was equivalent to +Mo+N. N supply 28 DAT did not recover root development. That is, in the omission of both nutrients the plant reduces root development, with the greatest effect attributed to N. A similar behavior was observed for aboverground, composed of stalk and leaves. However, it was noted that the Mo omission or supply by solution showed the same +Mo+N production, approximately 170 g pot^{-1}. The Mo addition in leaves reduced the DM accumulation by 17.8 % in the aboveground. This reduction possibly occurred due to the rate applied that resulted in toxicity. In this compartment also there was no response to the supply of N, which may be a result of the limited supply during the experiment, without complete nutrient omission.

The highest DM production was 216.6 g pot^{-1}, referring to +Mo+N, while the -Mo-N was 123.1 g pot^{-1}, showing that Mo and N omission combined resulted in a yield loss of 43.0 % (Table 3) (Suppl. Figure 4E). N supply 28 DAT did not recover DM, being equivalent to -Mo-N. The Mo supply by nutrient solution or Mo omission showed similar mean to +Mo+N, but it is noted that the Mo omission during the whole experiment had a DM 5.6 % lower than +MoR+N and +Mo+N. As well as the root and aboveground, the whole plant DM responded negatively to the foliar Mo supply, being 16.5% lower than the most productive treatments.

Table 3. Dry mass (DM) in roots, stalk and leaf (aboverground) compartments, and total of plant maize grown for 44 DAT on Mo and N omission and resupply

	DM		
	Roots	**Aboverground**	**Whole plant**
	g pot^{-1}		
-Mo +N	37.3±2.5 bc	166.7±12.5 a	204.4±8.9 a
-Mo$_L$ +N	39.4±1.6 b	141.5±6.9 b	180.9±5.1 b
-Mo$_R$ +N	47.4±2.7 a	166.7±2.6 a	214.1±2.2 a
-Mo +N$_R$	28.2±2.7 d	91.9±1.8 c	120.1±2.3 c
-Mo -N	30.6±0.5 cd	92.4±4.5 c	123.1±4.0 c
+Mo +N	44.2±3.2 ab	172.3±5.1 a	216.6±4.0 a
p-value	<0.01	<0.01	<0.01
CV (%)	12.6	9.5	7.6

-Mo+N: with out Mo, -Mo$_L$+N: Mo supply in leaves after 28 DAT, -Mo$_R$+N: Mo supply in roots after 28 DAT, -Mo+N$_R$: N supply in roots after 28 DAT, -Mo-N: control negative, and +Mo+N: control positive. Letters in the same column indicate difference between treatments by LSD test ($p<0.05$). Average followed by standard error.

The Mo content altered according to the addition of nutrient and to the time the plant was exposed to the supply, as expected. In the roots it was only possible to quantify Mo in the

+Mo+N and in the Mo supply via solution (-Mo_R+N), the other treatments showed values below the quantification limit of the mass spectrometer (1mg kg^{-1}). The Mo content of the supply was 60.4 % lower than +Mo+N in the roots. In the aboveground, the Mo supply resulted in 11.9 times higher than -Mo-N, while -Mo_R+N had 35.1 % less Mo (Table 4). In the treatments where Mo was not supplied and the N was supplied or not during the experiment, Mo amount in the plant was minimal. The Mo accumulation in +Mo+N was 3.28 mg while the root supply was 56.4 % lower and the foliar supply 106.7 % higher. In other words, the treatments with Mo accumulation between 1.43 mg and 3.28 mg had the highest yields (Table 3).

^{15}N percentage in the plant, e.i., the amount of N-NO_3^- absorbed was influenced in the roots and leaves throughout the omission and supply of nutrients. The root showed less sensitivity to the Mo availability, at 28 DAT the omission of Mo and N did not alter the N-NO_3^-, but at 35 and 44 DAT it was observed that the addition of N without Mo reduced the utilization of the N-NO_3^-, a difference of 5.2% (Figure 2A). In the leaves, N-NO_3^- increased with the Mo supply, N supply was the treatment with the least utilization at 35 and 44 DAT. Either Mo supply or not to the root showed no difference in the utilization of the nitrate in relation to the control.

In the plant compartments the percentage of N-NO_3^- was influenced by the treatments. In the root with Mo leaves supply and N root supply being the lowest percentages, while either supply or no of Mo was similar to the +Mo+N (Figure 2B). This behavior was different for the stalk, where the N-NO_3^- was higherin the supply of Mo via solution, being equal to the +Mo+N. On the other hand, the treatments without Mo showed a lower average utilization of 5 % compared to the +Mo+N. In the leaves there was a response to the addition or not of Mo, only the treatment with N supply showed less utilization of the nitrate. This indicates that in conditions of restricted N availability, Mo is an important factor for the utilization the N when it is made available to the plant (Figure 2B).

The addition of Mo in the leaves promoted less utilization of the N-NO_3^-, as well as N supply in the Mo omission. The treatments with Mo showed a N-NO_3^- uptake rate of 81 % while the treatments without Mo showed a N-NO_3^- uptake rate of 79 %. The absence of N and Mo favored the greatest absorption of ammonium by the plant, 22 %, while the treatments with N during the entire cycle was 19 % (Figure 2C).

Table 4. Mo content in roots, stalk and leaf (aboverground) compartments, and total of plant maize grown for 44 DAT on Mo and N omission and resupply

	Mo content		
	Roots	**Aboverground**	**Whole plant**
	——————— **mg pot^{-1}** ———————		
-Mo +N	< DL	< DL	< DL
-Mo$_L$ +N	< DL	6.78±a	6.78 a
-Mo$_R$ +N	1.07 b	0.36±b	1.43 c
-Mo +N$_R$	< DL	< DL	< DL
-Mo -N	< DL	< DL	< DL
+Mo +N	2.70 a	0.57± b	3.28 b
p-value	<0.01	<0.01	<0.01
CV (%)	39.7	38.0	34.5

DL: detection limit, -Mo+N: with out Mo, -Mo$_L$+N: Mo supply in leaves after 28 DAT, -Mo$_R$+N: Mo supply in roots after 28 DAT, -Mo+N$_R$: N supply in roots after 28 DAT, -Mo-N: control negative, and +Mo+N: control positive. Letters in the same column indicate difference between treatments by LSD test (p<0.05). Average followed by standard error.

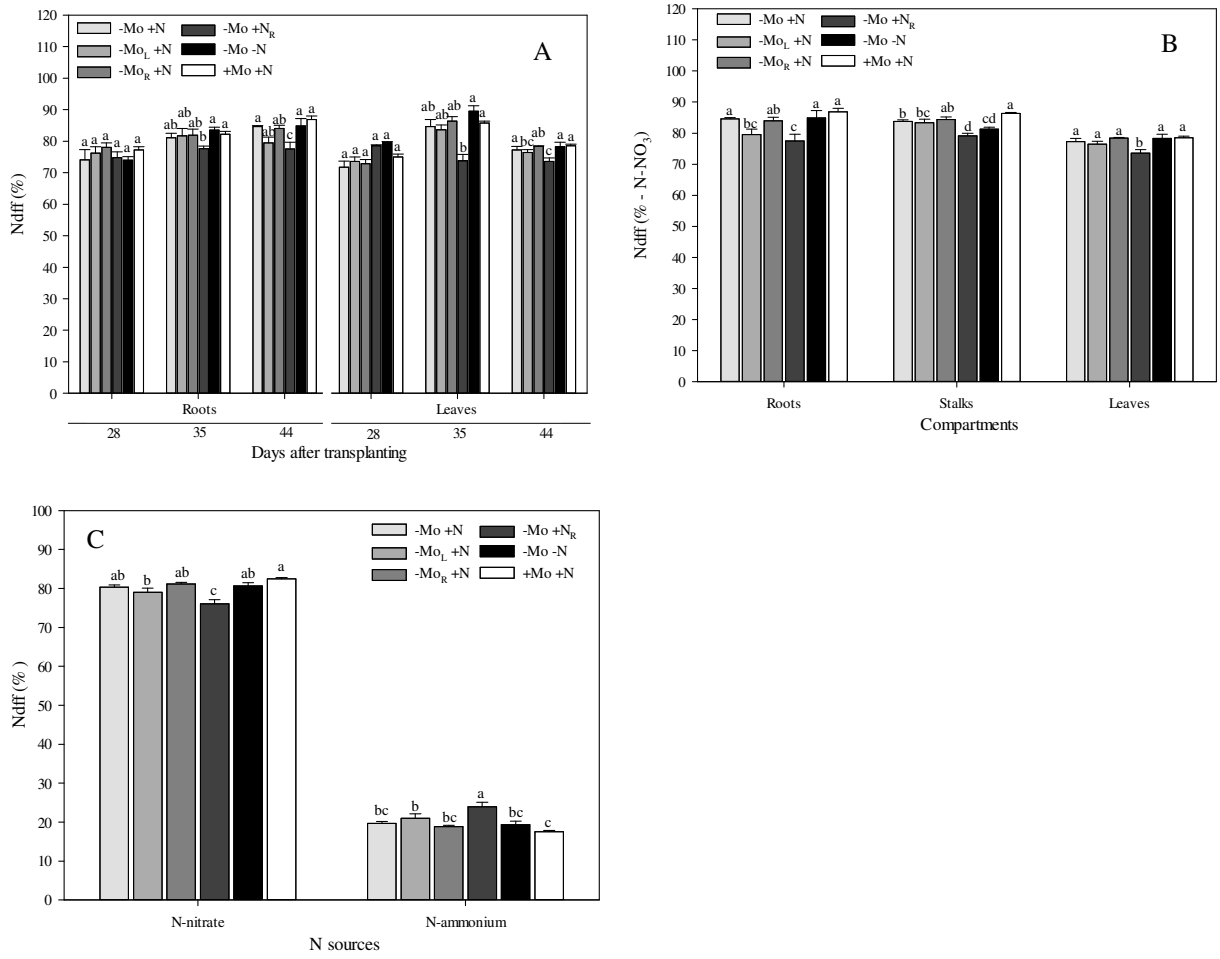

Figure 2. Nitrogen from nitric fertilizer during the experiment (A), in the compartments (B) and whole plant (C). -Mo+N: with out Mo, -Mo$_L$+N: Mo supply in leaves after 28 DAT, -Mo$_R$+N: Mo supply in roots after 28 DAT, -Mo+N$_R$: N supply in roots after 28 DAT, -Mo-N: control negative, and +Mo+N: control positive. Letters indicate difference between treatments in the same date or compartments by LSD test (p<0.05). Bars indicate the standard error.

The root N content was lower in the -Mo-N and the N supply, which was similar to the treatment without Mo. The highest N accumulation was in +Mo+N and in the root supply of Mo. Same responses were determined by the treatments for aboverground, but a smaller effect of Mo root supply was observed. N accumulation in the whole plant was lower in the treatment without N and Mo, and the addition of N did not restore the plant, with rates 3 times lower than the +Mo+N. The Mo omission also reduced N uptake, being 6.4 % lower than the +Mo+N. The addition of Mo in leaves resulted in a greater utilization of N compared to the root addition, in this treatment the contribution of the ammonia source was greater.

Table 5.N content in roots, stalk and leaf (aboverground) compartments, and total of plant maize grown for 44 DAT on Mo and N omission and resupply

	N content		
	Roots	**Aboverground**	**Whole plant**
	g pot^{-1}		
-Mo +N	0.80±0.06 ab	3.85±0.21 a	4.65±0.23 ab
-Mo$_L$ +N	0.87±0.04 a	3.73±0.13 ab	4.60±0.11 ab
-Mo$_R$ +N	0.96±0.05 a	3.44±0.18 b	4.41±0.22 b
-Mo +N$_R$	0.62±0.04 bc	2.54±0.12 c	3.16±0.15 c
-Mo -N	0.44±0.02 c	1.12±0.05 d	1.56±0.05 d
+Mo +N	0.99±0.10 a	3.98±0.08 a	4.97±0.13 a
p-value	<0.01	<0.01	<0.01
CV (%)	17.5	10.4	9.5

-Mo+N: with out Mo, -Mo$_L$+N: Mo supply in leaves after 28 DAT, -Mo$_R$+N: Mo supply in roots after 28 DAT, -Mo+N$_R$: N supply in roots after 28 DAT, -Mo-N: control negative, and +Mo+N: control positive. Letters in the same column indicate difference between treatments by LSD test (p<0.05). Average followed by standard error.

5.4. Discussion

The chlorophyll index in the plants was higher with the N presence, a result which is supported by the literature. It is known that at least 50 % of the N in leaves is present in chlorophyll, the pigment responsible for the green color measured (Erley et al., 2010). Furthermore, under nutritional stress conditions the plant will reduce the production of chlorophyll, a fact also demonstrated by Souza et al. (2021), and will compensate with the production of other pigments for photoprotection. Anthocyanins are pigments responsible for reducing free radical production and in the N omission their content is higher. Ravazzolo et al. 2020, also determined lower chlorophyll contents when there was greater availability of ammonium for plants, a recurrent fact in the treatment with N omission. This help to understand the gas exchange, where plants with N omission presented lower photosynthesis and stomatal conductance. Mo did not influence photosynthesis, but altered the conductance and, it is possible to observe that the Mo reduced the gs compared to the supply of N, and the Mo

supply resembled the gs to the control, indicating ease in plant adaptation to regulate stomatal conductance in the presence of molybdenum (Schwarz and Mendel, 2006). These results are also reported for wheat plants under stress condition (Sun et al. 2009, Wu et al. 2014).

Amino acid contents increased with the presence of molybdenum, especially those of reserve for N and those linked to plant development, being more expressive in leaves. Ravazzolo et al. 2020 determining amino acids found that plants with higher nitrate supply contained more free asparagine in the leaves and roots, also possibly originating from the redirection of glutamine to asparagine as a temporary measure to control excess ammonium. Such condition resembles the responses obtained, due to the higher production of both in the Mo presence and higher uptake of nitrate. Histidine was lower in plants leaves with higher uptake of the ammonium form, due to its relationship with genes negatively regulated by nitrate (Ingle, 2011). Huang et al. (2021) evaluating metabolite behavior in maize as a function of molybdenum trioxide rates also found that Mo addition resulted in higher amino acid production.

DM responded to root Mo supply, being the same yield as the +Mo+N, while leaves application reduced yield. It is believed that based on the amino acid profile, and the leaves Mo contents that the application of this nutrient resulted in toxicity to the plant, and it is necessary to review the rate performed. Kovács et al. (2015) observed that regardless of the Mo rate in nutrient solution the DM suffered a slight reduction compared to the Mo non-application, which showed low leaves contents, probably corresponding only to the content of the seed, as also reported in this experiment.

The nitrate uptake is not always evaluated when molybdenum application is performed, but in most of the literature the nitrate content in plants is quantified and often presents lower in the plants leaves with Mo (Li-Ping et al., 2007, Calonego et al., 2010, Kovács et al., 2015, Santos et al., 2018). Here we highlight that Mo application favors the uptake of nitrate sources, in addition to favoring its utilization by plants. That is, in well-aerated, nitrate-predominant soil conditions Mo may favor the utilization of the nitrate fertilizer or the available nitrate in the soil. Kovács et al. (2015) demonstrates that maize seedlings reduce the amount of nitrate in the leaves and increase the content in the roots as a function of the rate of ammonium molybdate in nutrient solution. The lower presence of free nitrate in the leaves is a constant reported when Mo is applied, regardless of the crop evaluated (Sun et al. 2009, Xiong et al. 2001, Ide et al. 2011).

The N accumulation in the plant was higher when both nutrients were available, and the Mo omission slightly compromised the N content, noting that the Mo supply did not recover the N content in the plant, as well as the N supply. Nitrogen fertilization in maize is normally divided into planting and top dressing, and the top dressing was performed in v4, i.e., before the period

of N supply in the plants grown in omission, which may have favored the impairment of the development of these plants. Mo is constantly omitted in grasses and here we demonstrate that its application contributes to the adequate development of the plants because it favors cellular metabolism and the production of metabolites involved in plant protection, stomata regulation, and N utilization.

5.5. Conclusions

N supply to maize is indispensable for plant development, especially at the beginning of the cycle. The late N supply does not recover the production of the plant despite the rapid recovery of metabolic pathways, indicated by the content of amino acids. Mo favors nitrate uptake and its late supply can recover the utilization of nitrate, mainly by root uptake, with this compartment showing the greatest difference in N content in the plant coming from the nitrate uptake. The Mo omission rapidly changed the stomatal conductance, evidencing the importance of this nutrient in the plant osmoregulation, which was confirmed by the amino acids and their distribution. The supply of Mo, as well as the Mo content provided greater production and improved the parameters evaluated or were equal to the positive control. The supply of Mo provided a greater uptake of nitrate by maize plants.

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6. FINAL REMARKS

The N use in agriculture is growing, as are strategies to improve the efficiency and management of this nutrient in agricultural crops (Kasim et al. 2009; Xu et al., 2013; Cancellier et al., 2016; Rech et al., 2017). N efficiency is dependent not only on the crop, but also on the N source used and the content of other nutrients in the plant, as well as many other factors (Doberman 2005; Ide et al., 2011). The higher N use efficiency is related not only to the increase of productivity, but also with the sustainability of crops, favoring mainly crops that result in ethanol production, such as maize and sugarcane (Börjesson, 2009). The development and evaluation of new formulations and technologies in these crops is essential for the evolution of the agricultural activity. In this context, this study contributed to the development of new strategies to increase N use efficiency in sugarcane and maize crops by the addition of Mo into urea granules.

In Chapter II we concluded that the Mo addition to urea at rates above 600 gper 100 kg^{-1} N resulted in a depletion of the physicochemical characterisctics of the fertilizer, independent of the type of Mo added. This result was most evident for the combined application of Mo trioxide and NBPT, revealing that a chemical reaction occurs after its mixture. The results indicates that is possible to add Mo into urea granules, but that the rate of Mo should not exceed 600 g per 100 kg of N ir order to maintain physicochemical characteristics. Under these conditions, the formulated product is promising for use and commercialization. The suggested degradation of the inhibitor was further evaluated, through the emission of ammonia, a factor that is also of paramount importance for the efficiency of urea fertilizers.

Chapter III showed that the addition of Mo to urea, regardless of the source of Mo, is feasible because the N lost to volatilization was the same regardless of the application when NBPT was not present in the mixture in a coated or incorporated manner. The addition of Mo trioxide with NBPT is not feasible because there is a loss of efficiency on the inhibitor, a result proven by the higher volatilization of N when both products were mixed compared to urea treated with NBPT. This fact proves the hypothesis formulated throughout chapter II, that the addition of Mo trioxide resulted in a reaction with the NBPT molecule and consequently there was a partial or total degradation of the inhibitor. The interaction between the compounds was more intense when both were used to coat the grains, probably due to the greater contact between the products. Clarification of the dateils of the reaction needs to be done, but our results are clear in showing that Mo can be added only to urea, without mixing with NBPT. Therefore,

the N efficiency of sugarcane plants was studied only with a mixture of Mo sources and the addition or not of Mo.

In Chapter IV we found that, different from expected, the addition of Mo to urea did not increase the amount of N derived from fertilizer over the 140 days of sugarcane growth. In opposite, as expected, the addition of Mo resulted in higher levels of Mo in treatments with Mo supply. Both sources (ammonium molybdate or molybdenum trioxide) showed similar potential to increase enzyme activity and N metabolism in the plant. The same fact was observed with the stomatal conductance data, indicating that that Mo supply favors the rapid adjustment of photosynthesis and gas exchange in the plant. Such conditions, with high availability of Mo, led to a reduction in the effect of Mo on biomass. Despite the low effect of Mo supply on sugarcane growth in the current study, in addition to the lack of effect of Mo in increasing NUE, the addition of Mo into urea granules is a feasible strategy to supply Mo to commercial crops, allowing distribution of low amounts of Mo homogeneously in the field.

In Chapter V, we identified that maize plants grown in nutrient solution responded to N and Mo as well as to the supply interaction of these nutrients. For N, a late addition did not recover the plant, although the metabolic pathways, indicated by the content of amino acids, recovered quickly. The presence of Mo and the late supply of this nutrient favored the uptake of nitrate, with the best effects by the application via nutrient solution and not foliar, which seems to have caused toxicity in the plants. The absence of Mo increased stomatal conductance, as it did in sugarcane plants grown in soil. Thus, in this condition the plant needed more water for its production, highlighting the importance of this nutrient in stomatal control, supported by the presence of higher concentrations of amino acids for stress control in plants with Mo. The supply of Mo, as well as the presence of Mo provided greater production and improved the parameters evaluated, and in the presence of this nutrient the plant absorbed a greater amount of nitrate.

This study is the first report on the possibility of adding Mo into urea granules, by granulation process or by coating. Few differences were observed between ammonium moybdate and molybdenum trioxide in terms of plant nutrition. Adding Mo into urea granules did not reduce ammonia volatilization. However, adding Mo as Mo trioxide combined with NBPT addition caused a chemical reaction that reduced the effectivity of NBPT in reducing ammonia volatilization. Despite the hyphotesys of this study was not confirmed, that Mo addition to urea granules would increase N use efficiency by sugarcane, the finding of this study is that is possible to produce a urea fertilizer enriched with Mo, with potential to supply homogeneously low amounts of the increasingly important Mo micronutrient to field crops.

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APPENDIX

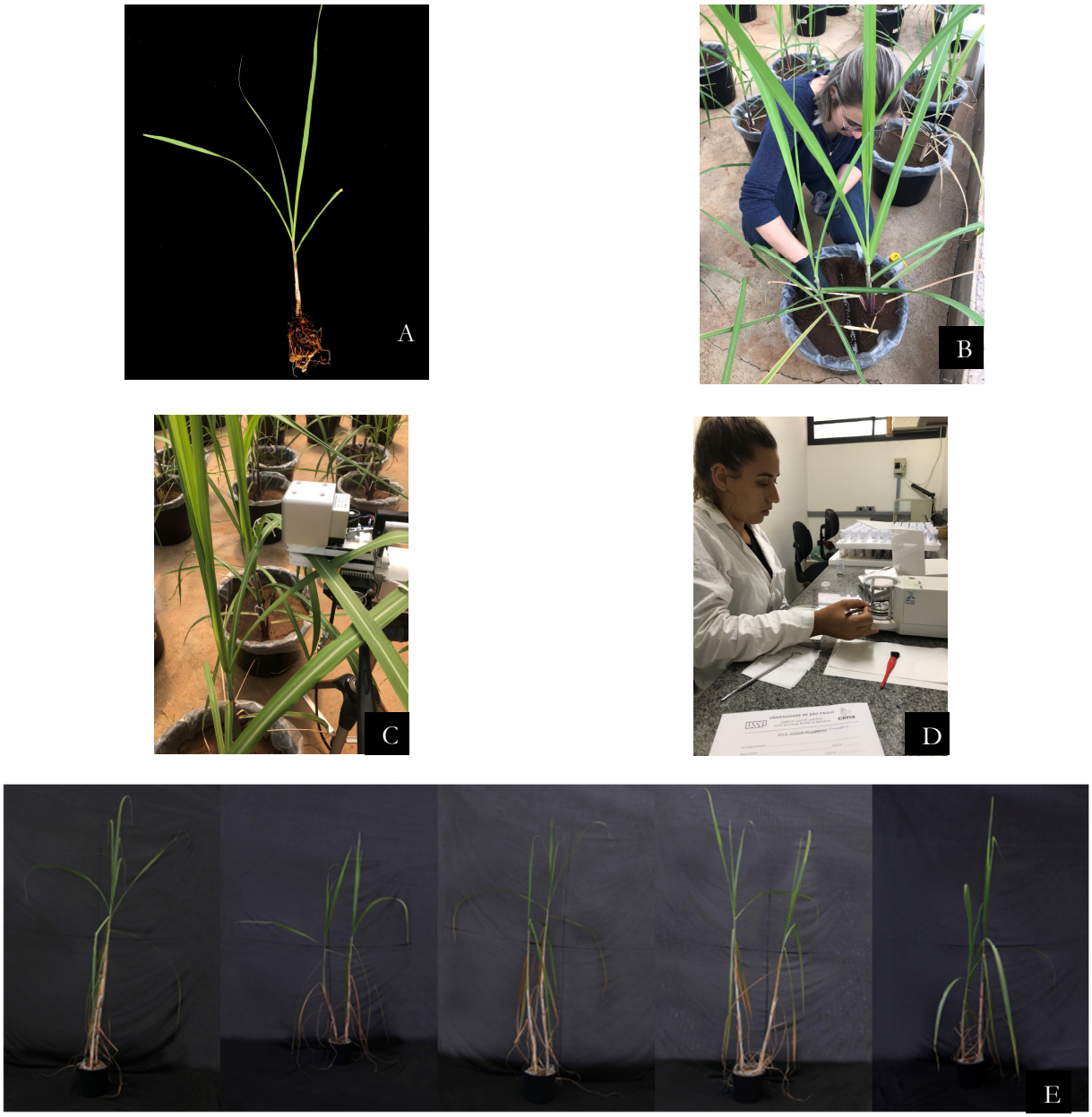
Supplementary results from section 2.

Suppl. Figure 1. Urea misture with molybdenum trioxide for granulation (A); Fertilizer granulation (B); Preparation for fertilizers drying (C); Mixture formulated before the granules are separated by size (D); Evaluation of granule hardness (E); and evaluation of the fertilizers hygroscopicity (F).

Supplementary results from section 3.

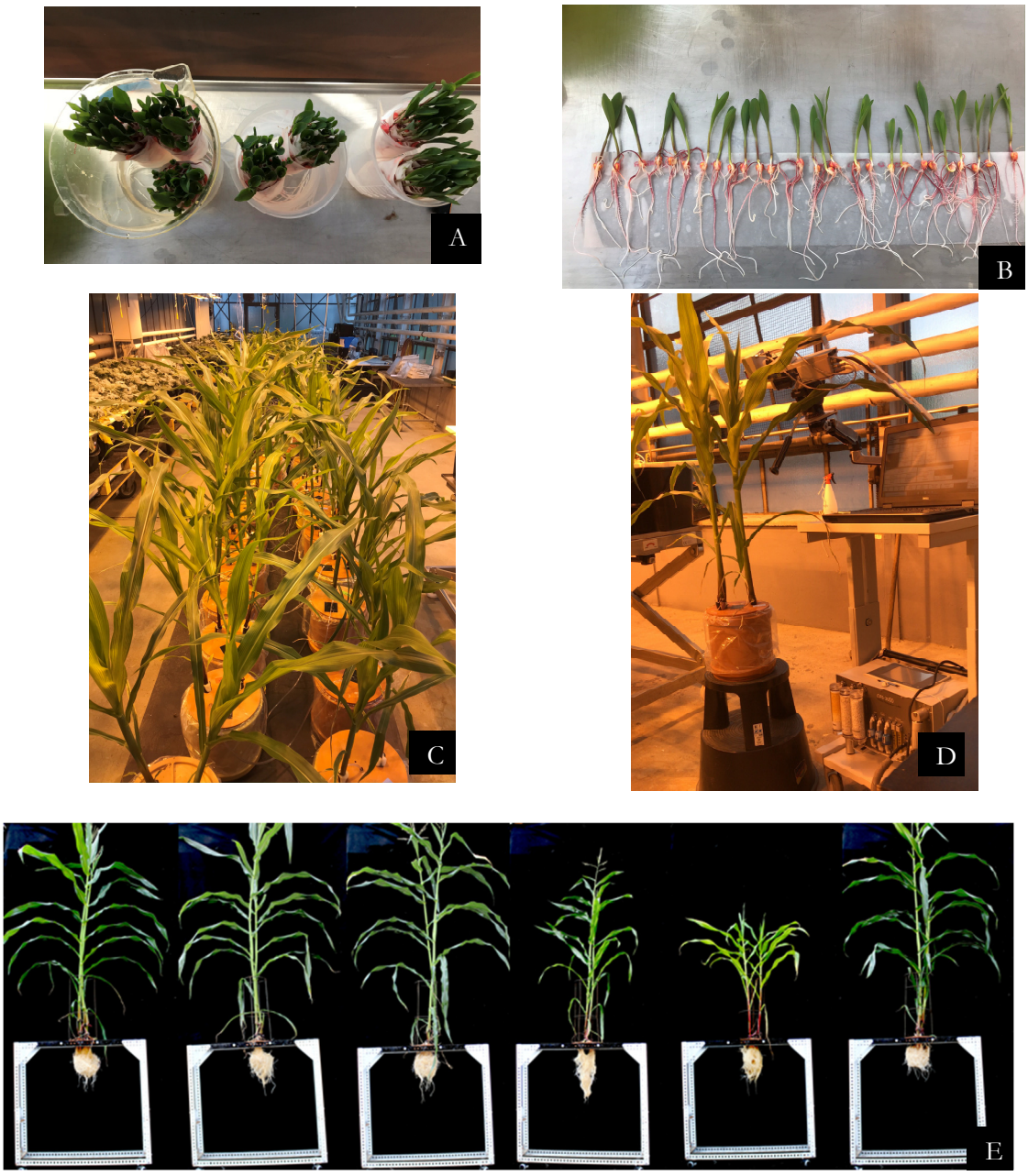
Suppl. Figure 2. Preparation of the bottles for the fertilizer application (A); Daily opening of the bottles to determine soils pH and volatilized N-NH_3. (B); pH evaluation using semisolids sensor (C); and determination of volatilized N-NH_3 using automatic titrator (D).

Supplementary results from section 4.



Suppl. Figure 3. Seedlings used in the experiment (A); ^{15}N-urea application (B); Assessment of gas exchange (C); Sample preparation for N (D); and UcAm600, UiAm600, UcMt600, UiMt600 and control (whiout Mo) treatments at the end of cultivation (E).

Supplementary results from section 5.



Suppl. Figure 4. Germination (A); Seedling selection (B); Plant growth during nutrient omission (C); Gas exchange assessment (D); and -Mo+N, -Mo_L+N, -Mo_R+N, -Mo-N_R and +Mo+N at the end of cultivation (E).