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

Proportions of nitrate and ammonium and their impact in two species of *Poaceae*: a study with nanoparticles and ionic nutrients in soil and nutrient solution

João Cardoso de Souza Junior

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

Piracicaba 2021 João Cardoso de Souza Junior Agronomist

Proportions of nitrate and ammonium and their impact in two species of *Poaceae*: a study with nanoparticles and ionic fertilizers in soil and nutrient solution versão revisada de acordo com a resolução CoPGr 6018 de 2011

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1. Assimilação de nitrogênio 2. Enzimas antioxidantes 3. Estresse oxidativo 4. Fotossíntese 5. Limitação de enxofre 6. *Megathyrsus maximum* 7. Nanopartículas de cobre 8. Prolina 9. Toxidez de manganês 10. *Zea Mays* I. Título

DEDICATION

To my parents Maria Salete and João Cardoso, for giving me the life, supporting me, loving me, everything that I needed to become the man that I am now; allowing me to see the world and all beautiful things; allowing me to live all my dreams. Everything happened and will happen because of you.

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EPIGRAPH

Imagination is more important than knowledge. For knowledge is limited to all we know and understand, while imagination embraces the entire world, and all there ever will be to know and understand. Albert Einstein

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RESUMO

Proporções de nitrato e amônio e seus impactos em duas espécies de *Poaceae*: um estudo com nanopartículas e nutrientes iônicos em solo e solução nutritiva

O nitrogênio (N) é o principal modulador do crescimento de gramíneas forrageiras. Estudos mostraram que o fornecimento de proporções de nitrato e amônio (NO₃⁻/NH₄⁺) melhoraram a performance do capim tanzânia [Panicum maximum Jacq. cv. Tanzania (syn. Megathyrsus maximus (Jacq.) B.K.Simon & S.W.L. Jacobs)] em situação de toxidez por excesso de cobre (Cu). No entanto, há poucos relatos indicando o efeito positivo de proporções de NO₃⁻/NH₄⁺ em gramíneas que receberam Cu via nanopartículas, excesso de manganês (Mn) ou sofreram limitação nutricional de S. Por isso, planejaram-se um experimento com milho (Zea mays L.) e dois com capim tanzânia com o objetivo de confirmar a hipótese que proporções de NO3-/NH4+ alteram a performance de gramíneas em condição de excesso e limitação de nutriente: i) um fatorial 2×4 em blocos completos ao acaso com quatro repetições, sendo os fatores: duas proporções de NO₃⁻/NH₄⁺ (100/0 e 50/50) combinada com quatro doses de nanopartículas de cobre (Cu) (0, 25, 50 e 100 mg) kg^{-1}) em um Espodossolo da Florida, Estados Unidos, cultivado com milho; ii) um fatorial 2 \times 4 em blocos completos ao acaso com quatro repetições, sendo os fatores: duas proporções de NO₃⁻ $/NH_4^+$ (100/0 e 70/30) combinadas com quatro doses de Mn (10, 500, 1.500 e 3.000 µmol L⁻¹) em solução nutritiva cultivada com capim tanzânia e; iii) um fatorial 2×3 em blocos completos ao acaso com quatro repetições, sendo os fatores: duas proporções de NO_3^-/NH_4^+ (100/0 e 70/30) combinadas com três doses de enxofre (S) (0,1, 1,0 e 2,0 mmol L⁻¹) em solução nutritiva cultivada com capim tanzânia. No experimento i) a produção de massa seca foi mais alta nas plantas de milho que receberam N na proporção 100/0 NO₃⁻/NH₄⁺, a qual propiciou maior disponibilidade de Cu e acumulação desse nutriente tanto na parte aérea quanto nas raízes, melhores crescimento, fotossíntese, absorção de nutrientes e aumento do pH do solo rizosférico, melhorando a disponibilidade de nutrientes importantes, bem como a diminuição de outros tóxicos. No experimento ii) o excesso de Mn 3.000 µmol L⁻¹ não afetou o crescimento do capim tanzânia. A máxima concentração de Mn foi obtida nas plantas que receberam todo o N na forma de NO₃-e Mn 3.000 µmol L⁻¹, com valores acima do reportado para hiper acumuladoras. Esse efeito induziu o estresse oxidativo no capim, afetando negativamente a atividade da glutamina sintetase e fotossíntese. No entanto, nessa condição, ocorreu aumento da concentração de prolina, a qual foi relacionada à melhor sobrevivência da planta em situação de alto Mn. No experimento iii) em situação de suficiência de S, plantas crescidas com N na forma de NO_3^- e NH_4^+ cresceram melhor e acumularam mais S do que plantas crescidas com N somente como NO₃⁻. A aplicação de NH₄⁺ em plantas sob limitação de S induziu maior acumulação de NO₃⁻ nas raízes do que nas plantas crescidas somente com NO3⁻. Apesar disso, a limitação de S diminuiu a atividade fotossintética das plantas que receberam N como NO3-, mas não nas plantas que receberam conjuntamente NO3- e NH4⁺. A limitação de S causou estresse oxidativo, o qual foi mitigado pela ação da guaiacol peroxidase estimulada pelo efeito positivo da aplicação conjunta de NO₃⁻ e NH₄⁺. Proporções de NO₃⁻/NH₄⁺ alteram a performance de duas espécies de *Poaceae* em condições de excesso e limitação de nutriente, mesmo na forma de nanopartículas, evidenciando que na fertilização nitrogenada deve-se levar em conta as proporções entre os íons de N e as condições abióticas impostas às plantas.

Palavras-chave: Assimilação de nitrogênio, Enzimas antioxidantes, Estresse oxidativo, Fotossíntese, Limitação de enxofre, *Megathyrsus maximum*, Nanopartículas de cobre, Prolina, Toxidez de manganês, *Zea mays*

ABSTRACT

Proportions of nitrate and ammonium and their impact in two species of *Poaceae*: a study with nanoparticles and ionic nutrients in soil and nutrient solution

Nitrogen (N) plays a key role in forage grass growth. Studies have shown that the proportions of nitrate and ammonium (NO₃⁻/NH₄⁺) improved the growth of Tanzania guinea grass [Panicum maximum Jacq. cv. Tanzania (syn. Megathyrsus maximus (Jacq.) B.K.Simon & S.W.L. [acobs)] under copper (Cu) excess. However, no reports are indicating the positive effects of proportions of NO₃⁻/NH₄⁺ for grasses that received Cu via nanoparticles (CuNP), excess manganese (Mn), or submitted to S limitation. For this reason, one experiment with maize (Zea mays L.) and two with Tanzania guinea grass were carried out to confirm the hypothesis that the proportions of NO₃⁻/NH₄⁺ affect the performance of the grasses under conditions of excess nutrient and limitation. The experiments were: i) a factorial 2×4 in complete randomized block design with four replications. Factors were: two proportions of NO₃⁻/NH₄⁺ (100/0 and 50/50) combined with four rates of Cu nanoparticles (0, 25, 50, and 100 mg kg⁻¹) in a Spodosol soil from Florida, United States, grown with corn; ii) a factorial 2×4 in complete randomized block design with four replications. Factors were: two proportions of NO_3^-/NH_4^+ (100/0 and 70/30) combined with four rates of Mn (10, 500, 1,500, and 3,000 μ mol L⁻¹) in nutrient solution grown with Tanzania guinea grass and; iii) a factorial 2×3 in complete randomized block design with four replications. Factors were: two proportions of NO_3^-/NH_4^+ (100/0 and 70/30) combined with three rates of sulfur (S) (0.1, 1.0, and 2.0 mmol L⁻¹) in nutrient solution grown with Tanzania guinea grass. Experiment i) the dry biomass of maize plants was high at 100/0 NO₃⁻/NH₄⁺, which provided greater concentration and accumulation of Cu in the shoots and roots, as well as better growth, photosynthesis, nutrient accumulation, and high rhizosphere soil pH, improving the availability of important nutrients, as well as mitigating toxic effects of CuNP. Experiment ii) The excess Mn at 3,000 µmol L⁻¹ did not affect the dry biomass of Tanzania guinea grass. The maximum concentration of Mn was obtained in the plants that received a combination of all N as NO3⁻ and Mn at 3,000 μ mol L⁻¹, with higher averages than that reported for hyperaccumulators plants. This effect induced oxidative stress in the grass, negatively affecting the activity of glutamine synthetase and photosynthesis. However, for that condition, there was an increase in the proline concentration, which was related to better plant survival in a situation of excess Mn. Experiment iii) in a situation with sufficient S, plants receiving 70/30 NO3-/NH4+ grown better and accumulated more S than those plants are grown with NO₃⁻ solely. The application of NH₄⁺ for plants under S limitation showed a high accumulation of NO₃⁻ in the roots. However, the S limitation decreased the photosynthetic activity in the plants grown with NO₃⁻ solely, but not in the plants that received both NO₃⁻ and NH₄⁺. The S limitation caused oxidative stress, which was mitigated by guaiacol peroxidase and stimulated by the positive effects of NO₃⁻ and NH₄⁺. Proportions of NO₃⁻/NH₄⁺ change the performance of two species of *Poaceae* in conditions of excess and nutrient limitation, even in the form of nanoparticles, indicating that N fertilization must be carefully applied, considering proportions between N ions and abiotic conditions imposed to plants.

Keywords: Antioxidant enzymes, Copper nanoparticles, Manganese toxicity, *Megathyrsus* maximum, Nitrogen assimilation, Oxidative stress, Photosynthesis, Proline, Sulfur limitation, *Zea mays*

1. INTRODUCTION

Proportions of NO₃⁻/NH₄⁺ can modulate N assimilation, biomass production, and tolerance of Tanzania guinea grass [*Panicum maximum* Jacq. cv. Tanzania (syn. *Megathyrsus maximus* (Jacq.) B.K.Simon & S.W.L. Jacobs)] to abiotic stress under copper (Cu) toxicity (Souza Junior et al., 2018; Souza Junior et al., 2019). This effect made our research group investigate the effect of other important nutrients, both in ionic and non-ionic forms, such as nanoparticles since they can also be uptake, assimilated, and used by plants. It was previously reported that proportions of NO₃⁻/NH₄⁺ can mitigate the oxidative stress caused by heat (Hu et al., 2017; Liu et al., 2017), excess cadmium (Cd) (Leite and Monteiro, 2019), and salt (Meng et al., 2016) in plants. However, it has not reported the effects of N forms associated with non-ionic nutrients, such as Cu nanoparticles, as well as these effects on stress mitigation caused by excess Mn or S limitation. Abiotic factors were selected as the following justification:

1. Cu nanoparticles: a) in recent years, several studies have been showing a positive effect of Cu nanoparticles in agriculture. Copper nanoparticles are less toxic than ionic Cu (Ameh and Sayes, 2019) and seem more efficient than Cu sulfate for plant uptake (Palit and Goswami, 2015); b) copper is an essential element for life, but very toxic for humans at concentrations aboverecommended limits, as it can cause Alzheimer, reduction in cognition, and neurodegeneration (Brewer, 2010); c) no reports are showing the relationship between proportions of NO₃⁻/NH4 ⁺ and Cu nanoparticles, especially by analyzing the rhizosphere soil since NO₃⁻ can especially affect the chemical reactions in this zone.

2. Excess Mn: a) industrial and urban residues containing heavy metals are often deposited on agricultural areas (Nogueirol et al., 2013), which causes contaminations of agricultural soil and water with Mn (Millaleo et al., 2010); b) manganese is a heavy metal essential for life, but its excess is very toxic. When entering the food chain, Mn can intoxicate humans and cause neurodegeneration, alteration of motor functions, reduced response to speed, problems with smell, mood changes, memory lack, and cognitive impairment (Santos et al., 2014); c) just a few plants can live in soils highly contaminated with Mn, such as areas next to mining (Boojar and Goodarzi, 2008), which encourages plant nutritionists to explore the potential of nutrients for increasing the plant tolerance against heavy metals, making Mn phytoextraction and phytostabilization even better (Souza Junior et al., 2018; Souza Junior et al., 2019).

3. Limitation of S: a) sulfur is a nutrient very important for grasses growth, especially when N fertilization is carrying out (De Bona et al., 2013). However, the preferences for more concentrated fertilizers that do not contain S and its exportation by crops due to intensive

cultivation can affect the plant growth (Gao et al., 2016); b) in recent years, global agreements have been signed to reduce the emissions of S gases (Stern and Rydge, 2012), which led to a low deposition of S from the atmosphere to the soil, aggravating the S deficiency in plants (Gao et al., 2016); c) sulfur is directly related to the tolerance of plants to abiotic stress and its deficiency in sensitivy plants can affect the growth and production in a global stressful context caused by climate and environmental changes (Bagheri et al., 2017; Rabêlo et al., 2016).

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2. NITRATE SUPPLY ENHANCES AVAILABILITY OF NUTRIENTS IN RHIZOSPHERE AND BULK SOIL AND COPPER NANOPARTICLES ACCUMULATION BY MAIZE PLANT

ABSTRACT

Maize (Zea mays L.) is one of the most responsive crops to nitrogen (N) fertilization, which is required in large amounts and can be supplied as a combination of NO3⁻ and NH4⁺. Copper (Cu) is a micronutrient for plants and Cu nanoparticles (CuNP) is one of the sources of Cu fertilizers used. However, there are no studies associating proportions of NO3-/NH4+ and CuNP for maize plants. The objective was to evaluate the effects of proportions of NO_3 -/NH₄⁺ combined with Cu nanoparticles on soil properties, nutrient availabilities, chlorophyll fluorescence, nutrient accumulation, and growth of maize plants. The experiment was performed in a greenhouse with a 4×2 factorial in a randomized complete block design with four replications. The treatments included proportions of NO3-/NH4+ (100/0 and 50/50) combined with applications of CuNP (0, 25, 50, and 100 mg kg-1) in a Spodosol soil. A two-way analysis of variance and the SNK test at 5 % was performed. The dry biomass production was the highest when the maize plants received a combination of $100/0 \text{ NO}_3$ -/NH4+ and CuNP 25 mg kg-1. Applications of CuNP did not increase the dry biomass production when the maize plants received 50/50 NO3-/NH4+. The maximum efficiency of photosystem II was high when the plants were grown under 100/0 NO₃-/NH₄+. Applications of CuNP decreased the quantum efficiency of photosystem II and photochemical quenching, as well as increased the non-photochemical quenching, but only when the plants received 50/50 NO₃-/NH₄+. The accumulations of C, N, P, K, Ca, Mg, Cu, Fe, Mn, and Zn were higher at the combination between 100/0 NO₃-/NH₄+ and CuNP 25 mg kg-1 than at the other combinations, which were related to the lowest availability of those nutrients, as well as pH and electric conductivity in the rhizosphere soil. There are several positive effects of CuNP when maize plants receive all N as NO3-. However, the positive effects of CuNP became nonsignificant when the plants receive 50/50 NO3-/NH4+. For a better uptake of Cu nanoparticles by maize plants, it is suggested an application of all N as NO₃-.

Keywords: Chlorophyll fluorescence; Copper nanoparticles; Nitrogen; Nutrient accumulation; *Zea mays*

2.1. INTRODUCTION

Maize (*Zea mays* L.) is one of the most important agricultural products and nutrientresponsive crops (Maresma et al., 2017), which is often studied as a plant model to understand the role of fertilizers in growth and production. The United States of America is leading the ranking of the world's largest maize producers with 370 million tons in 2017, ahead of China and Brazil with 215 and 97 million tons, respectively (Faostat, 2019). Maize plays a central role in global food security as a source of carbohydrate in the diet for humans and animals, as well as oil and sugar for industry (Adhikari et al., 2016; Zhang and Zhao, 2019).

Nitrogen (N) is required in a large amount by maize (Zhang and Zhao, 2019) since this nutrient is a constituent of all amino acids and proteins, nitrogenous bases, membranes, and plant

hormones (Heldt, 2005; Taiz et al., 2017). Nitrogen ions as NO_3^- and NH_4^+ are the major N forms applied as fertilizers to maize as they can be directly absorbed by plant roots (Zhang and Zhao, 2019). The proper proportion of NO_3^-/NH_4^+ for plant development depends on the crop species and environmental conditions (Liu et al., 2017; Nogueirol et al., 2018; Souza Junior et al., 2018). Pedersen et al. (2019) studied five combinations of NO_3^-/NH_4^+ for maize cultivated in sandy soil without application of micronutrients and they observed that the best proportion for plant development and nutrient accumulation was $50/50 NO_3^-/NH_4^+$. Going further, Souza Junior et al. (2019) showed that the use of NO_3^- solely improved the plant survival in a condition of excess Cu in the growth media.

Copper is an essential element for biota. In plants, Cu is related to electron transport in photosynthesis as a constituent of plastocyanin, the first electron donor from photosystem II to photosystem I. Copper also plays an essential role in the constitution of enzymes, respiration, and N metabolism (Epstein and Bloom, 2006; Mahanty et al., 2012). Since Cu is a micronutrient, it is required in a very small amount by most crops and there is a fine line between essentiality and toxicity (Liu et al., 2015). Therefore, Cu must be applied carefully and it is desirable to optimize other nutrients in the soil, which can raise the Cu accumulation or mitigate its possible toxicity in plants, as demonstrated by Souza Junior et al. (2019).

Several studies showed the positives effects of Cu nanoparticles (CuNP) in agriculture (Palit and Goswami, 2015; Rajput et al., 2018; Ameh and Sayes, 2019). Many techniques have been presented to produce nanoparticles, such as grinding, chemical reactions, and laser attacks (Rajput et al., 2018). Copper nanoparticles are different from regular Cu fertilizers in terms of physical, chemical, and biological characteristics (Ameh and Sayes, 2019). It was reported that CuNP are on average 10 times less toxic than ionic Cu (Ameh and Sayes, 2019) and more efficient than Cu sulfate for plant uptake (Palit and Goswami, 2015). This is due to their extremely small particle sizes, which allow greater reactivity and easy transport across cell membranes even when low rates are applied (Ameh and Sayes, 2019).

Copper nanoparticles can be a potential xenobiotic due to their high cell reactivity (Melegari et al., 2013; Song et al., 2015; Costa and Sharma, 2016) and its excess can inhibit various plant functions such as enzymatic activity, photosynthesis, chlorophyll synthesis, and plasma membrane integrity, culminating in low plant development (Nogueirol et al., 2018; Palit and Goswami, 2015; Souza Junior et al., 2019). The toxicity of CuNP depends on plant species, growth conditions, element exposure timing, metal concentration, and particle size (Rajput et al., 2018). However, an important point that is rarely mentioned in literature is the relationship between N and their ionic forms.

Copper availability in soils is known to be directly influenced by soil pH. Lower soil pH can increase Cu mobility and availability (Fan et al., 2011b, 2011a). However, rhizosphere pH is also known to be particularly important for Cu uptake and one of the ways to modify the rhizosphere soil pH is an adjustment in proportions of NO_3^-/NH_4^+ (Zhang and Zhao, 2019). Plant roots exposed to a high amount of NO_3^- exude HCO_3^- to rhizosphere soil to maintain a plant anionic balance, causing an increase in rhizosphere pH. Conversely, when plant roots uptake NH_4^+ from the soil they exude H⁺ to maintain a plant cation balance, thus lowering rhizosphere pH (Marschner, 2012).

The hypothesis is that the proper proportion of NO_3^-/NH_4^+ enhances the growth and production of maize grown under the rates of CuNP. The objective was to evaluate the effects of the proportions of NO_3^-/NH_4^+ combined with rates of CuNP on chlorophyll fluorescence, soil properties, nutrient availabilities, nutrient accumulations, and growth of maize plants.

2.2. MATERIAL AND METHODS

2.2.1. Experimental design

An experiment with maize was performed in a greenhouse with a 2 × 4 factorial in a randomized complete block design with four replications. Treatments included: proportions of NO_3^-/NH_4^+ at 100/0 and 50/50 combined with applications of CuNP at the rates of 0, 25, 50, and 100 mg kg⁻¹. The proportion of 50/50 NO₃⁻/NH₄⁺ was chosen since this is the same ratio present in ammonium nitrate fertilizer, one of the most important sources of N, and considered the proper proportion for maize development according to Pedersen et al. (2019). The rates of CuNP were chosen because those are the most responsive according to studies, with the high end (100 mg kg⁻¹) used to verify toxic effects (Du et al., 2018; Palit et al., 2019; Sun et al., 2018).

2.2.2. General conditions of the experiment

Greenhouse conditions were automatic air circulation and humidification. Twelve maize seeds were germinated directly in the pots, each contained 4 kg of air-dry Spodosol soil (sandy texture) collected at 0–15 cm depth from the University of Florida experimental farm in Fort Pierce, and the soil was sieved to <1 mm in particle sizes. Seeds were sown on February 19th, 2020, and germinated on February 22th, 2020. After four days of growth, four plants were removed and after five more days, another four plants were removed to keep four uniform plants per pot.

2.2.3. Fertilization, irrigation, and soil sampling

Soil water holding capacity was determined by the gravimetric method according to Abid et al. (2016) with some modifications. The average weight of three experimental units was determined. Afterward, the experimental units were saturated with deionized water and then drained for 48 h under shade. Differences between water drained and dry soil were considered as water mass 100% of total water retention (TWR). Thereby, the value that represents 70% of TWR was calculated and used in all phases of the experiment as a reference for irrigating.

Fertilizers were applied via nutrient solutions made from pure salts and the amounts of N, P, K, Ca, Mg, and S were 200, 100, 200, 100, 50, and 65 mg kg⁻¹, respectively, according to Pedersen et al. (2019). Stock solutions of these nutrients were used to compose a final solution that was applied to the soil. Then, the soil was placed in a closed individual plastic bag and homogenized for 5 min each pot. After seven days, the CuNP stock solution (Sigma-Aldrich[®], 25 nm particle size) was prepared and the rates of 0, 25, 50, and 100 mg kg⁻¹ were diluted in deionized water and applied to the soil, homogenized for 5 min, and then placed in 4.5-L plastic pots. Dicyandiamide was applied at a 5% of N rate to avoid converting NH₄⁺ into NO₃⁻ by nitrifying bacteria from the soil according to Ma et al. (2019).

A sampling of rhizosphere and bulk soil was performed separately according to Pedersen et al. (2019). After shoots harvesting, roots were gently separated from the bulk soil by placing roots on a 2-mm sieve, tapping the roots to take the soil off. Portions of the soil that passed through 2-mm were considered as the rhizosphere soil. The remaining soil in the pots was considered the bulk soil.

2.2.4. Visual observation

Visual observation of plants was performed, aiming to describe the positive effects of fertilizers and possible symptoms of toxicity. A photographic recording of the main effects and symptoms showed by the plants was performed.

2.2.5. Chlorophyll fluorescence

Chlorophyll fluorescence was measured on diagnostic leaves of maize (first and second fully expanded leaves from top to bottom) using a chlorophyll fluorescence meter (Opti-Sciences[®] OS5p). The maximum (Fv/Fm) and quantum efficiency of photosystem II (QEPSII),

photochemical (qP) and non-photochemical quenching (NPQ), and electron transport rate (ETR) were quantified.

2.2.6. Dry biomass production

Maize plants were harvested after 30 days of growth by cutting them next to the soil surface, on March 17^{th} , 2020. The shoot and roots were dried separately in a forced circulation oven at 75 °C until constant weight and then their dry biomass was recorded by using a digital scale. After recording, dried plant samples were ground by using a stainless micro-mill to < 1 mm particle size powder.

2.2.7. pH and electrical conductivity of the soil

Soil pH and electrical conductivity (EC) in the bulk and rhizosphere soil were measured in deionized water by using a pH/EC meter (AB 200, Fisher Scientific[®], Atlanta, USA). Ratios of 1:2.5 soil/water for pH and 1:5 for EC were used according to Carter and Gregorich (2007).

2.2.8. Availability of nutrients in the soil samples

 $N-NO_3^-$ and $N-NH_4^+$ in the air-dried rhizosphere and bulk soil were extracted by using potassium chloride (KCl 2 M) on a shaker for 30 min at 120 g and then determined their concentrations on a spectrophotometer at 520 nm according to Carter and Gregorich (2007). Labile P and exchangeable K, Ca, Mg, Cu, Fe, Mn, and Zn in the soil were extracted by using Mehlich–3 acid solution on a shaker for 5 min at 120 g, and then they were quantified on inductively coupled plasma-optical emission spectrometry (ICP-OES, Ultima[®], JY Horiba Group, Edison, USA) according to Carter and Gregorich (2007). A standard was used to verifying the accuracy of the method with the acceptable recovery of 90 – 110%.

2.2.9. Concentrations and accumulations of nutrients in the plant samples

Total C and N in the shoots and roots were determined by using the C/N 828 LECO auto-analyzer (Leco Inc.[®], St. Joseph, USA). The concentrations of P, K, Ca, Mg, Cu, Fe, Mn, and Zn in the shoots and roots were quantified after digesting with concentrated nitric acid at 180 °C

for 8 h and then they were analyzed on an ICP-OES according to Zhou et al. (2017). The accumulations of those minerals were calculated by multiplying their concentrations with the dry biomass of shoots and roots. Standards were used to verify the accuracy of the method with the acceptable recovery of 90 - 110 %.

2.2.10. Statistical analysis

A two-way analysis of variance (ANOVA) by using the "Statistical Analysis System" at 5% was performed (SAS[®] Institute, 2008). When the $NO_3^-/NH_4^+ \times Cu$ interaction was significant based on the F test, the main factor proportions of NO_3^-/NH_4^+ within the rates of CuNP and the rates of CuNP within the proportions of NO_3^-/NH_4^+ were further analyzed by the SNK test at 5%. When the interaction was not significant, averages of the main factors were compared separately by using the SNK test at 5%.

2.3. RESULTS

2.3.1. Visual observation

The germination and initial growth were the best when the maize plants were supplied with $100/0 \text{ NO}_3^-/\text{NH}_4^+$ and CuNP 25 mg kg⁻¹, among all treatments (Fig. 1a). Overall, the plants cultivated at $100/0 \text{ NO}_3^-/\text{NH}_4^+$ grew better than those at $50/50 \text{ NO}_3^-/\text{NH}_4^+$ (Fig. 1a). Additionally, the plant roots that grew at $100/0 \text{ NO}_3^-/\text{NH}_4^+$ were greater than those at other proportions (Fig. 1b, 1c). Finally, the plants that received $100/0 \text{ NO}_3^-/\text{NH}_4^+$ (Fig. 1a). Plant roots that grow has a $50/50 \text{ NO}_3^-/\text{NH}_4^+$ (Fig. 1a). Plant roots followed the same pattern of the shoots (Fig. 1b, 1c).



Figure 1. Shoots and roots of the maize plants grown under proportions of NO_3^-/NH_4^+ and rates of CuNP (mg kg⁻¹) at 25 days after germination. All treatments side by side **(a)**; maize roots under $100/0 NO_3^-/NH_4^+$ **(b)** and; maize roots under $50/50 NO_3^-/NH_4^+$ **(c)**.

2.3.2. Dry biomass production

The NO₃⁻/NH₄⁺ × CuNP interaction (from now on, it will be referred to as interaction only) was significant for the dry biomass of shoots and roots. The dry biomass of shoots (Fig. 2a) and roots (Fig. 2b) were the highest at the combination between 100/0 NO₃⁻/NH₄⁺ and CuNP at 25 mg kg⁻¹ among all treatments. Additions of CuNP did not increase the dry biomass of shoots and roots when the maize plants received 50/50 NO₃⁻/NH₄⁺ (Fig. 2). The dry biomass of shoot and roots decreased by applying CuNP at 50 and 100 mg kg⁻¹ regardless of the proportion of NO₃⁻/NH₄⁺ (Fig. 2).



Figure 2. Dry biomass of shoots (a) and roots (b) of maize grown under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil. The SNK test at 1% was applied. Lowercase letters compare rates of Cu nanoparticles within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each Cu nanoparticles rate. Coefficient of variation (%) = 8 (a) and 5 (b). Error bars indicate standard error.

2.3.3. Chlorophyll fluorescence

The interaction was significant for all parameters of chlorophyll fluorescence, except for the maximum efficiency of photosystem II (Fv/Fm), which was affected by the main factors (Table 1). Regarding the proportions of NO_3^-/NH_4^+ , a higher value at Fv/Fm at 100/0 than at 50/50 NO_3^-/NH_4^+ was verified (Table 1). Regarding the CuNP, a lower value at Fv/Fm was verified at CuNP 100 mg kg⁻¹ than at other rates (Table 1).

	Fv/Fm	C		N		C/N
	Leaf	Shoots	Roots	Shoots	Roots	Roots
			g	kg ⁻¹		
NO_{3}/N	H_{4}^{+}					
100/0	$0.788 \ ^{\rm a} \pm 0.004$	379 ^a ± 1.56	297 ª ± 5.54	47.1 $^{ns} \pm 0.62$	38.7 = 0.92	7.74 ^b \pm 0.20
50/50	$0.759 ^{\mathrm{b}} \pm 0.007$	347 ^ь ± 6.61	264 ^ь ± 10.6	$47.8 \text{ ns} \pm 1.58$	30.4 ^ь ± 1.25	$8.74 \ ^{a} \pm 0.19$
Cu nanoj	particles (mg kg ⁻¹))				
0	$0.784 \ ^{\rm a} \pm 0.011$	373 ^a ± 4.38	295 ª ± 8.28	45.9 ^ь ± 0.94	34.5 ^{ns} ± 1.86	$8.70 \text{ ns} \pm 0.32$
25	$0.781 \ ^{\rm a} \pm 0.008$	377 ^a ± 2.65	303 ª ± 6.85	43.7 ^ь ± 0.86	35.8 ^{ns} ± 1.04	$8.51 \text{ ns} \pm 0.25$
50	$0.772 \ ^{a} \pm 0.032$	347 ^ь ± 13.1	283 ª ± 9.08	46.9 ^ь ± 1.19	36.1 ^{ns} ± 1.01	$7.85 \text{ ns} \pm 0.13$
100	0.757 ^ь ± 0.010	355 ^ь ± 6.37	242 ^ь ± 15.1	53.3 ª ± 1.53	31.9 ^{ns} ± 3.35	$7.90 \text{ ns} \pm 0.42$
<i>p</i> value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
cv (%)	2.9	3.9	6.7	6.3	10.5	8.6

Table 1. Maximum efficiency of photosystem II (Fv/Fm), concentrations of C and N in the shoots and roots, and C/N ratio of the maize roots under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil.

The SNK test at 1% was applied. Different letters in each sub-column show significant differences. C/N ratio was calculated from the accumulation. ns not significant. \pm standard error. cv coefficient of variation.

The quantum efficiency of photosystem II (QEPSII) showed the lowest value at the rate of CuNP 100 mg kg⁻¹ (Fig. 3a). The rates of CuNP did not affect the QEPSII when the plants are grown under 100/0 NO₃⁻/NH₄⁺ (Fig. 3a). Additionally, regarding plants at CuNP 50 mg kg⁻¹, the average of QEPSII was higher at 100/0 NO₃⁻/NH₄⁺ than at 50/50 NO₃⁻/NH₄⁺ (Fig. 3a). The electron transport rate (ETR) in the maize plants was greater when the plants were grown at 50/50 NO₃⁻/NH₄⁺ combined with CuNP 50 mg kg⁻¹, as compared to the other treatments (Fig. 3b). However, the lowest ETR was verified when the plants were grown at 50/50 NO₃⁻/NH₄⁺ combined with CuNP 100 mg kg⁻¹, as well as at 100/0 NO₃⁻/NH₄⁺ combined with CuNP 0 mg kg⁻¹ (Fig. 3b).



Figure 3. Quantum efficiency of photosystem II (a), electron transport rate (b), photochemical (c), and non-photochemical quenching (d) of the maize plants under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil. The SNK test at 1% was applied. Lowercase letters compare rates of Cu nanoparticles within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each Cu nanoparticles rate. Coefficient of variation (%) = 8 (a), 18 (b), 7 (c), and 21 (d). Error bars indicate standard error.

The photochemical quenching was lower at the combination between $50/50 \text{ NO}_3^-/\text{NH}_4^+$ and CuNP 100 mg kg⁻¹ than at any other combination (Fig. 3c). A higher value of the nonphotochemical quenching (NPQ) was verified when the plants were exposed to $50/50 \text{ NO}_3^-/\text{NH}_4^+$ combined with CuNP 100 mg kg⁻¹ as compared to the other treatments (Fig. 3d).



Figure 4. Accumulations of C (a) and N (b) in the shoots and roots and C/N ratio (c) in the shoots of maize under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol. The SNK test at 1% was applied. Lowercase letters compare rates of Cu nanoparticles within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each Cu nanoparticles rate. Coefficient of variation (%) = 25 (a, shoots), 7 (a, roots), 24 (b, shoots), 26 (b, roots), 6 (c). Error bars indicate standard error.

2.3.4. Concentrations of C and N in the plant parts

The interaction was not significant for the concentrations of C and N in the shoots and roots. However, it was verified significance for the main factors (Table 1). The concentrations of C in the shoots and roots were decreased by the proportion of $50/50 \text{ NO}_3^-/\text{NH}_4^+$ (Table 1). Also, it has been verified the lowest concentration of C in the shoots and roots when the plants were supplied with CuNP at 100 mg kg⁻¹. Regarding the concentration of N in the shoots, it was verified the main effect of CuNP with higher values at CuNP 100 mg kg⁻¹ than at other rates (Table 1). About the concentration of N in the roots, it has been verified higher values at 100/0 than at $50/50 \text{ NO}_3^-/\text{NH}_4^+$ (Table 1).

The interaction was significant for the accumulations of C and N in the shoots and roots, as well as for the C/N ratio in the shoots (Fig. 4). However, the C/N ratio in the roots was significant for the main effect proportions of NO_3^-/NH_4^+ (Table 1). The accumulations of C and N in the shoots and roots were increased by CuNP 25 mg kg⁻¹, but only when the plants were grown at the proportion $100/0 NO_3^-/NH_4^+$ (Fig. 4a, 4b). The applications of CuNP did not increase the accumulation of C and N when the plants were grown with $50/50 NO_3^-/NH_4^+$ (Fig. 4a, 4b).

The C/N ratio in the shoots was affected by the rates of CuNP only when the plants were grown at 50/50 NO₃⁻/NH₄⁺ combined with CuNP at the rate of 50 and 100 mg kg⁻¹, showing a lower value at CuNP 100 mg kg⁻¹ than at any other combinations (Fig. 4c). The rates of CuNP did not affect the C/N ratio when the plants were grown at 100/0 NO₃⁻/NH₄⁺ (Fig. 4c). For the plant roots, the higher value of the C/N ratio was verified when the plants were grown at 50/50 than at 100/0 NO₃⁻/NH₄⁺ (Table 1).

2.3.5. Cu, Fe, Mn, and Zn in the plant parts

The interaction was not significant for the concentrations of Cu, Fe, Mn, and Zn in the shoots and roots. However, the main factors have affected those parameters (Table 2). Regarding the shoots, it was verified that only the Mn concentration was affected by the main factor proportions of NO_3^-/NH_4^+ , with the highest values occurring at $100/0 NO_3^-/NH_4^+$ (Table 2). The rates of CuNP affected the plant concentration of all micronutrients studied. The higher concentrations of Cu and Zn in the shoots were verified when the plants received CuNP at 25 mg kg⁻¹, as compared to the other rates (Table 2). Concentrations of Fe and Mn in the shoots without CuNP were higher than CuNP at 100 mg kg⁻¹ (Table 2).

In the roots, only the concentrations of Cu and Mn were affected by the proportions of NO_3^-/NH_4^+ with the higher values at 100/0 than at 50/50 NO_3^-/NH_4^+ , whereas the concentrations of Cu and Mn were increased with increasing the rates of CuNP (Table 2). An opposite trend was observed for the concentrations of Fe in the roots, which had lower values as the rates of CuNP increased (Table 2).

	Cu	Fe	Mn	Zn
		mg k	2g ⁻¹	
Shoots				
NO_3^-/NH_4^+				
100/0	$7.40 \text{ ns} \pm 0.93$	61.0 ^{ns} ± 6.21	156 ª ± 6.04	33.6 ^{ns} ± 1.69
50/50	$6.86 \text{ ns} \pm 0.52$	$58.9 \text{ ns} \pm 4.80$	130 ^b ± 8.02	$30.9 \text{ ns} \pm 2.75$
Cu nanoparticl	les (mg kg ⁻¹)			
0	$4.82 ^{\text{b}} \pm 0.46$	79.5 ^a ± 5.41	156 ª ± 7.96	35.3 ^ь ± 0.99
25	$9.95 \ ^{a} \pm 0.83$	$65.1 \text{ ab} \pm 5.95$	154 ª ± 5.17	41.3 ª ± 1.57
50	6.76 ^b ± 1.30	46.4 ^b ± 6.60	$138 ab \pm 11.3$	31.6 ^ь ± 2.57
100	$6.97 ^{\mathrm{b}} \pm 0.57$	48.7 ^ь ± 6.99	123 ^b ± 13.5	$20.8 \circ \pm 2.23$
<i>p</i> value	< 0.01	< 0.01	< 0.05	< 0.01
cv (%)	17.3	17.3	7.94	13.6
Roots				
NO_3^-/NH_4^+				
100/0	85.7 = 8.38	$274 \text{ ns} \pm 33.1$	140 ª ± 11.7	$49.0 \text{ ns} \pm 2.57$
50/50	$65.1 ^{\text{b}} \pm 5.05$	$231 \text{ ns} \pm 26.3$	$62.5 \text{ b} \pm 9.02$	43.9 ^{ns} ± 2.18
Cu nanoparticl	les (mg kg ⁻¹)			
0	38.1 ^ь ± 1.74	324 ª ± 35.8	78.4 ^b ± 15.1	47.9 ^{ns} ± 4.55
25	84.1 = 4.42	$224 \text{ ab} \pm 21.8$	88.1 ^ь ± 23.6	44.5 ns ± 2.64
50	94.1 ^a ± 8.51	311 ª ± 53.1	98.5 ^ь ± 16.7	48.8 ^{ns} ± 2.71
100	$85.4 \text{ a} \pm 10.1$	151 ^ь ± 18.3	141 ª ± 16.9	44.5 ns ± 3.42
<i>p</i> value	< 0.01	< 0.01	< 0.01	> 0.05
cv (%)	21.2	43.4	34.5	23.5

Table 2. Concentrations of Cu, Fe, Mn, and Zn in the shoots and roots of maize plants under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil.

The SNK test at 1% was applied. Different lowercase letters within each sub-column show significant differences. *ns* not significant; \pm standard error; *w* coefficient of variation.

The interaction was significant for the accumulations of Cu and Mn in the shoots and roots (Fig. 5), and for the accumulations of Fe and Zn in the shoots (Table 3). The accumulations of Fe and Zn in the roots were only affected by the main effects of NO_3^-/NH_4^+ and CuNP (Table 4). The highest accumulation of Cu in the shoots and roots occurred when the plants were grown at 100/0 NO_3^-/NH_4^+ combined with CuNP at 25 mg kg⁻¹ (Fig. 5a). The accumulations of Cu in the shoots and roots at both proportions of NO_3^-/NH_4^+ were the lowest as the rates of CuNP increased, starting from CuNP at 50 mg kg⁻¹ (Fig. 5a). The accumulation of Cu in the shoots (Fig. 5a). The rate of CuNP at 25 mg kg⁻¹ did not increase the accumulation of Cu in the shoots when the plants were treated with 50/50 NO_3^-/NH_4^+ compared to the other combinations (Fig. 5a).

The accumulations of Mn in the shoots and roots were higher when the plants received a combination of $100/0 \text{ NO}_3^-/\text{NH}_4^+$ and CuNP at 25 mg kg⁻¹, as compared to the other combinations (Fig. 5b). In both proportions of NO₃⁻/NH₄⁺, the accumulations of Mn decreased with increasing CuNP application, starting from CuNP at 50 mg kg⁻¹, except in the roots since it has not shown any significant increase by using CuNP (Fig. 5b). The accumulations of Mn in the shoots showed a higher value than those in the roots (Fig. 5b), whereas the accumulations of Mn were not affected by the rates of CuNP when combined with $50/50 \text{ NO}_3^-/\text{NH}_4^+$ (Fig. 5b).



Figure 5. Accumulations of Cu (a) and Mn (b) in the shoots and roots of maize plants under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil. The SNK test at 1% was applied. Lowercase letters compare rates of Cu nanoparticles within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each Cu nanoparticles rate. Coefficient of variation (%) = 23 (a, shoots), 34 (a, roots), 14 (b, shoots), and 25 (b, roots). Error bars indicate standard error.

The accumulation of Fe in the shoots was higher with a combination between $100/0 \text{ NO}_3^-/\text{NH}_4^+$ and CuNP at 0 or 25 mg kg⁻¹ than at any other combination (Table 3). The lowest accumulation of Fe was found when the plants received $50/50 \text{ NO}_3^-/\text{NH}_4^+$ with CuNP 50 or 100 mg kg⁻¹ (Table 3). The accumulation of Fe in the roots was higher at 100/0 than at $50/50 \text{ NO}_3^-/\text{NH}_4^+$ as well as at CuNP at a0 or 25 mg kg⁻¹ than at the other rates of CuNP (Table 4). The lowest accumulation of Fe in the roots occurred in either $50/50 \text{ NO}_3^-/\text{NH}_4^+$ and CuNP 100 mg kg⁻¹ (Table 4).

	Fe	Zn	Р	К	Ca	Mg
	—_µg ро	er pot ——		mg po	er pot —	
100/0 N	O ₃ ⁻ /NH ₄ +					
Cu nano	particles (mg kg	r ¹)				
0	468 $^{aA} \pm$ 45.5	$170 \text{ bA} \pm 16.4$	$18.1 ^{\mathrm{bA}} \pm 1.06$	$128 \text{ bA} \pm 11.4$	$16.9 ^{\mathrm{bA}} \pm 1.95$	$8.56~^{\mathrm{bA}}\pm1.05$
25	$402 ^{\text{aA}} \pm 44.7$	$257~^{\mathrm{aA}} \pm 10.9$	$23.7 ^{\text{aA}} \pm 1.72$	$156 ^{\mathrm{aA}} \pm 6.14$	$20.7 ^{\mathrm{aA}} \pm 1.31$	$10.9~^{\mathrm{aA}}\pm0.51$
50	86.9 $^{\rm bA} \pm$ 12.5	77.3 cA \pm 10.9	$7.36 \text{ cA} \pm 0.74$	52.6 cA \pm 7.56	7.21 cA \pm 0.87	$3.52 \text{ cA} \pm 0.34$
100	$29.3 \text{ cA} \pm 5.81$	$18.1 ^{\text{dA}} \pm 4.95$	$1.82 ^{\text{dA}} \pm 0.49$	$13.8 ^{\text{dA}} \pm 4.27$	$2.88 ^{\text{dA}} \pm 0.78$	$1.19~^{\rm dA}\pm0.33$
50/50 N	O_{3}^{-}/NH_{4}^{+}					
Cu nano	particles (mg kg	r ⁻¹)				
0	$119 \ ^{aB} \pm 9.09$	$66.5 {}^{\mathrm{bB}} \pm 4.44$	$8.54 \ ^{aB} \pm 0.56$	$46.3 a^{B} \pm 3.51$	$7.93 \ ^{\mathrm{aB}} \pm 0.78$	$2.64 \ ^{aB} \pm 0.43$
25	$156 aB \pm 35.6$	96.1 $^{aB} \pm 11.9$	$10.5 \ ^{aB} \pm 1.06$	$55.3 aB \pm 7.68$	$8.14 \ ^{aB} \pm 1.08$	$2.93 \ ^{\mathrm{aB}} \pm 0.21$
50	$15.3 ^{\mathrm{bB}} \pm 3.17$	$8.38 ^{\text{cB}} \pm 0.70$	$1.03 ^{\mathrm{bB}} \pm 0.22$	5.51 $^{\rm bB} \pm 0.88$	$1.31 {}^{\mathrm{bB}} \pm 0.11$	$0.36 \text{ bB} \pm 0.01$
100	7.01 $^{\rm bB}\pm$ 1.01	$2.35 {}^{\mathrm{cB}} \pm 0.13$	$0.32 {}^{\mathrm{bB}} \pm 0.02$	$1.91 {}^{\mathrm{bB}} \pm 0.17$	$0.61 {}^{\text{bB}} \pm 0.01$	$0.16 ^{\mathrm{bB}} \pm 0.01$
<i>p</i> value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	> 0.01
cv (%)	14.5	12.8	13.1	13.8	27.6	14.4

Table 3. Accumulations of Fe, Zn, P, K, Ca, and Mg in the shoots of maize plants under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil.

The SNK test at 1% was applied. Lowercase letters compare rates of Cu nanoparticles within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each Cu nanoparticles rate. *ns* not significant; \pm standard error; *cv* coefficient of variation.

The accumulation of Zn in the shoots was the highest with a combination of $100/0 \text{ NO}_3^-/\text{NH}_4^+$ and CuNP at 25 mg kg⁻¹ (Table 3), whereas the lowest accumulation of Zn occurred when the plants were applied with a combination between $50/50 \text{ NO}_3^-/\text{NH}_4^+$ and CuNP at 50 or 100 mg kg⁻¹ (Table 3). Regarding the accumulation of Zn in the roots, it has been verified higher values at 100/0 than at $50/50 \text{ NO}_3^-/\text{NH}_4^+$, as well as CuNP at 0 or 25 mg kg⁻¹ than at other rates of CuNP (Table 4). The lowest accumulation of Zn in the roots was observed at $50/50 \text{ NO}_3^-/\text{NH}_4^+$, as well as CuNP at 50 or 100 mg kg⁻¹ (Table 4).

	Fe	Zn	Р	K	Ca	Mg
	—_µg ро	er pot ——		mg p	er pot —	
NO3 ⁻ /NH	H_4^+					
100/0	234 ^a ± 36.9	$40.6 \ ^{a} \pm 5.01$	$3.08 \ ^{a} \pm 0.45$	$7.70 \ ^{a} \pm 1.11$	2.27 = 0.35	1.73 ^a ± 0.29
50/50	92.9 ^ь ± 16.1	17.3 ^ь ± 2.75	$1.84 ^{\text{b}} \pm 0.32$	$3.15 ^{\mathrm{b}} \pm 0.55$	1.00 ^b ± 0.13	$0.68 ^{\mathrm{b}} \pm 0.10$
Cu nanop	articles (mg kg-	¹)				
0	239 ^a ± 45.7	37.1 ª ± 6.82	$3.20 \text{ b} \pm 0.34$	7.29 ª ± 1.38	$2.18 \ ^{a} \pm 0.49$	1.60 = 0.34
25	235 ^a ± 39.7	46.1 ª ± 6.57	4.40 = 0.49	9.25 ª ± 1.37	$2.59 \ ^{a} \pm 0.45$	2.08 ª ± 0.39
50	135 ^b ± 44.4	19.2 ^ь ± 3.75	$1.46 \circ \pm 0.24$	3.03 ^b ± 0.63	0.86 ^b ± 0.13	$0.65 ^{\mathrm{b}} \pm 0.11$
100	43.9 ° ± 8.60	13.6 ^ь ± 2.85	$0.79 \circ \pm 0.14$	2.13 ^ь ± 0.47	0.90 ^b ± 0.13	$0.48 ^{\mathrm{b}} \pm 0.10$
<i>p</i> value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
cv (%)	24.9	35.9	15.1	38.5	49.3	49.5

Table 4. Accumulations of Fe, Zn, P, K, Ca, and Mg in the roots of maize plants under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil.

The SNK test at 1% was applied. Lowercase letters within each sub-column show significant differences. *ns* not significant; \pm standard error; *cv* coefficient of variation.

2.3.6. Concentrations of P, K, Ca, and Mg in the plant parts

The interaction was not significant for the concentrations of P, K, Ca, and Mg in the shoots and roots. Nonetheless, the main effects of NO_3^-/NH_4^+ and CuNP affected those response variables (Table 5). In the shoots, the concentrations of K and Mg were affected by proportions of NO_3^-/NH_4^+ with the highest value occurring at $100/0 NO_3^-/NH_4^+$ while the highest concentration of Ca was verified at $50/50 NO_3^-/NH_4^+$ (Table 5). However, the rates of CuNP affected the concentrations of P, K, and Ca in the shoots of maize with the lowest concentrations of P and K and the highest concentration of Ca occurring at CuNP 100 mg kg⁻¹ (Table 5). The proportions of NO_3^-/NH_4^+ had a significant effect on the concentrations of P and K in the roots, with both lowest and highest values, respectively, occurring at the proportion $100/0 NO_3^-/NH_4^+$ (Table 5). The concentrations of P, K, and Ca in the roots were affected by the rates of CuNP, with P and K being the lowest and Ca being the highest when the plants were applied with CuNP 100 mg kg⁻¹.

	Р	К	Ca	Mg
		g k	g ⁻¹	
Shoots				
NO_3^-/NH_4^+				
100/0	3.33 ^{ns} ± 0.16	23 .1 ^a ± 1.10	3.48 ^b ± 0.16	$1.66 \ ^{a} \pm 0.06$
50/50	$3.76 \text{ ns} \pm 0.33$	$20.2 ^{\text{b}} \pm 1.46$	4.13 ^a ± 0.13	$1.29 \text{ b} \pm 0.08$
Cu nanopartici	les (mg kg ⁻¹)			
0	$4.18 \ ^{a} \pm 0.20$	25.5 ª ± 0.33	$3.85 \text{ ab} \pm 0.24$	$1.57 \text{ ns} \pm 0.11$
25	4.21 ^a ± 0.30	24.3 $^{\rm ab} \pm 0.51$	3.41 ^b ± 0.15	$1.50 \text{ ns} \pm 0.06$
50	3.40 = 0.38	$20.9 \text{ b} \pm 2.04$	$3.76 \text{ ab} \pm 0.17$	$1.43 \text{ ns} \pm 0.13$
100	2.41 ^b \pm 0.18	15.8 ° ± 1.70	4.20 ª ± 0.28	$1.39 \text{ ns} \pm 0.15$
<i>p</i> value	< 0.01	< 0.01	< 0.01	< 0.01
cv (%)	18.6	16.4	14.3	17.1
Roots				
<i>NO₃⁻/NH</i> 4 ⁺				
100/0	$3.50 \text{ b} \pm 0.18$	8.92 ^a ± 0.49	$2.68 \text{ ns} \pm 0.19$	$1.70 \text{ ns} \pm 0.13$
50/50	4.37 ª ± 0.33	7.45 ^b ± 0.46	$2.73 \text{ ns} \pm 0.12$	$1.95 \text{ ns} \pm 0.09$
Cu nanopartici	les (mg kg ⁻¹)			
0	4.52 ª ± 0.33	9.56 ª ± 0.86	$2.85 \text{ ab} \pm 0.34$	$2.09 \text{ ns} \pm 0.25$
25	4.51 ª ± 0.44	$8.86 \ ^{a} \pm 0.30$	2.41 ^b ± 0.08	$1.89 \text{ ns} \pm 0.08$
50	3.97 ª ± 0.25	$7.62 \text{ ab} \pm 0.55$	$2.36 \text{ b} \pm 0.08$	$1.74 \text{ ns} \pm 0.12$
100	2.74 ^b ± 0.17	6.71 ^ь ± 0.58	3.20 ª ± 0.12	$1.57 \text{ ns} \pm 0.11$
<i>p</i> value	< 0.01	< 0.05	< 0.05	> 0.05
cv (%)	19.1	20.1	21.2	28.3

Table 5. Concentrations of P, K, Ca, and Mg in the shoots and roots of maize plants under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil.

The SNK test at 1% was applied. Lowercase letters within each sub-column show significant differences. *ns* not significant; \pm standard error; *cv* coefficient of variation.

The interaction was significant for the accumulations of P, K, Ca, and Mg in the shoots (Table 3). The accumulations of P, K, Ca, and Mg in the shoots was higher with a combination of $100/0 \text{ NO}_3^-/\text{NH}_4^+$ and CuNP at 25 mg kg⁻¹, as compared to the other combinations (Table 3), and decreased with increasing the rates of CuNP, starting from CuNP at 50 mg kg⁻¹, regardless of the proportions of $\text{NO}_3^-/\text{NH}_4^+$. The accumulations of P, K, Ca, and Mg in the shoots were higher when the plants were applied with $100/0 \text{ than } 50/50 \text{ NO}_3^-/\text{NH}_4^+$ (Table 3).

The main factors influenced the accumulations of P, K, Ca, and Mg in the roots of maize plants (Table 4). Regarding the proportions of NO_3^-/NH_4^+ , it was verified that the higher accumulations of P, K, Ca, and Mg in the plants occurred at 100/0 than at 50/50 NO_3^-/NH_4^+ (Table 4). Regarding the rates of CuNP, the higher accumulation of P occurred at

CuNP at 25 mg kg⁻¹ as compared to the other rates, whereas the accumulations of K, Ca, and Mg were higher at CuNP at 0 and 25 mg kg⁻¹ than at any other rates (Table 4).

2.3.7. Soil pH and EC

The interaction was not significant for the pH and EC in both bulk and rhizosphere soil. However, it has been verified the main effects of both NO_3^-/NH_4^+ and CuNP on the bulk soil pH, and NO_3^-/NH_4^+ on the rhizosphere soil pH (Table 6). The highest pH in both zones of the soil occurred when the plants were applied with $100/0 NO_3^-/NH_4^+$ (Table 6). The increases in the soil pH with $100/0 NO_3^-/NH_4^+$ were 3.5 and 10.3% respectively in the bulk and rhizosphere soil, relative to $50/50 NO_3^-/NH_4^+$ (Table 6). A higher bulk soil pH was observed at CuNP at 100 mg kg⁻¹ as compared to 0 mg kg⁻¹, but the rates of CuNP did not affect the rhizosphere soil pH (Table 6).

Table 6. Potential of hydrogen (pH in water 1:2.5), electrical conductivity (EC), and NO_3^- availability in the bulk and rhizosphere soil cultivated with maize under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil.

	pH		EC		NO ₃	
	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere
	soil	soil	soil	soil	soil	soil
			μS ι	cm ⁻¹	mg	kg ⁻¹
NO_{3}/N	H_{4}^{+}					
100/0	4.73 ^a ± 0.017	5.03 = 0.060	370.4 ^ь ± 12.9	349.4 ^b ± 34.2	89.9 ª ± 1.62	$164.7 = \pm 14.2$
50/50	$4.57 ^{\mathrm{b}} \pm 0.026$	4.56 ^ь ± 0.037	592.6 ª ± 13.1	668.4 ª ± 45.5	69.3 ^ь ± 1.12	113.2 ^ь ± 7.38
Си папо	particles (mg kg	-1)				
0	4.59 ^ь ± 0.040	$4.73 \text{ ns} \pm 0.112$	463.7 ^b ± 43.6	401.2 ° ± 57.1	$81.5 \text{ ns} \pm 4.09$	123.5 ^ь ± 9.15
25	$4.64 \text{ ab} \pm 0.047$	$4.84 \text{ ns} \pm 0.128$	447.7 ^ь ± 45.7	385.8 ° ± 52.9	76.4 ^{ns} ± 3.30	106.3 ^ь ± 12.7
50	$4.64 \text{ ab} \pm 0.045$	$4.82 \text{ ns} \pm 0.104$	509.5 ^a ± 44.8	513.5 ^ь ± 54.4	$81.7 \text{ ns} \pm 4.18$	143.6 ^b ± 18.0
100	4.72 ª ± 0.024	$4.84 \text{ ns} \pm 0.092$	505.2 ª ± 34.2	735.1 ª ± 83.2	$78.8 \text{ ns} \pm 4.58$	182.4 ª ± 19.8
<i>p</i> value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
cv (%)	1.5	3.1	9.1	17.6	6.7	12.42

The SNK test at 1% was applied. Lowercase letters within each sub-column show significant differences. *ns* not significant; \pm standard error; *cv* coefficient of variation.

The main effects of NO_3^-/NH_4^+ and CuNP on the soil EC were significant for both bulk and rhizosphere soil (Table 6). The lowest values of EC in the bulk and rhizosphere soil were observed when the plants were applied with $100/0 NO_3^-/NH_4^+$, whereas an increase in the rhizosphere soil EC occurred with increasing the CuNP rate, and the highest value was verified at CuNP at 100 mg kg⁻¹, except for the bulk soil by which the EC values were higher at both 50 and 100 mg kg⁻¹, as compared to the other rates (Table 6).

2.3.8. Extractable NO₃⁻ and NH₄⁺ in the soil

The interaction was not significant for the availability of NO_3^- in both bulk and rhizosphere soil. However, it was verified the main effects of NO_3^-/NH_4^+ and CuNP on the extractable NO_3^- and NH_4^+ in the soil (Table 6). The highest extractable NO_3^- was found in both bulk and rhizosphere soil at $100/0 NO_3^-/NH_4^+$ (Table 6). The increases in the extractable NO_3^- as a function of $100/0 NO_3^-/NH_4^+$ were 29.7 and 45.5%, respectively in the bulk and rhizosphere soil, and the highest value was found in the rhizosphere soil. Regarding the CuNP, the highest extractable NO_3^- was observed in the rhizosphere soil treated with CuNP at 100 mg kg⁻¹, but it was not verified any effects of the CuNP on extractable NO_3^- in the bulk soil.

The interaction was significant for the extractable NH_4^+ in both bulk and rhizosphere soil. The higher amounts of the extractable NH_4^+ were measured in both bulk and rhizosphere soil when the plants were applied with 50/50 NO_3^-/NH_4^+ than 100/0 NO_3^-/NH_4^+ and they were increased with the rates of CuNP (Fig. 6). However, the rates of CuNP did not affect the extractable NH_4^+ in the rhizosphere soil when the plants were supplied with 100/0 NO_3^-/NH_4^+ (Fig. 6). The increases were the most in the rhizosphere soil when the plants were applied with CuNP at 100 mg kg⁻¹.



Figure 6. Extractable NH_4^+ in the soil cultivated with maize plants grown under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil. The SNK test at 1% was applied. Lowercase letters compare rates of Cu nanoparticles within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each Cu nanoparticles rate. Coefficient of variation (%) = 5.3 (bulk soil) and 21 (rhizosphere soil).

2.3.9. Extractable Cu, Fe, Mn, and Zn in the soil

Regarding Mehlich-3 extractable Cu in the soil, it was found that the interaction was only significant for the rhizosphere soil. In the rhizosphere soil, the extractable Cu was higher when the plants received $50/50 \text{ NO}_3^-/\text{NH}_4^+$ combined with CuNP at 100 mg kg⁻¹, as compared to the other combination (Fig. 7). The rates of CuNP increased the extractable Cu in the soil regardless of the proportions of NO₃⁻/NH₄⁺ (Fig. 7). However, the proportion of $50/50 \text{ NO}_3^-/\text{NH}_4^+$ only increased the extractable Cu in the soil treated with CuNP at 100 mg kg⁻¹ (Fig. 7). Regarding the bulk soil, the extractable Cu was higher with CuNP at 100 mg kg⁻¹ compare to the other rates of CuNP (Table 7).



Figure 7. Mehlich 3 extractable Cu in the rhizosphere soil and Mn in the bulk soil cultivated with maize plants receiving proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil. The SNK test at 1% was applied. Lowercase letters compare rates of Cu nanoparticles within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each Cu nanoparticles rate. Coefficient of variation (%) = 10 (a) and 6.1 (b).

Extractable Mn was affected by the interaction in the bulk soil. The rates of CuNP affected the extractable Mn only when the plants were applied with $100/0 \text{ NO}_3^-/\text{NH}_4^+$ and the lower value was verified with CuNP at 25 mg kg⁻¹ than at any other combination (Fig. 7). The extractable Mn in the rhizosphere soil was affected by the rates of CuNP with a higher value at CuNP at 100 mg kg⁻¹ than at any other rate (Table 7). The extractable Zn was affected by the rates of CuNP only in the rhizosphere soil, with a higher value for CuNP at 100 mg kg⁻¹ than at the other rates of Mn (Table 7). The proportions of NO₃⁻/NH₄⁺ did not affect the extractable Zn in the bulk soil, whereas the extractable Fe was not affected by any main factors or interaction.
	Cu	Mn	Zn
	Bulk soil	Rhizosphere soil	
		mg kg ⁻¹	
NO_3^-/NH_4^+			
100/0	16.9 ^{ns} ± 2.45	$1.58 \text{ ns} \pm 0.13$	$2.70 \text{ ns} \pm 0.09$
50/50	16.9 ^{ns} ± 2.53	$1.75 \text{ ns} \pm 0.11$	$2.70 \text{ ns} \pm 0.18$
Cu nanoparticles (m	ng kg-1)		
0	$5.99 \text{ d} \pm 0.14$	1.60 ^в ± 0.11	2.49 ^b ± 0.12
25	11.5 ° ± 0.16	1.39 ^b ± 0.08	2.38 ^b ± 0.06
50	17.7 ^ь ± 0.34	1.66 ^в ± 0.10	2.68 ^b ± 0.14
100	32.6 = 0.47	2.01 ª ± 0.15	3.24 ª ± 0.26
<i>p</i> value	< 0.01	< 0.01	< 0.01
cv (%)	5.26	18.8	17.5

Table 7. Extractable Cu in the bulk soil, and extractable Mn and Zn in the bulk and rhizosphere soil cultivated with maize plants applied with proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil.

The SNK test at 1% was applied. Lowercase letters within each sub-column show significant differences. *ns* not significant; \pm standard error; *cv* coefficient of variation.

2.3.10. Extractable P, K, Ca, and Mg in the soil

The interaction was not significant for the extractable P, K, Ca, and Mg in both bulk and rhizosphere soil, but they were influenced by the main factors (Table 8). A larger amount of the extractable P was found in both bulk and rhizosphere soil treated with CuNP at 100 mg kg⁻¹ than at any other rate, but it was not affected by the proportions of NO_3^-/NH_4^+ (Table 8). The extractable K in the bulk soil was higher at CuNP at 50 or 100 mg kg⁻¹ than at the other rates (Table 8). In the rhizosphere soil, the extractable K was the highest at both 50/50 NO_3^-/NH_4^+ or CuNP at 100 mg kg⁻¹ (Table 8). The extractable Ca in the bulk soil was higher at 100/0 NO_3^-/NH_4^+ than at 50/50 NO_3^-/NH_4^+ , and under CuNP at 100 mg kg⁻¹ than CuNP at 25 mg kg⁻¹ (Table 8). In the rhizosphere soil, the extractable Ca was the highest for CuNP at 100 mg kg⁻¹ (Table 8). In the rhizosphere soil, the extractable Mg was not affected by the treatments (Table 8).

	P		K		Ca	
	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere
	soil	soil	soil	soil	soil	soil
			mg k	leg ⁻¹		
NO_{3}/N	TH_4^+					
100/0	83.9 ^{ns} ± 1.61	78.2 $^{ns} \pm 3.01$	$50.3 \text{ ns} \pm 3.41$	41.9 ^ь ± 3.63	236 ª ± 3.08	$247~^{\rm ns}\pm11.1$
50/50	85.1 $^{\rm ns}\pm 0.94$	$82.6 \text{ ns} \pm 2.41$	53.9 ^{ns} ± 1.73	68.7 ª ± 4.51	221 ^ь ± 3.28	$264 \text{ ns} \pm 14.4$
Cu nano	particles (mg kg	~1)				
0	84.6 ^ь ± 0.99	76.4 ^ь ± 2.55	46.9 ^ь ± 1.88	44.3 ° ± 4.19	$232 \text{ ab} \pm 4.10$	222 ° ± 6.22
25	79.6 ° ± 2.02	$73.2 ^{\text{b}} \pm 2.13$	42.9 ^ь ± 2.64	42.1 ° ± 5.04	219 ^ь ± 3.09	218 ° ± 3.92
50	83.3 $^{\rm bc} \pm 0.92$	82.5 $^{\mathrm{ab}} \pm$ 4.40	58.3 ª ± 4.07	56.6 ^ь ± 4.91	$230 \text{ ab} \pm 5.63$	251 ^ь ± 11.7
100	90.5 ° \pm 0.76	89.6 ª ± 3.49	$60.2 ^{\text{a}} \pm 2.54$	78.3 ª ± 7.37	234 ª ± 5.97	330 ª ± 12.1
<i>p</i> value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
cv (%)	4.4	9.1	15.3	15.6	4.63	9.81

Table 8. Extractable P, K, and Ca in the bulk and rhizosphere soil cultivated with maize plants under proportions of NO_3^-/NH_4^+ and rates of Cu nanoparticles in a Spodosol soil.

The SNK test at 1% was applied. Lowercase letters within each sub-column show significant differences. *ns* not significant; \pm standard error; *cv* coefficient of variation.

2.4. DISCUSSION

2.4.1. CuNP increase plant growth with NO₃⁻ fertilization

The dry biomass of shoots and roots were increased by the CuNP at 25 mg kg⁻¹. However, it was not verified any positive effect regarding the CuNP application when the plants were applied with a combination of NO_3^- and NH_4^+ (Fig. 2). This result proves the importance of N ionic forms when the maize plants are applied with Cu, especially in the form of nanoparticles, since the responses of plants to Cu depends on the proportions of NO_3^-/NH_4^+ in addition to the plant species and environmental conditions (Liu et al., 2017; Souza Junior et al., 2019). To maximize the growth of maize plants as a function of CuNP, it is necessary to prioritize the use of NO_3^- . According to Souza Junior et al. (2018), the development of Tanzania guinea grass [*Panicum maximum* Jacq. cv. Tanzania (syn. *Megathyrsus maximus* (Jacq.) B.K.Simon & S.W.L. Jacobs)] was proper with various rates of ionic Cu only when plants were applied with 100/0 NO_3^-/NH_4^+ since this proportion allowed the proper uptake of ionic Cu by plants, without Cu toxicity, as observed in the present study with CuNP. Pedersen et al. (2019) reported that the best proportion of NO_3^-/NH_4^+ to maximize the development of maize plants without Cu application was $50/50 NO_3^-/NH_4^+$, which disagreed with the present results. However, Zhang et al. (2019) reported a growth preference of maize plants for NO₃⁻ over NH₄⁺. A possible explanation can be related to the low soil pH found in the present study since the plants were grown in an acidic soil, which became more acid when applied a large amount of NH₄⁺, as evidenced by the decreases in the soil pH caused by NH₄⁺, thus inhibiting plant growth and nutrient accumulation (Roosta, 2014). When the plants were supplied with a high amount of NO₃⁻, an increase in the soil pH occurred due to the basic effect of this ion, thus improving the plant growth and nutrient accumulation (Zhang and Zhao, 2019). Excess NH₄⁺ can be also toxic to maize plants as this cation causes pH unbalanced intra-cell, thus stimulating high production of reactive oxygen species, decreasing photosynthesis, affecting accumulation of CuNP and other nutrients such as N, thus decreasing shoot and root development (Bittsánszkya et al., 2015; Esteban et al., 2016; Zhang et al., 2019).

Copper in the form of nanoparticles can be used by plants to improve the metabolic and physiologic functions, especially related to photosynthesis, interacting with other ionic nutrients to improve plant growth (Rajput et al., 2018). As reported by Pradhan et al. (2015), the rate of CuNP at 0.5 mg L⁻¹ for mung bean (*Vigna radiata* L.) under nutrient solution had a positive effect, improving plant growth. This result proves the beneficial use of CuNP as a fertilizer as evidenced by the present study for the maize plants. However, high rates of CuNP can be toxic for plants as reported by many authors (Melegari et al., 2013; Pradhan et al., 2015; Song et al., 2015; Costa and Sharma, 2016) and reported in the present study as well.

2.4.2. CuNP and NO₃⁻ fertilization have an opposite effect on the efficiency of PSII

The Fv/Fm was high when the plants were applied with $100/0 \text{ NO}_3^-/\text{NH}_4^+$ (Table 1). This result may also explain why the dry biomass of shoots and roots were high when the plants received all N as NO₃⁻, because those plants had high efficiency of turning sunlight into chemical energy, thus improving photosynthesis, and subsequent plant biomass production (Shang and Shen, 2018). The use of NO₃⁻ as an N source can increase nitrate reductase activity (Souza Junior et al., 2018) and then improves N accumulation. Nitrogen is one of the most important nutrients for the synthesis of proteins, chlorophyll, rubisco, plastocyanin, and other important compounds for photosynthesis, which can improve Fv/Fm (Shang and Shen, 2018; Souza Junior et al., 2019). Zhang et al. (2020) also reported high values of Fv/Fm when chili pepper (*Capsicum annum* L.) received $100/0 \text{ NO}_3^-/\text{NH}_4^+$ and low values at $50/50 \text{ NO}_3^-/\text{NH}_4^+$, and they suggested a downregulation of PSII induced by $50/50 \text{ NO}_3^-/\text{NH}_4^+$ supply because excess NH₄⁺ can be also toxic to plants.

A decrease in the Fv/Fm, QEPSII, and photochemical quenching occurred when the maize plants received CuNP at 100 mg kg⁻¹ (Table 1, Fig. 3a, 3c) because the plants that are grown with a combination of NO_3^- and NH_4^+ were affected by excess CuNP. Probably, the use of NO_3^- solely may improve the antioxidant defense against the CuNP toxicity, mitigating the negative effects of CuNP on photosynthesis. Souza Junior et al. (2019) suggested a better balance of photosynthetic parameters when Tanzania guinea grass was supplied with NO_3^- solely, which allowed proper development of plants even under high Cu toxicity as noticed in the present study. As expected, the NPQ (Fig. 3d) was increased by the CuNP at 100 mg kg⁻¹ since its excess combined with excess NH_4^+ might have disrupted the electron transport chain, causing photoinhibition of PSII, leading to energy dissipation by heat, thus increasing electron transport rate (Pradhan et al., 2015; Costa and Sharma, 2016). Costa and Sharma (2016) also reported an increase in NPQ when rice (*Oryza sativa* L.) plants received a high rate of CuNP at 1000 mg L⁻¹.

2.4.3. CuNP combined with NO3⁻ fertilization promotes accumulations of C and N

Concentrations of C and N in the plants were increased with NO₃⁻ fertilization and decreased by excess CuNP (Table 1). The accumulations of C and N in the shoots and roots were increased by CuNP at the rate of 25 mg kg⁻¹ (Fig. 4), but the increases occurred only when the plants were supplied with N as NO₃⁻ (Fig. 4a, 4b). There is an important synergetic interaction of NO₃⁻ × CuNP as CuNP at the proper rate of CuNP at 25 mg kg⁻¹ might upregulate genes that codify nitrate transporter (NRT), which has been reported for increasing N accumulation in wheat (*Triticum aestivum* L.) and subsequent plant development (Zhang et al., 2018). Moreover, the application of CuNP at a proper amount improved the activity of nitrate reductase, nitrite reductase, glutamine synthase, and glutamate synthase, thus improving N accumulation and development of mung bean (Pradhan et al., 2015). These enzymes are important for N assimilation and protein synthesis, which are required for many metabolic functions like carbon fixation, photosynthesis, DNA synthesis, and nutrient uptake (Pradhan et al., 2015). According to Souza Junior et al. (2018), plants of Tanzania guinea grass grown at 100/0 NO₃⁻/NH₄⁺ showed an increase in nitrate reductase activity as the ionic Cu increased, which improved N accumulation.

It was observed a decrease in the N accumulation when the plants received N of both ionic forms (Fig. 4a, 4b), and the decrease was high when the plants were exposed to high rates of CuNP (Fig. 4a, 4b). Abdel-Wahab et al. (2019) verified that the CuNP decreased the content of amino acids in black nightshade (*Solanum nigrum* L.) as its excess induced high synthesis of reactive oxygen species, which attack all types of biomolecules in the plant such as proteins, nucleic acids,

and amino acids, thus decreasing the C assimilation. In the present study, it is suggested that the CuNP also affect the synthesis of those compounds, which could explain those low accumulations of C and N in the maize plants when exposed to excess CuNP. Also, the CuNP toxicity decreased the photosynthesis and the acquisition of C and N, which could also explain the low plant growth and low accumulations of C and N. Maize plants with low C and N produced less biomass with low quality of grains (Zhang and Zhao, 2019), that is why it is important to determine which ionic form of N is better to combine with CuNP for maize plants.

The C/N ratio in the shoots was decreased by the rates of CuNP only when the plants received $50/50 \text{ NO}_3^-/\text{NH}_4^+$ (Fig. 4c). In plants, the C/N ratio is generally similar even under different environmental conditions, except for plants under high stress (Yang et al., 2018) since C fixation is affected in this situation due to an impairment on photosynthesis caused by stress. The amount of C was better when the plants received $100/0 \text{ NO}_3^-/\text{NH}_4^+$, which slows down the decomposition of the plant material. This result is favorable for no-tillage agriculture system since it is desirable a low decomposition rate of the mulch, which can induce a better soil coverage, reducing soil erosion, high temperature, and losses of nutrients, as well as improving soil moisture, soil structure, root development, and availability of nutrients, especially in tropical regions (Abro et al., 2011). However, the highest C/N ratio in the roots was observed when the plants were applied with $50/50 \text{ NO}_3^-/\text{NH}_4^+$ (Table 1). A possible explanation can be related to the low accumulations of N in the roots, thus leading to an increase in the C/N ratio. In this case, a high C/N ratio can be related to the low quality of the material relative to N accumulation, which is not desirable since the organic material can be an N source for microorganisms and plants (Abro et al., 2011).

2.4.4. CuNP improve accumulations of Cu, Fe, Mn, and Zn with NO₃⁻ fertilization

The concentrations of Fe, Mn, and Zn in the plants decreased as the concentration of Cu in the plant increased (Table 2). This result is expected since Cu has an important antagonistic effect in other micronutrients such as Fe, Mn, and Zn (Marschner, 2012; Rawat et al., 2018). When the concentrations of Cu in plant increase, concentrations of Fe, Mn, and Zn usually decreased due to an inhibitory effect of CuNP exposure (Rajput et al., 2018; Rawat et al., 2018) since Fe, Mn, and Zn share a common nutrient transporter with Cu to cross the cell membrane, such as heavy metal-transporting P-type ATPase (Deng et a., 2013).

The accumulations of Cu, Fe, Mn, and Zn in the shoots and roots were high with a combination of CuNP 25 mg kg⁻¹ and NO₃⁻ solely, especially for Cu, which was higher in the roots

than in the shoots (Fig. 5, Tables 3 and 4). Synergetic interactions of $NO_3^- \times CuNP$ can improve root development (Zhang et al., 2019), thus increasing the acquisition of Cu, Fe, Mn, and Zn from soil and fertilizers (Wang et al., 2006; Marschner, 2012; Griffiths and York, 2020). Plant roots with a large surface area can explore more soil and intercept more micronutrients in soil solution, thus increasing the accumulation of nutrients and subsequent translocation to shoots (Wang et al., 2006; Marschner, 2012; Griffiths and York, 2020). However, when the plants were supplied with a combination of NO_3^- and NH_4^+ the roots were short and the acquisition of nutrients was low, which led to the lowest accumulation of micronutrients by the plants. Du et al. (2018) subjected plants of oregano (*Origanum vulgare* L.) to rates of CuNP up to 200 mg kg⁻¹ and they observed different results from the present study since CuNP decreased the accumulations of Fe and Zn. This difference can be related to the different plant species and their effects on root development. Rawat et al. (2018) also found more Cu in roots than in shoots as it was noticed in the present study, which may be related to high concentrations of metallothionein and phytochelatins in roots, which are important organic compounds responsible for retaining Cu in roots.

Excess CuNP decreased the accumulations of Fe, Mn, and Zn in the plants (Fig. 5, Tables 3 and 4). Two possible hypotheses can explain it. The first is the same as that related to the concentration of micronutrients since there is an important antagonistic effect of Cu excess against Fe, Mn, and Zn (Marschner, 2011; Rajput et al., 2018; Rawat et al., 2018). The second is related to the poor root development due to Cu stress, which resulted in a low root dry biomass production, which suggest low root area, is not enough to uptake Cu, Fe, Mn, and Zn from the soil in proper amount (Wang et al., 2006; Marschner, 2012; Griffiths and York, 2020).

2.4.5. CuNP enhance accumulations of P, K, Ca, and Mg with NO₃⁻ fertilization

In the shoots, the concentrations of K and Mg were high while the concentration of Ca was low at $100/0 \text{ NO}_3^-/\text{NH}_4^+$ (Table 5). This result confirms the classic antagonistic effect of K and Mg against Ca because they compete for the same transporter to enter in plant cells, which is associated with an internal cation balance between K, Mg, and Ca (Marschner, 2012; Ertiftik and Zengin, 2017; Gaj et al., 2018). This antagonistic effect did not influence plant development since plant dry biomass production was high when receiving NO₃⁻ solely. Gaj et al. (2018) reported a high concentration of Mg when maize plants were grown with a deficiency of K. However, a low concentration of Mg was verified when K deficiency was suppressed, suggesting an antagonistic effect between those nutrients. Similarly, excess CuNP decreased the concentrations of P and K in the plants (Table 5). Rawat et al. (2018) also observed an inhibitory effect on P concentration due

to the high rate of CuNP at 125 mg kg⁻¹ in bell pepper (*Capsicum annuum* L.) and they related it to a possible downregulate effect of CuNP on P transporters since excess CuNP can induce oxidative stress, which affects the activity of nutrient transporters. Tamez et al. (2019) applied CuNP up to 60 mg kg^{-1} to sugarcane (*Saccharum officinarum* L.) and they also found lower concentrations of K in plants, as compared to control.

The accumulations of P, K, Ca, and Mg in the shoots and roots were the highest with $100/0 \text{ NO}_3^-/\text{NH}_4^+$ combined with CuNP at 25 mg kg⁻¹ (Tables 3 and 4). This may be related to a synergetic effect between NO₃⁻ and a moderate rate of CuNP, which improved the root development and then the accumulations of P, K, Ca, and Mg from the soil. According to Wang et al. (2006) and Griffiths and York (2020), a high root dry biomass and root surface area are responsible to improve the uptake of nutrients, especially associated with root interception, in addition to other processes such as the expression of genes that codify for nutrient transporters, which can also improve the active nutrient uptake.

2.4.6. NO₃⁻ fertilization increases the bulk and rhizosphere soil pH

The highest bulk and rhizosphere soil pH was observed when the plants received all N as NO_3^- (Table 6). This result is expected since the uptake of NO_3^- or NH_4^+ required a balance of charge inside of the plant cell and plant roots exudate ions to keep a proper cell internal balance (Marschner, 2012; Roosta, 2014). When plant roots acquire NH_4^+ they release H^+ to the soil, thus decreasing soil pH. However, when plant roots uptake NO_3^- they exude OH^-/HCO_3^- for the same reason, thus increasing soil pH (Roosta, 2014). These effects occurred especially in rhizosphere soil, the most active zone in regards to chemical activity and availability of nutrients, as it has been shown in the present study, and it can be especially important for uptake of nutrients such as P as their availability is pH-dependent (Pedersen et al. 2019). Pederson et al. (2019) also observed a decline in bulk and rhizosphere soil pH when maize plants were supplied with a high amount of NH_4^+ , as well as high soil pH (up to 5.5) due to a high NO_3^- application. However, for the rhizosphere soil, a decrease in soil pH can be caused by roots that release H^+ and/or organic acids, as well as an increase in soil pH due to the release of OH^-/HCO_3^- (Pederson et al., 2019).

Low soil EC was found in both bulk and rhizosphere soil when the plants were applied with all N as NO_3^- (Table 6). There are two possible explanations: the one related to the different saline effect of N ions, low from NO_3^- and high from NH_4^+ (Pederson et al., 2019), and the other related to the nutrient uptake because plants that received all N as NO_3^- showed high nutrient

accumulation, thus decreasing availabilities of nutrients in the soil and then its EC since the concentrations of nutrients in the soil are related to the saline effect (Marschner, 2012).

2.4.7. CuNP affect availabilities of NO₃⁻ and NH₄⁺ in the soil

The high availability of NO_3^- in the soil at $100/0 NO_3^-/NH_4^+$, as well as the high NH_4^+ availability at 50/50 NO_3^-/NH_4^+ (Table 6, Fig. 6) are expected since the fertilization with NO_3^- or NH_4^+ improve their availability in bulk and especially in rhizosphere soil. Pederson et al. (2019) and Zhang et al. (2019) reported an increase in NO_3^- or NH_4^+ due to an application of these ions, keeping the soil environment rich in either N ions. However, the highest rate of CuNP increased the availability of NO_3^- in the rhizosphere soil, as well as NH_4^+ in the bulk and rhizosphere soil. These results can be attributed to the toxic effects of CuNP, which inhibited plant growth and root development and thus led to the low uptake of N from the soil, keeping high these availabilities in the soil (Wang et al., 2006; Griffiths and York, 2020).

2.4.8. CuNP toxicity affect the availability of Mn and Zn in the soil

The application of CuNP increased the availability of Cu in the bulk and rhizosphere soil (Fig. 7, Table 7), indicating that the CuNP is an effective fertilizer to improve the availability of Cu in the soil, regardless of the zone, and its uptake by plant roots, as evidenced by the high concentration of Cu in the shoots and roots of maize plants. Similar results were reported by Pradhan et al. (2015) and Costa and Sharma (2016). However, Zuverza-Mena et al. (2015) did not find an increase in Cu accumulation when cilantro (*Coriandrum sativum* L.) received CuNP and they related it to the high availability of native Cu in the soil (27.1 mg kg⁻¹). However, there was a high correlation between rates of CuNP and extractable Cu by Mehlich-3 in the bulk and rhizosphere soil regardless of the proportions of NO_3^-/NH_4^+ ($R^2 = 0.99$, results not shown) and it means that the Mehlich-3 is a good extractor for CuNP.

The highest availabilities of Mn and Zn were noticed in the rhizosphere soil receiving the highest rate of CuNP at 100 mg kg⁻¹ (Fig. 7, Table 7). The highest availabilities of Mn and Zn may be associated with an antagonistic effect of the high rates of CuNP, which negatively affected the uptake of Mn and Zn from the soil, keeping a high amount of these nutrients in the soil solution. One of the most important effects of heavy metal toxicity is its secondary effect on uptake of other metal nutrients like Mn and Zn, aggravating the negative effect of toxicity since Cu has a strong

competition with other metal cations, which decreases the uptake of Mn and Zn (Marschner, 2012; Rajput et al., 2018; Rawat et al., 2018).

2.4.9. CuNP toxicity affect P, K, and Ca availability in the soil

The availability of K in the rhizosphere soil was low at $100/0 \text{ NO}_3^-/\text{NH}_4^+$ (Table 8). As discussed in the previous sections, maize plants that received $100/0 \text{ NO}_3^-/\text{NH}_4^+$ showed a high K accumulation (Table 3, 4, 5), which may explain the lowest available K in the soil. However, the availability of Ca in the bulk soil was high at $100/0 \text{ NO}_3^-/\text{NH}_4^+$ (Table 8). The largest accumulations of K and Mg in the plants applied with all N as NO₃⁻ might have decreased the Ca accumulation due to an antagonistic effect of cations (Marschner, 2012; Ertiftik and Zengin, 2017; Gaj et al., 2018). Consequently, the available Ca in the soil was high. Maize plants did not show any symptoms of Ca deficiency and plant growth was normal at $100/0 \text{ NO}_3^-/\text{NH}_4^+$, suggesting that a low Ca accumulation can be attributed to the high accumulations of K and Mg without problems for plant nutrition (Marschner, 2012). The availabilities of P and K was increased in both bulk and rhizosphere soil as the rate of CuNP increased (Table 8). Copper nanoparticles may also have an antagonistic effect on P uptakes, such as the formation of copper phosphates or inhibition of transporters for P and other macronutrients (Marschner, 2012; Rawat et al., 2018).

2.5. CONCLUSION

There are many positive effects of CuNP when the maize plants are applied with all N as NO_3^- . The plant growth, photosynthesis, and nutrient accumulations are high when the plants receive $100/0 NO_3^-/NH_4^+$ and the growth potential of maize plants can be improved by applying a proper rate of CuNP, which positively affects the plant performance. No beneficial effects from the application of CuNP are noticed when the plants are applied with $50/50 NO_3^-/NH_4^+$. This is particularly important for the calibration of N fertilization for the next studies with CuNP, opting to choose all N as NO_3^- to ensure that all beneficial effects from CuNP will be achieved.

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3. NITRATE FERTILIZATION ENHANCES MANGANESE PHYTOEXTRACTION BY TANZANIA GUINEA GRASS – A NOVEL HYPERACCUMULATOR PLANT?

ABSTRACT

Manganese (Mn) is essential for plants, but very toxic in high concentrations since it can cause oxidative stress and negatively affects plant growth. However, the use of NO₃⁻ may improve Mn phytoextraction and also the phytoremediation potential of Tanzania guinea grass [Panicum maximum Jacq. cv. Tanzania (syn. Megathyrsus maximus (Jacq.) B.K.Simon & S.W.L. Jacobs)]. The objective was to evaluate the effect of excess Mn on physiological, metabolic, nutritional, and productive indicators of Tanzania guinea grass cultivated with proportions of NO_3^{-}/NH_4^{+} . The experiment involved a 2 × 4 factorial in randomized complete block design with four replications, carried out in a growth chamber by using Hoagland and Arnon's nutrient solution. The factors were proportions of NO_3^-/NH_4^+ (100/0 and 70/30) combined with rates of Mn (10, 500, 1,500, and 3,000 µmol L⁻¹). Statistical analysis was performed by using the analysis of variance, comparing the averages by the Tukey test at 5%. Excess Mn did not affect the plant growth, relative chlorophyll index - SPAD (RCI), nitrate reductase, and the accumulation of N and its ions in the plant tissue. Nonetheless, the highest concentrations and accumulations of Mn were verified when the plants were grown with all N as NO₃-, reaching up to 5,500 and 21,187 mg kg⁻¹ (concentrations) in the shoots and roots, respectively, and 76.2 and 31.1 mg per pot (accumulations) in the shoots and roots, respectively (total Mn 107.3 mg per pot). The high concentrations of Mn in the plant tissue phytoextraction of increased H₂O₂ concentration, causing oxidative stress, which decreased glutamine synthetase activity, net photosynthesis, stomatal conductance, electron transport rate, photochemical quenching, as well as increased non-photochemical quenching. In that stress condition, proline synthesis seems to be more important to mitigate oxidative stress than the antioxidant enzymes, buffering excess H₂O₂ caused by excess Mn. Tanzania guinea grass is very tolerant to excess Mn, accumulating high amounts of this metal in the plant tissue, and NO₃- supply can increase even more its concentration, enhancing Mn accumulation as much as a hyperaccumulator plant. It is suggested to indicate Tanzania guinea grass as a Mn hyperaccumulator.

Keywords: Glutamine synthetase, *Megathyrsus maximus*, Nutrient accumulation, Photosynthesis, Phytoremediation

3.1. INTRODUCTION

Pastures occupy around 196 million hectares in Brazil, equivalent to 71% of the agricultural area or 23% of the territory (FAO, 2016). High productive pastures are compounded especially by Tanzania guinea grass [*Panicum maximum* Jacq. cv. Tanzania (syn. *Megathyrsus maximus* (Jacq.) B.K.Simon & S.W.L. Jacobs)] due to its high potential for nutrient extraction and biomass production (Silveira and Monteiro, 2011). Tanzania guinea grass has been also indicated as an important phytoremediation plant (Souza Junior et al., 2019; Leite and Monteiro, 2019; Cardoso and Monteiro, 2021). The tolerance of Tanzania guinea grass to excesses of copper (Cu), cadmium (Cd), and barium (Ba) and their high phytoextraction potential was previously confirmed (Gilabel

et al., 2014; Souza Junior et al., 2019; Leite and Monteiro, 2019; Cardoso and Monteiro, 2021). Recently, the importance of proportions of nitrate/ammonium (NO_3^-/NH_4^+) to improve the tolerance of Tanzania guinea grass to Cu and Cd stress was indicated, which affected their phytoextraction potential (Souza Junior et al., 2017; Souza Junior et al., 2018; Leite and Monteiro, 2019).

The major nutrient for the growth of forage grasses is nitrogen (N), which is uptake by plant roots in the ionic forms of NO_3^- and NH_4^+ . Nitrogen participates in the biosynthesis of all amino acids and proteins, as well as compounds related to plant development and tolerance to heavy metal excess in the growth media (Heldt, 2005; Taiz et al., 2017). Applications of proportions of NO_3^-/NH_4^+ led to a greater growth of *Megathyrsus maximus* cv. Aruana compared to plants grown with N as NO_3^- (Santos et al., 2013). The proper proportion of NO_3^-/NH_4^+ for Tanzania guinea grass grew without heavy metal toxicity was 70/30 (Souza Junior et al., 2018).

Proper proportions of NO_3^-/NH_4^+ increases both N use and efficiency and production of forage grasses, without any increase regarding rates of N (Santos et al., 2013). The positive effect of moderate concentrations of NH_4^+ on affinity-plants occurs because the uptake of NO_3^- and its chemical reduction in the plant cell by nitrate reductase (NR) requires metabolic energy and reducing power. Thus, free energy could be saved by plants when N assimilation is performed directly in the form of NH_4^+ since glutamine synthetase (GS) requires less reducing power and chemical energy than NR. Thus, the free energy could be used for other purposes such as plant growth or stress mitigation (Epstein and Bloom, 2006; Taiz et al., 2017). However, the effects of proportions of NO_3^-/NH_4^+ in forage grasses under heavy metal toxicity are poorly reported.

Excesses of heavy metals induce an excessive synthesis of reactive oxygen species (ROS) by plant cells, which are buffered through the action of dismutases, peroxidases, and osmoregulatory amino acids (Myouga et al., 2008). The enzymes are mainly: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and guaiacol peroxidase (GPX) (Gratão et al., 2008; Myouga et al., 2008); as well as the amino acid proline (Singh et al., 2010; Hayat et al., 2012).

Manganese is essential for plants since it has an important function in photosynthesis, mediating the electron transfer from water to pheophytin in a series of redox reactions named water photolysis or Hill's reaction (Taiz et al., 2017). In addition, Mn has a relevant role as an enzyme activator (Malavolta, 2006; Taiz et al., 2017). One of the classic effects of excess Mn is its influence on increasing oxidative stress, affecting N metabolism and photosynthesis (You et al., 2017; Pan et al., 2018; Rajpoot et al., 2020). However, no studies are reporting the effects of excess Mn on Tanzania guinea grass indicated as tolerant to metals in a controlled situation.

Many factors can influence stress intensity such as plant species and type of stress (Schulze et al., 2002). So far, only one research was found regarding the effect of N exclusively as NO_3^- or NH_4^+ (3.75 mmol L⁻¹) associated with rates of Mn, which studied the sexual dimorphism of *Populus cathayana* L. under Mn toxicity, without assessing the increases in the phytoextraction potential (Zhao et al., 2018). The results of that study indicated that proportions of NO_3^-/NH_4^+ can have the potential to increase the tolerance of plants to excess Mn.

The hypothesis is the Tanzania guinea grass is that the grass is highly tolerant to Mn contamination, but a proper proportion of NO_3^-/NH_4^+ can improve even more its tolerance and phytoextractor potential. The objective was to evaluate the effect of excess Mn on physiological, metabolic, and nutritional parameters, revealing the role of the proportions of NO_3^-/NH_4^+ as stress mitigators for Tanzania guinea grass.

3.2. MATERIAL AND METHODS

3.2.1. General conditions of the experiment

The experiment was carried out in a growth chamber with photosynthetically active radiation of 400 μ mol m⁻² s⁻¹, temperature of 29 °C, and humidity of 40%. The seeds were germinated in a plastic tray with washed sand by using deionized water. Nine seedlings (5 cm height) from the tray were transplanted into pots of 2 L containing the nutrient solution of Hoagland and Arnon (1950) prepared with deionized water. Plants were grown in a system named deep-water culture (Fig. 1), with oxygen constantly supplied by a mechanical pump.

Nutrient solution with 20% ionic strength for seven days after transplanting was used. The nutrient solutions contained proportions of NO_3^-/NH_4^+ and a fixed N rate of 15 mmol L⁻¹ (Hoagland and Arnon, 1950). After the initial seven days, a solution with 100% ionic strength was applied. Thinning of the seedlings was carried out for five days after using the nutrient solution with 100% ionic strength, remaining three plants per pot. Dicyandiamide nitrification inhibitor (7 µmol L⁻¹) to prevent NH_4^+ nitrification was used (Liu et al., 2017), and the nutrient solution was replaced every 14 days. Manganese was applied in the nutrient solution 36 days after transplanting with the plants exposed to excess Mn for 14 days. Tanzania guinea grass was harvested after 50 days of growth.

3.2.2. Experimental design and sampling

The experiment involved a 2 × 4 factorial in a randomized complete block design with eight replications, four for "nutritional and productive" analyzes and four for "metabolic and physiological" analyzes. The main factors were proportions of 100/0 and 70/30 NO₃⁻/NH₄⁺ combined with Mn at 10, 500, 1,500, and 3,000 µmol L⁻¹. The proportion of 70/30 NO₃⁻/NH₄⁺ was chosen because it contains NH₄⁺ enough to stimulates the maximum growth of Tanzania guinea grass (Souza Junior et al., 2018; Leite and Monteiro, 2019), proper to be compared to the supply of NO₃⁻ solely. Manganese rate at 10 µmol L⁻¹ is recommended by Hoagland and Arnon (1950) and the other rates were chosen according to related studies with other plants and heavy metals since no reports are showing similar results about Mn toxicity for tropical forage grasses (Rabêlo et al., 2016; Souza Junior et al., 2019; Rajpoot et al., 2020).

3.2.3. Visual observation

To describe the visual symptoms showed by plants, constant observation of the visual aspect was carried out. Main symptoms manifested such as chlorosis, wilting, necrosis, and growth inhibition were recorded as photographs.

3.2.4. Photosynthetic activity and relative chlorophyll concentration

The gas exchange and chlorophyll fluorescence on the diagnostic leaves (first and second leaves fully expanded from the top to the bottom) were evaluated by using a photosynthesis analyzer IRGA WALZ[®] GFS–3000 one day before harvesting. The quantification of relative chlorophyll index (RCI) was performed after measuring photosynthesis from the average of eight readings by using SPAD 502 MINOLTA[®] on the diagnostic leaves.

3.2.5. Plant sampling and production

The shoots and roots of plants called "nutritional and productive" were harvested and dried in a forced circulation oven at 65 °C for 72 h. After determining their dry biomass, the samples were ground in a Wiley mill. The shoots and roots dry biomass were determined by using a precision scale. The shoots and roots of plants called "metabolic and physiological" were

separated and packed in an aluminum bag, immediately frozen in liquid N, and then stored under low temperature – 80 °C. Afterward, cryogenic grinding was carried out.

3.2.6. Total N, NO₃⁻, NH₄⁺, and Mn in the plant samples

The concentrations of total N in the plant sample were obtained by the Kjeldahl method according to Sarruge and Haag (1974), while the concentrations of $N-NO_3^-$ and $N-NH_4^+$ were determined by distillation according to Tedesco et al. (1985). The concentrations of Mn were determined by atomic absorption spectrometry according to Sarruge and Haag (1974). The accumulation of nutrients was obtained considering the concentration of nutrients and the dry mass production.

3.2.7. Enzymes for N assimilation

Nitrate reductase activity (NR, EC 1.7.1.1) was performed according to Mulder et al. (1969). Fresh diagnostic leaves (0.1 g) were incubated in a solution containing sodium phosphate buffer (pH 7.5) and potassium nitrate (0.25 mol L^{-1}) at 35 °C for 2 h. Afterward, samples were centrifuged at 10,000 g and the supernatant was transferred to flasks. The reaction was stopped by using sulfanilamide (58 mmol L^{-1}) and N–1–naphthyl ethylenediamine dihydrochloride (0.77 mmol L^{-1}). For color development, sodium acetate (2 mol L^{-1}) was used. The quantification was performed on a spectrophotometer at 540 nm using a NO₂⁻ standard curve.

Glutamine synthetase activity (GS, EC 6.3.1.2) was done according to Elliot (1952). Samples were homogenized with tris–HCl (50 mmol L⁻¹), mercaptoethanol (2 mmol L⁻¹), ethylenediaminetetraacetic acid (EDTA, 1 mmol L⁻¹), centrifuging it at 10,000 g at 4 °C for 10 min. Then, extracts were reacted with tris–HCl (200 mmol L⁻¹), ATP (50 mmol L⁻¹), glutamic acid (500 mmol L⁻¹), magnesium sulfate (1 mol L⁻¹), hydroxylamine (100 mmol L⁻¹), and cysteine (100 mmol L⁻¹), incubating them for 30 min at 30 °C. Then, the activity was stopped by using iron chloride (616 mmol L⁻¹), trichloroacetic acid (TCA, 1.45 mol L⁻¹), and HCl (1 mol L⁻¹). Excesses of protein were precipitated by centrifuging the solution at 5000 g for 5 min, quantifying the activity in the supernatant using a standard curve with γ –glutamyl hydroxamate at 540 nm.

3.2.8. Oxidative stress indicators

Lipid peroxidation was evaluated by the 2–thiobarbituric acid (TBA) test, measuring in the form of malondialdehyde (MDA) in plant samples according to Health and Packer (1968). The ground plant samples were homogenized in TCA (1 g L⁻¹) and centrifuged at 10,000 g for 10 min at 4 °C. Trichloroacetic acid (200 g L⁻¹) and TBA (5 g L⁻¹) were added to the supernatant and incubated in a dry bath for 30 min at 95 °C. To stop the reaction, tubes were placed on ice for 10 min and then at room temperature for 15 min, quantifying it at 535 and 600 nm. The concentrations of MDA were determined from the difference in absorbances, using the extinction coefficient of 155 mmol⁻¹ cm⁻¹.

The concentrations of hydrogen peroxide (H_2O_2) in the shoots and roots were determined according to Velikova et al. (2000). An aliquot of the supernatant homogenized with a solution of TCA (1 g L⁻¹) was transferred to microtubes containing potassium phosphate buffer (KH₂PO₄, 100 mmol L⁻¹) and potassium iodide (KI, 1 mol L⁻¹), incubating it on ice in the dark for 60 min. The quantification in the supernatant was performed at 390 nm using a standard H₂O₂ curve.

3.2.9. Protein extraction and quantification

The extraction and quantification of soluble protein in the plant samples were carried out according to Monteiro et al. (2011). The plant material was homogenized with polyvinylpolypyrrolidone (PVPP) in a KH₂PO₄ (100 mmol L⁻¹, pH 7.5), containing EDTA (1 mmol L⁻¹), and dithiothreitol (3 mmol L⁻¹). The extracts were centrifuged at 10,000 g for 30 min at 4 °C, storing the supernatant at low temperature – 80 °C. The quantification was done following Bradford (1976).

3.2.10. Enzymes of the antioxidant system

Superoxide dismutase activity (SOD, EC 1.15.1.1) was done according to Azevedo et al. (1998). The protein extracts were mixed with a solution containing bromophenol blue (5 g L⁻¹), glycerol, and trizma (60 g L⁻¹), placing it in the gel containing acrylamide (400 g L⁻¹), trizma (60 g L⁻¹), N,N,N',N'-Tetramethylethylenediamine (TEMED), and ammonium persulfate (100 g L⁻¹). The running gel was the same as the previous one, but containing more trizma (363 g L⁻¹). Electrophoresis voltage was 15 mA per gel. At the end of the process, the gel was washed with deionized water and incubated for 30 min in a darkroom with KH₂PO₄ buffer solution

(100 mmol L⁻¹, pH 7.8), EDTA (1 mmol L⁻¹), tetrazolium nitroblue chloride (0.1 mmol L⁻¹), riboflavin (0.05 g L⁻¹), and TEMED. Then, those gels were washed with deionized water and illuminated with a fluorescent lamp for 20 min. The reaction was stopped by using glacial acetic acid. The bands were compared qualitatively to SOD bovine protein standard.

Catalase activity (CAT, EC 1.11.1.6) was determined according to Monteiro et al. (2011). At the time of determination, a solution of KH_2PO_4 buffer (100 mmol L⁻¹, pH 7.5) with concentrated H_2O_2 was prepared. An aliquot of this solution was mixed with the protein extracts in a glass cuvette, which initiates the reaction. The activity values were obtained from the decomposition of H_2O_2 for 1 min, performing the reading at 25 °C on a spectrophotometer at 240 nm.

Ascorbate peroxidase activity (APX, EC 1.11.1.1) was carried out according to Cakmak and Horst (1991). The protein extracts were added into a solution of KH₂PO₄ buffer (80 mmol L⁻¹, pH 7) with ascorbic acid (5 mmol L⁻¹) and EDTA (1.45 mmol L⁻¹) at 30 °C in a water bath. Then, concentrated H₂O₂ was added, initiating the reaction. The quantification was estimated by oxidation of ascorbate for 1 min in a quartz cuvette at 290 nm, using 2.8 mmol⁻¹ cm⁻¹ as ascorbate extinction coefficient.

Glutathione reductase activity (GR, EC 1.6.4.2) was done according to Gratão et al. (2008). A solution of KH_2PO_4 buffer (100 mmol L⁻¹, pH 7.5) containing 2–nitrobenzoic acid (3 mmol L⁻¹) was placed in glass cuvettes and then in the water bath at 30 °C. Afterward, NADPH (2 mmol L⁻¹) with oxidized glutathione (20 mmol L⁻¹) and the protein extracts were added to the cuvettes, initiating the reaction. The enzyme activity was quantified by estimating the reduction of oxidized glutathione for 1 min at 412 nm.

Guaiacol peroxidase activity (GPX, EC 1.11.1.7) was performed according to Matsuno and Uritani (1972). The protein extracts were mixed with sodium phosphate buffer (28.4 g L⁻¹, pH 5.0), citric acid (21 g L⁻¹), guaiacol, and concentrated H₂O₂, heating at 30 °C for 15 min. After the incubation, the reaction was stopped by using sodium metabisulfite (20 g L⁻¹) and a subsequent ice bath for 10 min. The quantification was performed on a spectrophotometer at 450 nm.

3.2.11. Proline concentration

Proline was determined according to Bates et al. (1973). The samples were homogenized with sulfosalicylic acid (30 g L^{-1}) and centrifuged at 10,000 g for 20 min at 15 °C. The supernatant was placed in glass tubes to react with ninhydrin (25 g L^{-1}), glacial acetic acid, and phosphoric acid (6 mol L⁻¹). The tubes were heated in water at 100 °C for 1 h. Then, concentrated toluene was

added into the tubes, shaken for 20 s, and waited for 10 min aiming the phase separation. The quantification was performed at 520 nm and quantified by using a standard proline curve.

3.2.12. Statistical analysis

Statistical analysis of the results was performed by using the software "Statistical Analysis System" (SAS Institute, 2008). The analysis of variance was initially performed by using the GLM procedure. When the F test was significant for $NO_3^-/NH_4^+ \times Mn$ interaction, the main factor proportions of NO_3^-/NH_4^+ was analyzed within the rates of Mn and the rates of Mn within the proportions of NO_3^-/NH_4^+ . In the cases when the interaction was not significant, the averages of the main factors were compared separately. To compare the averages, the Tukey test at 5% was applied.

3.3. RESULTS

3.3.1. Visual observation

The excess Mn caused chlorosis which was associated with points of necrosis on the leaves (Fig 1a). The plant extracts after nitric-perchloric digestion showed a strong pink color, suggesting a high concentration of Mn in the extracts (Fig 1b). The roots exposed to Mn 3,000 μ mol L⁻¹ (Fig 1c) have shown yellow color and high diameter compared to roots exposed to Mn 10 μ mol L⁻¹, suggesting that the excess Mn affects both shoots and roots of Tanzania guinea grass.



Figure 1. Leaves of Tanzania guinea grass at Mn 3,000 μ mol L⁻¹ (a), digestion extracts of leaves at Mn 3,000 μ mol L⁻¹ (b), and comparison of the roots under Mn 3,000 and 10 μ mol L⁻¹ (c).

3.3.2. Dry mass production and relative chlorophyll index (SPAD)

The NO₃⁻/NH₄⁺ × Mn interaction (from now on, it will be referred to as interaction only) was not significant for the dry biomass of shoots and roots, as well as the relative chlorophyll index (RCI). Nonetheless, the dry biomass production of shoots and roots, and the RCI were significant for the proportions of NO₃⁻/NH₄⁺, showing a higher value at the 100/0 NO₃⁻/NH₄⁺ than at 70/30 NO₃⁻/NH₄⁺ (Table 1).

Shoots Roots RCI NR GS g per pot $\mu g NO_2^- g^{-1} FW h^{-1}$ mg y-glutamyl g⁻¹ FW h⁻¹ unit NO_{3}/NH_{4}^{+} 100/012.7 ^b ± 0.76 1.93 ^b ± 0.21 44.0 ^b ± 0.82 26.1 ns ± 1.55 $28.2 \text{ }^{\mathrm{b}} \pm 1.83$ 70/30 14.8 $^{\mathrm{a}}\pm0.67$ $26.3 \text{ ns} \pm 2.50$ $2.71 \ ^{a} \pm 0.13$ $51.1 \text{ a} \pm 0.48$ $31.5~^{\rm a}\pm1.33$ $Mn \ (\mu mol \ L^{-1})$ $14.6 \text{ ns} \pm 0.87$ 10 $2.35 \text{ ns} \pm 0.25$ 48.4 ns ± 1.09 28.3 ns ± 1.99 $35.5 \ ^{a} \pm 1.59$ 34.3 ^a ± 1.69 500 13.9 ns ± 1.37 2.61 ns ± 0.41 48.3 ns ± 1.58 25.5 ns ± 3.93 $12.7 \text{ ns} \pm 1.17$ 2.15 ^{ns} ± 0.25 45.2^{ns} ± 1.62 27.0 ns ± 3.06 26.3 ^b ± 1.34 1,500 3,000 13.7 ns ± 0.61 2.17 ns ± 0.14 48.2 ns ± 1.61 24.2 ns ± 2.19 23.2 ^b ± 1.11 < 0.05 < 0.01 < 0.05 *p* value < 0.01 > 0.05 cv (%) 20 31 5.5 26 14

Table 1. Shoots and roots dry biomass production, relative chlorophyll index (RCI), nitrate reductase, and glutamine synthetase activity in the shoots and roots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of Mn in the nutrient solution.

The Tukey at 5% test was applied. Lowercase letters in each sub-column show significant differences. NR nitrate reductase. GS glutamine synthetase. FW fresh weight. ns not significant. ± standard error. cv coefficient of variation.

3.3.3. Activities of nitrate reductase and glutamine synthetase

The interaction was not significant for the activities of nitrate reductase (NR) and glutamine synthetase (GS) in the leaves of Tanzania guinea grass. However, the GS activity was affected by the main effects. The averages of GS activity were higher at $70/30 \text{ NO}_3^-/\text{NH}_4^+$ than at $100/0 \text{ NO}_3^-/\text{NH}_4^+$, as well as at Mn 1,500 and 3,000 µmol L⁻¹ compared to the other rates of Mn (Table 1). The main effects did not affect the NR activity (Table 1).

3.3.4. Total N, NO₃⁻, NH₄⁺, and Mn in the plant samples

The interaction was not significant for the concentrations of total N, NO_3^- , NH_4^+ and Mn in Tanzania guinea grass. It was verified that only the concentration of NH_4^+ in the roots was affected by the proportions of NO_3^-/NH_4^+ , showing a higher concentration in roots at 70/30 than at 100/0 NO_3^-/NH_4^+ (Table 2).

The interaction was significant for the concentrations of Mn in the shoots and roots (Fig. 2a). The concentrations of Mn in the shoots and roots of Tanzania guinea grass were higher with a combination of $100/0 \text{ NO}_3^-/\text{NH}_4^+$ and Mn at 3,000 µmol L⁻¹ than at the other combinations, reaching up to 5,500 mg kg⁻¹ and 21,187 mg kg⁻¹ in the shoots and roots, respectively (Fig. 2a). Additionally, it was verified higher concentrations of Mn in the roots than in the shoots, especially in those plants grown at $100/0 \text{ NO}_3^-/\text{NH}_4^+$ and Mn at 3,000 µmol L⁻¹ (Fig. 2a).

The interaction was not significant for the accumulations of total N, NO₃⁻, and NH₄⁺ in Tanzania guinea grass. The accumulations of these minerals in the shoots and roots were affected by the proportions of NO_3^-/NH_4^+ , showing the higher values at 70/30 than at $100/0 \text{ NO}_3^-/NH_4^+$ (Table 3). The rates of Mn did not affect the accumulations of total N, NO_3^- , and NH_4^+ (Table 3).

The interaction was significant for the Mn accumulation in the shoots (Fig. 2b). The accumulation of Mn in the shoots of Tanzania guinea grass was higher with a combination between $100/0 \text{ NO}_3^-/\text{NH}_4^+$ and Mn at 3,000 µmol L⁻¹ than at the other combinations (Fig. 2b), reaching 76.2 mg per pot. It was not verified any significant interaction for the accumulation of Mn in roots. However, it was verified the main effect of Mn rates in the roots regarding the Mn accumulation (Fig. 2c). The higher accumulation of Mn in the roots was verified with Mn at 3,000 µmol L⁻¹ compared to the other rates of Mn, reaching 31.1 mg per pot (Fig. 2c). The Mn accumulation was higher in the shoots compared to the roots (Fig. 1b, Fig. 2c).

	Ν	NO ₃ -	\mathbf{NH}_{4}^{+}
		mg kg ⁻¹	
Shoots			
NO_3^-/NH_4^+			
100/0	26.2 ^{ns} ± 1.64	$0.054 \text{ ns} \pm 0.001$	$0.159 \text{ ns} \pm 0.009$
70/30	$26.1 \text{ ns} \pm 0.77$	$0.055 \text{ ns} \pm 0.002$	$0.258 \text{ ns} \pm 0.012$
Mn (μmol L-1)			
10	26.1 ^{ns} ± 1.22	$0.053 \text{ ns} \pm 0.001$	$0.207 \text{ ns} \pm 0.017$
500	24.5 ^{ns} \pm 0.70	$0.056 \text{ ns} \pm 0.002$	$0.205 \text{ ns} \pm 0.023$
1,500	29.2 ^{ns} ± 2.90	$0.057 \text{ ns} \pm 0.002$	$0.226 \text{ ns} \pm 0.020$
3,000	24.9 ^{ns} ± 1.10	$0.054 \text{ ns} \pm 0.003$	$0.195 \text{ ns} \pm 0.030$
<i>p</i> value	> 0.05	> 0.05	> 0.05
c v (%)	17	16	23
Roots			
NO_3^-/NH_4^+			
100/0	$21.9 \text{ ns} \pm 0.667$	$0.061 \text{ ns} \pm 0.002$	$0.189 \text{ b} \pm 0.014$
50/50	$20.6 \text{ ns} \pm 0.399$	$0.057 \text{ ns} \pm 0.002$	0.386 ª ± 0.031
Mn (μmol L ⁻¹)			
10	21.3 ^{ns} ± 0.911	$0.060 \text{ ns} \pm 0.003$	$0.221 \text{ ns} \pm 0.036$
500	22.1 ^{ns} ± 1.01	$0.062 \text{ ns} \pm 0.003$	$0.308 \text{ ns} \pm 0.048$
1,500	$20.7 \text{ ns} \pm 0.596$	$0.056 \text{ ns} \pm 0.001$	$0.286 \text{ ns} \pm 0.026$
3,000	$20.9 \text{ ns} \pm 0.527$	$0.058 \text{ ns} \pm 0.004$	$0.335 \text{ ns} \pm 0.066$
<i>p</i> value	> 0.05	> 0.05	< 0.01
cv (%)	10	16	29

Table 2. Concentrations of N, NO_3^- , and NH_4^+ in the shoots and roots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of Mn in the nutrient solution.

The Tukey at 5% was applied. Lowercase letters in each sub-column show significant differences. ns not significant. \pm standard error. cv coefficient of variation.



Figure 2. Concentrations of Mn in the shoots and roots (a) and accumulation of Mn in the shoots (b) of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of Mn in the nutrient solution. The Tukey test at 1% was applied. Lowercase letters compare rates of Mn within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each Mn rate. Coefficient of variation (%) = 15 (a, shoots) and 19 (a, roots), 31 (b), and 21 (c). Error bars indicate standard error.

3.3.5. Photosynthetic activity

The interaction was not significant for the net photosynthesis (NP), evapotranspiration rate (ER), stomatal conductance (SC), and vapor pressure deficit (VPD). However, the NP, SC, and VPD were affected by the main effects of rates of Mn (Table 4). The higher value of NP was found at Mn at 10 and 500 μ mol L⁻¹ compared to the other rates (Table 4). Regarding the SC, the highest value was verified at Mn 500 μ mol L⁻¹ (Table 4). Finally, the VPD was higher at Mn 1,500 and 3,000 μ mol L⁻¹ than at 500 μ mol L⁻¹ (Table 4).

	Ν	NO ₃ -	\mathbf{NH}_{4}^{+}
		mg per pot	
Shoots			
<i>NO₃⁻/NH</i> ⁺			
100/0	324 ^b ± 18.6	$0.69 \text{ b} \pm 0.047$	$2.03 \text{ b} \pm 0.192$
70/30	382 ª ± 14.9	$0.81 \ ^{\rm a} \pm 0.041$	3.81 ª ± 0.229
Mn (μmol L-1)			
10	379 ^{ns} ± 25.9	$0.77 \text{ ns} \pm 0.056$	$3.08 \text{ ns} \pm 0.351$
500	335 ^{ns} ± 23.4	$0.77 \text{ ns} \pm 0.069$	$2.92 \text{ ns} \pm 0.461$
1,500	358 ^{ns} ± 31.3	$0.72 \text{ ns} \pm 0.067$	$3.01 \text{ ns} \pm 0.462$
3,000	$340 \text{ ns} \pm 18.5$	$0.74 \text{ ns} \pm 0.070$	$2.68 \text{ ns} \pm 0.441$
<i>p</i> value	< 0.05	< 0.05	< 0.01
cv (%)	20	21	29
Roots			
<i>NO3⁻/NH4⁺</i>			
100/0	41.6 ^ь ± 0.667	$0.11 \text{ b} \pm 0.002$	0.35 ^b ± 0.014
70/30	55.9 ª ± 0.399	$0.15 \ ^{a} \pm 0.002$	1.02 = 0.030
Mn (μmol L-1)			
10	48.8 ^{ns} ± 0.911	$0.14 \text{ ns} \pm 0.003$	$0.54 \text{ ns} \pm 0.036$
500	56.1 ^{ns} ± 1.011	$0.15 \text{ ns} \pm 0.003$	$0.78 \text{ ns} \pm 0.048$
1,500	44.4 ns ± 0.596	$0.12 \text{ ns} \pm 0.001$	$0.65 \text{ ns} \pm 0.026$
3,000	45.1 $^{ns} \pm 0.527$	$0.12 \text{ ns} \pm 0.004$	$0.77 \text{ ns} \pm 0.066$
<i>p</i> value	< 0.01	< 0.01	< 0.01
cv (%)	28	27	33

Table 3. Accumulations of N, NO_3^- , and NH_4^+ in the shoots and roots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of Mn in the nutrient solution.

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. ns not significant. \pm standard error. cv coefficient of variation.

	NP	ER	SC	VPD
	$\mu mol m^{-2} s^{-1}$	mmol m	1 ⁻² s ⁻¹	Ра Кра-1
NO_3^-/NH_4^+				
100/0	$16.4 \text{ ns} \pm 1.05$	$1.79 \text{ ns} \pm 0.11$	$80.9 \text{ ns} \pm 5.46$	22.4 ns \pm 0.16
70/30	$15.7 \text{ ns} \pm 1.12$	$1.61 \text{ ns} \pm 0.13$	$73.4 \text{ ns} \pm 6.61$	$22.3 \text{ ns} \pm 0.18$
Mn (μmol L-1)				
10	17.4 ª ± 1.02	$1.80 \text{ ns} \pm 0.13$	81.7 ^ь ± 6.34	$22.2 \text{ ab} \pm 0.21$
500	$19.2 \ ^{a} \pm 0.99$	$2.14 \text{ ns} \pm 0.13$	98.4 ª ± 6.99	21.9 ^ь ± 0.15
1,500	13.6 ^ь ± 1.55	$1.38 \text{ ns} \pm 0.15$	61.2 ° ± 7.27	22.8 $^{a} \pm 0.20$
3,000	14.1 ^ь ± 1.54	$1.50 \text{ ns} \pm 0.15$	$67.2 \text{ bc} \pm 7.53$	22.6 $^{a} \pm 0.25$
<i>p</i> value	< 0.01	> 0.05	< 0.01	< 0.01
cv (%)	12	18	20	2.3

Table 4. Net photosynthesis (NP), evapotranspiration rate (ER), stomatal conductance (SC), and vapor pressure deficit (VPD) of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of Mn in the nutrient solution.

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. ns not significant. \pm standard error. cv coefficient of variation.

The interaction was not significant for the maximum efficiency of photosystem II (Fv/Fm), quantum efficiency of photosystem II (QEPII), electron transport rate (ETR), and photochemical (qP) and non-photochemical quenching (qN). However, it was found that the ETR, qP, and qN were affected by the main effect rates of Mn (Table 5). Regarding the ETR and qP, it was verified higher values with Mn at 10 and 500 μ mol L⁻¹ than at the other rates of Mn (Table 5). Regarding the qN, a higher average with Mn at 1,500 μ mol L⁻¹ compared to Mn 10 μ mol L⁻¹ was verified (Table 5). The Fv/Fm and QEPSII were not affected by the main effects (Table 5).

0 0	1 1	•			
	Fv/Fm	QEPII	ETR	qP	qN
		<i>nit</i> ———	μ mol m ⁻² s ⁻¹	<i>u</i> r	nit
NO_{3}/N_{2}	H_4^+				
100/0	$0.780 \text{ ns} \pm 0.001$	$0.097 \text{ ns} \pm 0.004$	49.3 ns ± 2.02	$0.175 \text{ ns} \pm 0.007$	$0.824 \text{ ns} \pm 0.007$
70/30	$0.786 \text{ ns} \pm 0.002$	$0.103 \text{ ns} \pm 0.003$	51.9 ^{ns} ± 1.99	$0.188 \text{ ns} \pm 0.006$	$0.811 \text{ ns} \pm 0.006$
Mn (μma	ol L-1)				
10	$0.780 \text{ ns} \pm 0.004$	$0.107 \text{ ns} \pm 0.004$	54.1 ^a ± 2.30	0.197 = 0.008	$0.802 \text{ b} \pm 0.008$
500	$0.782 \text{ ns} \pm 0.002$	$0.109 \text{ ns} \pm 0.005$	55.0 ª ± 2.53	0.193 = 0.007	$0.806 \text{ ab} \pm 0.007$
1,500	$0.785 \text{ ns} \pm 0.002$	$0.091 \text{ ns} \pm 0.005$	46.2 ^ь ± 2.94	0.166 ^ь ± 0.010	$0.833 \ ^{\rm a} \pm 0.010$
3,000	$0.785 \text{ ns} \pm 0.001$	$0.093 \text{ ns} \pm 0.004$	47.0 ^ь ± 2.21	$0.170 \text{ b} \pm 0.006$	$0.829 \text{ ab} \pm 0.006$
<i>p</i> value	> 0.05	< 0.01	< 0.01	< 0.01	< 0.01
cv (%)	1.12	12	12	5.9	2.6

Table 5. Maximum (Fv/Fm) and quantum efficiency of photosystem II (QEPII), electron transport rate (ETR), and photochemical (qP) and non-photochemical quenching (qN) of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of Mn in the nutrient solution.

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. ns not significant. \pm standard error. cv coefficient of variation. Parameters with no unit.

3.3.6. Oxidative stress and proline

The interaction was not significant for the concentrations of malondialdehyde (MDA), hydrogen peroxidase (H₂O₂), and proline. However, the main effects affected the concentrations of H₂O₂ and proline in Tanzania guinea grass (Table 6). The rates of Mn affected the concentrations of H₂O₂ in the roots and proline in the shoots (Table 6). Regarding the concentrations of H₂O₂ in the roots, it was verified lower values in the plants that received Mn at 10 μ mol L⁻¹ compared to the plants exposed to the other rates of Mn (Table 6). Regarding the concentrations of proline in the shoots, it was found higher values with Mn at 1,500 μ mol L⁻¹ compared to Mn at 10 μ mol L⁻¹. The concentrations of MDA were not affected by the main effects (Table 6).

	MDA	H_2O_2	Proline
	$nmol g^{-1} FW$		g ⁻¹ FW
Shoots			
NO_3^-/NH_4^+			
100/0	$1.09 \text{ ns} \pm 0.06$	$2.75 \text{ ns} \pm 0.31$	$2.89 \text{ ns} \pm 0.34$
70/30	$1.00 \text{ ns} \pm 0.03$	$3.34 \text{ ns} \pm 0.27$	$3.07 \text{ ns} \pm 0.28$
Mn (μmol L ⁻¹)			
10	$1.13 \text{ ns} \pm 0.08$	$3.71 \text{ ns} \pm 0.47$	$2.04 \text{ b} \pm 0.24$
500	$1.10 \text{ ns} \pm 0.08$	$2.92 \text{ ns} \pm 0.39$	$2.72 \text{ ab} \pm 0.30$
1,500	$1.00 \text{ ns} \pm 0.06$	$2.59 \text{ ns} \pm 0.36$	3.71 ^a ± 0.53
3,000	$0.94 \text{ ns} \pm 0.04$	$2.97 \text{ ns} \pm 0.36$	$3.46 \text{ ab} \pm 0.41$
<i>p</i> value	> 0.05	> 0.05	< 0.01
cv (%)	15	38	20
Roots			
NO_3^-/NH_4^+			
100/0	$0.33 \text{ ns} \pm 0.02$	$1.21 \text{ ns} \pm 0.17$	$1.51 \text{ ns} \pm 0.07$
70/30	$0.31 \text{ ns} \pm 0.11$	$0.88 \text{ ns} \pm 0.18$	$1.49 \text{ ns} \pm 0.08$
Mn (μmol L-1)			
10	$0.36 \text{ ns} \pm 0.04$	$0.32 ^{\text{b}} \pm 0.06$	$1.56 \text{ ns} \pm 0.07$
500	$0.30 \text{ ns} \pm 0.02$	1.16 ^a ± 0.29	$1.35 \text{ ns} \pm 0.05$
1,500	$0.28 \text{ ns} \pm 0.01$	1.26 = 0.22	$1.43 \text{ ns} \pm 0.11$
3,000	$0.32 \text{ ns} \pm 0.02$	1.42 = 0.21	$1.67 \text{ ns} \pm 0.15$
<i>p</i> value	> 0.05	< 0.01	> 0.05
CV (%)	26	35	21

Table 6. Concentrations of malondialdehyde (MDA), hydrogen peroxide (H₂O₂), and proline in the shoots and roots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of Mn in the nutrient solution.

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. ns not significant. \pm standard error. cv coefficient of variation.

3.3.7. Antioxidant enzyme activity

The interaction was not significant for the activities of catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX). However, it was verified the main effects of the glutathione reductase (GR) activity in the plants (Table 7). Regarding the shoots, the GR activity was the highest at $100/0 \text{ NO}_3^-/\text{NH}_4^+$ (Table 7). Also, it was verified higher values of GR activity in the shoots and roots with Mn at $10 \,\mu\text{mol L}^{-1}$ than at 3,000 $\mu\text{mol L}^{-1}$ (Table 7). It was not verified any effects regarding superoxide dismutase activity. The activities of CAT, GPX, and APX were not affected by the main factors (Table 7).

	CAT	GPX	APX	GR		
	μmol min ⁻¹ mg ⁻¹ protein					
Shoots						
NO_3^-/NH_4^+						
100/0	90.6 ^{ns} ± 5.83	$5.59 \text{ ns} \pm 0.84$	901 ns \pm 75.5	20.9 ª ± 1.01		
70/30	$82.2 \text{ ns} \pm 7.24$	$5.97 \text{ ns} \pm 0.77$	798 ns \pm 60.4	17.7 ^ь ± 0.84		
Mn (µmol L-1)						
10	$70.6 \text{ ns} \pm 8.38$	$4.85 \text{ ns} \pm 0.84$	772 ^{ns} \pm 70.6	22.5 ^a ± 1.27		
500	$100 \text{ ns} \pm 8.48$	6.63 ns ± 1.33	924 ^{ns} ± 129	19.8 $^{\rm ab}\pm 0.74$		
1,500	85.3 ^{ns} ± 8.63	$6.77 \text{ ns} \pm 0.91$	$865 \text{ ns} \pm 80.6$	$18.1 ^{\text{ab}} \pm 1.24$		
3,000	$89.2 \text{ ns} \pm 8.97$	4.88 ns ± 1.25	838 ^{ns} ± 95.4	16.9 ^ь ± 1.54		
<i>p</i> value	> 0.05	> 0.05	> 0.05	< 0.05		
cv (%)	25	47	32	18		
Roots						
NO_{3}/NH_{4}^{+}						
100/0	$242 \text{ ns} \pm 20.4$	$5.15 \text{ ns} \pm 0.42$	1331 ^{ns} ± 95.1	$4.27 \text{ ns} \pm 1.02$		
70/30	$311 \text{ ns} \pm 40.8$	$6.39 \text{ ns} \pm 0.77$	1610 ^{ns} ± 173	$6.44 \text{ ns} \pm 1.22$		
Mn (µmol L-1)						
10	242 ^{ns} ± 32.3	$5.59 \text{ ns} \pm 0.54$	1484 ^{ns} ± 157	8.03 ^a ± 1.36		
500	289 ^{ns} ± 42.5	$5.92 \text{ ns} \pm 0.95$	1555 ^{ns} ± 172	$5.79 \text{ ab} \pm 1.52$		
1,500	$245 \text{ ns} \pm 25.9$	$5.42 \text{ ns} \pm 0.62$	1291 ^{ns} ± 151	$5.32 \text{ ab} \pm 2.1$		
3,000	329 ^{ns} ± 68.7	6.16 ^{ns} ± 1.30	1553 ^{ns} ± 289	$2.28 \text{ b} \pm 0.57$		
<i>p</i> value	> 0.05	> 0.05	> 0.05	< 0.05		
CV (%)	44	45	39	39		

Table 7. Activities of catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR) in the shoots and roots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of Mn in the nutrient solution.

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. ns not significant. \pm standard error. cv coefficient of variation.

3.4. DISCUSSION

3.4.1. Excess Mn does not affect dry biomass production

The excess Mn caused chlorosis and necrosis on the leaves and affected the root system, which altered its morphology, colors, and length (Fig. 1). However, these visual effects were not associated with any negative effects on the dry biomass production, even under high rates of Mn at 3,000 μ mol L⁻¹ (Table 1). This suggests a high tolerance of Tanzania guinea grass to excess Mn. Tanzania guinea grass has been reported as an important plant that can tolerate high concentrations of Cd, Cu, and Ba (Leite and Monteiro, 2019; Souza Junior et al., 2019; Cardoso and Monteiro,

2021). Plants with high stress tolerance for heavy metals are desirable in phytoremediation's program since one of the major criteria for being a phyextractor plant is the ability to survive under the high availability of heavy metals (Schulze et al., 2002; Taiz et al., 2017). One of the major indications to use a plant species for phytoremediation is its dry biomass production under excess heavy metal. Pan et al. (2018) reported a decrease in dry biomass when the hyperaccumulator *Xanthium strumarium* L. was exposed to Mn up to 20,000 μ mol L⁻¹. According to Pan et al. (2018), dry biomass is one of the more important indicators of Mn toxicity because the total phytoextraction is also a function of dry biomass production.

Ammonium addition increased both shoots and roots dry biomass, as well as the RCI value (Table 1). This positive effect is expected and was previously reported for Tanzania guinea grass (Silveira e Monteiro, 2011; Souza Junior et al., 2018; Leite and Monteiro, 2019). Many plant species can assimilate part of N as NH_4^+ instead of NO_3^- , improving N use efficiency and releasing free energy which can act to improve chlorophyll synthesis, as well as plant growth and development (Marschner, 2012). Glutamine synthetase requires less energy and reduced power than NR and the free energy released from this balance can be used for NH_4^+ affinity plants as growth-enhancing (Epstein and Bloom, 2006; Taiz et al., 2017; Marschner, 2012).

3.4.2. Mn excess decreases GS activity

Few studies are reporting the relationships between Mn toxicity and GS activity for plants. Plants of *Citrus grandis* M. exposed to Mn 600 μ mol L⁻¹ showed a higher GS activity compared to non-intoxicated plants (You et al., 2017), disagreeing with the present study. Those authors related that result to the fact that Mn toxicity may increase photorespiration. In that situation, plants can express genes that code for GS, increasing its activity and reassemble the NH₄⁺ lost in photorespiratory metabolism. In the present study, it was verified a clear effect of inhibition on GS activity due to excess Mn, suggesting that the excess Mn downregulates genes that code for GS, decreasing its expression, likely due to oxidative stress caused by Mn (Rajpoot et al., 2020). It has been previously reported that the GS activity in plants grown with a combination of NO₃⁻ and NH₄⁺ was increased by using excess Cu in nutrient solution (Souza Junior et al., 2018), agreeing with You et al. (2017). Likely, the photorespiration caused by excess Mn and suggested by You et al. (2017) might also occur for Cu under specific conditions of N forms, but not for Mn.

3.4.3. NO₃⁻ supply improves the concentration and accumulation of Mn as a hyperaccumulator

The effects of N forms on concentrations and accumulations of Mn in Tanzania Guinea grass have not been yet reported. However, there are some reports regarding the effects of proportions of NO₃⁻/NH₄⁺ on the concentrations and accumulation of Cu and Cd for Tanzania guinea grass (Souza Junior et al., 2018; Leite and Monteiro, 2019). In the present study, it was found high concentrations and accumulations of Mn when the plants were grown with N as NO3⁻ and high rates of Mn (Fig. 2). Manganese hyperaccumulators are plants that can concentrate more than 10,000 mg kg⁻¹ Mn in their biomass (Baker and Brooks, 1989). In the present study, it was verified an average concentration of 13,345 mg kg⁻¹ Mn (5,500 and 21,187 mg kg⁻¹ in the shoots and roots, respectively) (Fig. 2), which is higher than those plants indicated as Mn hyperaccumulator (Baker and Brooks, 1989). This suggests that Tanzania guinea grass is a Mn hyperaccumulator and can be highly recommended as a Mn phytoremediator. Pan et al. (2018) found concentrations of Mn in leaves and roots around 30,000 and 15,000 mg kg-1, respectively, for the hyperaccumulator Xanthium strumarium. However, these plants received Mn up to 20,000 mmol L⁻¹, almost seven-fold higher than the rates applied in the present study, which suggests that Tanzania guinea grass has a high potential to accumulate Mn even under lower rates of Mn than those applied to hyperaccumulators.

In the present study, plants grown under NO₃⁻ solely exposed to high rates of Mn showed the highest concentrations and accumulation of Mn (Fig. 2). This result was not expected since Tanzania guinea grass showed the lowest concentrations and accumulations of Cu and Cd when plants were grown under N as NO₃⁻ solely (Souza Junior et al., 2018; Leite and Monteiro, 2019). Cheng et al. (2016) reported that the NH₄⁺ application improved the accumulation of Cd in two hyperaccumulators named Carpobrotus rossii S. and Solanum nigrum L. However, aiming to evaluate the effect of N supply exclusively as NO3⁻ or NH4⁺ (3.75 mmol L⁻¹) on responses to sexual dimorphism of Populus cathayana exposed to excess Mn, Zhao et al. (2018) reported that plants cultivated with all N as NO3⁻ showed the highest Mn concentration in the plant parts. However, plants supplied with all N as NH4⁺ showed the lowest Mn concentration in the plant parts. These authors did not explain the reasons why those plants accumulated more Mn under NO₃⁻ supply as it was found in the present study. Therefore, there is a possibility of NO₃⁻ upregulates the heavy metal transporters, such as heavy metal-transporting P-type ATPase (Deng et al., 2013). Other studies showed an increase in cadmium (Cd) phytoextraction by rates of NO₃-applied to sorghum plants (Sorghum bicolor L.) and the authors related it to several nitrate transporters such as NRT 1.1, which seems to play a central role to increase Cd accumulation in sorghum plants according to mechanisms not clear yet (Bai et al., 2021). There are other studies with alfalfa plants (*Medicago sativa* L.) that showed a direct relationship between NO₃⁻ supply and phytoextracting potential of mercury (Hg) (Carrasco-Gil et al., 2012) and Cd (Hattab et al., 2014).

The highest accumulations of NO_3^- , NH_4^+ , and N in the shoots and roots of Tanzania guinea grass supplied with NO_3^- and NH_4^+ were verified (Table 3). This result is expected and means that the NH_4^+ supply might improve the N use efficiency and energy acquired, improving the N accumulation, as previously reported for Tanzania guinea grass (Santos et al., 2013; Souza Junior et al., 2018, Leite and Monteiro, 2019). The highest accumulation of N from the nutrient solution influenced by NH_4^+ addition led those plants to grow more. However, it did not have any relation with the highest accumulation of Mn verified in the present study since the plants that showed the lowest dry biomass also showed the highest concentration and accumulation of Mn. This effect reinforces the hypothesis that NO_3^- solely enhances the heavy metal-transporting P-type ATPase related to Mn uptake (Deng et al., 2013).

3.4.4. Excess Mn toxicity decreases photosynthetic activity

The excess Mn led to a decrease in the NP, SC, ETR, and qP, as well as an increase in the NPQ (Table 4). In the plant cells, excess Mn can cause the excessive synthesis of ROS, which attack cell membranes, especially in thylakoids where oxidative photophosphorylation occurs and the electron transport chain is located (Ribeira et al., 2013; Pan et al., 2018). Excess Mn can negatively affect NP and it occurs because excess Mn affects the photosynthetic electron transport, causing electron impairment and then disruption in thylakoid membrane function, which increases the energy losses by heat and fluorescence (Chatzistathis et al., 2010), as it showed in the present study. The stomatal conductance was also affected by the excess Mn as a result of excess ROS, which can be associated with disruptions in membranes containing aquaporins, decreasing the water use efficiency and transportation (Pan et al., 2018; Inostroza-Blancheteau et al., 2017). Rajpoot et al. (2020) reported that rice plants (*Oryza sativa* L.) grown with Mn at 1 and 2 mmol L⁻¹ for eight days showed the lowest NP and SC because excess Mn destroyed thylakoid membranes due to oxidative stress. Pan et al. (2018) also found the lowest NP, SC, ETR, and qP in *Xanthium strumarium* due to excess Mn supplied up to 20 mmol L⁻¹ for the same reasons.

3.4.5. Oxidative stress increases proline synthesis but decreases GPX activity

It was found a high H_2O_2 concentration in the roots due to the excess Mn (Table 6). Oxidative stress plays a central role in heavy metal toxicity in plants. Excessive ROS may cause lipid peroxidation, inducing membrane injuries that modify its permeability, ion leakage, and finally cell death (Rajpoot et al., 2020). Rajpoot et al. (2020) also verified that rice plants grown with Mn at 2 mmol L⁻¹ in nutrient solution showed higher concentrations of H₂O₂ compared to control plants supplied with 10 µmol L⁻¹. Hydrogen peroxide is one of the main ROS synthesized by plant cells under stress conditions, which cause lipid peroxidation in cell membranes (Myouga et al., 2008; Singh et al., 2010; Hayat et al., 2012). However, antioxidant enzymes can turn H₂O₂ into H₂O, based on redox reactions, called Fenton and Haber–Weiss reaction, culminating in stress mitigation (Liu et al., 2015). In this context, turning H_2O_2 into H_2O stands out the enzyme GPX (Carrasco-Gil et al., 2012). In the present study, the excess Mn decreases the GPX activity in the plants (Table 7), as well as increases the concentrations of proline in the shoots (Table 6). These results suggest that the non-enzymatic antioxidant system has higher importance on mitigating oxidative stress caused by excess Mn in Tanzania guinea grass than the enzymatic antioxidant system. For that reason, Tanzania guinea grass likely saved energy from GPX synthesis to be used in proline synthesis.

An increase in proline concentration in the shoots due to excess Mn was verified (Table 6). Proline is the main osmotic regulator in plant cells, playing a central role to mitigate ROS production and then stabilizes cell structure (Huang et al., 2019) due to its direct importance as an antioxidant and osmoregulator (Hayat et al., 2012). As an osmoregulator, an increase in proline concentration can confer cellular homeostasis in a situation of low stomatal conductance (Hayat et al., 2012), as verified in the present study (Table 4). In this context, an increase in proline concentration in plant cells leads to a decrease in cell Ψ_{water} , which allows continuous absorption of water by plant roots (Taiz et al., 2017) even under the high availability of heavy metals. Huang et al. (2019), studying physiological alterations of *Broussonetia papyrifera* L. to excess Mn, also found an increase in concentrations of proline due to exposure of Mn up to 2 mmol L⁻¹.

Osmotic stress caused by heavy metals has been reported for Cu in Tanzania guinea grass (Souza Junior et al., 2018). This grass exposed to excess Cu reached a permanent wilt point as a defense mechanism, avoiding absorbing water and then excess Cu solubilized in the nutrient solution. This effect led to an inhibition of water absorption and then all mineral nutrients dissolved in water, which are crucial for plant development, leading to plant death (Souza Junior et al., 2018). In the present study, the wilting effect caused by Mn was practically null, probably due to the high

proline synthesis in a situation of high Mn contamination, suggesting that proline may play a central role to mitigate the wilting effect from excess heavy metal.

As an antioxidant, proline can act as a ROS scavenger and also enables proper NADP/NADPH ratio in stressed cells (Singh et al., 2010; Hayat et al., 2012), which can improve carbon fixation and N assimilation under adverse conditions (Hayat et al., 2012). Pan et al. (2018) found an increase in proline concentration due to the application of Mn at 5 mmol L^{-1} in *Xanthium strumarium*, which was associated with stress mitigation and heavy metal tolerance. Proline can eliminate hydroxyl radicals, aiming to protect the plant from osmotic stress and preventing membrane disruption (Pan et al., 2018). In the present study, proline can also be increased ROS mitigation in Tanzania guinea grass exposed to excess Mn.

3.5. CONCLUSION

Tanzania guinea grass is very tolerant to excess Mn. This plant can concentrate Mn in its biomass as much as a hyperaccumulator plant and even under excess Mn the growth is not affected by its excess. However, to maximize the phytoextraction and the heavy metal tolerance, the grass should be fertilized with all N as NO_3^{-} . The excess Mn negatively affects photosynthesis and glutamine synthetase activity due to the oxidative stress caused by H₂O₂. In this context, proline synthesis seems to play a key role as a ROS mitigator, protecting plants from metabolic impairment caused by excess Mn. Tanzania guinea grass has the potential to be recognized as a Mn hyperaccumulator and this recognition will improve its utilization in phytoremediation programs.

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4. AMMONIUM ADDITION MITIGATES OXIDATIVE STRESS CAUSED BY SULFUR LIMITATION IN TANZANIA GUINEA GRASS

ABSTRACT

Nitrogen is the major stimulator of forage grass growth and a proper proportion of NO₃-/NH₄⁺ can improve the uptake of N and S and plant production. However, no reports are showing the role of N forms to mitigate S limitation for Tanzania guinea grass [Panicum maximum Jacq. cv. Tanzania (syn. Megathyrsus maximus (Jacq.) B.K.Simon & S.W.L. Jacobs)]. The objective was to evaluate the effect of S limitation and S sufficiency on metabolic, physiological, nutritional, and productive parameters, revealing the role of N forms as a stress mitigator for Tanzania guinea grass. A 2×3 factorial experiment in a growth chamber and randomized complete block design with four replications was carried out. Factors were proportions of 100/0 and 70/30 NO₃-/NH₄+ combined with rates of S 0.1 (limitation), 1 (intermediate), and 2 mmol L-1 (sufficient). The Hoagland and Arnon's nutrient solution in a hydroponic system was used. The analysis of variance and Tukey test at 5% to compare averages were applied. In a situation of S sufficiency, the shoots and roots dry biomass were higher in the plants grown at 70/30 NO₃⁻/NH₄⁺ than at NO₃⁻ solely, showing a high relative chlorophyll index under 70/30 NO₃-/NH₄⁺. The supply of S decreased the glutamine synthetase activity in the plants grown at NO3- solely but did not affect the glutamine synthetase activity in the plants that received 70/30 NO₃-/NH₄+. The plants cultivated with NO3- solely and S limitation showed the highest NH4+ concentration in the shoots. The application of 100/0 NO₃-/NH₄+ on the plants that are grown under S limitation showed a higher NO₃⁻ accumulation in the shoots than at 70/30 NO₃⁻/NH₄⁺, but under S sufficiency it was verified the opposite effect. Under S sufficiency, the accumulation of S in the shoots and roots was higher in the plants grown at $70/30 \text{ NO}_3^-/\text{NH}_4^+$ than at NO₃- solely. The rates of S improved the accumulations of N and NH4+ in the shoots and roots. Under S limitation, the quantum efficiency of photosystem II and electron transport rate were higher in the plants grown at $70/30 \text{ NO}_3^-/\text{NH}_4^+$ than NO_3^- solely. Overall, the highest net photosynthesis was verified in the plants cultivated with 70/30 NO₃-/NH₄+ or S at 1 and 2 mmol L⁻¹. The malondialdehyde concentration was lower in the root plants grown at 70/30 NO₃-/NH₄+ than at NO3- solely, regardless of the rates of S, and similar effects were found for H2O2 concentration in plants grown under S at 1 mmol L⁻¹. These results are consistent with guaiacol peroxidase activity in the shoots since its activity was higher at S limitation or sufficiency combined with NO₃-/NH₄+ than at NO₃- solely. The rates of S decreased the ascorbate peroxidase activity in the plants grown at $100/0 \text{ NO}_3^-/\text{NH}_4^+$, but the opposite effect was verified in the plants grown at 70/30 NO3⁻/NH4⁺. Tanzania guinea grass has a great tolerance to S limitation. However, the combination of NO3- and NH4+ further improves plant tolerance. The proportion of 70/30 NO₃-/NH₄⁺ mitigates S starvation, ensuring proper growth of Tanzania guinea grass even under low S availability

Keywords: Antioxidant enzymes, Glutamine synthetase, *Megathyrsus maximus*, Nitrate, Photosynthesis

4.1. INTRODUCTION

Brazil is one of the most important countries for world beef production with 196 million hectares of pastures for animal production (FAO, 2016). Pastures with high production

are represented by plants such as Tanzania guinea grass [*Panicum maximum* Jacq. cv. Tanzania (syn. *Megathyrsus maximus* (Jacq.) B.K.Simon & S.W.L. Jacobs)] because of its high growth potential and forage quality (Silveira and Monteiro, 2011). The growth of forage grasses can be affected by many abiotic stressors such as nutrient deficiency (Schulze et al., 2002). However, few studies investigated the effects of abiotic stressors for Tanzania guinea grass, one of the greatest species of tropical forage grass.

A topic with great importance is related to sulfur (S) limitation since this nutrient has an interesting correlation with nitrogen (N), the major nutrient for forage grass production (De Bona et al., 2013, Schmidt et al, 2013). Nitrogen is the major stimulator for Tanzania guinea grass production (Silveira and Monteiro, 2011). This is because N is an integral part of all amino acids and proteins such as nitrogenous bases, membranes, hormones, and other compounds (Heldt, 2005; Marschner, 2012; Taiz et al., 2017). Nitrogen is assimilated in the form of NO_3^- and NH_4^+ and adjustments in their proportions can also improve Tanzania guinea grass development (Souza Junior et al., 2018; Leite and Monteiro, 2019).

Proper proportions of NO₃⁻/NH₄⁺ can improve N uptake and then the forage grass production without any increase in N rate, as previously reported (Santos et al., 2013; Souza Junior et al., 2018; Leite and Monteiro, 2019). The positive effects of NH₄⁺ addition occur because the assimilation of N via NO₃⁻ and its chemical reduction in the plant cell requires a high investment of ATP and NADPH, majors metabolic energy used for plants. When N assimilation occurs via NH₄⁺, less amount of ATP and NADPH are required (Epstein and Bloom, 2006; Taiz et al., 2017), saving free energy that can be used to mitigates stress from nutrient starvation or limitation (Schulze et al., 2002; Meng et al., 2016). The best proportion of NO₃⁻/NH₄⁺ for Tanzania guinea grass was 70% NO₃⁻ combined with 30% NH₄⁺ (Souza Junior et al., 2018; Leite and Monteiro, 2019), or just referred to as 70/30 NO₃⁻/NH₄⁺, which raised the nutrient accumulation and plant production and improved the antioxidant defense against abiotic stress.

Sulfur is an essential element for plants and very important when N is optimized in growth media (De Bona et al., 2013; Schmidt et al., 2013; Schmidt and Monteiro, 2014) since S has great importance in protein synthesis and plays a unique role as an integral constituent of amino acids like cysteine and methionine, as well as coenzyme A, thiamine pyrophosphate, glutathione, biotin, vitamin B, and pantothenic acid, essentials for plant metabolism (Taiz et al., 2017). Preferences of using fertilizers without S, sulfur exportation by crops, and international agreements aiming to decrease the emissions of S gases also decreases S deposition and may induce S limitation in plants, which decrease the efficiency of N assimilation and induces oxidative stress in plants, affecting

photosynthesis (Stern and Rydge, 2012; Khan et al., 2015; Sorin et al., 2012). So far, there is no report regarding the role of N ionic forms to mitigate S limitation.

Sulfur limitation can induce oxidative stress by acting of reactive oxygen species (ROS) (Schulze et al., 2002; Khan et al., 2015). Hydrogen peroxide (H₂O₂) is one of the major ROS induced by abiotic stress (Gratão et al., 2008; Myouga et al., 2008) that damages cellular membranes, causing lipid peroxidation. This process is also indicated by high concentrations of malondialdehyde (MDA), which may decrease plant growth and development (Sing et al., 2010). In response to this, plants developed an efficient antioxidant defense system, acting from enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and guaiacol peroxidase (GPX), turning H₂O₂ into H₂O by a redox reaction named Fenton and Harber-Weiss reaction (Liu et al., 2015). A non-enzymatic defense system was also developed for plants to fight abiotic stress such as the amino acid proline, an important compound that can eliminate ROS directly and act as an osmoregulator in the plant cells (Sing et al., 2010).

The hypothesis is the combination of NO_3^- and NH_4^+ mitigates oxidative stress from S limitation more than plants receiving NO_3^- solely. The objective was to compare the effect of S limitation and sufficiency on metabolic, physiological, nutritional, and productive indicators, revealing the role of NO_3^- and NH_4^+ as a stress reliever and growth-enhancing for Tanzania guinea grass.

4.2. MATERIAL AND METHODS

4.2.1. Experimental design and treatments

A 2 × 3 factorial experiment with Tanzania guinea grass in a randomized complete block design with four replications was carried out. Factors were proportions of 100/0 and 70/30 NO₃⁻/NH₄⁺ combined with rates of S 0.1 (limitation), 1 (intermediate), and 2 mmol L⁻¹ (sufficient). The proportion containing 30% of N as NH₄⁺ was selected since it has NH₄⁺ in a proper amount to stimulates high growth of Tanzania guinea grass, preferred to be compared to NO₃⁻ solely (Souza Junior et al., 2018; Leite e Monteiro, 2019). Regarding the rates of S, it was chosen 2 mmol L⁻¹ since it is recommended by Hoagland and Arnon (1950). The rate of S at 0.1 mmol L⁻¹ instead of S 0.0 mmol L⁻¹ was chosen to simulate a high limitation without killing the plants because S is an essential element, needing to be applied at least in a minimum amount to ensure some plant growth (Marschner, 2012). It was chosen S at 1 mmol L⁻¹ since this rate is half the amount of the recommended rate. These rates of S were consistent with another study with Tanzania guinea grass (Schmidt and Monteiro, 2014).

4.2.2. Overall conditions of the experiment

The experiment was carried out in a growth chamber with photosynthetically active radiation of 400 μ mol m⁻² s⁻¹, humidity of 40%, and temperature of 29 °C. The seeds were germinated and pre-cultivated on a plastic tray, containing washed sand with deionized water. Afterward, nine seedlings (around 5 cm height) were put into pots of 2 L with Hoagland and Arnon's (1950) nutrient solution prepared with deionized water (Fig. 1a). Plants were grown in a growth system called deep water culture. This substrate-free system allows monitoring of root growth (Fig. 1b), but oxygen must be constantly supplied by using a mechanical air pump.

For seven days after transplanting, a Hoagland and Arnon's nutrient solution with 20% ionic strength was used for plant establishment. This initial nutrient solution was applied considering the proportions of NO_3^-/NH_4^+ studied with an N rate of 15 mmol L⁻¹ (Hoagland and Arnon, 1950). For five days after using this nutrient solution, three plants were taken off the pots and then more three, keeping three final overall plants per pot. After seven initial days, a nutrient solution with 100% ionic strength containing all treatments was applied. The nutrient solutions were replaced every ten days. A nitrification inhibitor dicyandiamide (7 µmol L⁻¹) was used in all phases and treatments, aimed to prevent NH_4^+ nitrification (Liu et al., 2017). Tanzania guinea grass was harvested after 38 days of growth.

4.2.3. Visual observation

Symptoms of nutrient starvation or related effects were described by watching the plants. Common symptoms as chlorosis, necrosis, wilt, and growth inhibition were recorded as photographs.

4.2.4. Photosynthetic activity and relative chlorophyll index

Using a photosynthesis analyzer IRGA WALZ[®] GFS–3000, gas exchange and chlorophyll fluorescence on diagnostic leaves (first and second leaves fully expanded from the top to the bottom) were evaluated one day before harvesting. The quantifications of relative chlorophyll index

(RCI) were performed on the diagnostic leaves after measuring photosynthesis, calculating the final value from an average of eight readings by using SPAD–502 MINOLTA[®].

4.2.5. Plant sampling and production

The experiment had four replications for "nutritional and productive" analyzes and another four for "metabolic and physiological" analyzes. At the harvest time, the samples called "nutritional and productive" were separated and dried in a forced circulation oven at 65 °C for 72 h. After measuring dry biomass production, samples were ground in a Wiley mill. The shoots and roots dry biomass were determined by using a precision scale. The samples called "metabolic and physiological" were separated and packed in an aluminum bag, immediately frozen in liquid N, and then stored under low temperature – 80 °C. Afterward, cryogenic grinding was done.

4.2.6. Total N, N–NO₃⁻, N–NH₄⁺, and S in plant samples

The concentrations of total N in the shoots and roots of Tanzania guinea grass were quantified by the Kjeldahl method (Sarruge and Haag, 1974), and concentrations of N–NO₃⁻ and N–NH₄⁺ were quantified by using magnesium oxide and Devarda's alloy (Tedesco et al., 1985). The concentrations of S were determined by using barium chloride for turbidimetry according to Sarruge and Haag (1974). The accumulations of nutrients were obtained from the multiplication between its concentration and the respective dry biomass.

4.2.7. The activity of enzymes for N assimilation

Nitrate reductase enzyme activity (NR, EC 1.7.1.1) was determined according to Mulder et al. (1969). Fresh diagnostic leaves (0.1 g) were placed into a solution containing potassium phosphate buffer (KH₂PO₄, pH 7.5) and potassium nitrate (0.25 mol L⁻¹) at 35 °C for 2 h. After centrifuging at 10,000 g, supernatants were transferred to flasks. The reaction was stopped by using a solution containing sulfanilamide (58 mmol L⁻¹) and N–1–naphthyl ethylenediamine dihydrochloride (0.77 mmol L⁻¹). Sodium acetate (2 mol L⁻¹) was used for color development. The quantification was carried out on a spectrophotometer at 540 nm with a NO₂⁻ curve.

Glutamine synthetase enzyme activity (GS, EC 6.3.1.2) was carried out according to Elliot (1952). Tris–HCl (50 mmol L^{-1}), mercaptoethanol (2 mmol L^{-1}), and ethylenediaminetetraacetic acid (EDTA, 1 mmol L^{-1}) were homogenized with the plant samples and then centrifuged at 10,000

g at 4 °C for 10 min. Afterward, the extracts were reacted with TRIS–HCl (200 mmol L⁻¹), ATP (50 mmol L⁻¹), glutamic acid (500 mmol L⁻¹), magnesium sulfate (1 mol L⁻¹), hydroxylamine (100 mmol L⁻¹), and cysteine (100 mmol L⁻¹), incubating it for 30 min at 30 °C. Then, a solution containing iron chloride (FeCl₂ 616 mmol L⁻¹), trichloroacetic acid (TCA, 1.45 mol L⁻¹), and hydrochloric acid (HCl 1 mol L⁻¹) was used to stop the reaction. Centrifuging at 5,000 g for 5 min was done to precipitate the protein excess. It was determined the activity in the supernatant by using a standard curve with γ –glutamyl hydroxamate at 540 nm.

4.2.8. Malondialdehyde and hydrogen peroxide

The test of 2–thiobarbituric acid (TBA) to measure lipid peroxidation was done, quantifying it as malondialdehyde (MDA) in the plant samples (Health and Packer, 1968). The ground plant samples were mixed with TCA (1 g L⁻¹) and centrifuged at 10,000 g for 10 min at 4 °C. Trichloroacetic acid (200 g L⁻¹) and TBA (5 g L⁻¹) were mixed with the supernatant and incubated in a dry bath for 30 min at 95 °C. Tubes containing that solution were placed on ice for 10 min to stop reacting and then seated at room temperature for 15 min, running at 535 and 600 nm. The concentrations of MDA were quantified by using an extinction coefficient of 155 mmol⁻¹ cm⁻¹.

The concentrations of hydrogen peroxide (H_2O_2) in the shoots and roots were determined (Velikova et al., 2000). Microtubes containing KH_2PO_4 buffer (100 mmol L⁻¹) and potassium iodide (1 mol L⁻¹) received an aliquot of supernatant homogenized with TCA (1 g L⁻¹) and then they were incubated on ice in the dark for 60 min. The quantification was done at 390 nm by using a standard of H_2O_2 .

4.2.9. Protein extraction and quantification

The soluble proteins from the plant samples were extracted and quantified according to Monteiro et al. (2011). Polyvinylpolypyrrolidone (PVPP) in a KH₂PO₄ buffer solution (100 mmol L⁻¹, pH 7.5), containing EDTA (1 mmol L⁻¹), and dithiothreitol (3 mmol L⁻¹) were homogenized with the plant samples. The extracts were centrifuged at 10,000 g for 30 min at 4 °C, keeping the supernatants at low temperature – 80 °C. The quantification was performed according to Bradford (1976).

4.2.10. Antioxidant enzymatic system

Guaiacol peroxidase activity (GPX, EC 1.11.1.7) was determined according to Matsuno and Uritani (1972). The protein extracts were homogenized with sodium phosphate buffer (Na₃PO₄ 28.4 g L⁻¹, pH 5.0), citric acid (21 g L⁻¹), guaiacol, and H₂O₂ concentrated, heating them at 30 °C for 15 min. Afterward, the reaction was stopped by using sodium metabisulfite (20 g L⁻¹) and a subsequent ice bath for 10 min. The quantification was done on a spectrophotometer at 450 nm.

Glutathione reductase activity (GR, EC 1.6.4.2) was performed according to Gratão et al. (2008). Glass cuvettes were used to place a solution containing KH₂PO₄ (100 mmol L⁻¹, pH 7.5) and 2–nitrobenzoic acid (3 mmol L⁻¹) and then led them to a water bath at 30 °C. Afterward, the protein extracts were added to the cuvettes, which contained NADPH (2 mmol L⁻¹) and oxidized glutathione (20 mmol L⁻¹), starting the reaction. The quantification was estimated by analyzing a reduction of oxidized glutathione for 1 min at 412 nm.

Catalase activity (CAT, EC 1.11.1.6) was performed according to Monteiro et al. (2011). An aliquot of KH_2PO_4 buffer (100 mmol L⁻¹, pH 7.5) with concentrated H_2O_2 was homogenized with the protein extracts in a glass cuvette, starting the reaction. The quantification was obtained from the decomposition of H_2O_2 for 1 min at 25 °C, running on a spectrophotometer at 240 nm.

Ascorbate peroxidase activity (APX, EC 1.11.1.11) was performed according to Cakmak and Horst (1991). The protein extracts were placed into a cuvette containing KH₂PO₄ buffer (80 mmol L⁻¹, pH 7) with ascorbic acid (5 mmol L⁻¹) and EDTA (1.45 mmol L⁻¹) at 30 °C in a water bath. Afterward, concentrated H₂O₂ was mixed with that solution, starting the reaction. The quantification was done by the oxidation of ascorbate for 1 min in a quartz cuvette at 290 nm. The ascorbate extinction coefficient of 2.8 mmol⁻¹ cm⁻¹ was used.

Superoxide dismutase activity (SOD, EC 1.15.1.1) was performed according to Azevedo et al. (1998). The protein extracts were homogenized with a solution of bromophenol blue (5 g L⁻¹), glycerol, and trizma (60 g L⁻¹), ejecting it on top of the gel containing acrylamide (400 g L⁻¹), trizma (60 g L⁻¹), N,N,N',N'-Tetramethylethylenediamine (TEMED), and ammonium persulfate (100 g L⁻¹). The running gel was similar to the previous one, but contained more trizma (363 g L⁻¹). Electrophoresis voltage 15 mA per gel was used. After running, the gel was washed with deionized water and then incubated for 30 min under darkroom in a KH₂PO₄ buffer solution (100 mmol L⁻¹, pH 7.8) with EDTA (1 mmol L⁻¹), nitroblue tetrazolium chloride (0.1 mmol L⁻¹), riboflavin (0.05 g L⁻¹), and TEMED. Afterward, gels were cleaned with deionized water and illuminated by using a fluorescent lamp for 20 min. Glacial acetic acid was used to stop the reaction. Superoxide dismutase bands were compared qualitatively with SOD bovine protein standard.

4.2.11. Proline concentration

Proline quantification was performed according to Bates et al. (1973). Samples were mixed with sulfosalicylic acid (30 g L⁻¹) and centrifuged at 10,000 g for 20 min at 15 °C. The supernatants were put into glass tubes to react with ninhydrin (25 g L⁻¹), glacial acetic acid, and phosphoric acid (6 mol L⁻¹). The tubes were warmed up in the water at 100 °C for 1 h. Then, toluene was used to separate the phases by using a shaker for 20 s and then waited for 10 min. The quantification was performed at 520 nm by using a standard proline curve.

4.2.12. Statistical analysis

Statistical analysis was done using the "Statistical Analysis System" (SAS Institute, 2008). It was applied the F test to verify if the $NO_3^-/NH_4^+ \times Mn$ interaction was significant and then the main effect proportions of NO_3^-/NH_4^+ was analyzed within the rates of S and the rates of S within the proportions of NO_3^-/NH_4^+ . The average of the main factors was compared separately when the interaction was not significant. The Tukey test at 5% to compare averages was used.

4.3. RESULTS

4.3.1. Visual observation

The growth system "deep water culture" worked very well to grow Tanzania guinea grass, indicating that it can be used as an experimental system under growth chamber for that grass since it is possible to watch both shoots and roots development and save more than 95% of substrate (Fig. 1a, 1b). However, the system spends a high amount of nutrient solution per pot.

The S limitation did not cause any negative visual effects on the seedling at the very early stages (Fig. 1a). Differences were observed in the root structure, color, ramification, and length, highlighting the plants that are grown at S at 0.1 mmol L^{-1} and NO_3^- solely since they showed very high root length (Fig. 1b). The plants grown under 70/30 NH₃⁻/NH₄⁺ and S at 2 mmol L^{-1} showed high shoot development, high leaf expansion, and very strong green (Fig. 1c). The plants growing under the S limitation did not show any deficiency or nutrient imbalance caused by starvation.



Figure 1. Details of the pots cultivated with Tanzania guinea grass at the very early stage (a), plant roots grown with S at 0.1 mmol L⁻¹ and NO₃⁻ solely (b), and comparison of shoots of plants grown under S at 0.1 mmol L⁻¹ with NO₃⁻ solely (left) or 70/30 NO₃⁻/NH₄⁺ (right) at 38 days after transplanting (c).

4.3.2. Dry biomass production, plant growth, and relative chlorophyll index

The NO₃⁻/NH₄⁺ × S interaction (from now on, it will be referred to as interaction only) was significant for the shoots (SDM) and roots dry biomass (RDM) (Fig. 1a, 1b). The higher SDM was verified in the plants grown with 70/30 NO₃⁻/NH₄⁺ and S at 2 mmol L⁻¹ compared to all other combinations (Fig. 1a). The other combinations did not show any significance as a function of the treatments (Fig. 1a). Regarding the RDM, the higher values were found in the plants grown with 70/30 NO₃⁻/NH₄⁺ and S at 2 mmol L⁻¹ compared to those plants grown with NO₃⁻ solely or other rates of S (Fig. 1c). However, the higher values of RDM in the plants grown under S at 0.1 mmol L⁻¹ and NO₃⁻ solely than under 70/30 NO₃⁻/NH₄⁺ were found (Fig. 1 c).



Figure 2. Shoots (a) and roots (b) dry biomass production of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution. The Tukey test at 5% was applied. Lowercase letters compare rates of S within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each S rate. Coefficient of variation (%) = 15 (a) and 18 (b). Error bars indicate standard error.

The relative chlorophyll index (RCI) was significant for the main effects only (Table 1). The highest RCI was verified in the plants that received NO_3^- and NH_4^+ compared to those which received all N as NO_3^- (Table 1). The number of leaves (NL) and tillers (NT) were not significant for the interaction or the main factors (Table 1).

Table 1. Number of leaves (NL), number of tillers (NT), relative chlorophyll index (RCI), and nitrate reductase (NR) of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution.

	NL	NT	RCI	NR
	<i>u</i>	nit ———	unit	µg NO2 ⁻ g ⁻¹ FW h ⁻¹
NO_3^-/NH_4^+				
100/0	42.4 ^{ns} ± 1.56	$7.50 \text{ ns} \pm 0.38$	42.1 ^ь ± 0.97	$36.8 \text{ ns} \pm 0.51$
70/30	45.1 ^{ns} ± 1.17	$7.66 \text{ ns} \pm 0.27$	$50.8 \ ^{a} \pm 0.48$	39.1 ^{ns} ± 1.01
Sulfur (mmol	L-1)			
0.1	42.2 ^{ns} ± 1.91	$7.37 \text{ ns} \pm 0.49$	47.9 ^{ns} ± 1.11	$36.8 \text{ ns} \pm 0.84$
1	43.7 ns ± 1.61	$7.62 \text{ ns} \pm 0.35$	46.2 ns ± 1.27	$37.6 \text{ ns} \pm 0.99$
2	45.4 ^{ns} ± 1.56	$7.75 \text{ ns} \pm 0.34$	47.5 ^{ns} ± 1.47	39.5 ns ± 1.09
<i>p</i> value	> 0.05	> 0.05	< 0.01	> 0.05
cv (%)	9.4	15	7.1	6.9

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. FW fresh weight. ns not significant. \pm standard error. cv coefficient of variation.

4.3.3. Activities of nitrate reductase and glutamine synthetase

The interaction or main effects were not significant for the nitrate reductase activity (NR) in the shoots (Table 1). However, the interaction was significant for the glutamine synthetase activity (GS) in the shoots. The plants submitted to S starvation at 0.1 mmol L⁻¹ grown under N as NO_3^- solely showed higher GS activity than under other rates of S (Fig. 3a). However, the rates of S did not affect the GS activity when the plants received a combination of NO_3^- and NH_4^+ (Fig. 3a). The plants that received S at 2 mmol L⁻¹ showed higher GS activity when they had NO_3^- and NH_4^+ as N source compared to those plants grown with NO_3^- solely (Fig. 3a).



Figure 3. Glutamine synthetase activity in the shoots (a), concentrations of NH_4^+ in the shoots (b), accumulation of NO_3^- in the shoots (c) and roots (d), and accumulation of N in the roots (e) of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution. The Tukey test at 5% was applied. Lowercase letters compare rates of S within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each S rate. Coefficient of variation (%) = 4 (a), 15 (b), 15 (c), 33 (d), and 15 (e)

4.3.4. Total N, NO₃⁻, NH₄⁺, and S in the plant parts

Regarding the concentration of nutrients, the interaction was only significant for the concentrations of NH_4^+ in the shoots (Fig. 3b). The concentrations of N, NH_4^+ , NO_3^- , and S were affected by the main factors (Table 2). In the shoots, the concentration of NH_4^+ was higher at the combination of NO_3^- and S at 0.1 mmol L⁻¹ compared to the other treatments (Fig. 3b). The concentration of NO_3^- was higher in the shoots of plants grown with S at 0.1 mmol L⁻¹ compared to the other rates (Table 2). In the roots, the concentration of N was higher in the plants that received S at 2 mmol L⁻¹ than those that received S at 0.1 mmol L⁻¹ (Table 2). However, the highest

concentration of NH_4^+ was verified in the roots of plants grown with S at 0.1 mmol L⁻¹ compared to the other rates of S (Table 2).

	Ν	$\mathrm{NH_{4}^{+}}$	NO₃ [−]	S		
			g kg ^{_1}			
Shoots						
NO_3^-/NH_4^-	+					
100/0	$32.2 \text{ ns} \pm 0.61$	*	$0.041 \text{ ns} \pm 0.002$	$0.80 \text{ ns} \pm 0.06$		
70/30	$31.9 \text{ ns} \pm 0.73$	*	$0.043 \text{ ns} \pm 0.002$	$0.86 \text{ ns} \pm 0.05$		
Sulfur (mme	of L^{-1})					
0.1	$33.9 \text{ ns} \pm 0.69$	*	$0.050 \ ^{a} \pm 0.002$	$0.80 \text{ ns} \pm 0.06$		
1	$32.1 \text{ ns} \pm 0.88$	*	$0.040 \text{ b} \pm 0.002$	$0.83 \text{ ns} \pm 0.07$		
2	$31.1 \text{ ns} \pm 0.57$	*	$0.037 \text{ b} \pm 0.002$	$0.87 \text{ ns} \pm 0.08$		
<i>p</i> value	> 0.05	*	< 0.01	> 0.05		
cv (%)	7.5	*	16.7	23.3		
Roots						
NO_3^-/NH_4^-	+					
100/0	24.6 $^{ns} \pm 1.05$	$0.39 \text{ ns} \pm 0.04$	$0.101 \text{ ns} \pm 0.008$	$1.75 \text{ ns} \pm 0.11$		
70/30	$36.8 \text{ ns} \pm 0.80$	$0.34 \text{ ns} \pm 0.04$	$0.111 \text{ ns} \pm 0.003$	$1.89 \text{ ns} \pm 0.14$		
Sulfur (mmol L-1)						
0.1	23.5 ^ь ± 0.79	$0.53 \ ^{\rm a} \pm 0.02$	$0.097 {}^{\rm ns} \pm 0.005$	$1.77 \text{ ns} \pm 0.19$		
1	$25.9 \text{ ab} \pm 1.36$	0.29 ^b ± 0.01	$0.111 \text{ ns} \pm 0.009$	$1.84 \text{ ns} \pm 0.13$		
2	$27.6 \ ^{a} \pm 0.92$	$0.28 \text{ b} \pm 0.04$	$0.110 {}^{\rm ns} \pm 0.008$	$1.84 \text{ ns} \pm 0.15$		
<i>p</i> value	< 0.05	< 0.01	> 0.05	> 0.05		
cv (%)	11	27	25	26		

Table 2. Concentrations of N, NH_4^+ , NO_3^- , and S in the shoots and roots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution.

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. ns not significant. \pm standard error. cv coefficient of variation. * graphically represented.

The interaction was significant for the accumulation of NO_3^- in the shoots and roots, as well as N in the roots (Fig. 3). The accumulations of N and NH_4^+ in the shoots and NH_4^+ in the roots were affected by the main effects (Table 3). In the shoots, the accumulations of N and NH_4^+ were higher in the plants grown with S at 2 mmol L⁻¹ than S at 0.1 mmol L⁻¹ (Table 3). The accumulation of NO_3^- in the shoots was the highest at 70/30 NO_3^-/NH_4^+ with S at 2 mmol L⁻¹ and the lowest at 100/0 NO_3^-/NH_4^+ with S at 2 mmol L⁻¹ (Fig. 3c). The accumulation of S in the shoots was higher in the plants grown at 70/30 NO_3^-/NH_4^+ that received S at 2 mmol L⁻¹ compared to those plants grown at NO_3^- solely (Fig. 4a). In the roots, the accumulations of NO_3^- (Fig. 3d) and N (Fig. 3e) showed higher value in the plants grown with 70/30 NO_3^-/NH_4^+ and S at 2 mmol L⁻¹ than the plants grown with S at 0.1 mmol L⁻¹, and the rates of S increased the accumulations of NO_3^- and N in the roots of plants grown at 70/30 NO_3^-/NH_4^+ (Fig. 3d, Fig. 3e). The accumulations of NH_4^+ in the roots were higher with S at 2 mmol L⁻¹ than at other rates of S (Table 3). The accumulation of S in the roots was higher in the plants grown with 70/30 NO_3^-/NH_4^+ that received S at 2 mmol L⁻¹ compared to those plants grown at NO_3^- solely (Fig. 4b). The rates of S increased the accumulation of S in the plants that received NO_3^- and NH_4^+ as N source (Fig. 4b).

	Ν	\mathbf{NH}_{4}^{+}	\mathbf{NH}_{4}^{+}
	Shoots	Shoots	Roots
		mg per pot	
NO_3^-/NH_4^+			
100/0	$187 \text{ ns} \pm 6.42$	$1.02 \text{ ns} \pm 0.09$	$0.48 \text{ ns} \pm 0.09$
70/30	$208 \text{ ns} \pm 18.1$	$0.97 \text{ ns} \pm 0.07$	$0.42 \text{ ns} \pm 0.07$
Sulfur (mmol L	-1)		
0.1	169 ^b ± 11.0	0.74 ^b ± 0.05	0.27 ^b ± 0.05
1	$189 ab \pm 15.5$	$0.98 \text{ ab} \pm 0.10$	0.37 ^b ± 0.10
2	234 ª ± 15.3	1.26 ª ± 0.04	0.72 = 0.04
<i>p</i> value	< 0.01	< 0.01	< 0.01
cv (%)	19	23	34

Table 3. Accumulations of N, NH_4^+ , and NO_3^- in the shoots or roots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution.

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. ns not significant. \pm standard error. cv coefficient of variation.



Figure 4. Accumulations of S in the shoots (a) and roots (b) of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution. The Tukey test at 5% was applied. Lowercase letters compare rates of S within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each S rate. Coefficient of variation (%) = 30 (a) and 31 (b).

4.3.5. Photosynthetic activity

The interaction was significant for the stomatal conductance (SC), quantum efficiency of photosystem II (QEPSII), and electron transport rate (ETR) (Fig. 5). The SC was raised by applying S at 1 mmol L⁻¹ when the plants were cultivated with 70/30 NO₃⁻/NH₄⁺, which were also higher compared to those plants grown with N in the form of NO₃⁻ and S at 1 mmol L⁻¹ (Fig. 5a). The rates of S did not affect the SC when the plants were cultivated with NO₃⁻ solely (Fig. 5a). The QSPII and ETR in Tanzania guinea grass were increased as the rates of S increased in the plants that received 70/30 NO₃⁻/NH₄⁺, with the highest value at 70/30 NO₃⁻/NH₄⁺ and S at 2 mmol L⁻¹ (Fig. 5b).



Figure 5. Stomatal conductance (a), quantum efficiency of photosystem II (b), and electron transport rate (c) of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution. The Tukey test at 5% was applied. Lowercase letters compare rates of S within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each S rate. Coefficient of variation (%) = 8.9 (a), 4.7 (c), and 4.5 (c).

The net photosynthesis (NP), internal CO₂ (ICO₂), and Fv/Fm were affected by the main effects (Table 4). The NP and Fv/Fm were higher at 70/30 NO₃⁻/NH₄⁺ compared to 100/0 NO₃⁻/NH₄⁺ (Table 4). On the other hand, the ICO₂ was higher at 100/0 NO₃⁻/NH₄⁺ compared to 70/30 NO₃⁻/NH₄⁺ (Table 4). The NP was lower with S at 0.1 mmol L⁻¹ compared to S at 1 and 2 mmol L⁻¹ (Table 4). Evapotranspiration rate, vapor pressure deficit, photochemical quenching, and

non-photochemical quenching were not significantly affected by the interaction or the main effects (Table 4).

Table 4. Net photosynthesis (NP), evapotranspiration rate (ER), internal CO₂ (ICO₂), vapor pressure deficit (VPD), maximum efficiency of photosystem II (FvFm), photochemical quenching (qP), and non-photochemical quenching (NPQ) of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution.

	NP	ER	ICO ₂	VPD	Fv/Fm	qP	NPQ
	µmol m ⁻² s ⁻¹	mmol $m^{-2} s^{-1}$	ppm	Pa/Kpa		unit —	
NO_3^-/NH_4^+							
100/0	23.0 ь	2.72 ns	88.2 ª	20.8 ns	0.84 ^b	0.25 ns	0.74 ^{ns}
	± 0.3	± 0.1	± 9.5	± 0.09	± 0.01	± 0.01	± 0.01
70/30	25.9 ª	2.72 ns	60.4 ^b	20.7 ns	0.85 ^a	0.27 ^{ns}	0.72 ^{ns}
	± 0.7	± 0.1	± 4.9	± 0.08	± 0.01	± 0.01	± 0.01
S (mmo	$(1 L^{-1})$						
0.1	23.2 ь	2.66 ns	82.3 ns	20.8 ns	0.84 ^{ns}	0.26 ^{ns}	0.73 ^{ns}
	± 0.5	± 0.1	± 13	± 0.10	± 0.01	± 0.01	± 0.01
1	25.6 ª	2.83 ns	74.9 ns	20.7 ns	0.84 ^{ns}	0.26 ns	0.73 ns
	± 0.9	± 0.1	± 5.1	± 0.09	± 0.01	± 0.01	± 0.01
2	24.5 a	2.67 ns	65.7 ^{ns}	20.7 ns	0.84 ^{ns}	0.26 ns	0.73 ^{ns}
	± 0.6	± 0.1	± 10	± 0.13	± 0.01	± 0.01	± 0.01
<i>p</i> value	< 0.05	> 0.05	< 0.01	> 0.05	< 0.05	> 0.05	> 0.05
cv (%)	7.1	12	36	1.6	0.49	10	3.7

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. ns not significant. \pm standard error. cv coefficient of variation.

4.3.6. Oxidative stress and proline

The interaction was only significant for the malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) in the roots (Fig. 6). The concentration of MDA in the plant roots was lower at $70/30 \text{ NO}_3^-/\text{NH}_4^+$ than at $100/0 \text{ NO}_3^-/\text{NH}_4^+$, regardless of the rates of S (Fig. 6a). Regarding the concentrations of H₂O₂, the highest values were found in the plants grown with NO₃⁻ solely and S at 1 mmol L⁻¹ (Fig. 6b). However, the application of NO₃⁻ and NH₄⁺ in the plants that received S at 1 mmol L⁻¹ decreased the concentrations of H₂O₂ (Fig. 6b). The concentrations of MDA in the shoots were affected by the main effects (Table 5). The MDA concentration was higher in the shoots of plants grown with an association between NO₃⁻ and NH₄⁺ than the plants grown under N as NO₃⁻ solely (Table 5). The interaction or the main factors did not affect the concentrations of proline (Table 5).



Figure 6. Concentrations of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) in the roots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution. The Tukey test at 1% was applied. Lowercase letters compare rates of S within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each S rate. Coefficient of variation (%) = 10 and 29 (b).

Table 5. Concentrations of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and proline in the shoots and proline in the roots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution.

	MDA	H_2O_2	Proline	Proline
		Shoots		Roots
	nmol g ⁻¹ FW		μmol g ⁻¹ FW	
NO_3^-/NH_4^+				
100/0	$0.33 \text{ b} \pm 0.01$	$1.55 \text{ ns} \pm 0.09$	$2.37 \text{ ns} \pm 0.14$	$2.05 \text{ ns} \pm 0.10$
70/30	$0.54 \ ^{a} \pm 0.02$	$1.61 \text{ ns} \pm 0.10$	$2.41 \text{ ns} \pm 0.15$	$2.47 \text{ ns} \pm 0.22$
Sulfur (mmol	L-1)			
0.1	$0.62 \text{ ns} \pm 0.03$	$1.57 \text{ ns} \pm 0.11$	$2.43 \text{ ns} \pm 0.16$	$1.85 \text{ ns} \pm 0.15$
1	$0.56 \text{ ns} \pm 0.03$	$1.50 \text{ ns} \pm 0.12$	$2.52 \text{ ns} \pm 0.13$	2.49 ^{ns} ± 0.16
2	$0.62 \text{ ns} \pm 0.02$	$1.68 \text{ ns} \pm 0.11$	$2.22 \text{ ns} \pm 0.21$	$2.44 \text{ ns} \pm 0.27$
<i>p</i> value	< 0.01	> 0.05	> 0.05	> 0.05
C V (%)	10	23	22	25

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. FW fresh weight. ns not significant. \pm standard error. cv coefficient of variation.

4.3.7. Antioxidant enzyme activity

The interaction was significant for the activities of guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) in the shoots of Tanzania guinea grass (Fig. 7). In the shoots, the highest value of GPX was found in the plants grown under 70/30 NO_3^-/NH_4^+ and S at 2 mmol L⁻¹ (Fig. 7a). The GPX activity was higher in the shoots of plants cultivated with 70/30 NO_3^-/NH_4^+ and S at 0.1 mmol L⁻¹ compared to those plants grown with NO_3^- solely (Fig. 7a). The APX activity was higher in the shoot of plants grown with 70/30 NO_3^-/NH_4^+ and S

at 2 mmol L⁻¹ than plants grown under NO_3^- solely and S at 2 mmol L⁻¹ (Fig. 7b). However, the activities of APX in the shoots of plants grown under $70/30 NO_3^-/NH_4^+$ or NO_3^- solely combined with S at 0.1 mmol L⁻¹ were similar (Fig. 7b). The activities of catalase (CAT) and glutathione reductase (GR) in the shoots were not affected by the main factors (Table 6). The superoxide dismutase (SOD) activity in the shoots did not show visible bands.



Figure 7. Activities of guaiacol peroxidase (GPX, a) and ascorbate peroxidase (APX, b) in the shoots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution. The Tukey test at 1% was applied. Lowercase letters compare rates of S within each NO_3^-/NH_4^+ proportion and capital letters compare proportions of NO_3^-/NH_4^+ within each S rate. Coefficient of variation (%) = 17 (a) and 15 (b).

In the roots, the activities of CAT, GPX, and APX were affected by the main factors (Table 6). Activities of CAT and APX in the plant roots were higher at $70/30 \text{ NO}_3^-/\text{NH}_4^+$ than at NO_3^- solely (Table 6). It was also verified a higher activity of APX in the plant roots grown with S at 1 mmol L⁻¹ than S at 2 mmol L⁻¹ (Table 6). The GPX activity was higher in the plants that received S at 1 mmol L⁻¹ compared to the other rates of S (Table 6). The SOD in the roots was not affected by the treatments since its band was not visible.

	CAT	GPX	APX	GR		
	μmol min ⁻¹ mg ⁻¹ protein					
Shoots						
NO_3^-/NH_4^+						
100/0	88.3 ^{ns} ± 3.4	*	*	$6.20 \text{ ns} \pm 0.28$		
70/30	$102 \text{ ns} \pm 7.5$	*	*	$6.38 \text{ ns} \pm 0.44$		
Sulfur (mmol L ⁻¹)						
0.1	$100 \text{ ns} \pm 9.1$	*	*	$6.62^{\text{ ns}} \pm 0.66$		
1	103 ^{ns} ± 6.9	*	*	6.49 ^{ns} ± 0.31		
2	$82.6 \text{ ns} \pm 3.5$	*	*	$5.76 \text{ ns} \pm 0.21$		
<i>p</i> value	> 0.05	*	*	> 0.05		
cv (%)	22	*	*	21		
Roots						
NO_3^-/NH_4^+						
100/0	175 ^ь ± 5.74	$6.01 \text{ ns} \pm 0.50$	816 ^ь ± 26.1	$5.03 \text{ ns} \pm 0.48$		
70/30	272 ª ± 11.4	$7.56 \text{ ns} \pm 0.66$	1114 ª ± 22.3	$12.6 \text{ ns} \pm 0.70$		
Sulfur (mmol L ⁻¹)						
0.1	$210 \text{ ns} \pm 17.5$	5.41 ^b ± 0.52	$974 \text{ ab} \pm 51.4$	$7.30 \text{ ns} \pm 1.56$		
1	231 ^{ns} ± 14.1	$8.07 \ ^{a} \pm 0.62$	1025 ^a ± 59.6	$10.7 \text{ ns} \pm 1.60$		
2	229 ns ± 26.6	6.87 ^ь ± 0.83	896 ^b ± 61.1	8.42 ^{ns} ± 1.13		
<i>p</i> value	< 0.05	< 0.05	< 0.01	< 0.01		
cv (%)	13	27	8.1	14		

Table 6. Activities of catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR) in the shoots and roots of Tanzania guinea grass under proportions of NO_3^-/NH_4^+ and rates of S in the nutrient solution.

The Tukey test was applied. Lowercase letters in each sub-column show significant differences. ns not significant. \pm standard error. cv coefficient of variation. * graphically represented.

4.4. DISCUSSION

4.4.1. NH4⁺ addition improves plant growth under S sufficiency

In a situation of S limitation (0.1 mmol L^{-1}), Tanzania guinea grass that received 70/30 NO₃^{-/}NH₄⁺ showed similarly lower SDM and RDM than plants that received NO₃⁻ solely (Fig. 2). Tanzania guinea grass under a growth chamber did not develop as fast as under sunlight, which means that the positive effect of NH₄⁺ addition on plant growth under S limitation might happen after more days of growth. With more days of S starvation, the stress would become stronger, and then the real effect of NO₃⁻ and NH₄⁺ to mitigates S deficiency might be more noticeable in the plant dry biomass. Honsel et al. (2012) did not find any decrease in plant

production when they submitted poplar hybrid plants (*Populus tremula* x *Populas alba* L.) to S depletion. A study carried out with Tanzania guinea grass cultivated in an Entisol soil that received S rates from 0 to 40 mg dm⁻³ combined with N from 0 to 400 mg dm⁻³ showed an only main effect for N, at the first harvest (Artur and Monteiro, 2014). However, at the second harvest, the dry biomass production was higher in plants that received high rates of S compared to plants without S fertilization. This suggests that S limitation in Tanzania guinea grass is more severe at the late stages of growth such as the second harvest, especially under nutrient solution where the concentration of nutrients is higher than in soil solution.

Plants that grown under S sufficient (2 mmol L⁻¹) showed higher dry biomass of shoots and roots when exposed to NO₃⁻ and NH₄⁺ than NO₃⁻ solely (Fig. 2a, Fig. 2b). The NH₄⁺ addition stimulated the production of RDM under S sufficiency (Fig. 2b), which may help the plant roots to accumulate more nutrients, suggesting that the NH₄⁺ administration may improve the uptake, storage, transportation, and S utilization, which culminates in better plant growth (Maruyama-Nakashita et al., 2015; Sorin et al., 2015; Souza Junior et al., 2018). The plants growing under 70/30 NO₃⁻/NH₄⁺ showed the highest RCI value (Table 1), which confirms the improvement in the plant dry biomass caused by NH₄⁺ addition. The chlorophyll was associated with the highest dry biomass production when Tanzania guinea grass was cultivated with NH₄⁺ in nutrient solution (Souza Junior et al., 2018). De Bona et al. (2013), testing proportions of NO₃⁻ and NH₄⁺ for Marandu palisade grass (*Urochloa brizantha* Hochst. ex A.Rich.) under S limitation and sufficiency in an Entisol, concluded that a fertilizer containing both ions NO₃⁻ and NH₄⁺ are highly recommended for forage grasses since this association can increase the accumulations of N and S, improves plant growth, and then the dry biomass production even under S limitation.

4.4.2. Sulfur supply affects glutamine synthetase and NH_4^+ concentration under NO_3^- supply

The S supply decreased the GS activity in the plants grown with N as NO_3^- , but did not affect GS activity in plants receiving NO_3^-/NH_4^+ (Fig. 3a). The plants cultivated with N as $NO_3^$ under S limitation showed a high NH_4^+ concentration in the shoots and low concentration when S was applied (Fig. 3b). According to Yu et al. (2018), three differential expression genes related to glutamine synthetase in wheat (*Triticum aestivum* L.) located on chromosomes 6AL, 6BL, and 6DL are down-regulated in a situation of high S supply and up-regulated under S deficiency, which means that the S limitation enhances GS activity in wheat. Furthermore, KEGG pathway analysis showed that three pathways related to metabolisms of N and amino acid were down-regulated under high S supply. It means that plants under high S supply had low GS activity, which led to less accumulation of free nitrogenous compounds. Results found by Yu et al. (2018) might explain why rates of S improved the concentrations of N in the roots of Tanzania guinea grass while concentrations of NH₄⁺ in the shoots were decreased (Table 2, Table 3). Organic N can be stored by plants as an N stock for adverse conditions and less NH₄⁺ is assimilated due to a possible down-regulation in GS genes.

Transportation of NO_3^- by plant cells occurs through active transporters (Wang et al., 2012; Taiz et al., 2017). After crossing membranes, NO₃⁻ is reduced to NO₂⁻ in the cytosol by NR, using NADH or NADPH as an electron donor. Then, a reduction of NO₂⁻ into NH₄⁺ in chloroplasts (leaves) or plastids (roots) by nitrite reductase (NiR) occurs by using reduced ferredoxin (Fd_{red}) as an electron donor (Xiong et al., 2006; Taiz et al., 2017). Afterward, the reaction of turning NH4⁺ into glutamine is catalyzed by GS, combining NH4⁺ with glutamate in the presence of both ATP and manganese in the root cytosol and plastids, as well as in shoots chloroplasts. Enzyme glutamine 2-oxoglutarate aminotransferase (glutamate synthase, GOGAT) transfers an amide group from glutamine to 2-oxoglutarate, producing two glutamates, first amino acid synthesized by plants from N assimilation by using NADH and Fd_{red} as reducing power (Thomsem et al., 2014; Taiz et al., 2017). Uptake of NH₄⁺ by plant cells occurs through active transporters (Wacker et al., 2014; Taiz et al., 2017), directly assimilated into glutamate via GS-GOGAT pathway (saving energy required by NR and NiR). Ammonium can also be re-assimilated by low-affinity enzyme glutamate dehydrogenase (GDH), which catalyzes a reaction that combines NH₄⁺ with 2oxoglutarate, synthesizing glutamate by using NADH and NADPH as reducing power (Epstein and Bloom, 2006; Taiz et al., 2017).

4.4.3. NH₄⁺ administration raises accumulations of N, NO₃⁻ and S under S sufficiency

The plants grown under NO_3^- solely and S limitation (0.1 mmol L⁻¹) showed a higher accumulation of NO_3^- in the shoots and roots compared to the plants grown under S at 2 mmol L⁻¹ or 70/30 NO₃⁻/NH₄⁺ and S at 2 mmol L⁻¹ (Fig. 3c). Under S limitation, it has been reported that one of the most ion accumulated in plants is NO₃⁻ (Koralewska et al., 2009; Kaur et al., 2011; Sarda et al., 2014). Schmidt et al. (2013) reported a high accumulation of NO₃⁻ and free amino acids in Tanzania guinea grass under S limitation, especially in the form of asparagine, one of the major amino acids accumulated under S limitation since this amino acid plays an important role as N stock that can delivery soluble N in different plant parts. High concentrations of asparagine indicate that S limitation might affect the pathways of methionine and aspartate synthesis (Schmidt et al., 2013). It has been also shown that rapeseed (*Brassica napus* L.) under S deprivation at the early stages (up to 13 days of starvation) induces a decrease in the contribution of SO_4^- as an osmotic regulator. However, this lack of SO_4^{2-} was fully compensated by NO_3^- , keeping stable the osmotic potential during the early stages of S deprivation (Sorin et al., 2015). Nitrate has an important function as an osmotic regulator since this ion plays a key role in osmotic balance and its accumulation can be detected when another mineral with osmotic function is limited (Sorin et al., 2015).

In a situation of S sufficiency, the NH₄⁺ addition in the nutrient solution improved the accumulation of NO₃⁻ in the shoots and roots even higher than NO₃⁻ supply, which led to a high N and S accumulation in the roots (Fig. 3c, Fig. 3d, Fig. 3d). Thus, it can be suggested that the free energy generated by assimilating NH₄⁺ instead of NO₃⁻ can be used to improve the uptake of important nutrients such as N and S used to raise plant growth (Marschner, 2012; De Bona et al., 2013). Under S sufficiency, the accumulations of S in the shoots and roots were higher in the plants that are grown under $70/30 \text{ NO}_3^-/\text{NH}_4^+$ compared to those which received NO_3^- solely (Fig. 4). In wheat, there are four sulfate transporters genes families (SULTR), located on the plasma membrane and induced by sulfate administration in roots. Sulfur supply improves gene expression that codes for those transporters, which improves S uptake (Yu et al., 2018). One of these sulfate transporters was recognized as a high-affinity sulfate transporter, significantly up-regulated under S limitation and related to a high efficiency on S uptake, named SULTR 1;1 (Yu et al., 2018). Thus, the NH_4^+ administration might up-regulate the expression of SULTR, which may explain the highest accumulation of S in the plants grown under S sufficiency. A second hypothesis is related to root development since nutrient limitation generally enhances root growth and morphology (Honsel et al., 2012; Gao et al., 2016). In the present study, it was indicated that RDM was higher under S at 2 mmol L⁻¹ associated with 70/30 NO₃⁻/NH₄⁺ than plants grown under the same S rate and NO₃⁻ solely (Fig. 2b), Further, it was noticed that the roots grew under NO₃⁻ and NH₄⁺ were developed very fast, especially regarding their length, secondary roots, and fine roots. Wheat plants submitted to S sufficiency showed a higher area and high dry biomass, as well as S accumulation per unit root mass than plants exposed to S limitation (Carciochi et al., 2017).

4.4.4. NH4⁺ supply mitigates oxidative stress on photosynthesis under S limitation

Under S limitation and sufficiency, it was reported that the QEPSII and ETR were higher in the plants grown at 70/30 NO_3^-/NH_4^+ than NO_3^- solely (Fig. 5b, 5c), and the same effect has occurred for the SC in the plants grown under S at 1 mmol L⁻¹ (Fig. 5a). These results partially explain the highest dry biomass, nutrient accumulation, and performance of Tanzania guinea grass under NO_3^- and NH_4^+ since high QEPSII and ETR are related to the high efficiency of photosynthesis and better plant growth, and both are raised by combing NO_3^- and NH_4^+ (Khan et al., 2015; Souza Junior et al., 2019). However, it has not been reported why NH_4^+ addition can alleviate stress from S limitation on photosynthesis and plant metabolism. Overall, it was found the highest NP in the plants cultivated with $70/30 NO_3^-/NH_4^+$ or S at 1 and 2 mmol L^{-1} (Table 4). Plants of Tanzania guinea grass grown at $70/30 NO_3^-/NH_4^+$ showed a higher NP than at 100/0 NO_3^-/NH_4^+ , at the first harvest (Leite and Monteiro, 2019). Some hypotheses can be indicated to explain this result. The major one is related to the positive effects of NH_4^+ to enhance antioxidant activity and mitigates oxidative stress, which may affect photosynthesis, causing electron impairment and then loss of thylakoid membrane function (Schulze et al., 2002; Myouga et al., 2008).

Khan et al. (2015) found that wheat grown on a substrate (compost + sand) without S fertilization showed lower NP, SC, QEPSII, ETR, and dry biomass production than plants grown with S at a200 mg kg⁻¹. They also found high concentrations of MDA and H₂O₂ in plant tissue due to S limitation (Khan et al., 2015). Bagheri et al. (2017) reported that spinach plants (*Spinacia oleracea* L.) grown in a growth chamber under nutrient solution with S at 30 µmol L⁻¹ (deficiency) had a higher MDA content than plants fertilized with 300 µmol L⁻¹ (sufficiency). Sulfur limitation caused a decrease in S concentration in plants and then imbalance on NO₃⁻, NH₄⁺, and SO₄²⁻, which result in less protein synthesis (De Bona et al., 2013; Schmidt et al., 2013; Postles et al., 2016). This effect was confirmed by Saha et al. (2016) for Chinese beans (*Vigna radiata* L.), which indicated that S deficiency induces a state of permanent oxidative stress. These reports have shown that S limitation causes oxidative stress, which negatively affects photosynthesis. However, the present study has been showing that the combination between NO₃⁻ and NH₄⁺ mitigates the negative effects of S limitation on photosynthesis, likely due to the free-energy released by N assimilation directly as NH₄⁺, which might be used by plants to improve the antioxidant defense system.

The concentration of MDA in the roots was lower in the plants grown at $70/30 \text{ NO}_3^-/\text{NH}_4^+$ than at NO_3^- solely, regardless of the rates of S (Fig. 6a). These results are consistent with GPX activity in the shoots since this enzyme was indicated as higher at S limitation and sufficiency when the plants received $70/30 \text{ NO}_3^-/\text{NH}_4^+$ than NO_3^- solely (Fig. 7a). It suggests that GPX activity was an important antioxidant enzyme that acted to mitigate lipid peroxidation caused by S limitation and even S sufficiency (Khan et al., 2015), an effect that was stimulated by NH_4^+ addition. In this case, NH_4^+ application might up-regulate genes that codify for antioxidants such as GPX, CAT, and APX which were higher in the roots when the plants received

 $70/30 \text{ NO}_3^-/\text{NH}_4^+$ than NO_3^- solely (Table 6). This result confirms that NH_4^+ application can improve the antioxidant defense system in Tanzania guinea grass under S limitation.

Abiotic stress such as S limitation induces metabolic processes that converge to a single point in common: an excessive synthesis of ROS by plant cells. Thus, plants interacting with stressful environments developed mechanisms to eliminate excess ROS, related to the action of dismutases and peroxidases (Myouga et al., 2008). Antioxidant enzymes can turn ROS into nontoxic forms from redox reactions, which culminate in stress mitigation (Liu et al., 2015). In this context, turning H_2O_2 into H_2O , both CAT and GPX stand out (Carrasco-Gil et al., 2012). Another important mechanism of plants to tolerate oxidative stress is the ascorbate-glutathione cycle, in which the APX enzyme catalyzes a reduction of H_2O_2 into H_2O by using reduced glutathione (GSH) as reducing power, forming glutathione oxidized. Glutathione reductase then acts by reducing oxidized glutathione to GSH once again, completing the cycle (Drazkiewicz et al., 2003).

4.5. CONCLUSION

The S limitation is an adverse condition that causes oxidative stress, negatively affecting photosynthesis. However, Tanzania guinea grass has a great tolerance to S limitation. The tolerance of Tanzania guinea grass to S limitation is better manifested when the plants grow at a proper proportion of NO_3^- and NH_4^+ since a combination between these ions decreases oxidative stress by stimulating mainly guaiacol peroxidase, improving the photosynthesis. In this context, a combination of $70/30 NO_3^-/NH_4^+$ for Tanzania guinea grass is desirable to mitigate the oxidative stress caused by S limitation. It is suggested a combination of $70/30 NO_3^-/NH_4^+$ to ensure the proper development of Tanzania guinea grass even under S limitation.

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