RAFAELLA DE PAULA AVELAR

NITROGEN DYNAMICS IN SUGARCANE: NUTRITIONAL STRATEGIES UNDER ELEVATED CO_2 AND THE SPATIAL DISTRIBUTION ALONG THE LEAF BLADE

LAVRAS – MG

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Thesis submitted to the Federal University of Lavras as part of the requirements for the Postgraduate Program in the area of concentration in Plant Physiology, for obtaining the title of Doctor.

Orientador
Prof. Dr. Paulo Eduardo Ribeiro Marchiori

Coorientador
Prof. Dr. Fernando Cesar Bachiega Zambrosi

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Coorientador: Fernando Cesar Bachiega Zambrosi

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RAFAELLA DE PAULA AVELAR

NITROGEN DYNAMICS IN SUGARCANE: NUTRITIONAL STRATEGIES UNDER ELEVATED CO_2 AND THE SPATIAL DISTRIBUTION ALONG THE LEAF BLADE

DINÂMICA DO NITROGÊNIO NA CANA-DE-AÇÚCAR: ESTRATÉGIAS NUTRICIONAIS SOB CO_2 ELEVADO E A DISTRIBUIÇÃO ESPACIAL AO LONGO DA LÂMINA FOLIAR.

Thesis submitted to the Federal University of Lavras as part of the requirements for the Postgraduate Program in the area of concentration in Plant Physiology, for obtaining the title of Doctor.

APPROVED in 24th September of 2025.

Dr. Paulo Eduardo Ribeiro Marchiori	UFLA
Dr. Fernando Cesar Bachiega Zambrosi	IAC
Dra Cristina Rodrigues Gabriel Sales	UC – Reino Unido
Dr. Vitor de Laia Nascimento	UFLA
Dra Zulma Catherine Cardenal Rubio	UPTC – Colômbia

Orientador
Prof. Dr. Paulo Eduardo Ribeiro Marchiori

Coorientador
Prof. Dr. Fernando Cesar Bachiega Zambrosi

LAVRAS – MG

2025

To my parents, Ivone and Sebastião, my brothers Luiz Otávio and Gabriel, my husband Ronan, and my daughter Sofia.

I dedicate

.

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I would like to thank God first and foremost for granting me strength, wisdom, and serenity throughout this journey. In times of uncertainty and exhaustion, it was faith that sustained me and kept me going. Without His constant presence, this work would not have been possible. The completion of this thesis represents not only the realization of an academic project, but also the reflection of the support, encouragement, and inspiration of many people throughout this journey.

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I would like to deeply thank my family, especially my parents Ivone and Sebastião, and my brothers Luis Otávio and Gabriel for their unconditional love, understanding, and support at all times, including the most challenging ones. I would especially like to thank my husband Ronan for his constant presence on this journey. Thank you for your unconditional support, for believing in me even when I doubted, for encouraging me in difficult times and celebrating with me every small achievement. Your love, patience and understanding were fundamental for me to get here. I would also like to thank my daughter Sofia, for filling my life with joy and inspiration, and my unborn baby, whose presence already fills my heart with hope and love. Even without fully understanding this journey, your smile, your affection and your purity made each day lighter and full of meaning. In the most difficult moments, all I had to do was look at you to find reasons to keep going. May this work be proof that dreams can be achieved with dedication and love. Everything I do, I also do for you. To the friends who were by my side, offering words of encouragement, listening attentively and moments of lightness, my sincere thanks. Finally, I dedicate this work to everyone who believed in me and, in some way, contributed to making this dream come true.

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“Determination, courage and self-confidence are decisive factors for success. If we are possessed by unshakable determination, we will be able to overcome them. Regardless of the circumstances, we must always be humble, modest and devoid of pride.”

(Dalai Lama)

ABSTRACT

Nitrogen (N) is an essential macronutrient involved in critical plant metabolic processes, including the synthesis of amino acids, proteins, nucleic acids, and pigments. Elevated atmospheric CO_2 (eCO_2) generally enhances photosynthesis but often reduces leaf N content, a phenomenon known as CO_2 acclimation. Adjusting the NO_3^-:NH_4^+ ratio in nutrient solutions may help mitigate this decline, particularly in sugarcane (*Saccharum* spp.), which exhibits genotypic variation in NH_4^+ uptake and tolerance. Besides this understanding N assimilation, redistribution, and utilization along the leaf can reveal adaptive strategies to optimize N use and photosynthetic capacity, supporting sustainable agronomic practices. This study tested two hypotheses: (i) eCO_2 enhances NH_4^+ assimilation in sugarcane, and increasing NH_4^+ supply alleviates N decline, potentially mediated by chloroplast positioning due to its role in photosynthetic efficiency; and (ii) total N, NH_4^+, and NO_3^- follow a gradient along the sugarcane leaf blade, which is modified under increased NH_4^+ supply. Sugarcane stem nodes (var. RB 855536) were sprouted and grown in 10 L pots with washed sand under greenhouse conditions (28.8°C, 79% RH) for 45 days. Plants were fertigated every two days with a complete nutrient solution at two NO_3^-:NH_4^+ ratios (87.5:12.5 and 50:50), gradually increasing ionic strength during acclimation. Afterward, plants were transferred to open-top chambers (OTCs) for nine days for acclimatation, before exposure to either ambient CO_2 (400 µmol mol^{-1}) or elevated CO_2 (800 µmol mol^{-1}) for 35 days. CO_2 concentrations inside the OTCs were monitored every three hours using an infrared gas analyzer. A 2 × 2 factorial design with nine replicates per treatment was employed. Results show that eCO_2 enhanced NH_4^+ assimilation, with higher NH_4^+ supply increasing total leaf N. Both eCO_2 and elevated NH_4^+ shifted chloroplasts toward a centripetal arrangement, suggesting enhanced PEPCK activity and photosynthetic efficiency. Biochemical analyses revealed gradients of total N, NH_4^+, and NO_3^- along the leaf blade, and increased NH_4^+ promoted redistribution of inorganic N, potentially optimizing N use and reducing toxicity. These findings provide mechanistic insights into N metabolism under eCO_2 and inform strategies to improve N use efficiency in sugarcane under future climate scenarios.

Key words: nitrogen metabolism; chloroplast; photosynthesis; leaf blade.

RESUMO

O nitrogênio (N) é um macronutriente essencial envolvido em processos metabólicos críticos das plantas, incluindo a síntese de aminoácidos, proteínas, ácidos nucleicos e pigmentos. A elevação do CO_2 atmosférico (eCO_2) geralmente aumenta a fotossíntese, mas frequentemente reduz o teor de N nas folhas, um fenômeno conhecido como aclimatação ao CO_2. Ajustar a razão NO_3^-:NH_4^+ nas soluções nutritivas pode ajudar a mitigar essa redução, particularmente na cana-de-açúcar (*Saccharum* spp.), que apresenta variação genotípica na absorção e tolerância ao NH_4^+. Além disso, compreender a assimilação, redistribuição e utilização do N ao longo da lâmina foliar pode revelar estratégias adaptativas para otimizar o uso de N e a capacidade fotossintética, apoiando práticas agronômicas sustentáveis. Este estudo testou duas hipóteses: (i) o eCO_2 aumenta a assimilação de NH_4^+ na cana-de-açúcar, e o aumento do fornecimento de NH_4^+ alivia a redução de N, potencialmente mediado pelo posicionamento dos cloroplastos devido ao seu papel na eficiência fotossintética; e (ii) o N total, NH_4^+ e NO_3^- seguem um gradiente ao longo da lâmina foliar da cana-de-açúcar, o qual é modificado pelo aumento do fornecimento de NH_4^+. Os nós caulinares da cana-de-açúcar (var. RB 855536) foram germinados e cultivados em vasos de 10 L com areia lavada em casa de vegetação sob condições de estufa (28,8°C, 79% UR) por 45 dias. As plantas foram fertirrigadas a cada dois dias com uma solução nutritiva completa em duas razões NO_3^-:NH_4^+ (87,5:12,5 e 50:50), aumentando gradualmente a força iônica durante a aclimatação. Em seguida, as plantas foram transferidas para câmaras abertas (OTCs) por nove dias para aclimatação, antes da exposição a CO_2 ambiente (400 µmol mol^{-1}) ou CO_2 elevado (800 µmol mol^{-1}) por 35 dias. As concentrações de CO_2 dentro das OTCs foram monitoradas a cada três horas usando um analisador de gás por infravermelho. Foi utilizado um delineamento fatorial 2 × 2 com nove repetições por tratamento. Os resultados mostram que o eCO_2 aumentou a assimilação de NH_4^+, sendo que o maior fornecimento de NH_4^+ elevou o N total nas folhas. Tanto o eCO_2 quanto o aumento de NH_4^+ deslocaram os cloroplastos para um arranjo centrípeto, sugerindo maior atividade da PEPCK e eficiência fotossintética. Análises bioquímicas revelaram gradientes de N total, NH_4^+ e NO_3^- ao longo da lâmina foliar, e o aumento de NH_4^+ promoveu a redistribuição do N inorgânico, potencialmente otimizando o uso de N e reduzindo a toxicidade. Esses achados fornecem esclarecimentos sobre o metabolismo do N sob eCO_2 e orientam estratégias para melhorar a eficiência do uso de N na cana-de-açúcar em cenários climáticos futuros.

Palavras-chave: metabolismo do nitrogênio; cloroplasto; fotossíntese; lâmina foliar.

IMPACT INDICATORS

The increase in atmospheric carbon dioxide concentration is one of the main challenges of climate change and directly affects agricultural productivity. This thesis investigated the effects of elevated CO_2 on nitrogen dynamics in sugarcane, evaluating nutritional strategies to mitigate the reduction of leaf nitrogen frequently observed under these conditions. Experiments were conducted in open-top chambers with different proportions of nitrate and ammonium as nitrogen sources, allowing the assessment of physiological, biochemical, and anatomical changes in the plants. The results demonstrated that increasing the proportion of ammonium can restore nitrogen levels and improve photosynthetic efficiency even under conditions of elevated CO_2. These findings have both direct and potential impacts in multiple dimensions. From a social and economic perspective, they contribute to maintaining sugarcane productivity, a strategic crop for Brazil, highly relevant to the sugar-energy sector, which generates thousands of jobs and drives the national economy through the production of sugar, ethanol, and bioenergy. From a technological perspective, the research provides fertigation management protocols that can be applied in the field to reduce nitrogen losses, increase fertilizer use efficiency, lower production costs, and support precision agriculture systems and sustainable practices. From an environmental perspective, the results align with global climate change mitigation goals, as more efficient nitrogen use contributes to reducing emissions associated with fertilizer use and to ensuring agricultural resilience in scenarios of increasing CO_2 concentration. Furthermore, the research is consistent with the thematic areas of Environment, Technology and Production, and Education of the National Extension Policy, and aligns with several United Nations Sustainable Development Goals, including SDG 2 (Zero hunger and sustainable agriculture), SDG 7 (Affordable and clean energy), SDG 12 (Responsible consumption and production), SDG 13 (Climate action), and SDG 15 (Life on land). Therefore, this work presents both concrete and potential impacts, strengthening agricultural sustainability, technological innovation, and the connection between science, society, and territory.

INDICADORES DE IMPACTO

O aumento da concentração de dióxido de carbono atmosférico é um dos principais desafios das mudanças climáticas e afeta diretamente a produtividade agrícola. Esta tese investigou os efeitos do CO_2 elevado na dinâmica do nitrogênio na cana-de-açúcar, avaliando estratégias nutricionais para mitigar a redução de nitrogênio foliar frequentemente observada nessas condições. Os experimentos foram conduzidos em câmaras abertas (OTCs) com diferentes proporções de nitrato e amônio como fontes de nitrogênio, permitindo a avaliação de alterações fisiológicas, bioquímicas e anatômicas nas plantas. Os resultados demonstraram que o aumento da proporção de amônio pode restaurar os níveis de nitrogênio e melhorar a eficiência fotossintética, mesmo sob condições de CO_2 elevado. Esses achados possuem impactos diretos e potenciais em múltiplas dimensões. Do ponto de vista social e econômico, contribuem para a manutenção da produtividade da cana-de-açúcar, uma cultura estratégica para o Brasil, altamente relevante para o setor sucroenergético, que gera milhares de empregos e movimenta a economia nacional por meio da produção de açúcar, etanol e bioenergia. Do ponto de vista tecnológico, a pesquisa fornece protocolos de manejo de fertirrigação que podem ser aplicados em campo para reduzir perdas de nitrogênio, aumentar a eficiência do uso de fertilizantes, reduzir custos de produção e apoiar sistemas de agricultura de precisão e práticas sustentáveis. Do ponto de vista ambiental, os resultados estão alinhados com os objetivos globais de mitigação das mudanças climáticas, uma vez que o uso mais eficiente do nitrogênio contribui para a redução das emissões associadas ao uso de fertilizantes e para assegurar a resiliência agrícola em cenários de aumento da concentração de CO_2. Além disso, a pesquisa é consistente com as áreas temáticas de Meio Ambiente, Tecnologia e Produção, e Educação da Política Nacional de Extensão, e está alinhada com vários Objetivos de Desenvolvimento Sustentável das Nações Unidas, incluindo ODS 2 (Fome zero e agricultura sustentável), ODS 7 (Energia acessível e limpa), ODS 12 (Consumo e produção responsáveis), ODS 13 (Ação contra a mudança global do clima) e ODS 15 (Vida terrestre). Portanto, este trabalho apresenta impactos concretos e potenciais, fortalecendo a sustentabilidade agrícola, a inovação tecnológica e a conexão entre ciência, sociedade e território.

SUMÁRIO

FIRST PART

1 INTRODUCTION

Carbon dioxide (CO_2) is one of the main greenhouse gases emitted by the combustion of fossil fuels and a significant contributor to global climate change (Bhargava & Mitra, 2021). Atmospheric CO_2 concentration has increased from 315 μmol mol^{-1} in 1960 to 424 μmol mol^{-1} in 2025, and projections indicate a continued rise (NOAA, 2025). Different developmental trajectories point to contrasting scenarios by the end of the century. In an intermediate scenario, characterized by moderate policies and emission levels, atmospheric CO_2 concentrations could reach 650–700 μmol mol^{-1} by 2100. In high-emission scenarios, with continued reliance on fossil fuels, concentrations may exceed 1000 μmol mol^{-1} (Meinshausen et al., 2020; IPCC, 2021). The current rate of increase, exceeding 2.5 ppm per year, highlights the urgency of implementing effective climate mitigation measures (NOAA, 2025) and the need to better understand how plants respond to elevated CO_2.

Initially, the rise in CO_2 concentration (eCO_2) stimulates photosynthesis and biomass accumulation in plants (Kant et al., 2012; De Souza et al., 2008). However, long-term exposure to eCO_2 often results in a decline in photosynthesis, a phenomenon known as CO_2 acclimation (Nowak et al., 2004; Ainsworth, 2005). This response is frequently associated with a decrease in leaf nitrogen (N) concentration and subsequent disruption of the photosynthetic machinery (Tausz et al., 2017; Cardenal-Rubio et al., 2025). Therefore, identifying strategies to maintain adequate N nutrition under eCO_2 is critical for sustaining crop productivity.

In this regard, altering the proportion of N sources (NO_3^- and NH_4^+) in fertilization may be a viable alternative, as studies indicate that, under eCO_2, plants show a greater preference for NH_4^+ over NO_3^- (Domiciano et al., 2020; Asensio, 2015). In this context, the present study aims to understand the effects of increased atmospheric CO_2 concentration on sugarcane, as well as to evaluate whether adjustments in fertilization can mitigate the N reduction observed in plants grown under eCO_2. Sugarcane is a crop of great global economic relevance, particularly for Brazil, which stands out as the world's largest producer. For the 2025/26 harvest, Brazilian production is estimated at 663.4 million tons (CONAB, 2025). This crop is notable for its industrial versatility, serving as a raw material for the production of sugar, ethanol, cachaça, and various other products of strategic importance to both the economy and the national energy matrix.

This thesis will be presented in article format, comprising two scientific articles. The experiment was conducted as follows: plants were grown in open-top chambers for 35 days

under two CO_2 concentrations: aCO_2 (400 µmol mol^{-1}) and eCO_2 (800 µmol mol^{-1}), and two NO_3^-:NH_4^+ ratios (87.5:12.5 and 50:50) to examine their effects on N metabolism in sugarcane (Saccharum spp., variety RB 855536).

The first article addresses the effect of altering the NH_4^+:NO_3^- ratio on mitigating the N reduction observed in plants grown under eCO_2. The second article investigates the variation of N, NH_4^+, and NO_3^- along the leaf blade, as well as the effect of increasing NH_4^+ concentration in fertigation on this gradient.

2 2. THEORETICAL FRAMEWORK

2.1 Nitrogen Metabolism and Plant Responses to Elevated CO_2

Plants absorb N primarily in inorganic forms, mainly NO_3^- and NH_4^+, though the relative preference for each one is species-dependent (Zayed, 2023; Chen, 2024). In general, the combined supply of both forms has shown enhance growth and productivity, due to their complementary physiological roles (Tao et al., 2017; Masood, 2023, Chen, 2024). Typically, NO_3^- is the dominant N source in well-aerated soils, while NH_4^+ prevails in acidic or poorly oxygenated soils (Miller, 2005). Sugarcane (*Saccharum* spp.) is a crop of major economic importance, widely used for the production of sugar, ethanol, cachaça, bioenergy, and other plant-based products. Although sugarcane has been considered an ammonium-preferring crop (Robinson et al. 2007), other studies have shown that NH_4^+ proportions of 30% or 50% can be toxic reduce biomass accumulation (Boschiero et al., 2019; Pissolato et al., 2019). Part of these conflicting results might be explained by the considerable genotypic variation in NH_4^+ uptake and tolerance (Kölln et al., 2022), which underscores the need for genotype-specific N management strategies.

After absorption, NO_3^- is first reduced to nitrite (NO_2^-) in the cytosol by nitrate reductase (NR), transferred to plastids, then to NH_4^+ in the plastids by nitrite reductase (NiR). The resulting NH_4^+ is incorporated into amino acids through the glutamine synthetase (GS) and glutamate synthase (GOGAT) pathways, producing glutamine (GLN), glutamate (GLU) (Crawford, 1995). In contrast, NH_4^+ is assimilated directly into amino acids through the same GS-GOGAT pathway, typically in roots (Miller, 2007; Xu, 2012). While energetically more efficient, excess NH_4^+ can be toxic, potentially impairing growth (Cao and Li, 2004, Domínguez-Valdivia, 2008; Chen, 2024).

Recent evidence suggests that eCO_2 may alter N uptake and metabolism. In tobacco and Arabidopsis, for example, eCO_2 increased NH_4^+ uptake relative to NO_3^- (Domiciano et al.,

2020, Asensio, 2015). Plants supplied solely with NO_3^- under eCO_2 showed a reduced in total N content, whereas those that received only NH_4^+ maintained N status (Asensio, 2015). Furthermore, eCO_2 has been shown to attenuate NH_4^+ toxicity (Torralbo et al., 2019; Domiciano et al., 2020; Wang and Dang, 2024), and reduce NO_3^- assimilation (Bloom, 2014, Bloom, 2010). These findings underscore the importance of optimizing the NO_3^-: NH_4^+ to sustain crop performance in future high-CO_2 environments (Dong, 2024).

2.2 Anatomical Responses under Elevated CO_2

The eCO_2 induces changes in leaf anatomy, including a reduction in stomatal density (Hetherington, 2003; Ainsworth, 2007; Cardenal-Rubio et al., 2025) and modifications in cell wall structure (Taylor, 2001). However, there is still limited information regarding the effects of eCO_2 on chloroplasts distribution in the cells. These organelles, which play a central role in photosynthesis and various biochemical and biophysical processes, are particularly sensitive to a wide range of environmental stresses (Wang et al., 2024; Adamiec et al., 2024). One study reported that eCO_2 led to an increase in the number, width, and cross-sectional area of chloroplasts (Teng, 2006). Nonetheless, available data remain scarce. For the best of my knowledge, no studies have addressed the effects of eCO_2 on chloroplast positioning in C4 grasses, like sugarcane. It is known, however, that chloroplast position can be influenced by environmental factors such as shading, and that such rearrangements are associated with changes in the CO_2 decarboxylation mechanism and enhanced photosynthetic efficiency (Sales et al., 2018).

C_4 species are classified into three biochemical subtypes based on their predominant decarboxylation enzyme: NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK). The spatial distribution of chloroplasts within bundle sheath cells, as well as the transport of metabolites, varies among these subtypes (Sage et al., 2014). In general, NADP-ME subtype grasses tend to exhibit centrifugally positioned chloroplasts in bundle sheath cells. In the PEPCK subtype, chloroplasts may be distributed either centrifugally or centripetal, while the NAD-ME subtype typically displays chloroplasts in a centripetal position (Gutierrez, 1974; Sales, 2018). Although sugarcane is traditionally classified as a NADP-ME subtype, molecular, biochemical, and physiological evidence suggests some flexibility in the decarboxylation routes used by C_4 plants. This indicates that the classification of C_4 subtypes may not be strictly genetically determined (Furbank, 2011). Therefore, multiple chloroplast positioning patterns may be expected within this species.

In sugarcane, transcriptomic analyses indicate that PEPCK-mediated decarboxylation may predominate over NADP-ME in mature leaves (Calsa, 2007). According to Bellasio and Griffiths (2014) and Yin and Struik (2018), activation of the PEPCK pathway can contribute to enhanced photosynthetic performance in C_4 species, both in terms of biochemical efficiency (CO_2 fixed per ATP consumed) and quantum yield [$Y(CO_2)$, or CO_2 fixed per absorbed photon]. This advantage is largely due to the fact that PEPCK regenerates phosphoenolpyruvate (PEP) using only one ATP, whereas pyruvate phosphate dikinase (PPDK), a more commonly employed enzyme for this regeneration, requires two ATP molecules per cycle. Although there is evidence showing changes in chloroplast position in response to environmental variations (Sales et al., 2018), there is still no information available regarding the effects of CO_2 on the positioning of these organelles.

2.3 Nitrogen Metabolism along the Leaf Blade

Information on the spatial dynamics of N metabolism along the leaf blade is still limited. C_4 plants exhibit a developmental gradient along the leaf blade: while basal cells are undifferentiated and immature, cells become progressively more mature and specialized toward the leaf apex (Cahoon et al., 2008; Pick et al., 2011). Most studies on leaf blade physiology in C_4 species have focused primarily on maize (*Zea mays*) (Cahoon et al., 2008; Bräutigam et al., 2010; Bogart & Myers, 2015). In contrast, few investigations have addressed these aspects in sugarcane (Matiello et al., 2015). Although variations in total N concentration and photosynthetic activity along the sugarcane leaf blade have been documented, spatial variations in NO_3^- and NH_4^+ concentrations remain poorly understood. Furthermore, the effect of differential N fertilization on N metabolism throughout the leaf blade has yet to be elucidated.

SECOND PART

Article 1 - Written in accordance with the scientific journal's standards - Preliminary version	
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Nitrate-to-Ammonium Ratio Drives Sugarcane Nitrogen Status Under Elevated CO_2: A Compensatory Nutritional Strategy

Rafaella de Paula Avelar[1]; Orivaldo Benedito da Silva[1]; Rafaela Andrade von Bentzeen[1], Valdelice Oliveira Lacerda, Taís Teixeira das Neves[1]; Fernando Júnior Marques[1], Mewael Kiros Assefa[1]; Evaristo Mauro de Castro[1]; Fernando Cesar Bachiega Zambrosi[2]; Paulo Eduardo Ribeiro Marchiori[1].

*Corresponding author: - paulo.marchiori@ufla.br

[1]Institute of Natural Sciences, Department of Biology, Graduate Program in Agronomy/Plant Physiology, Federal University of Lavras, 37200-900, Lavras, MG, Brazil.

[2] Agronomic Institute of Campinas (IAC).

Abstract

Purpose

The continuous increase in atmospheric CO_2 concentrations, lead to physiological changes in plants, resulting in a phenomenon known as CO_2 acclimation. This response is often linked to leaf nitrogen (N) decreasing, although the cause of underlying mechanisms are not yet fully understood. One strategy is to adjust N fertilization regimes, particularly the balance between nitrate (NO_3^-) and ammonium (NH_4^+).

Methods

This study examined the effects of eCO_2 (800 µmol mol^{-1}) and two NO_3^-:NH_4^+ ratios (87.5:12.5 and 50:50) on N metabolism in sugarcane (*Saccharum spp*., variety RB 855536). Plants were cultivated in open-top chambers for 35 days under high CO_2 and distinct fertigation conditions. The concentrations of total N, NO_3^-, and NH_4^+ were evaluated, along with the activity of the enzymes nitrate reductase (NR), glutamine synthetase (GS), and glutamate synthase (GOGAT). In addition, anatomical analyses were performed to assess chloroplast positioning.

Results

Although, eCO_2 did not significantly affect photosynthetic rates, it altered N metabolism, including total leaf N content and enzymes activities. Under eCO_2, plants that received the lower proportion of NH_4^+ showed a reduced leaf N. In contrast, increasing the NH_4^+ proportion restored N levels to values like the control. Anatomical changes, such as chloroplast repositioning, were also observed and may be linked to changes in photosynthetic efficiency.

Conclusion

These results suggest that adjusting the NO_3^-:NH_4^+ ratio in fertilization may be an effective strategy to maintain N status in sugarcane grown under eCO_2 conditions.

Key words: Anatomy, Chloroplast, Nitrogen metabolism, Photosynthesis.

1 Introduction

Carbon dioxide (CO_2) is a major greenhouse gas emitted through fossil fuel combustion and is a key contributor to global climate change (Bhargava & Mitra, 2021). Atmospheric CO_2 concentration has risen from 315 μmol mol^{-1} in 1960 to 424 μmol mol^{-1} in 2025 and projections indicate continued increase (NOAA, 2025). Different developmental trajectories point to contrasting scenarios by the end of the century. In an intermediate scenario, characterized by moderate policies and emission levels, atmospheric CO_2 concentrations could reach between 650 and 700 μmol mol^{-1} by 2100. In high-emission pathways, with continued reliance on fossil fuels, concentrations may exceed 1000 μmol mol^{-1} (Meinshausen et al., 2020; IPCC, 2021). The current rate of increase, exceeding 2.5 ppm per year, underscores the urgency of implementing effective climate mitigation measures (NOAA, 2025) and achieving a better understanding of how the plants responds to elevated CO_2.

Initially, the elevation in [CO_2] (eCO_2) stimulates photosynthesis and biomass accumulation in plants (Kant et al., 2012; De Souza et al., 2008). However, under long term exposure to eCO_2 often results in a decline in photosynthesys, a phenomenon named CO_2 acclimation (Nowak et al., 2004, Ainsworth, 2005). This response is frequently associated with a decrease in leaf nitrogen (N) concentration and the subsequent disarrangement of photosynthetic machinery (Tausz et al., 2017; Cardenal-Rubio et al., 2025). Therefore, identifying strategies to maintain proper N nutrition under eCO_2 is a critical priority to sustain crop productivity under eCO_2.

Plants absorb N primarily in inorganic forms, mainly NO_3^- and NH_4^+, though the relative preference for each one is species-dependent (Zayed, 2023; Chen, 2024). In general, the combined supply of both forms has shown enhance growth and productivity, due to their complementary physiological roles (Tao et al., 2017; Masood, 2023, Chen, 2024). Typically, NO_3^- is the dominant N source in well-aerated soils, while NH_4^+ prevails in acidic or poorly oxygenated soils (Miller, 2005). Sugarcane (Saccharum spp.) is a crop of major economic importance, widely used for the production of sugar, ethanol, cachaça, bioenergy, and other plant-based products. Although sugarcane has been considered an ammonium-preferring crop (Robinson et al. 2007), other studies have shown that NH_4^+ proportions of 30% or 50% can be toxic reduce biomass accumulation (Boschiero et al., 2019; Pissolato et al., 2019). Part of these conflicting results might be explained by the considerable genotypic variation in NH_4^+ uptake and tolerance (Kölln et al., 2022), which underscores the need for genotype-specific N management strategies.

After absorption, NO_3^- is first reduced to nitrite (NO_2^-) in the cytosol by nitrate reductase (NR), transferred to plastids, then to NH_4^+ in the plastids by nitrite reductase (NiR). The resulting NH_4^+ is incorporated into amino acids through the glutamine synthetase (GS) and glutamate synthase (GOGAT) pathways, producing glutamine (GLN), glutamate (GLU) (Crawford, 1995). In contrast, NH_4^+ is assimilated directly into amino acids through the same GS-GOGAT pathway, typically in roots (Miller,2007; Xu,2012) While energetically more efficient, excess NH_4^+ can be toxic, potentially impairing growth (Cao and Li, 2004, Domínguez-Valdivia, 2008; Chen, 2024).

Recent evidence suggests that eCO_2 may alter N uptake and metabolism. In tobacco and Arabidopsis, for example, eCO_2 increased NH_4^+ uptake relative to NO_3^- (Domiciano et al 2020, Asensio, 2015). Plants supplied solely with NO_3^- under eCO_2 showed a reduced in total N content, whereas those that received only NH_4^+ maintained N status (Asensio, 2015). Furthermore, eCO_2 has been shown to attenuate NH_4^+ toxicity (Torralbo et al., 2019; Domiciano et al 2020; Wang and Dang, 2024), and reduce NO_3^- assimilation (Bloom, 2014, Bloom, 2010). These findings underscore the importance of optimizing the NO_3^-: NH_4^+ to sustain crop performance in future high-CO_2 environments (Dong, 2024).

In addition, eCO_2 induces changes in leaf anatomy, including a reduction in stomatal density (Hetherington, 2003; Ainsworth, 2007; Cardenal-Rubio et al., 2025) and modifications in cell wall structure (Taylor, 2001). However, there is still limited information regarding the effects of eCO_2 on chloroplasts distribution in the cells. These organelles, which play a central role in photosynthesis and various biochemical and biophysical processes, are particularly sensitive to a wide range of environmental stresses (Wang et al., 2024; Adamiec et al., 2024). One study reported that eCO_2 led to an increase in the number, width, and cross-sectional area of chloroplasts (TENG, 2006). Nonetheless, available data remain scarce. To date, no studies have addressed the effects of eCO_2 on chloroplast positioning. It is known, however, that chloroplast position can be influenced by environmental factors such as shading, and that such rearrangements are associated with changes in the CO_2 decarboxylation mechanism and enhanced photosynthetic efficiency (Sales et al., 2018).

Based on this background, we hypothesized that eCO_2 enhances NH_4^+ assimilation in sugarcane, and that increasing the proportion of NH_4^+ in the nutrient solution may mitigate the decline in nitrogen status commonly observed under eCO_2 conditions. We further propose that chloroplast positioning may play a role in this mitigation process, given its direct association with photosynthetic efficiency and cellular adaptation to environmental changes. To test this, we evaluated sugarcane responses to two atmospheric CO_2 concentrations (400 and 800 μmol

mol^{-1}) and two NO_3^-:NH_4^+ ratios (87.5:12.5 and 50:50), with a focus on physiological, biochemical, and anatomical responses.

2.Materials and Methods

2.1 Experimental Setup

The experiment was conducted at the Federal University of Lavras, Brazil, using six open-top chambers (OTCs) measuring 1.8 m height x 1,06 m diameter. Sugarcane stem-node (*Saccharum* spp.) of the variety RB 855536 were obtained from nodes collected from mother plants an experimental field. Fifty days after sprouting, seedlings were transplanted into 10 L pots filled with washed sand and maintained in a greenhouse for 45 days with an average temperature of 28,8 C° and an average humidity of 79% inside the OTCs. During this period, plants were fertigated every two days with 500 ml of a complete nutrient solution containing mM N (87.5% NO_3^- and 12.5% NH_4^+), 0.5 mM P, 3.0 mM K, 5.0 mM Ca, 1.3 mM Mg, 1.3 mM S, 41.6 µM B, 1.0 µM Cu, 46.7 µM Fe, 10.0 µM Mn, 1.3 µM Mo, and 3.5 µM Zn (Zambrosi et al. 2023). The ionic strength of the solution was gradually increased from 25% to 100% during the acclimation phase, being increased by 25% every 5 days.

Following the greenhouse phase, plants were transferred to the OTCs for nine days of acclimation. The acclimation period in OTCs is essential for allowing plants to adjust to the microclimatic conditions within the chamber (such as temperature, humidity, ventilation, and light) before the onset of eCO_2 exposure, thereby preventing initial stress from interfering with the physiological and anatomical responses being assessed. Plants were then exposed for 35 days to two atmospheric CO_2 concentrations: ambient CO_2 (aCO_2, 400 µmol mol^{-1}) and elevated CO_2 (eCO_2, 800 µmol mol^{-1}). Each CO_2 level was combined with two NO_3^-: NH_4^+ ratios in the nutrient solution: 87.5%:12.5% and 50%:50%. The experiment was conducted using a 2 × 2 factorial design with nine replicates per treatment (n = 36), distributed across six OTCs.Four repetitions were used for biomass and growth analysis, and five for biochemical and anatomical measurements. Leaves developed under eCO_2 conditions were selected for anatomical analyses. To ensure consistency in developmental stage, +1 leaves were marked immediately after the acclimatization period. Open-top chambers (OTCs) are systems that permit continuous gas exchange with the atmosphere. Therefore, external air entry and temperature fluctuations can significantly affect the CO_2 concentration inside the chamber, highlighting the need for constant monitoring to maintain experimental consistency. In this study, CO_2 concentrations inside the OTCs were monitored every three hours using an infrared gas analyzer (SBA-5, PP Systems, USA).

2.2 Gas exchange measurements

Gas exchange parameters: Photosynthesis (A), internal CO_2 concentration (Ci), or transpiration (E) were measured on the +3 leaf one day before harvest using an infrared gas analyzer (IRGA, model LI-6400, LICOR Inc., USA), between 8:00 AM and 12:00 PM. Measurement conditions were set at 1,200 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR), and 400 µmol mol^{-1} internal CO_2 concentration (Ci), leaf temperature of 28 °C and the airflow of 500 µl s^{1}.

2.3 Growth and biomass assessment

At harvest, the number of fully expanded leaves per plant (NL) and total leaf area (LA) were recorded. Leaf area was estimated using the equation (Francis et al., 1969): $LA = L\ x\ W\ x\ 0{,}75$. Where: L is leaf length, W is the maximum width, and 0.75 is a shape correction factor. Plants were then separated into shoot and root components, oven-dried at 65 °C until constant weight, and weighed to determine dry mass.

2.4 Pigments, proteins, and amino acids

Leaves +3 that grew during CO_2 exposure were used for biochemical evaluations. Pigments and amino acids were extracted in ethanol according to López-Hidalgo (2021). Chlorophyll a and b were quantified spectrophotometrically (Lichtenthaler & Buschmann, 2001), and free amino acids were determined using the ninhydrin method (Yemm & Cocking, 1955). Proteins were quantified from the extraction of pellet using the Bradford (Bradford, 1976).

2.5 Nitrogen quantification

Leaf (+3) and root NO_3^- concentrations were determined from dried tissue according to (Cataldo et al., 1975). NH_4^+ was quantified from fresh samples following McCullough (1967). Total N was determined using the Kjeldahl method (Malavolta et al., 1997), which involved sample digestion with concentrated sulfuric acid, distillation with NaOH, and titration with HCl.

2.6 Enzymatic activity related to N metabolism

To determine enzyme activity when using fresh tissue, leaf (+3) and root. Nitrate reductase (NR) activity was measured following the method of Berges & Harrison (1995), glutamine synthetase (GS) activity was determined following Ratajczack et al. (1981), and glutamate synthase (GOGAT) activity was assessed using the method of Groat & Vance (1981).

2.7 Photosynthetic N use efficiency (PNUE)

PNUE was calculated following Pinto et al. (2016) using the equation:$\mathrm{PNUE\ (mmol\ CO_2\ mol-1\ N\ s-1)} = \mathrm{A/SLN}$. Where A is the net photosynthesis rate, and *SLN* is the specific leaf N concentration (g N m^{-2})**.**

2.8 Leaf anatomical analyses

At harvest, +3 leaves developed under eCO_2 were collected. Tissue was excised 1 cm from the midrib and fixed FAA solution (formaldehyde: acetic acid: 70% ethanol, 1:1:18) for 72 hours, then transferred to 70% ethanol (Johansen, 1940). For cross-sectioning, tissues were dehydrated through an ethanol series (70, 80, 90, and 100%), embedded in historesin (Leica Microsystems, Heidelberg, Germany), and sectioned at 7 µm thickness using rotary microtome, stained with 0.05% toluidine blue (w/v) (Feder and O'Brien, 1968), and mounted in Entellan (Merck, Darmstadt, Germany) for permanent slides. Paradermal sections of the adaxial and abaxial surfaces were made with a steel blade, clarified with 1% sodium hypochlorite, rinsed, stained with 1% safranin, and mounted in 50% glycerol (Johansen, 1940). The slides were photographed using a camera coupled to a microscope (Eclipse E100-LED; Nikon, Tokyo, Japan). The number of chloroplasts in centrifugal and centripetal positions was quantified using ImageJ software. The percentage of each position was calculated relative to the total number of bundle sheath cells analyzed.

2.9 Statistical Analysis

Data were analyzed by two-way ANOVA, and treatment means were compared using the Scott-Knott test at a 5% significance level. Statistical analyses were performed using RStudio, and graphs were created with Prism software.

3. Results

3.1 Photosynthesis

The eCO_2 did not significantly affect gas exchange parameters of sugarcane. There were no significant effect or interactions between CO_2 concentration and N source on A, Ci, or E (Table S1). However, stomatal conductance (gs) was reduced in plants receiving the 50 % NH_4^+, regardless of CO_2 level.

3.2 Growth biomass partitioning

Plant growth parameters were not significantly influenced by eCO_2. The number of leaves, total leaf area, and shoot and root dry biomass were similar across treatments. However, plants grown with 50% NH_4^+ showed higher shoot dry mass and greater leaf area compared to those receiving 12.5% NH_4^+ (Table S2).

3.3 Nitrogen metabolism and Pigment concentration

There was a significant interaction ($p<0.05$) between CO_2 concentration and N source for total N, NH_4^+ and NO_3^- concentrations in leaves and roots in leaves and roots. Under eCO_2, plants that received 12.5% NH_4^+ had reduced total N in leaves (Fig. 1A), while plants receiving 50% NH_4^+ maintained leaf N concentration similar to aCO_2. Under aCO_2, plants with 50% NH_4^+

showed lower leaf N and higher root N accumulation. eCO_2 had no effect on leaf NO_3^- in plants receiving 12.5% NH_4^+ (Fig. 2A), but it reduced root NO_3^- in the same treatment (Fig. 2B). In plants that received 50% NH_4^+, eCO_2 led to a reduction in the leaf NO_3^- relative to aCO_2. eCO_2 did not alter root NH_4^+ in the 12,5% NH_4^+ treatment. (Fig. 2D), but it reduced NH_4^+ concentration in leaves (Fig. 2C). Under aCO_2, plants with 50% NH_4^+ showed larger NH_4^+ in roots and lower NH_4^+ in leaves compared to the 12.5% NH_4^+.

There was no interaction between CO_2 and N source for nitrate reductase (NR) or glutamate synthase (GOGAT) activity in either leaves or roots. However, NR and GOGAT activity in leaves increased under eCO_2 (Fig.s 3A and 3E), while NR activity in roots decreased under the same condition (Fig. 3B). GOGAT activity in roots was higher in plants receiving 50 % NH_4^+ (Fig. 3F). A significant interaction between CO_2 level and N source was observed for GS in both leaves and root. In the leaves, GS activity increased under eCO_2 and in the 50% NH_4^+ treatment (Fig. 3C). In contrast, GS activity decreased in plants with 50% NH_4^+ under aCO_2. In the root, GS activity was higher under eCO_2 in plants receiving 50% NH_4^+ (Fig. 3D), while NR activity was reduced in both NH_4^+ treatments in eCO_2. Plants grown under eCO_2 and those received 50% NH_4^+ showed higher showed higher PNUE compared to other treatments (Fig. 5), indicating more efficient use of N for photosynthetic carbon assimilation under these conditions. Chlorophyll a and b levels were significantly reduced by eCO_2 in plants receiving 12.5% NH_4^+. No such reductions were observed in the 50% NH_4^+ treatment (Fig. 4).

3.4 Leaf anatomical features

Anatomical analyses showed alterations in chloroplast positioning under eCO_2 and 12.5% NH_4^+ and aCO_2 and 50% NH_4^+ treatments, with the most pronounced change occurring under aCO_2 + 50% NH_4 (Fig. 6). In Fig. 7 there are images of the sections, where it is possible to observe the difference in the position of the chloroplasts between the treatments.

4. Discussion

On the best of our knowledge, this the first study revealing that: i) plant N nutritional status is influenced by the NO_3-:NH_4^+ ratio in the rooting medium under eCO_2 and, ii) sugarcane may acclimate to its photosynthetic energetic statusthrough decarbolxilation metabolism shifts, as demonstrated by changes in chloroplast positioning.

Under eCO_2, plants supplied with 12.5% NH_4^+ exhibited reduced total N concentration in the leaves, while those receiving 50% NH_4^+ maintained N levels comparable to aCO_2 (Fig. 1). The absence of significant changes in biomass (Table S2) suggests that the observed reduction in leaf N under eCO_2 in 12.5% NH_4^+ plants was not caused by a dilution effect due to

increased growth. This suggests that a higher proportion of NH_4^+ improves N uptake efficiency and/or translocation to shoots under eCO_2.

Under eCO_2, plants receiving 50% NH_4^+ showed reduced NH_4^+ concentrations in both leaves and roots (Fig. 2 C and D), along with increased GS activity (Fig. 3 D), indicating enhanced NH_4^+ assimilation compared to 12.5% plants. Since NO_3^- levels remained unchanged in these plants (Fig. 2 A), the increase in total leaf N was likely driven by rapid NH_4^+ assimilation. In contrast, under eCO_2 and 12.5% NH_4^+, GS activity remained unchanged, and NH_4^+ accumulation was larger indicating a limited assimilation capacity in this treatment. It is suggested that eCO_2 can shift plant preference toward NH_4^+ and reduce NO_3^- assimilation (DOMICIANO at el., 2020, ASENSIO, 2015; Bloom et al., 2010, 2014). Additionally, although high NH_4^+ levels are often associated with toxicity in sugarcane (Boschiero et al., 2019; Pissolato et al., 2019), no toxicity symptoms and growth depression were observed in this study. Part of this phenomenon could be ascribed to the fact that tolerance to NH_4^+ is genotype-dependent (Boschiero et al., 2019; Pissolato et al., 2019, Kölln et al., 2022).

It is noteworthy that the change in N concentration was not accompanied by a corresponding change in photosynthetic activity (Table S1). It is well established that exposure to eCO_2 initially enhances photosynthesis, but this stimulation subsequently declines, in parallel with a reduction in leaf N (Taub, 2008). However, in the present study, alterations in N metabolism were observed at both 12.5% and 50% NH_4^+ proportions, despite the absence of changes in photosynthesis (Fig.s 1 and 3). Although the responses in N metabolism varied between the different ammonium treatments, both showed clear metabolic shifts. This may represent an important finding, as previous studies have primarily linked changes in N metabolism under eCO_2 to increased photosynthetic activity, causing greater growth and dilution of N (Norby,2004; Bloom, 2010; Bloom, 2012). Our results suggest that N reduction can occur independently of photosynthetic stimulation, underscoring the need to explore strategies to mitigate this effect. Several studies have reported enhanced photosynthesis in C_4 plants exposed to eCO_2 (Prins, 2011; Prasad, 2009; Cardenal-Rubio et al., 2025), even though these species possess a CO_2-concentrating mechanism. Notably, a previous study using the same sugarcane variety demonstrated increased photosynthesis and growth under eCO_2 (Cardenal-Rubio et al., 2025).

A plausible explanation for the lack of a photosynthetic response in the current study may lie in the air temperature conditions and the duration of the experiment. The average temperature during the trial was 28.8 °C, which lies within the optimal range for sugarcane growth (25 °C to 35 °C) (Matsuoka, 2011). Nevertheless, previous studies have shown that

higher temperatures can intensify the response of C_4 plants to eCO_2 (Prasad, 2009; Vu et al., 2006). Additionally, this was a short-term experiment, with plants exposed to eCO_2 for only 35 days. In a previous study using the same variety, exposure lasted 70 days and resulted in increased photosynthesis (Cardenal-Rubio et al., 2025). Other investigations evaluating eCO_2 responses over longer periods have also reported enhanced photosynthetic rates (Ottman, 2001; De Souza, 2008).

Beyond changes in N metabolism, notable anatomical alterations were detected. The eCO_2 and the NO_3^-:NH_4^+ ratio affected the spatial distribution of chloroplasts in sugarcane (Fig. 6). This change, which is directly related to the type of decarboxylation mechanism employed by C_4 plants (GUTIERREZ, 1974).C_4 species are classified into three biochemical subtypes based on their predominant decarboxylation enzyme: NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK). The spatial distribution of chloroplasts within bundle sheath cells, as well as the transport of metabolites, varies among these subtypes (Sage et al., 2014). In general, NADP-ME subtype grasses tend to exhibit centrifugally positioned chloroplasts in bundle sheath cells. In the PEPCK subtype, chloroplasts may be distributed either centrifugally or centripetal, while the NAD-ME subtype typically displays chloroplasts in a centripetal position (Gutierrez, 1974; Sales, 2018). Although sugarcane is traditionally classified as a NADP-ME subtype, molecular, biochemical, and physiological evidence suggests some flexibility in the decarboxylation routes used by C_4 plants. This indicates that the classification of C_4 subtypes may not be strictly genetically determined (Furbank, 2011). Therefore, multiple chloroplast positioning patterns may be expected within this species.

There is a reduction in the proportion of the centrifugal position in plants grown under eCO_2 and 12.5% NH_4^+, and also a reduction in plants grown under aCO_2 and 50% NH_4^+ (Fig. 6). In sugarcane, transcriptomic analyzes suggest that PEPCK-mediated decarboxylation may dominate over NADP-ME in mature leaves (Calsa, 2007). We hypothesize that the increased proportion of chloroplasts in the centripetal position is related to the increased proportion of PEPCK activity. According to Bellasio and Griffiths (2014) and Yin and Struik (2018), the involvement of the PEPCK pathway can contribute to enhanced photosynthetic performance in C_4 species, both in terms of biochemical efficiency (CO_2 fixed per ATP) and quantum yield [$Y(CO_2)$, or CO_2 fixed per absorbed photon]. This advantage is largely due to the fact that PEPCK regenerates phosphoenolpyruvate (PEP) using only one ATP, whereas pyruvate phosphate dikinase (PPDK), a more commonly utilized enzyme for this regeneration, requires two ATP molecules per cycle.

The alteration in chloroplast positioning observed under eCO_2 and 12.5% NH_4^+ treatment (Fig. 6) suggests an enhancement in photosynthetic efficiency, potentially serving as a compensatory mechanism to offset the reductions in nitrogen and pigment concentration commonly induced by elevated CO_2. The more pronounced change in chloroplast positioning observed in the aCO_2 + 50% NH_4^+ treatment may be attributed to the absence of increased NH_4^+ assimilation in this condition, necessitating greater investment in photosynthetic efficiency to compensate for the reduction in total N.

5.Conclusion

We conclude that under eCO_2, NH_4^+ assimilation increases regardless of the proportion supplied. However, when 50% of the N is provided as NH_4^+, there is a greater assimilation of this ion, along with an increase in the total N concentration in the leaves. These findings suggest that adjusting the NO_3^-:NH_4^+ ratio in fertilization may be a viable strategy to mitigate the N reduction typically induced by eCO_2. Furthermore, both eCO_2 and higher NH_4^+ proportions induce changes in chloroplast positioning. The observed increase in the proportion of cells with chloroplasts in the centripetal position, compared to the centrifugal position, suggests enhanced PEPCK activity and greater photosynthetic efficiency. The predominance of centripetal positioning under eCO_2 implies that, despite the lack of a clear photosynthetic response, photosynthetic efficiency may have increased. Similarly, the greater centripetal positioning in plants grown under aCO_2 with 50% NH_4^+ indicates a compensatory increase in photosynthetic efficiency, potentially offsetting reductions in leaf N concentration. Interestingly, the changes observed in N metabolism under both N proportions occurred independently of any increase in photosynthesis.

Declaration

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Availability of data and material (data transparency) The datasets generated and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

Authors' Contributions

Conceptualization: Avelar, P.A, Marchiori, P.E.R., Zambrosi, F.C.B, **Data curation**: Avelar, P.A, Marchiori, P.E.R**. Formal analysis**: Avelar, P.A, Neves, T.T, Marchiori, P.E.R., Orivaldo Benedito da Silva, Rafaela Andrade von Bentzeen, **Funding acquisition**: P.A, Marchiori. **Investigation**: Avelar, P.A, Neves, T.T, Lacerda, V.O, Junior,F,M. **Project administration**:

Avelar, P.A, Marchiori, P.E.R., Zambrosi, F.C.B. **Writing review & editing:** Avelar, P.A, Marchiori, P.E.R., Zambrosi, F.C.B., Mewael Kiros Assefa, Evaristo Mauro de Castro.

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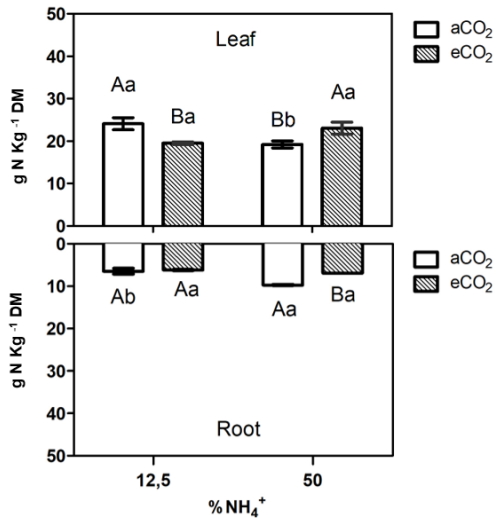
649 **Figures**
650 **Fig.1**



651
652 Total nitrogen (N) concentration in sugarcane leaves and roots. White bars represent aCO_2 (400
653 µmol mol^{-1}), dashed bars represent eCO_2 (800 µmol mol^{-1}). Data were subjected to analysis of
654 variance (ANOVA) by the Scott Knot test at a probability level of 5%. Capital letters compare
655 pCO_2 levels and lowercase letters compare treatments. Bars represent the standard error.
656
657 **Fig.2**
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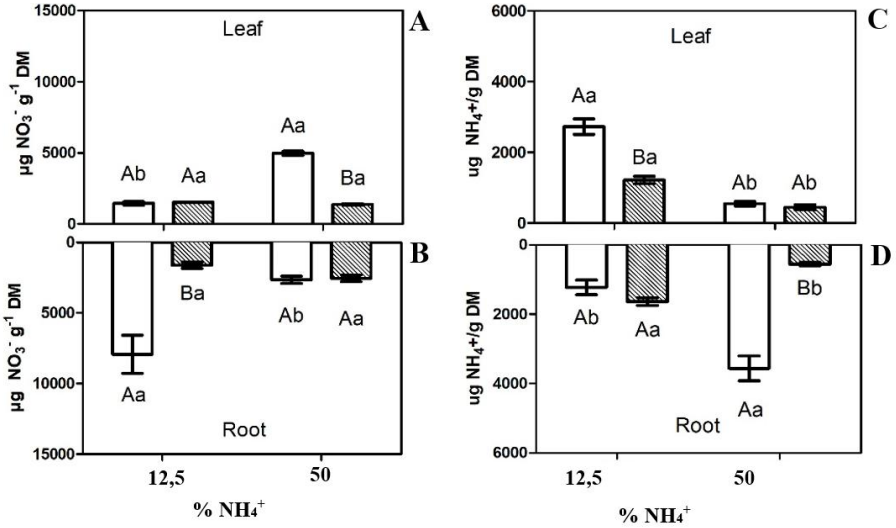

659
660 Nitrate (A and B) and ammonium (C and D) concentration in sugarcane leaves and roots. White
661 bars represent aCO_2 (400 µmol mol^{-1}), dashed bars represent eCO_2 (800 µmol mol^{-1}). Capital
662 letters compare pCO_2 levels and lowercase letters compare treatments. Bars represent the
663 standard error.
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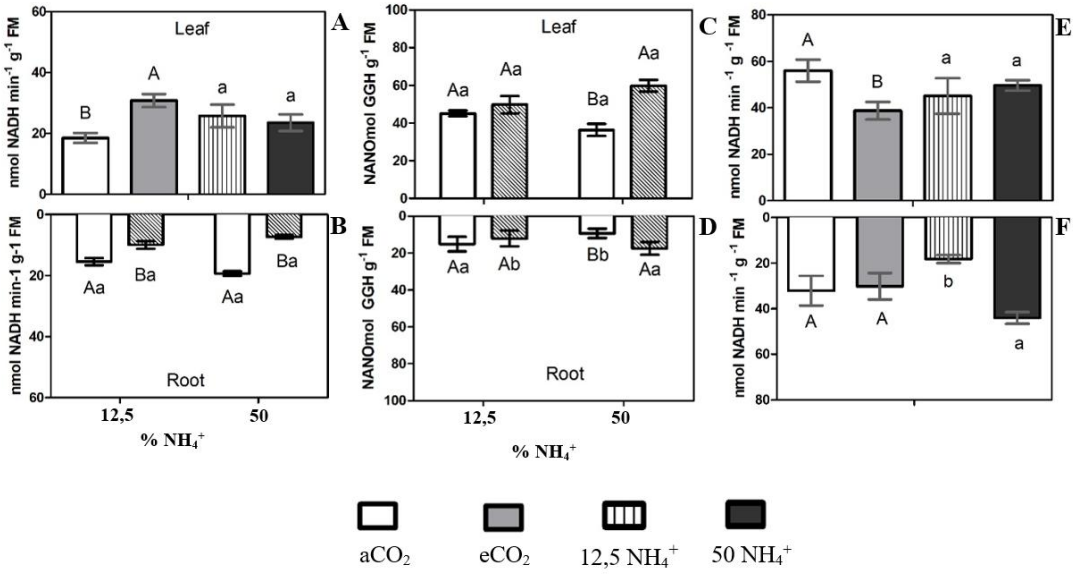
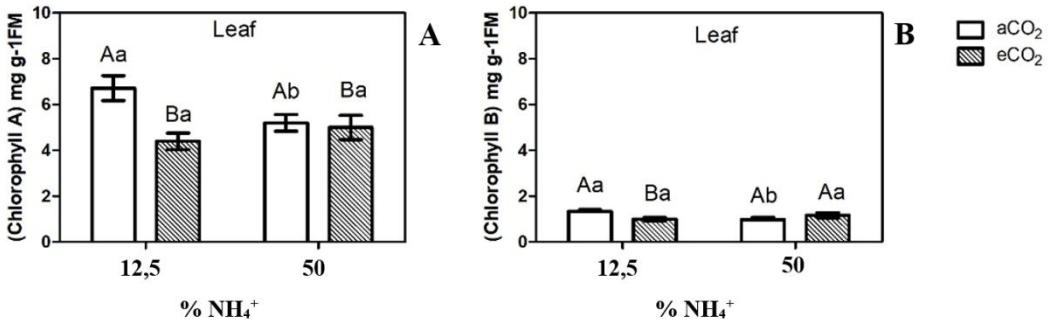
Fig.3



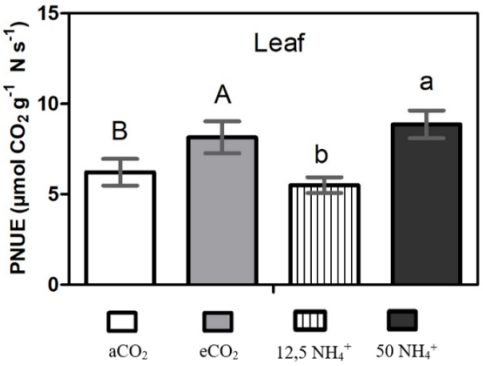
Nitrate reductase activity in leaves and roots (A and B), Gs activity in leaves and roots (C and D), Gogat activity in leaves and roots (E and F). In the graphs with interaction white bars represent aCO_2 (400 µmol mol^{-1}), dashed bars represent eCO_2 (800 µmol mol^{-1}). In the graphs without interaction effects, white bars represent aCO_2, gray bars (eCO_2), the dashed bar (12.5% NH_4^+), and the black bar (50% NH_4^+).Capital letters compare pCO_2 levels and lowercase letters compare treatments. Bars represent the standard error.

Fig.4



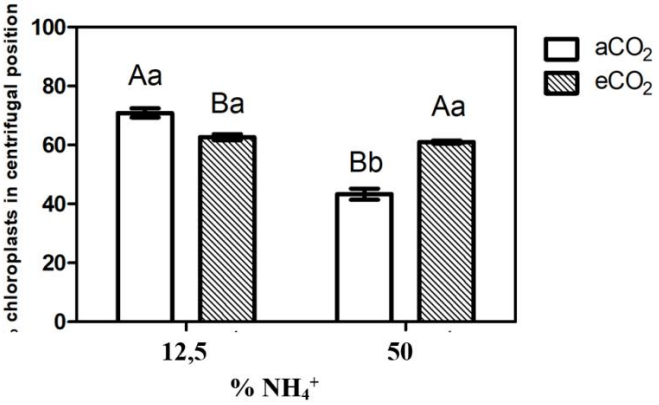
Chorophyll A (A) and Chorophyll B (B) in sugarcane leaves and roots. White bars represent aCO_2 (400 µmol mol^{-1}), dashed bars represent eCO_2 (800 µmol mol^{-1}). Capital letters compare pCO_2 levels and lowercase letters compare treatments. Bars represent the standard error.

Fig.5



Product Nitrogen Use Efficiency (PNUE) in sugarcane. white bars represent aCO_2 (400 µmol mol^{-1}), gray bars eCO_2 (800 µmol mol^{-1}), the dashed bar (12.5% NH_4^+), and the black bar (50% NH_4^+). Capital letters compare pCO_2 levels and lowercase letters compare treatments. Bars represent the standard error.

Fig.6



Percentage of cells with chloroplast distribution in the centrifugal position. White bars represent aCO_2 (400 µmol mol^{-1}), dashed bars represent eCO_2 (800 µmol mol^{-1}). Capital letters compare pCO_2 levels and lowercase letters compare treatments. Bars represent the standard error.

Fig.7

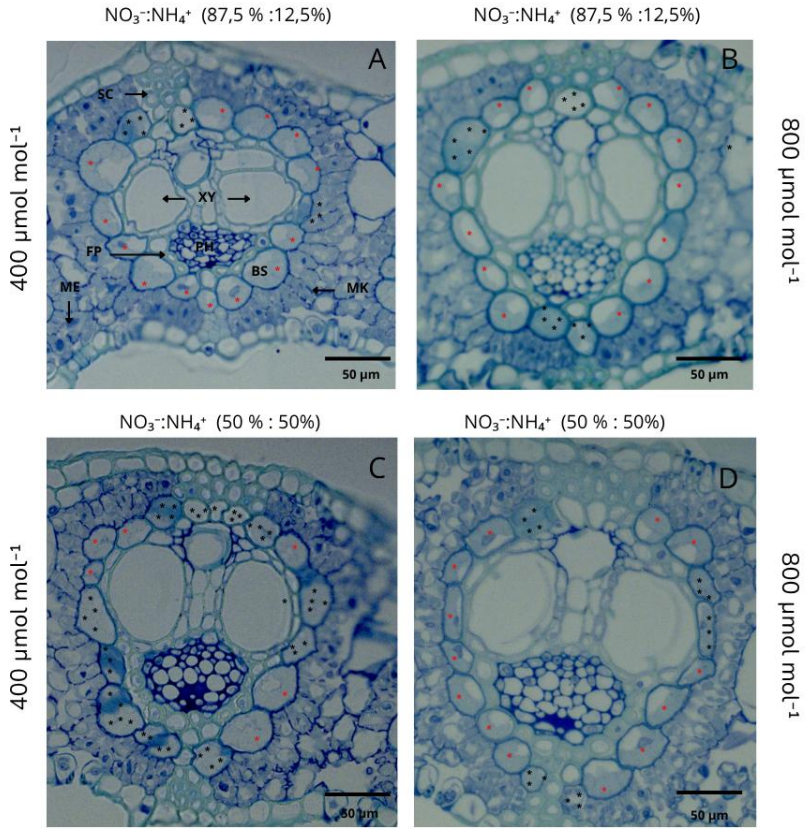


Image of cross-sectioning the leaves. (A) 400 µmol mol^{-1} and 12.5% NH_4^+, (B) 800 µmol mol^{-1} and 12,5% (C) NH_4^+,400 µmol mol^{-1} and 50% (D) NH_4^+,800 µmol mol^{-1} and 50% NH_4^+. SC-Sclerenchyma, FP-Pericyclic fibers, ME-Mesophyll, MK-Mesofilo Kranz, XY-Xylem, PH-Phloem, BS-bundle sheath cells

Supplementary data

Supplementary tables

Table S1

	(A) µmol CO_2 $m^{-2}s^{-1}$		(Ci) µmol CO_2 mol^{-1}		E (mmol H_2O $m^{-2}s^{-1}$)		gs (mol H_2O m^{-2} s^{-1})	
Trat	**Mean**	**(SEM)**	**Mean**	**(SEM)**	**Mean**	**(SEM)**	**Mean**	**(SEM)**
aCO_2 (400 µmol mol^{-1})	18.02 A	1.58	145.12 A	18.75	2.77 A	0.11	0,14 A	0,01
eCO_2(800 µmol mol^{-1})	22.43 A	3.00	177.29 A	19.63	2.96 A	0.23	0,13 A	0,02
12,5% NH_4^+	19.33 a	2.73	181.62 a	20.89	3.07 a	0.16	0,16 a	0,01
50% NH_4^+	17.6 a	2.00	140.79 a	15.97	2.66 a	0.17	0,11 b	0,01

Photosynthesis (A), internal carbon (Ci), Transpiration (E) and stomatal conductance (Gs) in sugarcane. Capital letters compare pCO_2 levels and lowercase letters compare treatments.

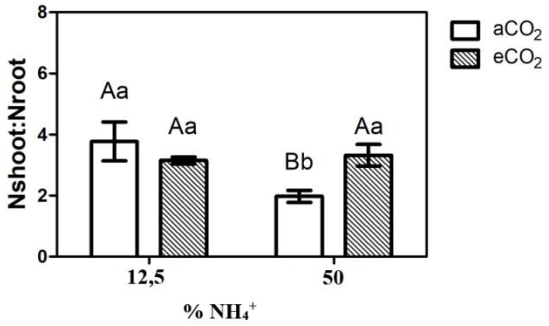
Table S2

	Number of leaves		Leaf area (m2)		Dry mass shoot (g)		Dry mass root(g)	
Trat	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
aCO_2 (400 µmol mol^{-1})	24.16 A	0,9	0,160 A	0,013	23,32 A	2,14	7,47 A	0,88
eCO_2(800 µmol mol^{-1})	26,38A	1,64	0,145A	0,006	22,11 A	1,52	8,24 A	0,38
12,5% NH_4^+	24,83 a	1,66	0,137b	0,007	19,28 b	0,92	7,21 a	0,71
50% NH_4^+	25,71a	1,08	0,16a	0,011	26,15 a	1,22	8,5 a	0,55

Table S2: Number of leaves, leaf area, dry mass shoot and dry mass of the root in sugarcane. Capital letters compare pCO_2 levels and lowercase letters compare treatments

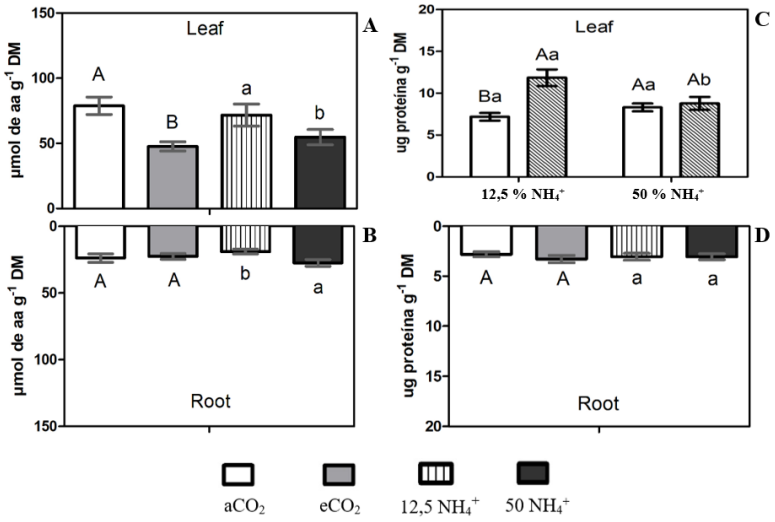
Supplementary Fig.s

Fig. S1



Ratio of N in the shoot and N in the root. White bars represent aCO_2 (400 µmol mol^{-1}), dashed bars represent eCO_2 (400 µmol mol^{-1}).
Capital letters compare pCO_2 levels and lowercase letters compare treatments. Bars represent the standard error.

Fig. S2



Amino acids and proteins in sugarcane leaves and roots. White bars represent aCO_2 (400 µmol mol^{-1}), gray bars and the longitudinal dashed bars eCO_2(800 µmol mol^{-1}), vertical dashed bar T1 and black bar T2. Fig. (A and B) Amino acids in leaves and roots, (C and D) proteins in leaves and roots. Capital letters compare pCO_2 levels and lowercase letters compare treatments. Bars represent the standard error

Article 2 - Prepared in accordance with standard NBR 6022	
Article title:	Nitrate-to-Ammonium Ratio Influences the Gradient Distribution of Total and Inorganic Nitrogen Along the Sugarcane Leaf Blade
Authors:	Rafaella de Paula Avelar[1]; Taís Teixeira das Neves[1]; Fernando Marques Júnior[1]; Valdelice Oliveira Lacerda[1]; Mewael Kiros Assefa[1]; Fernando Cesar Bachiega Zambrosi[2]; Paulo Eduardo Ribeiro Marchiori[1]

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UNIVERSIDADE FEDERAL DE LAVRAS

ARTIGO 2 - Nitrate-to-Ammonium Ratio Influences the Gradient Distribution of Total and Inorganic Nitrogen Along the Sugarcane Leaf Blade

Rafaella de Paula Avelar
https://orcid.org/0009-0004-8889-4519

Taís Teixeira das Neves

Abstract:

Understanding plant physiology is essential for optimizing crop productivity, with nitrogen (N) availability being directly linked to biomass accumulation, enhanced photosynthesis, and yield. Although the biochemical and physiological mechanisms of N metabolism are well documented, little is known about its dynamics along the leaf blade, particularly in sugarcane. This C4 crop, of considerable economic relevance especially in Brazil has been understudied in this context. Evidence indicates variation in photosynthesis and total N content along the leaf blade. However, the distribution of nitrate (NO_3^-) and ammonium (NH_4^+), as well as the effects of different NH_4^+ proportions in fertilization on this gradient, remain unclear. The effects of NH_4^+ on sugarcane growth and metabolism remain controversial in the literature. Although certain studies report potential toxicity associated with NH_4^+, others suggest that higher NH_4^+:NO_3^- ratios may be beneficial. Understanding N dynamics along the leaf blade and the impact of increased NH_4^+ supply through the soil may support the development of more efficient fertilization strategies, contributing to yield maximization. Therefore, this study aimed to test the hypothesis that a gradient exists in the concentrations of total N, ammonium, and nitrate along the sugarcane leaf blade, and that this gradient is altered by increased NH_4^+ supply. To test this, we evaluated sugarcane responses to two NO_3^-:NH_4^+ ratios (87.5:12.5 and 50:50), focusing on physiological and biochemical responses. Our findings demonstrate distinct differences along the leaf blade in total N, pigment, NO_3^-, and NH_4^+ concentrations. When NH_4^+ supply was increased, plants showed an ability to adjust by redistributing inorganic N forms along the leaf, thereby maximizing utilization and preventing ammonium toxicity.

Keywords: Nitrate; Ammonium; Nitrogen Metabolism; Nitrogen Distribution.

Introduction

N is essential for plant growth and development. It is the element plants require in greatest quantity (Kusano,2011). Plants absorb N primarily in inorganic forms, mainly nitrate NO_3- and NH_4^+, although the relative preference for each form varies by species (Zayed, 2023; Chen, 2024). In general, the combined supply of both forms has shown enhance growth and productivity, due to their complementary physiological roles (Tao et al., 2017; Masood, 2023).

Typically, NO_3^- is the dominant N source in well-aerated soils, while NH_4^+ prevails in acidic or poorly oxygenated soils (Miller, 2005). Although both forms are taken up by the roots, they differ in their biochemical and energetic properties (Jackson, 2008). NO_3^- is first reduced to nitrite (NO_2^-) in the cytosol by nitrate reductase (NR), then to NH_4^+ in the plastids by nitrite reductase (NiR). The resulting NH_4^+ is incorporated into amino acids through the glutamine synthetase (GS) and glutamate synthase (GOGAT) pathways, producing glutamine (GLN), glutamate (GLU) (Crawford, 1995; Howitt, 2000). In addition to the GS-GOGAT cycle, NH_4^+ can also be assimilated through an alternative pathway mediated by the NADH-dependent glutamate dehydrogenase (GDH), which catalyzes the synthesis of glutamate in the cytosol from ammonium and 2-oxoglutarate (Tercé-Laforgue et al., 2004; Cruz et al., 2006; Sarasketa et al., 2014). While energetically more efficient, excess NH4+ is can be toxic, potentially impairing growth (Cao and Li, 2004, Domínguez-Valdivia, 2008; Chen, 2024).

N metabolism is closely integrated with carbon (C) metabolism. C metabolism provides not only the energy required for N assimilation but also the carbon skeletons necessary for the incorporation of N into organic compounds, such as 2-oxoglutarate. This intermediate of the tricarboxylic acid (TCA) cycle is produced during cellular respiration and serves as the carbon acceptor in the synthesis of glutamate via the enzyme GOGAT. The source of energy and reducing power for these reactions depends on the tissue type and subcellular compartment. While glutamine synthesis consistently requires ATP, glutamate formation via GOGAT can utilize either NADH (in heterotrophic tissues, such as roots) or reduced ferredoxin (in photosynthetic tissues, such as leaves), showing that the metabolism of C and N are coordinated (ZHENG, 2009).

The photosynthetic rate is directly related to the N concentration in the leaves, partly because N is a key component of essential molecules involved in photosynthesis, such as chlorophyll and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Evans, 1989; LUO, 2021). The N allocated to RuBisCO accounts for a substantial fraction of the total leaf N (Evans, 1989). In C_3 plants, approximately 25 to 30% of leaf N is associated with RuBisCO, whereas in C_4 species this proportion is significantly lower, ranging from 10 to 15% (Sage, 1987). Enhancing photosynthesis is considered a potential strategy to improve productivity in C_4 crops, as it may lead to greater biomass accumulation and yield under optimal and stress conditions (Zhu et al., 2010; Leakey et al., 2019). Extensive research has elucidated the biochemical and physiological mechanisms underlying N metabolism, including N acquisition, translocation, assimilation, remobilization, and its regulatory crosstalk with C metabolism (Xu et al., 2012; Tegeder & Masclaux-Daubresse, 2018; Wang et al., 2023).

Despite this progress, limited information is available regarding the spatial dynamics of N metabolism along the leaf blade. C_4 plants exhibit a developmental gradient along the leaf blade. While basal cells are undifferentiated and immature, as one moves toward the leaf apex, the cells become progressively more mature and specialized. (Cahoon et al., 2008; Pick et al., 2011). Most studies on leaf blade physiology in C_4 species have focused primarily on maize (Zea mays) (Cahoon et al., 2008; Bräutigam et al. ,2010; Bogart & Myers ,2015). In contrast, few investigations have addressed these aspects in sugarcane (Saccharum spp.), a C_4 crop of significant economic importance, particularly in Brazil, the world's largest producer (Conab, 2024). Although variations in total N concentration and photosynthetic activity along the sugarcane leaf blade are documented, spatial variations in NO_3^- and NH_4^+concentrations remain poorly understood. Furthermore, the impact of differential N fertilization on N metabolism throughout the leaf blade has yet to be elucidated.

Investigating how N is assimilated, redistributed, and utilized along the leaf blade can reveal spatial patterns that reflect adaptive strategies for optimizing N use and enhancing photosynthetic capacity. Such knowledge has the potential to support the development of more sustainable agronomic practices and contribute to yield improvement in sugarcane, particularly under conditions that demand efficient use of N fertilizers. In this context, adjusting the NO_3^-:NH_4^+ ratio may represent a promising strategy for improving N use efficiency. However, particular attention must be given to the proportion of NH_4^+ supplied. Although some studies suggest that sugarcane exhibits a preference for NH_4^+, excessive proportions, such as 30% or 50%, have been associated with toxicity symptoms and reduced biomass (Boschiero et al., 2019; Pissolato et al., 2019). Furthermore, genotypic variation in NH_4^+ uptake and tolerance has been documented (Kölln et al., 2022), reinforcing the need for genotype-specific N management strategies. Therefore, this study aimed to test the hypothesis that a gradient exists in the concentrations of total N, NH_4^+, and nitrate NO_3^- along the sugarcane leaf blade, and that increasing NH_4^+ supply alters this gradient. To address this, we evaluated sugarcane responses to two NO_3^-:NH_4^+ ratios (87.5:12.5 and 50:50), with a focus on physiological and biochemical parameters related to N metabolism.

Materials and Methods

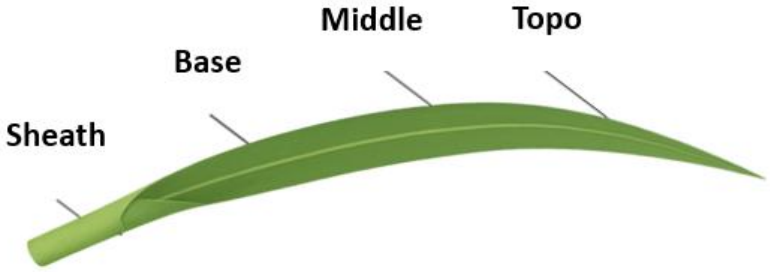
Experimental Setup

The experiment was conducted at the Federal University of Lavras, Brazil. Sugarcane seedlings (Saccharum spp.) of the variety RB 855536 were obtained from the university's experimental field. Fifty days after sprouting, seedlings were transplanted into 10 L pots filled with washed sand and maintained in a greenhouse for 45 days. During this period, plants were

fertigated every two days with 500 ml of a complete nutrient solution containing 87.5% NO3- and 12.5% NH4+, at 6 mM N 0.5 mM P, 3.0 mM K, 5.0 mM Ca, 1.3 mM Mg, 1.3 mM S, 41.6 µM B, 1.0 µM Cu, 46.7 µM Fe, 10.0 µM Mn, 1.3 µM Mo, and 3.5 µM Zn as per Zambrosi (2023). The ionic strength of the solution was gradually increased from 25% to 100% during the acclimation phase.

After this period, the experiment was installed, which lasted 35 days. A completely randomized design (CRD) was used with two factors: NO_3^-:NH_4^+ ratio in the nutrient solution (87.5%/12.5% and 50%: 50%) and plant region (apex, middle, base, leaf sheath and root). Each treatment included nine replicates, totaling 18 plants. After 35 days they were collected to analyses, segments the top, middle, base and sheath of the leaves + 1 (Figure 1).

Figure 1: Image representing the location where material was collected for biochemical analysis: Sheath, base, middle and topo



Source: By the author

Pigments

Ethanolic extraction was performed following the methodology of López-Hidalgo (2021). The extract was used to quantify pigments. Pigments were quantified according to Lichtenthaler & Buschmann (2001).

Ammonium and Nitrate Quantification

NO_3^- was determined in dried leaf and root samples using the method described by Cataldo et al. (1975). NH_4^+ quantification was performed by extracting 60 mg of fresh material in 1.5 mL of ultrapure water, incubating at 80 °C for 10 minutes, and centrifuging at 4,000 rpm for 20 minutes at 4 °C. The NH_4^+ concentration was determined following McCullough (1967).

Total Nitrogen

The Kjeldahl method was used to determine total N. The process involved three steps: sample digestion with concentrated sulfuric acid, N distillation using NaOH, and final titration with HCl to determine N concentration (Malavolta, Vitti & Oliveira, 1997).

Enzymatic Activity of Nitrogen Metabolism

To assess enzyme activity related to N metabolism, 1.25 g of fresh plant tissue was homogenized in 5 mL of incubation buffer containing 100 mM phosphate buffer (pH 7.5), 2 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 5 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 16,000 g for 20 minutes at 4 °C, and the supernatant was used for enzyme assays. Nitrate reductase (NR) activity was measured according to Berges & Harrison (1995); Glutamine synthetase (GS) activity was determined following Ratajczack et al. (1981).

Statistical Analysis

Data were subjected to analysis of variance (ANOVA), and means were compared using the Scott-Knott test at a 5% probability level in RStudio. Graphs were created using the software Prism.

Results

There was a differential distribution of total N, photosynthetic pigments, NO_3^-, and NH_4^+ along the leaf blade under both NO_3^-:NH_4^+ ratios. The distribution patterns of total N (Figure 2) and pigments (Figure 6) were similar along the leaf, regardless of the NO_3^-:NH_4^+ ratio supplied. Total N concentration was higher in the middle and apical regions of the leaves. In contrast, roots of plants receiving 50% NH_4^+ showed a lower total N concentration. Regarding photosynthetic pigments, higher concentrations of chlorophyll a, chlorophyll b, and carotenoids were observed in the middle region of leaves from plants supplied with 12.5% NH_4^+, and in the apical region of those treated with 50% NH_4^+. Overall, plants receiving 50% NH_4^+ accumulated more pigments in the apical region of the leaf blade compared to those supplied with 12.5% NH_4^+.

Variations in NH_4^+ and NO_3^- concentrations (Figure 3 and 4) were observed along the leaf blade under both NO_3^-:NH_4^+ ratios, as well as distinct responses depending on the proportion of N supplied. At the 12.5% NH_4^+ ratio, NH_4^+concentration was higher in the middle region of the leaf. In contrast, under the 50% NH_4^+ treatment, the highest NH_4^+ concentration was found at the leaf apex. These results indicate that the NO_3^-:NH_4^+ ratio influenced the spatial distribution of NH_4^+ within the leaf. In plants supplied with 50% NH_4^+, a higher accumulation of NH_4^+ was detected in the apical region and a lower concentration in the middle region, compared to plants receiving 12.5% NH_4^+. Regarding NO_3^-, plants treated with 12.5% NH_4^+ showed greater NO_3^- concentrations in the sheath and basal regions of the leaves. In contrast, those supplied with 50% NH_4^+ exhibited increased NO_3^- accumulation in the sheath, base, and middle regions of the leaf blade. Notably, NO_3^- concentration in the middle region was higher in plants receiving 50% NH_4^+ than in those treated with 12.5% NH_4^+. No interaction between

factors was observed for glutamine synthetase (GS) and nitrate reductase (NR) activities (Figure 5) . GS activity was lower in the leaf sheath, while NR activity remained unchanged along the leaf blade.

Discussion

N concentration varied along the leaf blade, with higher concentrations observed in the middle and apical regions (Figure 2). This pattern was consistent across both NH_4^+ supply proportions tested. Similar results were reported by Mattiello (2015), who observed greater N accumulation in the middle portion of the sugarcane leaf. There is evidence that C_4 plants allocate approximately 30% of their foliar N to thylakoid components, including chlorophylls (Ghannoum, 2005). The distribution of photosynthetic pigments (Figure 6) followed a similar pattern to that of N, with higher levels of chlorophyll a, chlorophyll b, and carotenoids detected in the middle region of the leaf blade, regardless of the NH_4^+ proportion supplied. These findings suggest that photosynthetic metabolism is spatially variable along the leaf blade, reinforcing the strong association between N concentration and photosynthetic efficiency.

Previous studies have shown that the photosynthetic rate in sugarcane increases linearly with foliar N concentration (Meinzer & Zhu, 1998), likely due to the direct involvement of N in the composition of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) and chlorophyll, key molecules for photosynthetic activity. Rubisco quantity and activity are correlated with leaf N concentration (Nakano et al., 1997; Sage et al., 1987). Additionally, chlorophyll concentration is widely recognized as a reliable indicator of pigment concentration involved in light absorption and energy transfer during the photochemical phase of photosynthesis (Bassi, 2018). Supporting the present findings, Mattiello (2015) also reported an increase in N concentration from the leaf base to the apex, along with enhanced photosynthetic activity in the middle region of the sugarcane leaf blade.

It is noteworthy that, although the total N concentration is higher in the middle portion of the leaf, the distribution patterns of NO_3^- and NH_4^+ (Figure 3 and 4) differ along the leaf blade and are influenced by the supplied NO_3^-:NH_4^+ ratio. In plants treated with 12.5% NH_4^+, higher NO_3^- concentrations were observed in the basal portion and the sheath of the leaf. A similar response was reported in sugarcane by Mattiello (2015), suggesting increased NO3- assimilation in the leaf base, possibly followed by its transport to other regions. In maize, the basal regions of the leaves have been shown to play a key role in N assimilation (Hirel et al., 2001). Likewise, in sugarcane, genes encoding NO_3^- transporters were found to be upregulated in the basal region of the leaves (Mattiello, 2015).

In contrast, NH_4^+ (Figure 3) showed a different accumulation pattern, with higher concentrations detected in the middle portion of the leaves, surpassing those observed in the roots. Although it is commonly assumed that NH_4^+ is primarily assimilated in the roots, with limited transport to the shoot (Tobin & Yamaya, 2001; Fukumorita, 1982), several studies have demonstrated that NH_4^+ can indeed be translocated via the xylem and has been detected at considerable levels in aerial tissues (Schjoerring, 2002; Koltun, 2022; Maniero, 2023). The higher NH_4^+ concentration in the middle part of the leaf, combined with increased glutamine synthetase (GS) activity (Figure 5) in the leaves compared to the roots, indicates that NH_4^+ was effectively transported and assimilated in the aerial parts. This accumulation may be related to a higher metabolic activity in this leaf region, particularly in terms of photosynthesis, as observed by Mattiello (2015) and Bassi (2018). Ammonium assimilation is closely linked to carbon metabolism, as it depends on the supply of carbon skeletons, especially 2-oxoglutarate, for its incorporation (Matt et al., 2001). Since NH_4^+ cannot be stored and excessive levels can be toxic to plant cells (Cao & Li, 2004; Domínguez-Valdivia, 2008; Chen, 2024), rapid assimilation in the shoot is essential to prevent toxicity and maintain cellular homeostasis.

Increasing the proportion of NH_4^+ led to changes in the spatial distribution of NH_4^+ and NO_3^- along the leaf blade (Figure 3 and 4). NO_3^-, which was previously concentrated mainly in the sheath and basal regions of the leaf (Figure 4), also showed higher concentrations in the middle portion under elevated NH_4^+ conditions. This shift may be associated with enhanced NH_4^+ assimilation in the roots, possibly prompting the plant to redirect NO_3^- to the leaves for assimilation. This hypothesis is supported by the fact that the energetic cost of NO_3^- assimilation is lower in the leaves (Bredemeier, 2000). Meanwhile, NH_4^+, which initially accumulated in the middle portion of the leaf, showed a shift in concentration toward the apical region. This pattern may reflect increased NO_3^- assimilation in the middle portion, leading to a redistribution of NH_4^+ assimilation to the apex. Consequently, NO_3^- assimilation became more prominent in the basal and middle leaf regions, while NH_4^+ assimilation predominated in the apex.

Although some studies have reported that NH_4^+ may be toxic to sugarcane, no signs of toxicity were observed in the present study. It is possible that NH_4^+ was rapidly assimilated in the apical region, preventing toxic accumulation. Although photosynthetic activity was not directly measured, the increased proportion of NH_4^+ was associated with higher concentrations of chlorophyll a, chlorophyll b, and carotenoids in the apical leaf region. This increase in pigment concentration may indicate a potential enhancement of photosynthetic capacity. Carotenoids, in particular, may have played an important role in protecting against potential

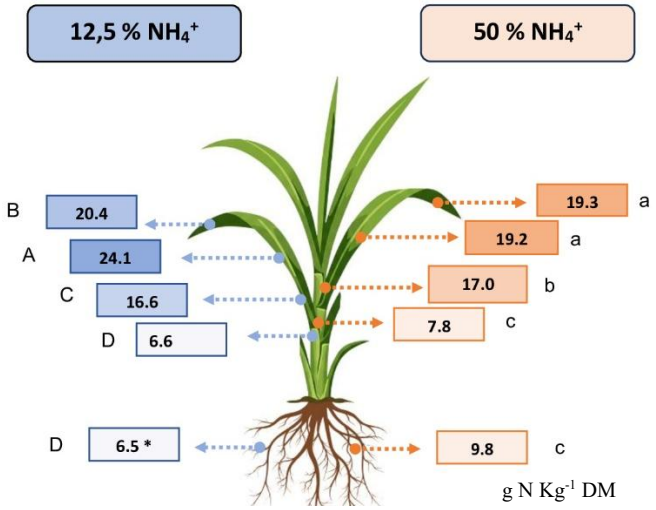
NH_4^+ toxicity. These compounds constitute a large family of lipophilic antioxidants whose main function is to scavenge free radicals through electron transfer to their conjugated double bonds. Additionally, they are crucial for chlorophyll protection, quenching the excited triplet state and singlet oxygen, thereby preventing lipid peroxidation and membrane collapse in chloroplasts. This action contributes to the structural and functional integrity of these organelles (Kenneth et al., 2000).

The effects of NH_4^+ on sugarcane growth and metabolism remain controversial in the literature. Recent evidence at physiological and molecular levels demonstrates that sugarcane exhibits a marked capacity to absorb and assimilate NH_4^+. Preferential uptake of NH_4^+ over NO_3^- has been observed (Otto, R. 2022; Sanglard, 2019) and functional characterization of transporters ScAMT2;1 and ScAMT3;3 confirms specialized molecular mechanisms for efficient NH_4^+ uptake and remobilization (Koltun etal.2022; Maniero etal.2023). However, some studies report that certain proportions of NH_4^+ can induce toxicity symptoms in sugarcane. For example, the variety CTC15 exhibited toxicity symptoms when supplied with 50% NH_4^+ (Boschiero, 2019), while the variety IACSP95-5000 showed similar responses at 30% NH_4^+ (Pissolato, 2019). Given the controversial responses of sugarcane to ammonium (NH_4^+), further research is necessary to investigate its effects across different varieties. Such studies will help clarify varietal differences in tolerance and assimilation mechanisms, contributing to optimized N management strategies.

The results indicate that at lower NH_4^+ proportions, NO_3^- predominantly accumulates in the sheath and basal regions of the leaf, while ammonium is primarily assimilated in the middle section. Conversely, increasing the NH_4^+ supply enhanced the translocation of NO_3^- toward the aerial parts of the plant, likely due to elevated NH_4^+ assimilation by the roots. This shift resulted in higher NO_3^- concentrations extending from the sheath to the mid-leaf region, whereas NH_4^+ was preferentially transported to the leaf apex. Although metabolic activity in the apical region is generally lower than in the middle portion, no toxicity symptoms were detected. This tolerance may be linked to increased carotenoid levels in the apex, which potentially confer protection against stress.

Figures

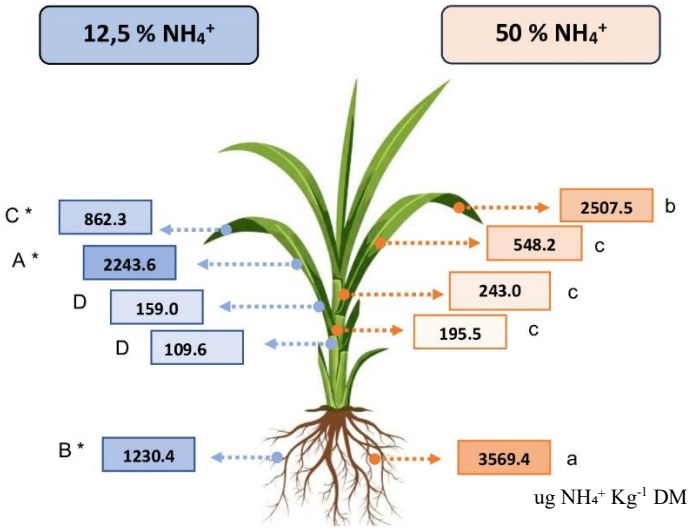
Figure 2 Total N concentration in sugarcane leaf blade and roots.



Caption: The left panel shows plants grown under a 12.5% NO_3^- / 50% NH_4^+ ratio (T1), while the right panel shows plants grown under a 50% NO_3^- / 50% NH_4^+ ratio (T2). Uppercase letters indicate significant differences among segments within T1, and lowercase letters indicate differences within T2. Asterisks (*) denote significant differences between corresponding segments of T1 and T2. Data were analyzed by analysis of variance (ANOVA), and means were compared using the Scott-Knott test at a 5% significance level in RStudio.

Source: By the author

Figure 3 Amonium concentration in sugarcane leaf blade and roots.

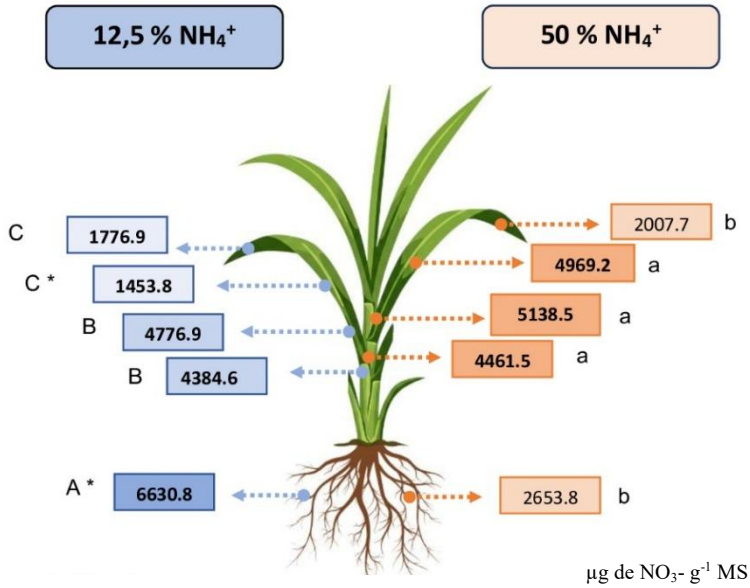


Caption:The left panel shows plants grown under a 12.5% NO_3^- / 50% NH_4^+ ratio (T1), while the right panel shows plants grown under a 50% NO_3^- / 50% NH_4^+ ratio (T2). Uppercase letters indicate significant differences among segments within T1, and lowercase letters indicate differences within T2. Asterisks (*) denote significant differences between corresponding segments of T1 and T2. Data were

analyzed by analysis of variance (ANOVA), and means were compared using the Scott-Knott test at a 5% significance level in RStudio.

Source: By the author

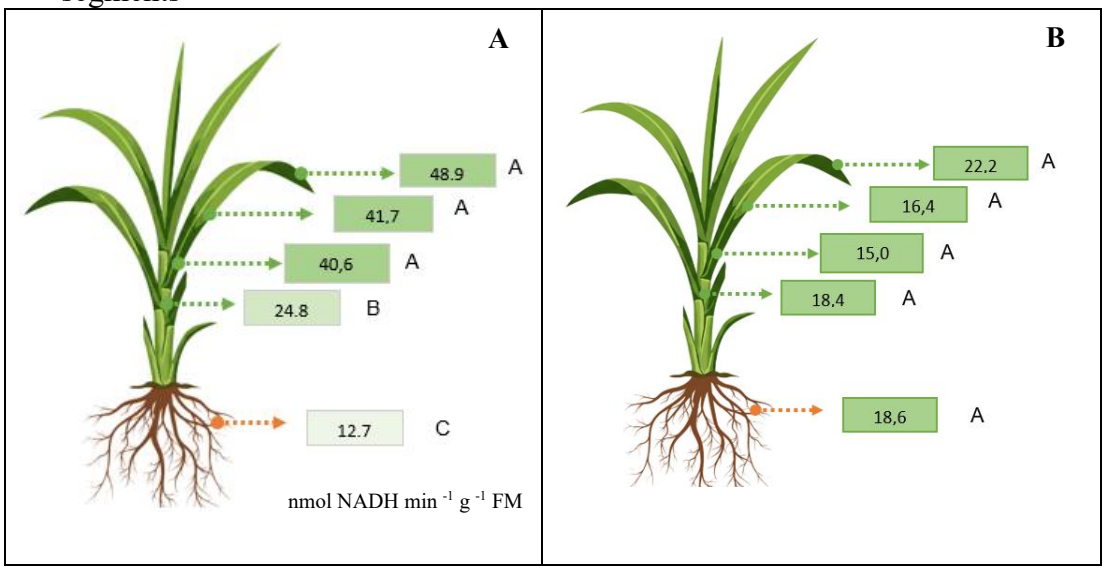
Figure 4 Nitrate concentration in sugarcane leaf blade and roots



Caption: The left panel shows plants grown under a 12.5% NO_3^- / 50% NH_4^+ ratio (T1), while the right panel shows plants grown under a 50% NO_3^- / 50% NH_4^+ ratio (T2). Uppercase letters indicate significant differences among segments within T1, and lowercase letters indicate differences within T2. Asterisks (*) denote significant differences between corresponding segments of T1 and T2. Data were analyzed by analysis of variance (ANOVA), and means were compared using the Scott-Knott test at a 5% significance level in RStudio.

Source: By the author

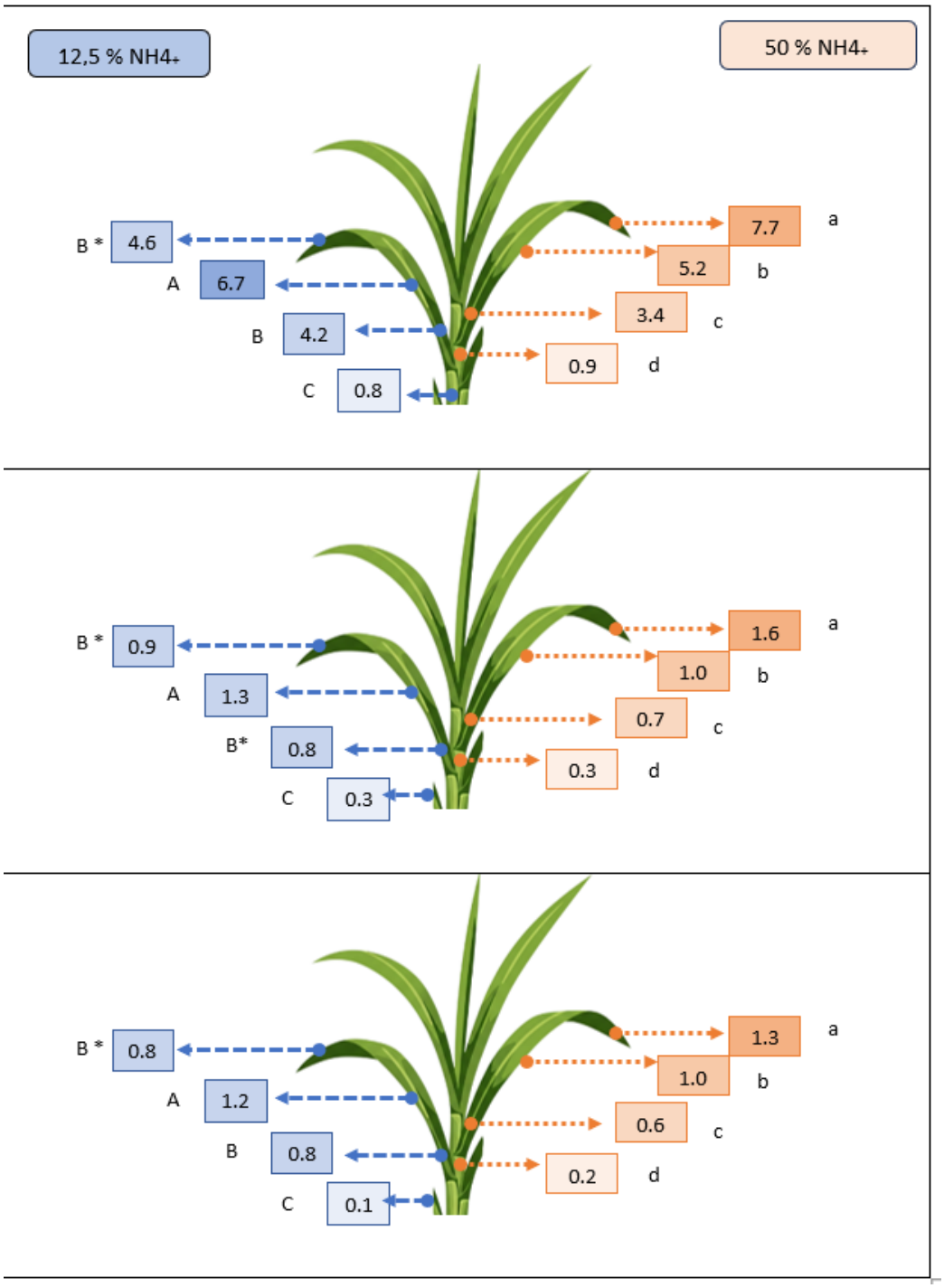
Figure 5 Glutamine synthetase (GS) and nitrate reductase (NR) activity in sugarcane leaf segments



Caption: (A) Gs activity, (B) NR activity. Different letters indicate difference between leaf and root segments. Data were subjected to analysis of variance (ANOVA) using the Scott Knot test at a probability level of 5% in the Rstudio program.

Source: By the author

Figura 6 Chlorophyll a, chlorophyll b, and carotenoids in different segments of the sugarcane leaf.



Caption: (A)Chlorophyll a, (B) chlorophyll b and (C) carotenoids. The left panel shows plants grown under a 12.5% NO_3^- / 50% NH_4^+ ratio (T1), while the right panel shows plants grown under a 50% NO_3^- / 50% NH_4^+ ratio (T2). Uppercase letters indicate significant differences among segments within

T1, and lowercase letters indicate differences within T2. Asterisks (*) denote significant differences between corresponding segments of T1 and T2. Data were analyzed by analysis of variance (ANOVA), and means were compared using the Scott-Knott test at a 5% significance level in RStudio.

Source: By the author

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THIRD PART

3 FINAL CONSIDERATIONS

This work provided a comprehensive understanding of nitrogen metabolism in sugarcane under elevated atmospheric CO_2 conditions (eCO_2). In the first study, it was observed that both total N and its inorganic forms were affected by eCO_2, which appears to favor greater NH_4^+ assimilation at the expense of NO_3^-. Supplying a higher proportion of NH_4^+ via fertigation proved effective in reducing nitrogen loss induced by eCO_2.

Changes in chloroplast positioning were also observed in response to eCO_2 and to the increased NH_4^+ proportion, indicating morphophysiological adjustments associated with enhanced photosynthetic efficiency. These findings are particularly relevant in the context of the ongoing rise in atmospheric CO_2 and its impacts on plant physiology.

In the second study, changes in N metabolism along the leaf blade were identified, with a gradient of total N, NH_4^+, and NO_3^- from the leaf base to the apex, which was modified by increased NH_4^+availability. This spatial distribution suggests that sugarcane optimizes the use of available N.

The study of the N gradient along the leaf blade is essential for understanding the interaction between mineral nutrition, leaf development, and photosynthetic efficiency, providing insights for nutrient management strategies and breeding programs.

In summary, this work advances the understanding of fundamental aspects of N metabolism in sugarcane and provides valuable tools for the development of agricultural and genetic practices aimed at increasing productivity and sustainability under changing environmental conditions.

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