RAQUEL YELLIN CASTAÑEDA VENEGAS

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Dissertation presented to the Federal University of Lavras, as part of the requirements of the Graduate Program in Agronomy/Plant Physiology, to obtain the title of Master.

Prof. Dr. Paulo Eduardo Ribeiro Marchiori
Advisor

Prof. Dr. Mewael Kiros Assefa
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REMOBILIZAÇÃO DE NITROGÊNIO-15 EM SACCHARUM SPP. DURANTE O CRESCIMENTO VEGETATIVO SOB ESTRESSE HÍDRICO E CONDIÇÕES DE REIDRATAÇÃO

Dissertation presented to the Federal University of Lavras, as part of the requirements of the Graduate Program in Agronomy/Plant Physiology, to obtain the title of Master.

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In fields of sugar and silence,
I grew up with those who held my roots.
This work is for them
and for the strength I found in the drought.

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ABSTRACT

Drought stress is a major constraint on sugarcane (*Saccharum* spp.) productivity, negatively affecting nitrogen uptake and assimilation. Under such conditions, internal nitrogen remobilisation becomes essential for sustaining plant growth and development. This study investigated the effects of drought stress and subsequent rehydration on nitrogen remobilisation in sugarcane (variety CTC9001bt), with a specific focus on the interaction between nitrogen and carbon metabolism. Sugarcane plants were cultivated under controlled greenhouse conditions and subjected to well-watered (~90% water holding capacity, WHC) and water-deficient (~20% WHC) treatments. ^{15}N-labelled ammonium sulphate was applied to the +1 leaf at 25 days after transplanting (DAT) to trace nitrogen remobilisation patterns. Physiological, biochemical, and isotopic analyses were performed at maximum stress (38 DAT) and following rehydration (43 DAT).

Drought stress significantly reduced plant height, leaf water potential, stomatal conductance, photosynthetic rate, and transpiration. These physiological changes were accompanied by a decline in carbon isotope discrimination ($\Delta^{13}C$), particularly in roots, leaves, and ^{15}N-labelled leaves. In contrast, $\Delta^{13}C$ values in stalks increased under drought. Nitrogen remobilisation was enhanced under water deficit conditions, with preferential allocation to roots and sheath leaves, contributing to improved nitrogen use efficiency (NUE). The accumulation of starch and osmoprotectants, such as proline, soluble sugars, and carotenoids, supports photoprotection and metabolic balance. Following rehydration, nitrogen was redistributed to the leaf blades, promoting partial recovery of chlorophyll content and photosynthetic performance. However, gas exchange parameters remained below those of well-watered plants, indicating incomplete recovery. Key enzymes involved in nitrogen metabolism, including glutamine synthetase (GS) and nitrate reductase (NR), were modulated by water availability. Changes in the carbon-to-nitrogen ratio reflected dynamic resource allocation in response to stress and recovery.

These findings underscore the importance of integrated nitrogen and carbon metabolic responses for drought resilience in sugarcane. Importantly, understanding nitrogen remobilisation dynamics has practical agronomic implications, including the potential to optimise fertiliser management, reduce nitrogen losses, and enhance yield stability under drought conditions. Moreover, these insights can support the development of precision agriculture strategies and inform breeding programmes aimed at improving NUE and environmental sustainability in sugarcane production systems.

Keywords: Nitrogen remobilisation, ^{15}N isotopic labelling, drought stress, rehydration, nitrogen-use efficiency, sugarcane, Saccharum spp., carbon metabolism.

RESUMO

O estresse hídrico é uma das principais limitações à produtividade da cana-de-açúcar (*Saccharum* spp.), afetando negativamente a absorção e assimilação de nitrogênio. Nessas condições, a remobilização interna de nitrogênio torna-se essencial para sustentar o crescimento e o desenvolvimento das plantas. Este estudo investigou os efeitos do estresse hídrico e da reidratação subsequente sobre a remobilização de nitrogênio na cana-de-açúcar (variedade CTC9001bt), com foco específico na interação entre os metabolismos de nitrogênio e carbono. As plantas foram cultivadas em casa de vegetação sob condições controladas e submetidas a tratamentos bem irrigados (~90% da capacidade de retenção de água, CRA) e com deficiência hídrica (~20% da CRA). Sulfato de amônio enriquecido com ^{15}N foi aplicado na folha +1 aos 25 dias após o transplante (DAT) para rastrear os padrões de remobilização de nitrogênio. Análises fisiológicas, bioquímicas e isotópicas foram realizadas no estresse máximo (38 DAT) e após a reidratação (43 DAT).

O estresse hídrico reduziu significativamente a altura das plantas, o potencial hídrico foliar, a condutância estomática, a taxa fotossintética e a transpiração. Essas alterações fisiológicas foram acompanhadas por uma redução na discriminação isotópica de carbono ($\Delta^{13}C$), principalmente em raízes, folhas e folhas marcadas com ^{15}N. Em contrapartida, os valores de $\Delta^{13}C$ aumentaram nos colmos sob estresse. A remobilização de nitrogênio foi intensificada em condições de déficit hídrico, com alocação preferencial para raízes e bainhas foliares, contribuindo para o aumento da eficiência do uso de nitrogênio (EUN). O acúmulo de osmorreguladores, como prolina, açúcares solúveis, amido e carotenoides, favoreceu a fotoproteção e o equilíbrio metabólico. Após a reidratação, o nitrogênio foi redistribuído para os limbos foliares, promovendo uma recuperação parcial do teor de clorofila e do desempenho fotossintético. No entanto, os parâmetros de troca gasosa permaneceram inferiores aos das plantas bem irrigadas, indicando recuperação incompleta. Enzimas chave do metabolismo do nitrogênio, como a glutamina sintetase (GS) e a redutase do nitrato (NR), foram moduladas pela disponibilidade hídrica. Alterações na razão carbono-nitrogênio refletiram a alocação dinâmica de recursos em resposta ao estresse e à recuperação.

Esses achados ressaltam a importância das respostas metabólicas integradas de nitrogênio e carbono para a resiliência da cana-de-açúcar frente ao estresse hídrico. Compreender a dinâmica da remobilização de nitrogênio possui implicações agronômicas práticas, incluindo o potencial para otimizar o manejo da adubação, reduzir perdas de nitrogênio e aumentar a estabilidade da produtividade em condições de seca. Além disso, esses conhecimentos podem subsidiar o desenvolvimento de estratégias de agricultura de precisão e orientar programas de melhoramento voltados à melhoria da EUN e à sustentabilidade ambiental na produção de cana-de-açúcar.

Palavras-chave: Remobilização de nitrogênio, marcação isotópica com ^{15}N, estresse hídrico, reidratação, eficiência do uso de nitrogênio, cana-de-açúcar, Saccharum spp., metabolismo do carbono

IMPACT INDICATORS

This work significantly contributes to scientific progress in the field of plant physiology, particularly in the understanding of nitrogen remobilisation in sugarcane (*Saccharum* spp.) under drought and rehydration conditions. Through isotopic techniques and physiological and biochemical analyses, relevant data were obtained that support more efficient nutrient management strategies, especially for varieties adapted to tropical regions facing water limitations. The potential impacts include improvements in nitrogen use efficiency (NUE), reduced input losses, and technical knowledge applicable to the sugar-energy sector. Although no direct engagement with external communities occurred, the results have the potential to benefit farmers, agricultural technicians, researchers, and policymakers through scientific dissemination. The impact territory includes sugarcane-producing regions in Brazil and other tropical countries. It fits within the thematic areas of 'Environment' and 'Technology and Production' of the National Extension Policy. The work aligns with the United Nations Sustainable Development Goals, especially SDG 2 (Zero Hunger and Sustainable Agriculture) and SDG 13 (Climate Action). The project involved 3 professors, 1 master's student, and 1 undergraduate student, contributing to scientific training and relevant academic-technological impact.

INDICADORES DE IMPACTO

Este trabalho contribui significativamente para o avanço científico na área de fisiologia vegetal, especialmente no entendimento da remobilização de nitrogênio em cana-de-açúcar (*Saccharum* spp.) sob condições de estresse hídrico e reidratação. Através de técnicas isotópicas e análises fisiológicas e bioquímicas, foram obtidos dados relevantes que podem embasar estratégias de manejo nutricional mais eficientes, principalmente para variedades adaptadas a ambientes tropicais com limitação hídrica. Os impactos potenciais abrangem a melhoria da eficiência do uso de nitrogênio (EUN), redução de perdas de insumos e aplicação de conhecimentos técnicos ao setor sucroenergético. Embora não tenha havido envolvimento direto com comunidades externas, os resultados possuem potencial de beneficiar agricultores, técnicos agrícolas, pesquisadores e formuladores de políticas públicas por meio da disseminação científica. O território de impacto inclui regiões produtoras de cana-de-açúcar no Brasil e em países de clima tropical. Classifica-se nas áreas temáticas de ‘Meio Ambiente’ e ‘Tecnologia e Produção’ da Política Nacional de Extensão. Alinha-se aos Objetivos de Desenvolvimento Sustentável da ONU, especialmente o ODS 2 (Fome zero e agricultura sustentável) e o ODS 13 (Ação contra a mudança global do clima). O trabalho envolveu a participação direta de 3 docentes, 1 mestranda, e 1 estudante de graduação, promovendo formação científica e impacto acadêmico-tecnológico relevante.

SUMMARY

1. INTRODUCTION

Nitrogen (N) is an essential and mobile element crucial for plant growth, playing a fundamental role in the biosynthesis of amino acids, proteins, nucleic acids, and chlorophyll (MASCLAUX-DAUBRESSE et al., 2010; WANG et al., 2024). In sugarcane (*Saccharum* spp.), nitrogen is vital due to the crop's high biomass production and rapid growth cycle. The main nitrogen sources available to plants are nitrate (NO_3^-) and ammonium (NH_4^+), which are absorbed by the roots and assimilated through enzymatic pathways. Nitrate reductase (NR) first converts nitrate to nitrite (NO_2^-), which is subsequently reduced to ammonium by nitrite reductase (NiR). Ammonium is then assimilated into amino acids via the glutamine synthetase–glutamate synthase (GS–GOGAT) pathway, forming the key nitrogen carrier's glutamine and glutamate (MASCLAUX-DAUBRESSE, 2018). This assimilation process is closely connected to carbon metabolism, relying on carbon skeletons and energy generated by photosynthesis, photorespiration, and respiration (TEGEDER & MASCLAUX-DAUBRESSE, 2018).

Under optimal conditions, photosynthesis provides the energy and reducing equivalents (NADH, NADPH, and ferredoxin) required for nitrate reduction and ammonium assimilation (BUCHANAN et al., 2000). Carbon fixation through the Calvin cycle efficiently generates triose phosphates, which serve as precursors for the synthesis of carbohydrates, organic acids, and amino acids. The availability of these carbon compounds supports nitrate reduction and ammonium assimilation, ensuring an adequate nitrogen supply for plant growth.

Drought stress reduces nitrogen uptake by limiting root activity and enzymatic efficiency (KANT, 2018). This triggers several physiological adjustments aimed at maintaining carbon and nitrogen balance (MARCHIORI et al., 2017). As a result, plants rely more heavily on internal nitrogen remobilisation from older, senescing tissues to younger, actively growing organs. Sugarcane, a C4 crop, has relatively high nitrogen use efficiency (NUE) compared to C3 species, which enables it to optimise nitrogen remobilisation under water-limiting conditions (MASCLAUX-DAUBRESSE et al., 2001). However, nitrogen remobilisation is an energy-intensive process that requires coordination with carbon metabolism, particularly in synthesising and transporting amino acids (MARTINS et al., 2016).

During drought stress, stomatal closure restricts CO_2 uptake, reducing photosynthetic carbon fixation and limiting ATP and NADPH production. This energy imbalance directly affects nitrogen metabolism by decreasing nitrate reduction efficiency and restricting the assimilation of ammonium into amino acids (MASCLAUX-DAUBRESSE et al., 2010). Metabolic adjustments in response to drought stress involve not only nitrogen remobilisation

but also the accumulation of key carbon metabolites. Soluble sugars, such as sucrose and glucose, function as osmoprotectants and energy sources, helping to maintain cellular homeostasis under stress conditions (YEMM & WILLIS, 1954).

Proline, a stress-responsive amino acid, accumulates in drought-stressed plants and serves multiple functions. It acts as an osmoprotectant by stabilising proteins and membranes, reduces oxidative stress by scavenging reactive oxygen species (ROS), and serves as a nitrogen storage molecule that can be reutilised upon rehydration (BATES et al., 1973).

Despite the recognised role of nitrogen remobilisation in drought adaptation, little is known about how this process is regulated during rehydration. While previous studies have focused on nitrogen movement during stress periods, nitrogen redistribution following water availability remains unclear (FARIAS, 2017). Given that rehydration marks a shift from stress-induced metabolic constraints to recovery, nitrogen reassimilation is expected to be closely linked to carbon metabolism. Understanding how sugarcane rebalances its nitrogen and carbon economy during rehydration is critical for improving NUE and enhancing resilience under fluctuating water availability.

NUE refers to the plant's capacity to absorb, assimilate, remobilise, and utilise nitrogen efficiently to sustain growth and productivity. It is a key determinant of biomass accumulation and yield. High NUE enables plants to maintain metabolic efficiency under nitrogen-limiting conditions, ensuring optimal nitrogen allocation between source and sink tissues while minimising nitrogen losses. In crops like sugarcane, NUE is particularly important due to the high nitrogen demand required for rapid biomass production. Recent studies have shown that after an initial lag phase, sugarcane enters a period of rapid and linear nitrogen uptake, reflecting an increase in nitrogen demand (ZHAO et al., 2017). This makes nitrogen remobilisation a critical mechanism for sustaining growth under fluctuating water availability.

Understanding the dynamics of nitrogen remobilisation under drought and subsequent rehydration holds significant agronomic value for sugarcane cultivation. Recent studies have highlighted the role of optimised nitrogen management in improving nitrogen use efficiency (NUE), particularly under abiotic stress conditions (ALBERT et al., 2024; RU et al., 2024). Synchronising nitrogen fertiliser applications with key developmental stages or periods of high nitrogen demand, such as recovery following drought stress, can enhance nitrogen uptake and allocation efficiency (LI et al., 2016; ULLAH et al., 2023). Split nitrogen applications, in contrast to a single basal dose, have been shown to improve NUE by aligning external nitrogen supply with temporal crop requirements, reducing losses due to leaching and volatilisation (OLŠOVSKÁ et al., 2024; RU et al., 2024). Moreover, these strategies contribute to sustainable

agriculture by minimising environmental impacts such as nitrate leaching and greenhouse gas emissions (LI et al., 2024; LÓPEZ et al., 2023). Integrating knowledge of nitrogen remobilisation and carbon-nitrogen interactions into fertiliser management practices can therefore support sugarcane productivity and resilience under fluctuating water availability, offering both economic and environmental benefits (ALBERT et al., 2024; OLŠOVSKÁ et al., 2024).

In this study, we investigate the effects of drought stress and rehydration on nitrogen remobilisation in sugarcane (*Saccharum* spp. cv. CTC9001bt), with a specific focus on the interaction between nitrogen and carbon metabolism. We hypothesise that water availability significantly influences nitrogen remobilisation in sugarcane, with drought conditions promoting the redistribution of nitrogen from source tissues to sink tissues in coordination with carbon metabolism. By examining nitrogen fluxes through ^{15}N isotopic labelling and conducting biochemical analyses of key carbon metabolites, such as soluble sugars, starch, and proline, this research aims to elucidate the physiological mechanisms that enable sugarcane to maintain nitrogen and carbon metabolic balance under fluctuating water conditions. Furthermore, we propose that the modulation of key nitrogen metabolism enzymes, particularly glutamine synthetase and nitrate reductase, contributes to the efficient recycling and utilisation of nitrogen within the plant, thereby improving physiological nitrogen use efficiency (NUE) and supporting adaptation and recovery during drought and subsequent rehydration.

2. MATERIALS AND METHODS

2.1 Experimental conditions, plant materials, and treatments

The experiment was conducted in a greenhouse with controlled conditions. Average light (529 µmol m^{-2} s^{-1}), temperature (29.5 °C), and relative humidity (54%). Three-month-old sugarcane plants (variety CTC9001bt; Centro de Tecnologia Canavieira, Piracicaba, SP, Brazil) were transplanted into 10 L polyethylene pots. The substrate consisted of a 2:1 mixture of unwashed sand and clayey Oxisol, collected from the UFLA experimental station. The soil had a stable aggregate structure and low natural fertility, with a texture composed of 66% clay, 18% silt, and 16% sand. Chemical characterisation indicated an acidic pH (KCl) of 4.9, low base saturation (V = 15.7%), and high aluminium saturation (m = 23.8%). The cation exchange capacity (CEC) at pH 7.0 was 2.02 cmolc dm^{-3}, and the organic matter content was 1.73 dag kg^{-1}. Nutrient availability was limited, with phosphorus (10.91 mg dm^{-3}) and potassium (0.15 cmolc dm^{-3}) at low levels, and calcium and magnesium contents of 0.22 and 0.07 cmolc dm^{-3},

respectively. Soil amendments were applied following MALAVOLTA (2006) to correct nutrient deficiencies and support optimal plant development. Plants received nutrients at the following rates: nitrogen (100 mg N kg^{-1} soil), phosphorus (300 mg P kg^{-1} soil), sulphur (50 mg S kg^{-1} soil), potassium (150 mg K kg^{-1} soil), calcium (75 mg Ca kg^{-1} soil), and magnesium (15 mg Mg kg^{-1} soil). Micronutrients were supplied as follows: boron (0.81 mg B kg^{-1} soil), copper (1.50 mg Cu kg^{-1} soil), iron (1.50 mg Fe kg^{-1} soil), manganese (3.66 mg Mn kg^{-1} soil), molybdenum (0.15 mg Mo kg^{-1} soil), and zinc (5.00 mg Zn kg^{-1} soil).

Water holding capacity (WHC) was determined using standardised gravimetric methods involving soil saturation followed by free drainage, as described by ROBERTSON et al. (1999). Plants were subjected to two watering regimes: well-watered [WW, ~90% water holding capacity (WHC)] and water-deficient (WD, 20% of WHC) (Figure 1a) (BARBOSA et al., 2015). At 25 d after transplanting (DAT), the +1 leaf was labelled with ^{15}N-enriched ammonium sulfate (10 ^{15}N atom %) (Figure 2), following the method described by Putz et al. (2011), using 50 mg N $leaf^{-1}$, as described by ALTARUGIO (2023). Water deficit was initiated at 27 DAT, with soil moisture gradually decreasing until maximum stress (MS; 20% of WHC) at 38 DAT, followed by complete rehydration (R) at 45 DAT (Figure 1b). Physiological, biochemical, isotopic, and biomass assessments were performed at these critical points (MS and R). The experiment followed a randomised complete design, consisting of four replicates per treatment, and samples were taken twice: at maximum stress and after rehydration.

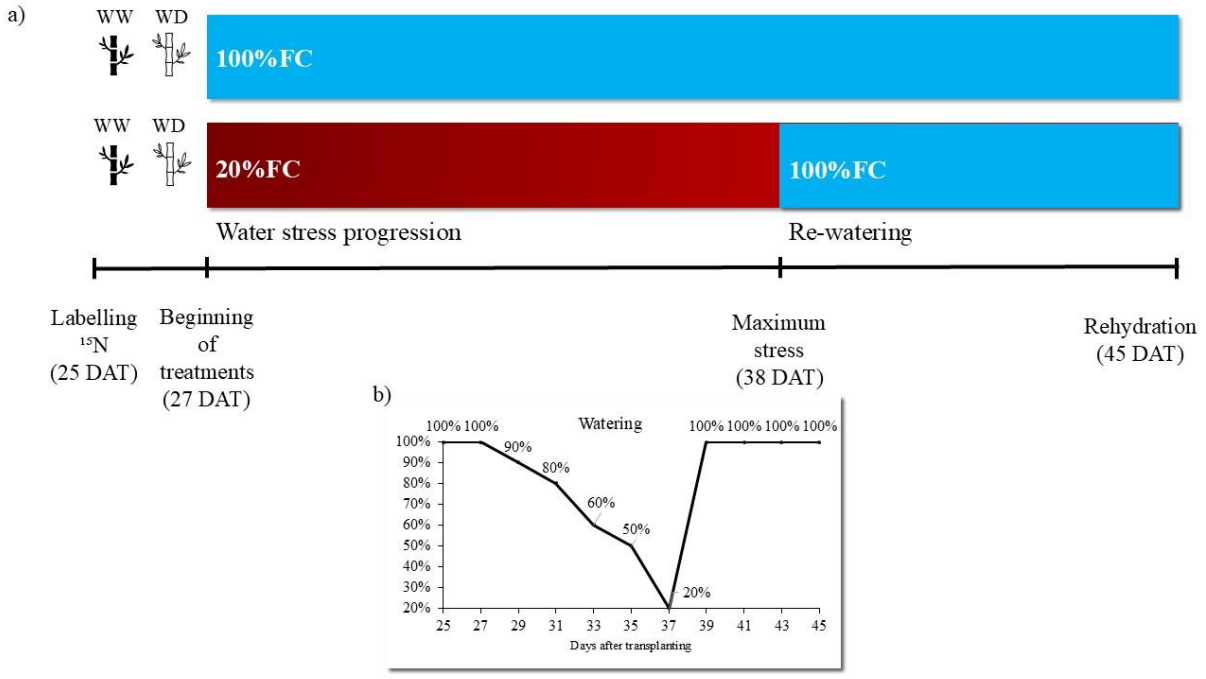

Figure 1. The experimental timeline and soil water availability. (a) Plants were subjected to two water regimes: well-watered [WW, ~90% water holding capacity (WHC)] and water-deficient (WD, 20% WHC). A water deficit was imposed 27 days after transplanting (DAT), maintained until maximum stress was reached at 38 DAT, and

followed by rehydration at 45 DAT. (b) Soil moisture under WD conditions reached 20% WHC before rehydration, while WW plants remained at ~90% WHC throughout.

2.2 Sampling

Sampling involved separating plant organs into roots, stalks, sheath leaves, ^{15}N-labelled leaves (including both blade and sheath), top leaves (those directly above the ^{15}N-labelled leaves), and all remaining leaves (Figure 2). Samples were subjected to dry mass, isotopic, and biochemical analyses (fresh and dry matter) to determine nitrogen content, distribution, and enzyme activity. Each treatment included four biological replicates (n = 4), and a representative portion of each organ was collected from each plant for analysis.

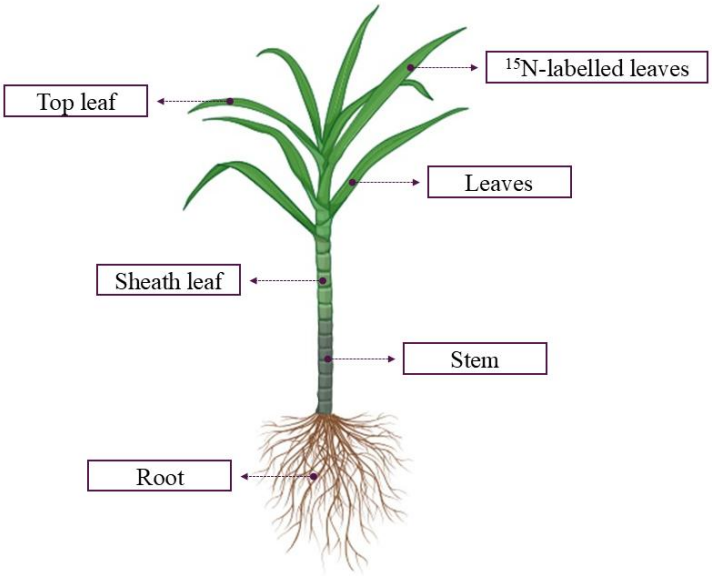

Figure 2. Schematic representation of the sugarcane plant shows the plant organ separation for sampling. Identified structures include roots, stalks, sheath leaves, ^{15}N-labelled leaves (blade and sheath), top leaf (blade and sheath directly above the labelled leaf), and remaining leaves. The +1 leaf was labelled with ^{15}N-enriched ammonium sulphate (50 mg N leaf^{-1}).

2.3 Biometric measurements

Plant height was measured as described by MAIA JÚNIOR *et al*. (2019). Dry mass was determined by oven-drying plant parts (roots, stalks, sheath leaves, 15N-labelled leaves, top leaves, and other leaves) at 65°C until a constant weight was achieved (BOSCHIERO et al., 2019). Relative growth rate (RGR) was calculated to assess biomass accumulation over time, using the equation:

$$Equation\ 1: RGR = \frac{\overline{lnW_2} - \overline{lnW_1}}{t_2 - t_1}$$

where W_1 and W_2 are the dry weights at times t_1 and t_2 (HOFFMANN & POORTER, 2002).

2.4 Leaf water potential (Ψw)

Leaf water potential (Ψw) was measured at predawn (pdΨ) and midday (mdΨ) using a Scholander pressure chamber on two sections of the second leaf above the one labelled with ^{15}N. The leaf was cut in half. In the basal section, measurements were taken within the first 15 cm from the base to the centre of the leaf. In the apical section, measurements were taken within 15 cm of the centre to the tip of the leaf. Measurements were performed on the 38th and 43rd DAT, corresponding to the maximum stress and rehydration phases, respectively (DOS SANTOS et al., 2015).

2.5 Chlorophyll a and b

Chlorophyll content was estimated non-destructively using a portable chlorophyll meter (Falker ClorofiLOG CFL 1030; Falker Agricultural Automation, Brazil). Measurements were taken from the topmost leaf, corresponding to the one immediately above the leaf labelled with ^{15}N. Each leaf was divided into three sections: the tip, the middle, and the base. In each section, chlorophyll measurements were taken at the midpoint of the area, without cutting or damaging the tissue. Values were recorded in Falker Chlorophyll Index (FCI) units.

2.6 Leaf gas exchange parameters

Two approaches were used to assess CO_2 assimilation rate (A) and related gas exchange parameters. A gas exchange analyser (SBA-5, PP Systems, Amesbury, MA, USA) measured A at the tip and base of the +1 leaf. The leaf was divided into two sections: in the basal section, measurements were taken within the first 15 cm from the base to the centre of the leaf; in the apical section, measurements were taken within 15 cm from the centre to the tip of the leaf. Measurements were performed on the same occasion as chlorophyll readings. Photosynthesis was calculated using the modified Mitchell equation (CHAVES et al., 2024; MITCHELL, 1992).

$$Equation\ 2: A = \frac{\frac{C_1 - C_2}{T_1 - T_2} * V * 1}{L} * T * p$$

where A is the CO_2 assimilation in terms of leaf area and time; C_1 and C_2 are the delta of CO_2 concentration µmol/mol; T_1 and T_2 are the delta of time (s); V is the total volume of the chamber (m^3); L is the leaf area (m^2); T is the temperature (K); and p is the pressure (MPa), with these two last units standardised to 273 K and 0.1013 MPa.

An infrared gas analyser (IRGA) (ADC BioScientific LCpro-SD, Geddings Rd, Hoddesdon, United Kingdom) was used to measure additional photosynthetic parameters along three different regions of the +1 leaf tip, middle, and base: each leaf was divided into three sections (base, middle, and tip), and measurements were taken at the midpoint of each section without damaging the tissue. It recorded the following parameters: *E*, transpiration rate (mmol H_2O m^{-2} s^{-1}); *gs*, stomatal conductance (mol H_2O m^{-2} s^{-1}); and *A*, net CO_2 assimilation rate (µmol CO_2 m^{-2} s^{-1}). Instantaneous water use efficiency (WUEi) was calculated as the ratio of *A* to *E*. Measurements were conducted on six dates throughout the experiment: 11, 14, 19 (maximum stress), 24, 27, and 28 November 2024 (complete rehydration). These evaluations were performed across all three regions of the +1 leaf (tip, middle, and base), in alignment with the gas exchange assessments. Reference CO_2 concentration was derived from the environment, as the system continuously drew atmospheric air through the chamber. Photosynthetically active radiation (PAR) was maintained at 1000 µmol m^{-2} s^{-1} inside the leaf chamber, following GRAÇA et al. (2010), to simulate optimal light conditions for sugarcane photosynthesis.

2.7 Isotopic analysis

Sugarcane samples were dried at 65 °C for 48 h and ground in a Wiley mill to pass through a 0.5 mm mesh, following BOSCHIERO et al. (2019). Composite samples were prepared from six plant parts: roots, stalks, sheath leaves, 15N-labelled leaves, top leaves, and other leaves. For isotopic analysis, 0.2 mg of each composite sample was used for carbon analysis, while 5 mg was used for nitrogen analysis. Measurements were performed using an elemental analyser (Flash 2000, ThermoFisher Scientific Corp.) connected to an isotope ratio mass spectrometer (EA-IRMS; Delta V Advantage, ThermoFisher Scientific Corp.), according to BARRIE and PROSSER (1996). The accumulation of ^{15}N in plants and the degree of enrichment (mg pot-¹) were calculated using the isotopic abundance of the labelled material (^{15}N-ammonium sulphate) and the natural abundance of ^{15}N (0.3663% ^{15}N atoms) (IAEA, 2001).

The partitioning of ^{15}N among plant parts (roots, stalks, sheath leaves, 15N-labelled leaves, top leaves, and leaves) was calculated following the procedures described by MARMAGNE et al., (2024). The percentage of ^{15}N in each tissue relative to the whole plant was determined by:

$$Equation\ 3: \%^{15}Npartition = \left(\frac{At\%^{15}N_{tissue} - 0.3663}{At\%^{15}N_{totalplant} - 0.3663}\right) * 100$$

where $At\%^{15}N_{tissue}$ represents the ^{15}N atom percent in a plant organ; and $At\%^{15}N_{totalplant}$ represents the total atom percent of ^{15}N in the whole plant. The value 0.3663% corresponds to the ^{15}N natural abundance and was used as a baseline correction.

To quantify the total nitrogen content (g) in each plant tissue, the following equation was used:

$$Equation\ 4: Totalnitrogen(g) = TotalN(\%) * 10 * Drybiomass(g)$$

where *Total N (%)* represents the nitrogen concentration in the dry biomass, obtained through EA-IRMS analysis; and *Dry biomass (g)* is the mass of dried plant material. The factor of 10 is used to convert total nitrogen content from percentage (%) to milligrams per gram (mg/g) of dry biomass. CASTRO et al., (2023).

2.8 Nitrogen use efficiency index

The Partition Index (PI), Nitrogen Harvest Index (NHI), and Nitrogen Use Efficiency (NUE) were calculated based on biomass and nitrogen content in plant tissues, according to the approach defined by HAVÉ et al., (2017). This method was selected due to its suitability for tissue-specific analysis, allowing for the assessment of nitrogen partitioning and remobilisation across different leaf sections. The equations used are provided below:

$$Equation\ 5: Partition\ index(PI) = \frac{Biomassofspecifictissue(g)}{Totalplantbiomass(g)}$$

$$Equation\ 6: Nitrogenharvestindex(NHI) = \frac{Nitrogencontentofspecifictissue(g)}{Totalplantnitrogencontent(g)}$$

$$Equation\ 7: Nitrogenuseefficiency(NUE) = \frac{PI}{NHI}$$

2.9 Carbon analysis

To determine the total carbon content and carbon isotope discrimination ($\Delta^{13}C$), sugarcane samples were dried at 55°C for 48 h and then ground to a 25-mesh size. Carbon analysis was performed using the mentioned IRMS to measure total C concentration (%) and $\delta^{13}C$ values (BOSCHIERO et al. 2019). The total carbon content in each plant tissue was calculated as:

$$Equation\ 8: Totalcarbon(g) = \frac{TotalC(\%)}{100} * Drybiomass(g)$$

Carbon isotope discrimination ($\Delta^{13}C$) was determined using the following:

$$Equation\ 9: \Delta^{13}C(‰) = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \frac{\delta^{13}C_p}{1000}}$$

where $\delta^{13}C_a$ is the atmospheric CO_2 isotopic composition (-8.0‰) consistent with standard values reported in the literature (KÖLLN et al., 2016) and $\delta^{13}C_p$ is the isotopic composition of the sugarcane leaves.

2.10 Nitrogen metabolism enzymes: extraction and analysis

All enzyme activity data were normalised per gram of fresh weight (FW) to enable comparison across treatments. Fresh tissue samples (0.3 g) were macerated in liquid nitrogen with 10% PVPP and homogenised in 5 mL extraction buffer (100 mM potassium phosphate pH 7.5, 5 mM EDTA, 2 mM DTT, 1 mM PMSF). The homogenate was centrifuged at 16,000 g for 20 min at 4°C, and the resulting supernatant was used for enzyme assays. The glutamine synthetase (GS, EC 6.3.1.2) activity was determined following the method of RATAJCZAK et al., (1981). Enzyme extract (75 µL) was mixed with 175 µL reaction buffer (500 mM Tris-HCl pH 7.5, 100 mM mercaptoethanol, $MgSO_4$, 400 mM NH_2OHCl, 500 mM monosodium glutamate, 100 mM ATP), incubated at 30°C for 30 min, and the reaction was stopped with a $FeCl_3$-HCl-TCA solution. Following centrifugation, the absorbance was measured at 540 nm to quantify GS activity, expressed as µmol of γ-glutamyl hydroxamate (GHA) per gram of fresh weight per minute. For glutamate synthase (GOGAT, EC 1.4.1.14) activity, measurements were made according to GROAT & VANCE (1981). Enzyme extract aliquots (14 µL) were mixed with incubation buffer (83 mM TRIS-HCl, pH 7.8, 117 mM 2-oxoglutarate, and 117 mM L-glutamine), incubated at 35°C for 5 min, and then β-NADH was added. Absorbance was recorded at 340 nm every minute for 10 min. Nitrate reductase (NR, EC 1.7.1.1) activity was assessed using the protocol described by BERGES & HARRISON (1995). Enzyme extract samples (14 µL) were mixed with potassium phosphate buffer (100 mM pH 7.5, potassium nitrate), incubated at 30°C for 3 min, then β-NADH was added, and absorbance at 340 nm was recorded every min for 10 min.

2.11 Ammonium quantification

The extract for NH_4^+ determination was prepared from 60 mg of dry mass, to which 1.5 mL of ultrapure water was added. The samples were incubated at 80°C in a water bath for 10

minutes, followed by centrifugation at 4000 rpm for 20 min. The supernatant was kept on ice for quantification. The extraction was based on MCCULLOUGH (1967). The samples were then placed in a water bath at 37°C for 60 min. After the samples reached room temperature, readings were taken at 625 nm. Ammonium concentrations were expressed as micrograms (µg) of NH_4^+ per gram of dry mass (DM).

2.12 Nitrate quantification

The extract for NO_3- determination was prepared by adding 2 mL of deionised water to 20 mg of dry mass. Samples were incubated at 45°C in a water bath for 1 h and then centrifuged at 5000 g for 15 min. The resulting supernatant was collected for analysis. The extraction procedure followed the method described by CATALDO et al. (1975). Quantification was performed using a colorimetric reaction with salicylic acid in a sulfuric acid medium. A solution of 5% salicylic acid in concentrated sulphuric acid was prepared and stored at -4°C until use. A nitrate standard solution (100 µg mL^{-1}) was prepared by dissolving 0.041 g of KNO_3 in 250 mL of deionised water. A standard curve was established by mixing various concentrations of KNO_3 with the reaction mix and incubating at room temperature for 20 min. After incubation, 2 M NaOH was slowly added to stabilise the reaction. Absorbance was measured at 410 nm after the samples reached room temperature. Nitrate concentrations were expressed as micrograms (µg) of NO_3^- per gram of dry mass (DM).

2.13 Carbon metabolites

2.13.1 Extraction

Briefly, 0.03 g of dry tissue was placed in 2.0 mL centrifuge tubes with 350 µL of 100% ethanol. The mixture was vortexed, incubated at 70-75°C for 20 min, and then centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant (350 µL) was transferred to a new tube, while the pellet underwent two additional extractions with 80% and 50% ethanol, respectively, under the same conditions. The final supernatant was used for quantifying pigments, total soluble sugars, starch, sucrose, proteins, amino acids, reducing sugars, and proline. All metabolite concentrations were expressed as micrograms (µg) per gram of dry mass (DM).

2.13.2 Pigment quantification

In a microplate, 170 µL of 100% ethanol was added to the first two wells (for the blank), and 50 µL of supernatant with 120 µL of ethanol was added to the remaining wells in duplicate.

Absorbance was measured at 647 nm for chlorophyll a, 623 nm for chlorophyll b, and 450 nm for carotenoids (LICHTENTHALER & BUSCHMANN, 2001).

2.13.3 Total soluble sugars, starch, and sucrose

Based on YEMM and WILLIS (1954), 40 mg of anthrone reagent was dissolved in 1 mL of distilled water and mixed with 20 mL of H_2SO_4. The supernatant from the samples substituted glucose for the glucose in the glucose standard curve. Absorbance was read at 620 nm.

2.13.4 Proteins

Following BRADFORD (1976), 400 µL of 0.1 M NaOH was used to dissolve the pellet, which was then incubated at 75°C for 1 h and subsequently cooled on ice. Coomassie Blue was dissolved in ethanol and phosphoric acid, and the mixture was left overnight before being filtered. A BSA standard curve was prepared, using the sample extract as a substitute for BSA for analysis. Absorbance was read at 595 nm.

2.13.5 Amino acids

The reagent, comprising sodium citrate buffer (0.2M, pH 5.0), 5% ninhydrin, and 2% KCN, was mixed with glycine and water and then heated at 100°C for 20 min. The sample was substituted with glycine, and a glycine standard curve was then analysed. Absorbance was read at 570 nm (YEMM *et al*., 1955).

2.13.6 Reducing sugar

The DNS reagent was prepared by mixing NaOH, DNS, and Rochelle salt in distilled water, resulting in an unstable mixture sensitive to light and CO_2. A standard glucose curve was prepared, with glucose replaced by the sample for analysis. Absorbance was read at 540 nm (MILLER, 1959).

2.13.7 Proline

Two solutions were prepared: a ninhydrin acid solution (2.5 g of ninhydrin in 40 mL of 6M phosphoric acid and acetic acid) and a 500 µg mL^{-1} proline standard solution. The proline solution was further diluted to 100 µg mL^{-1}, and a standard curve was generated. The samples were treated similarly, with absorbance measured at 520 nm (BATES *et al*., 1973).

2.14 Nutrient content

After acid digestion, nutrient content in sugarcane tissues was determined using inductively coupled plasma optical emission spectrometry (ICP-OES). Dried, ground plant tissue samples (500 mg) were digested with 5 mL of concentrated nitric acid (HNO_3) and 1 mL of perchloric acid ($HClO_4$) until complete oxidation was achieved. After cooling, the digested solution was diluted to 50 mL with ultrapure water and stored in polyethylene tubes before analysis. The analysis used ICP-OES (Blue, SPECTRO Analytical Instruments, Mahwah, New Jersey, USA). Calibration curves were prepared using certified standard solutions, and nutrient concentrations were expressed as g kg^{-1} for macronutrients and mg kg^{-1} dry mass for micronutrients (SILVA, 2009). Analytical accuracy was verified using the certified reference material NIST 1515 (apple leaf), with elemental recoveries of 92% for phosphorus, 93% for potassium, 94% for magnesium, 97% for calcium, 98% for manganese, 100% for iron, 101% for zinc, and 102% for copper.

2.15 Data analysis

All statistical analyses were performed in R (version 4.3.1). Descriptive statistics were used to analyse the isotopic data. The remaining data were analysed using ANOVA to assess treatment effects. The Shapiro-Wilk test was applied to the residuals of the models to check for normality, while Bartlett's test assessed residual variance homogeneity. When assumptions of normality were not met, data transformations (log, square root, or Box-Cox) were applied before analysis. When significant differences were detected, Tukey's test was applied for pairwise comparisons. Statistical significance ($p < 0.05$) was indicated with asterisks (*) in the plots. Principal component analysis (PCA) was performed using the FactoMineR package (LÊ et al., 2008), with the factoextra (KASSAMBARA & MUNDT, 2020) and ggplot2 (WICKHAM, 2016) packages for data visualisation and biplot construction. PCA included physiological, biochemical, isotopic, enzymatic, and nutritional traits, such as gas exchange (A, gs, E), pigments (chlorophyll a, b, carotenoids), total nitrogen, ^{15}N partitioning, NUE, $\delta^{13}C$, C:N ratio, GS, NR, GOGAT, ammonium, nitrate, proline, soluble sugars, starch, sucrose, proteins, amino acids, and macro and micronutrients (P, K, Ca, Mg, S, Cu, Fe, Mn, Zn).

3. RESULTS

3.1 Biometric traits

3.1.1 Plant height

At MS, plant height declined under WD (145 cm) compared to WW plants (158 cm) (Figure 3). Despite rehydration, WD plants (mean = 147 cm) did not fully recover to the height of WW plants (mean = 160 cm), indicating limited recovery following the WD.

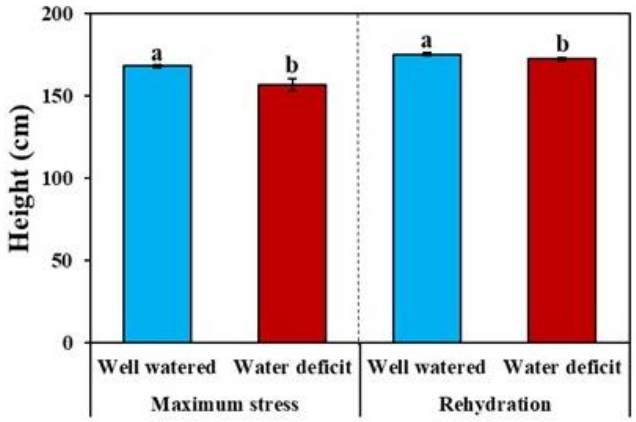

Figure 3. Plant height (cm) under well-watered (WW, ~90% of WHC) and water-deficient (WD, 20% of WHC) conditions at maximum stress (38 DAT) and after rehydration (43 DAT). Data are mean (n = 4) ± SEM. Different letters indicate significant differences between treatments ($p \leq 0.05$, Tukey's test).

3.1.2 Biomass

Biomass under MS and WD plants showed reduced biomass in stalks, leaves, and top leaves compared to WW plants, although root and leaf biomass remained similar between treatments (Figure 4). After rehydration (R), WD plants showed reduced biomass in roots, sheath leaves, ^{15}N-labelled leaves, and top leaves compared to WW plants.

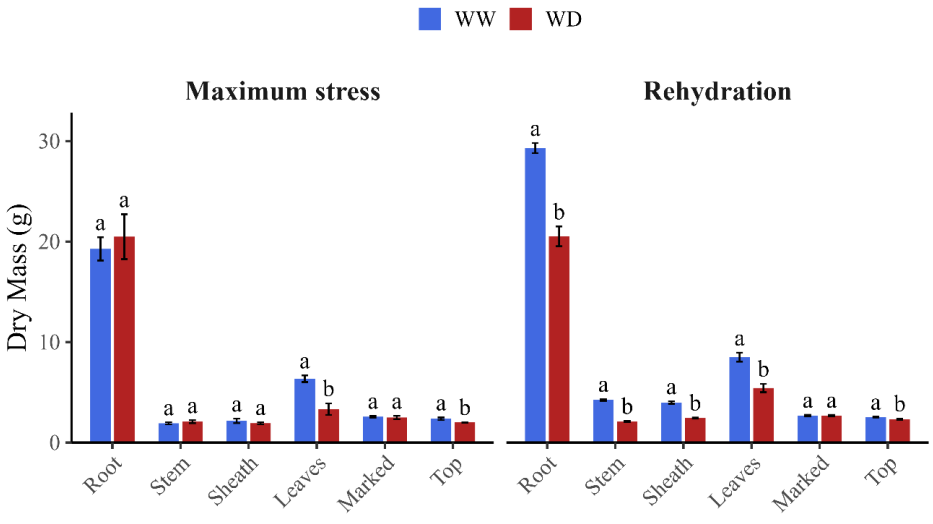

Figure 4. Dry mass (g) of plant organs under well-watered (WW, ~90% of WHC) and water-deficient (WD, 20% of WHC) conditions at maximum stress (38 DAT) and after rehydration (43 DAT). Data are mean (n = 4) ± SEM.

Different letters indicate significant differences between treatments ($p \leq 0.05$, Tukey's test). Biomass was measured in roots, stalks, sheath leaves, leaves, 15N-labelled leaves, and top leaves.

3.1.3 Relative growth rate

Relative growth rates differed between organs and water regimes during the recovery phase (Figure 5). In WW plants, the highest RGR values were observed in sheath leaves (0.090 g g^{-1} d^{-1}), stalks (0.082 g g^{-1} d^{-1}), and roots (0.058 g g^{-1} d^{-1}). In WD plants, leaves showed the highest RGR (0.067 g g^{-1} d^{-1}), followed by sheath leaves (0.034 g g^{-1} d^{-1}) and top leaves (0.032 g g^{-1} d^{-1}). Negative RGR values were recorded in stalks of WD plants (-0.005 g g^{-1} d^{-1}) and top leaves of WW plants (-0.035 g g^{-1} d^{-1}). ^{15}N-labelled leaves exhibited the lowest RGR in both treatments.

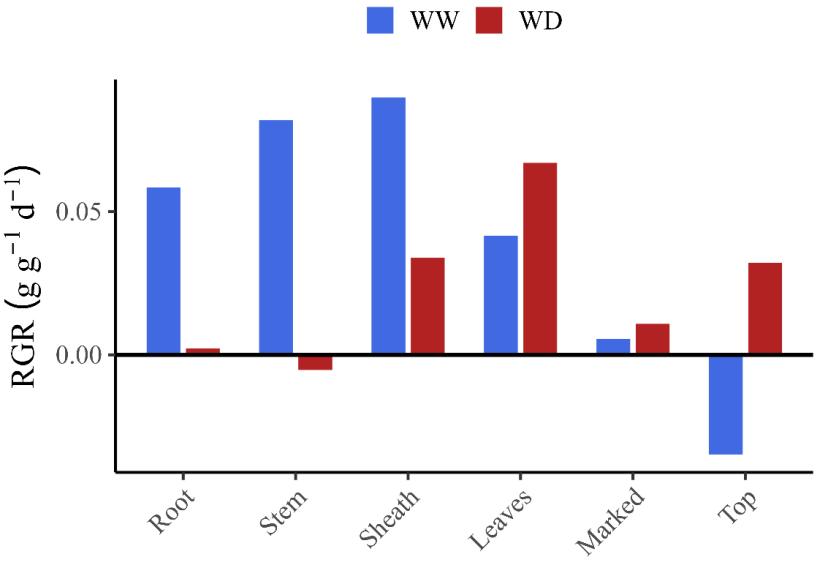

Figure 5. Relative growth rate (RGR; g g^{-1} d^{-1}) of plant organs under water deficit (WD) and well-watered (WW) conditions. RGR was calculated between maximum stress (MS, 38DAT) and rehydration (R, 43DAT) for roots, stalks, sheath leaves, leaves, 15N-labelled leaves (Marked), and top leaves. Statistical comparisons were not applied because RGR was obtained from the difference in the mean of the natural logarithm-transformed dry weights at two time points, resulting in a single value per treatment and organ.

3.2 Water relations

3.2.1 Leaf water potential (Ψw)

At MS, predawn and midday Ψw were more negative in WD plants compared to WW, indicating substantial water stress (Figure 6). Following rehydration, significant differences persisted at predawn in both leaf sections. Midday Ψw was more negative at the tip in WW plants, and at the base in WD plants, indicating a partial recovery.

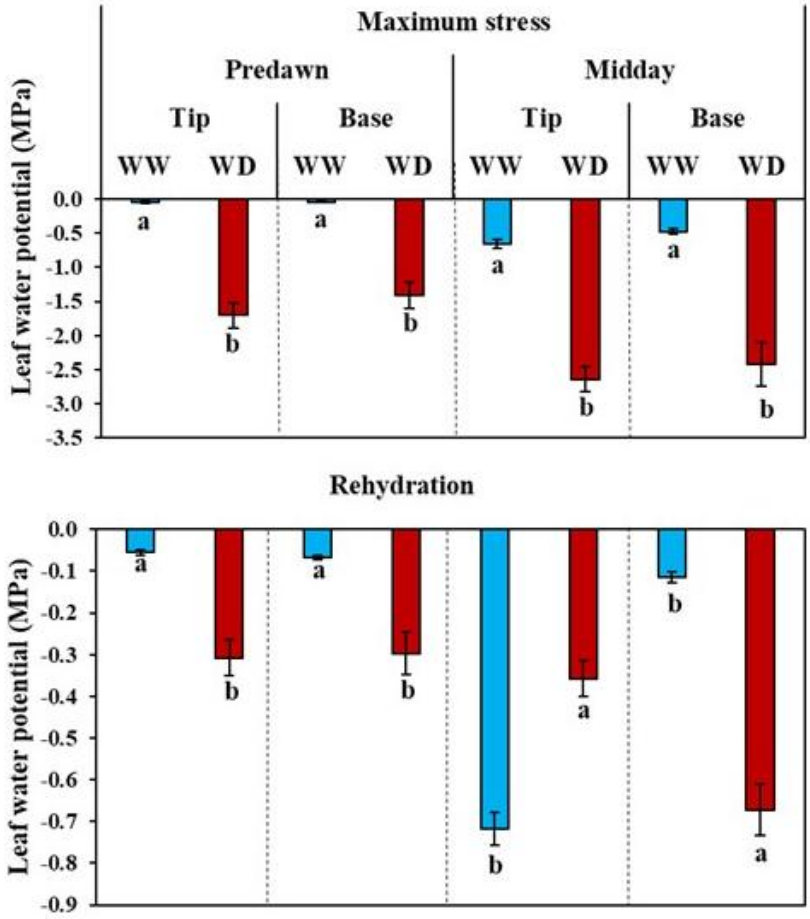

Figure 6. Leaf water potential at the tip and base of the +1 leaf under well-watered (WW, ~90% WHC) and water-deficient (WD, 20% WHC) conditions at predawn and midday during maximum stress (MS, 38DAT) and rehydration (R, 43DAT). Data are means (n=4). Different letters indicate significant differences ($p \leq 0.05$, Tukey's test).

3.2.2 Transpiration (*E*)

On 11 November, *E* was similar between WW and WD plants. However, as the water deficit progressed, differences began to emerge. By 14 November, transpiration in WD plants had already declined compared to WW. On 19 November, during maximum stress, the difference became more pronounced, with WD plants exhibiting the lowest transpiration rates (1.35 mmol m^{-2} s^{-1} in the middle and 1.05 mmol m^{-2} s^{-1} at the tip) as stomatal closure intensified to minimise water loss. Meanwhile, WW plants maintained higher transpiration (2.46 mmol m^{-2} s^{-1} at the middle and 1.70 mmol m^{-2} s^{-1} at the tip), indicating sustained water availability and stomatal opening. Between 19 and 27 November, transpiration remained lower in WD plants. On 28 November (rehydration), WW plants exhibited an increase in transpiration, exceeding 4.0 mmol m^{-2} s^{-1}, while WD plants showed only partial recovery.

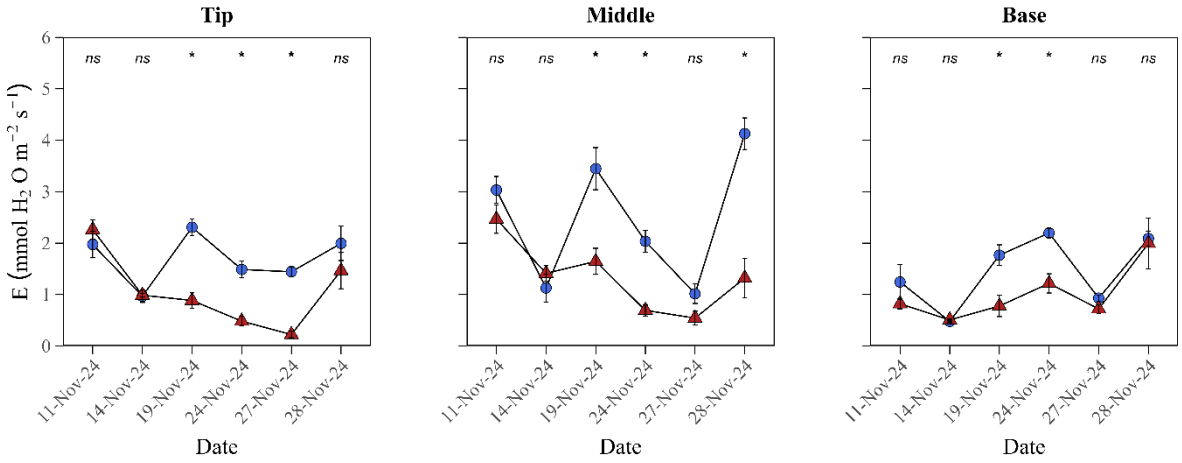

Figure 7. Transpiration rate (E) (mmol H_2O m^{-2} s^{-1}) over time in well-watered (WW) and water-deficient (WD) sugarcane plants. Data are presented for three leaf sections at six-time points: November 11, 2024, November 14, 2024, November 19, 2024, November 24, 2024, November 27, 2024, and November 28, 2024. Maximum stress (MS) occurred on 19 November 2024, while full rehydration (R) was completed by 28 November 2024. Blue circle markers indicate WW plants: (●). WD plants are shown with red triangle markers: (▲). Black lines connect the data points for each treatment and leaf section. Significant differences between WW and WD treatments are denoted by asterisks (*, $p < 0.05$). Error bars represent the standard error of the mean (SEM).

3.3.3 Stomatal conductance (*gs*)

On 11 November 2024, *gs* was similar between WW and WD plants. By 14 November, *gs* began to decline in WD plants. On 19 November (MS), significant reductions were observed in WD plants, particularly in the middle and base sections of the leaf, indicating restricted stomatal opening. The lowest values in WD plants were recorded at this time point (0.06 mol m^{-2} s^{-1} at the middle, 0.05 mol m^{-2} s^{-1} at the tip, and 0.04 mol m^{-2} s^{-1} at the base), while WW plants maintained higher conductance (0.10 mol m^{-2} s^{-1} at the middle and 0.06 mol m^{-2} s^{-1} at the tip). Between 19 and 27 November, *gs* remained lower in WD plants, with significant differences observed on 24 and 27 November. On 28 November (R), WW plants exhibited higher *gs* values, while WD plants showed only partial recovery, remaining lower than WW.

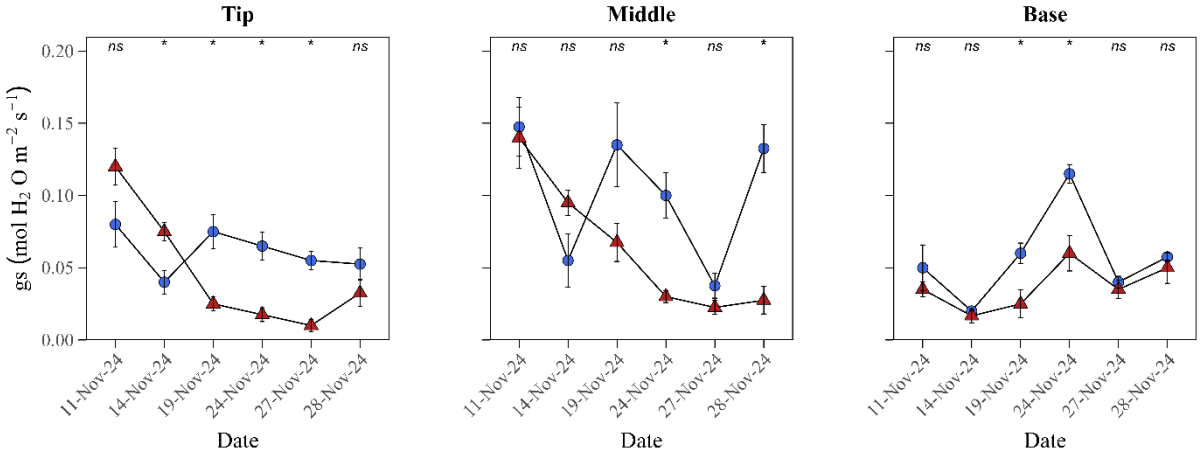

Figure 8. Stomatal conductance (gs) (mol H_2O m^{-2} s^{-1}) over time in well-watered (WW) and water-deficient (WD) sugarcane plants. Data are presented for three leaf sections at six-time points: November 11, 2024, November 14,

2024, November 19, 2024, November 24, 2024, November 27, 2024, and November 28, 2024. Maximum stress (MS) occurred on 19 November 2024, while full rehydration (R) was completed by 28 November 2024. Blue circle markers indicate WW plants: (●). WD plants are shown with red triangle markers: (▲). Black lines connect the data points for each treatment and leaf section. Significant differences between WW and WD treatments are denoted by asterisks (*, $p < 0.05$). Error bars represent the standard error of the mean (SEM).

3.4 Photosynthetic performance

3.4.1 Photosynthesis in maximum stress and rehydration

At MS, WD plants exhibited reduced *A* at the leaf base compared to WW plants, while the leaf tip showed stable rates across treatments (Figure 9). After rehydration, photosynthesis fully recovered at the base and remained stable at the leaf tip in both treatments.

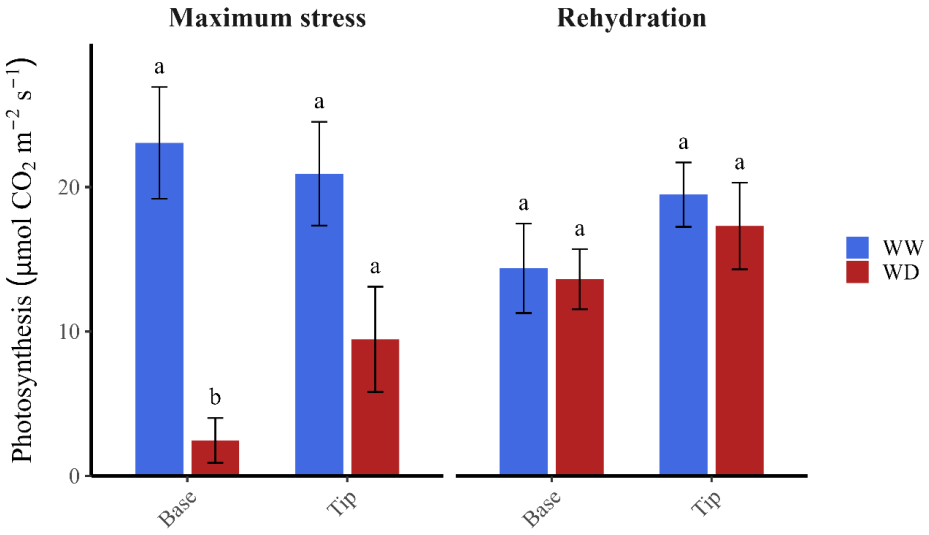

Figure 9. Photosynthesis at the base and tip of the +1 leaf under well-watered (WW) and water-deficient (WD) conditions during Maximum Stress (MS) and Rehydration (R). Bars represent mean ± SEM. Different letters indicate significant differences between treatments ($p < 0.05$, Tukey's HSD test).

3.4.2 Photosynthesis over time

On 11 November, photosynthetic rates were similar between WW and WD plants. On 19 November (MS), photosynthetic rates in WD plants reached their lowest values (4.17 µmol m^{-2} s^{-1} at the base, 3.95 µmol m^{-2} s^{-1} at the middle, and 5.73 µmol m^{-2} s^{-1} at the tip), lower than in WW plants, which maintained higher rates (5.20 µmol m^{-2} s^{-1} at the base, 12.01 µmol m^{-2} s^{-1} at the middle, and 6.16 µmol m^{-2} s^{-1} at the tip). Between 19 and 27 November, photosynthesis remained lower in WD plants, with significant differences continuing on 24 and 27 November. On 28 November (R), WW plants showed an increase in photosynthetic rates. In contrast, WD plants exhibited only partial recovery, remaining lower than WW, showing a progressive decline in photosynthesis under water deficit, with significant reductions during stress and an incomplete recovery following rehydration.

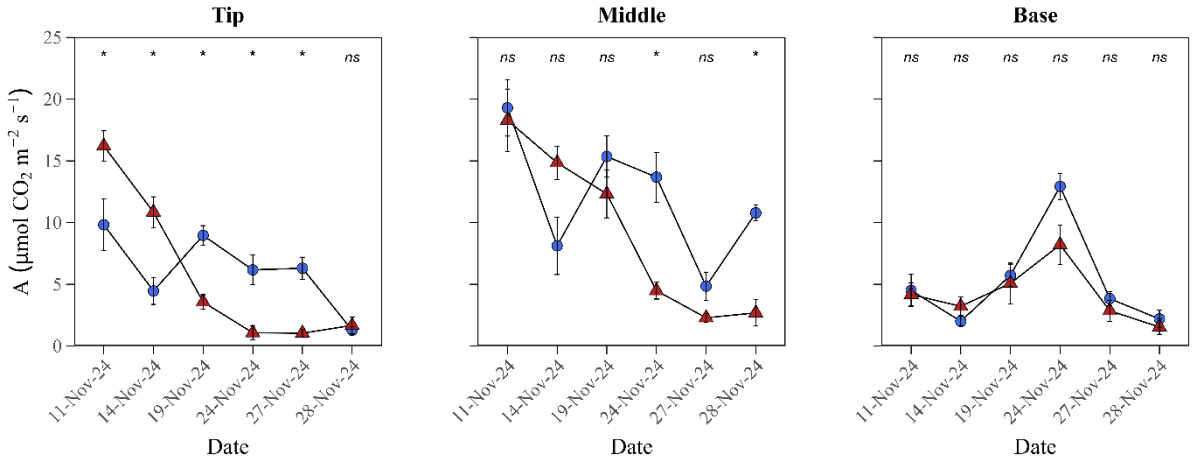

Figure 10. The photosynthesis (A) (µmol CO_2 m^{-2} s^{-1}) was measured over time in well-watered (WW) and water-deficient (WD) sugarcane plants. Data are presented for three leaf sections at six time points: November 11, 2024, November 14, 2024, November 19, 2024, November 24, 2024, November 27, 2024, and November 28, 2024. Maximum stress (MS) occurred on November 19, 2024, while complete rehydration (R) was reached by November 28, 2024. Blue circle markers indicate WW plants: (●). WD plants are shown with red triangle markers: (▲). Black lines connect the data points for each treatment and leaf section. Significant differences between WW and WD treatments are marked with asterisks (*, $p < 0.05$). Error bars represent the standard error of the mean (SEM).

3.4.3 Instantaneous water use efficiency

At the leaf tip and middle, WUEi was higher in WD plants during the early stages (11 and 14 November) compared to WW plants. However, WUEi declined in WD plants by 19 November (MS), remaining lower or like WW plants until rehydration. At the base, WUEi differences between treatments were minimal, with an increase in WD plants on 19 November. After rehydration on 28 November, WUEi values converged across treatments in all leaf regions.

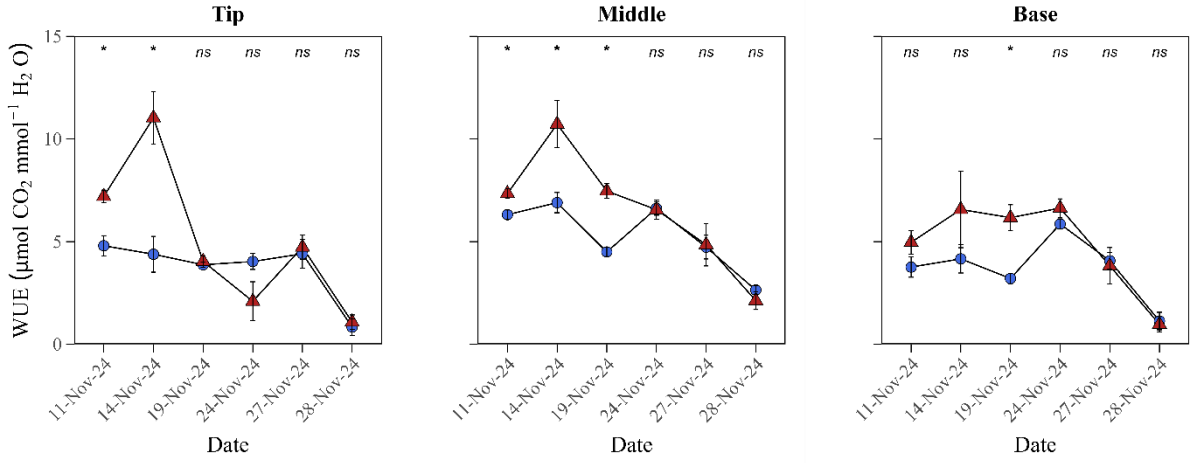

Figure 11. The water use efficiency (WUE) (µmol CO_2 mmol-^{1} H_2O) was measured over time in well-watered (WW) and water-deficient (WD) sugarcane plants. Data are presented for three leaf sections at six time points: November 11, 2024, November 14, 2024, November 19, 2024, November 24, 2024, November 27, 2024, and November 28, 2024. Maximum stress (MS) occurred on November 19, 2024, while complete rehydration (R) was reached by November 28, 2024. Blue circle markers indicate WW plants: (●). WD plants are shown with red triangle markers: (▲). Black lines connect the data points for each treatment and leaf section. Significant

differences between WW and WD treatments are marked with asterisks (*, $p < 0.05$). Error bars represent the standard error of the mean (SEM).

3.4.5 Chlorophyll a and b over time

During MS, WD plants exhibited a consistent reduction in chlorophyll a and b content across all leaf sections, with the leaf tip showing the most pronounced decline (Figure 12). In contrast, WW plants maintained relatively stable chlorophyll levels, particularly in the leaf base and midsections. After rehydration, WD plants showed a recovery in chlorophyll a and b content, particularly in the leaf base, where levels nearly approached those of WW plants. However, the leaf tip remained lower in chlorophyll content compared to WW plants.

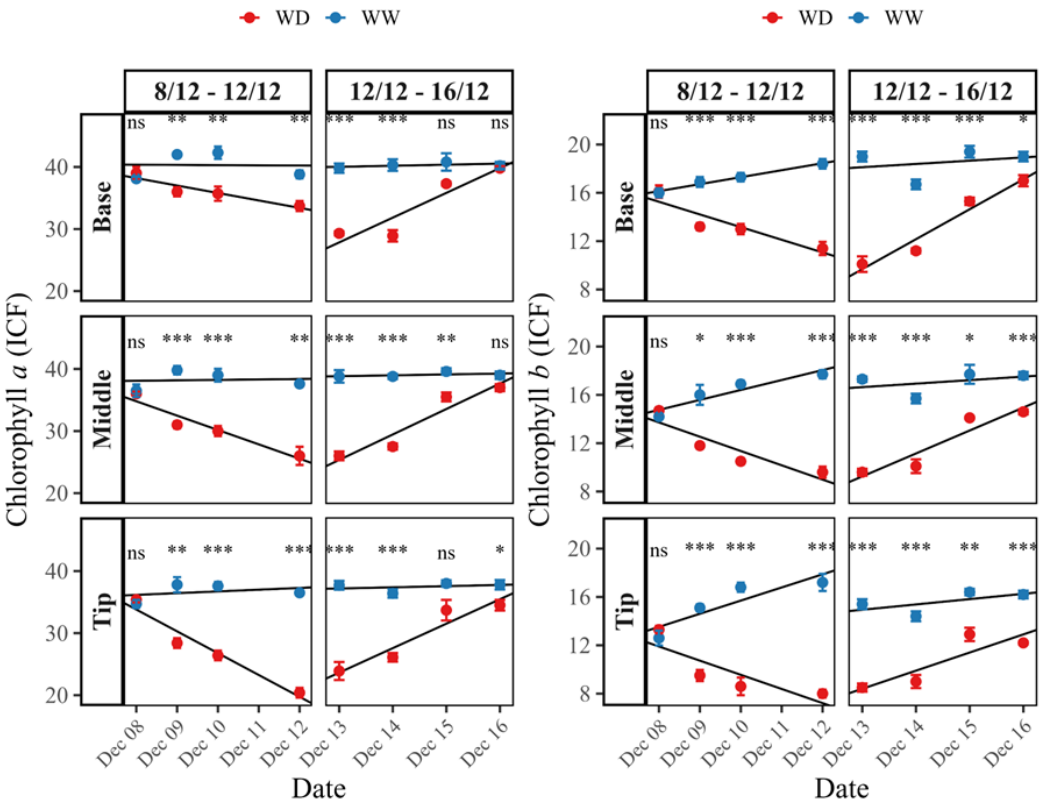

Figure 12. Chlorophyll a and b content under well-watered (WW, ~90% WHC) and water-deficient (WD, 20% WHC) conditions. Measurements were taken in two time intervals: 8/12-12/12 (Maximum stress) and 12/12-16/12 (Rehydration) across three leaf sections (Base, Middle, and Tip) of the +1 leaf. Bars represent the mean ± SEM (n = 4). Red circles: WD; blue circles: WW. Black lines show linear regression trends. Significant differences between WW and WD treatments are marked with asterisks (*, $p < 0.05$). The regression equations corresponding to each line are presented in Annex A.

3.4.6 Chlorophyll a and b, and carotenoids at maximum stress and rehydration

At MS, chlorophyll a content was reduced in WD plants compared to WW plants, particularly in the top leaves and ^{15}N-labelled leaves, where reductions reached 0.21-0.28 mg

g^{-1} FW under WD, whilst WW plants maintained values between 1.68-0.50 mg g^{-1} FW (Table 1). A similar trend was observed in the sheath leaves, where WD plants showed higher chlorophyll a levels (2.16 mg g^{-1} FW) than WW plants (1.39 mg g^{-1} FW). No significant differences were detected in the stalks between treatments. Following rehydration, chlorophyll a levels partially recovered across most tissues, with WW plants maintaining higher values in the top leaves (2.22 mg g^{-1} FW) compared to WD plants (1.59 mg g^{-1} FW). In 15N-labelled leaves, WD plants showed slight recovery (0.88 mg g^{-1} FW) relative to WW plants (2.30 mg g^{-1} FW). No significant differences were found between treatments in the stalks and sheath leaves following R. For chlorophyll b, MS led to higher concentrations in WD plants compared to WW plants, particularly in the stalks (1.22 vs. 0.83 mg g^{-1} FW) and sheath leaves (1.31 vs. 0.98 mg g^{-1} FW). However, no differences were detected between treatments in the leaves, 15N-labelled leaves, or top leaves. After R, chlorophyll b remained elevated in WD plants in the sheath leaves (0.94 mg g^{-1} FW) compared to WW (0.65 mg g^{-1} FW), whilst no differences were observed in other tissues, showing a partial recovery of pigment stability. Regarding carotenoids, WD plants exhibited higher contents in the top leaves under MS (0.36 mg g^{-1} FW) compared to WW plants (0.17 mg g^{-1} FW). No differences were observed in the leaves and 15N-labelled leaves between treatments. After rehydration, carotenoid levels had no significant differences, including in top leaves, where both WW and WD plants presented similar concentrations (0.17 mg g^{-1} FW).

Table 1. Chlorophyll a, chlorophyll b, and carotenoid contents (mg g^{-1} FW) in different plant tissues under to well-watered (WW, ~90%WHC) and water-deficit (WD, 20%WHC) conditions at Maximum Stress (MS) and after Rehydration (R). Data are mean ± SEM (n = 4). Different lowercase letters within the same tissue and time point indicate significant differences between treatments ($p \leq 0.05$, Tukey's test). A dash (-) indicates unavailable data.

Part/ time/ treatments	Chlorophyll a	Chlorophyll b	Carotenoids
STALK			
Maximum stress			
WW	1.04±0.31[a]	0.83±0.08[b]	-
WD	1.12±0.11[a]	1.22±0.05[a]	-
Rehydration			
WW	0.30±0.06[a]	0.63±0.08[a]	-
WD	0.31±0.05[a]	0.53±0.01[a]	-
SHEATH			
Maximum stress			
WW	1.39±0.67[a]	0.98±0.09[b]	-
WD	2.16±0.08[a]	1.31±0.05[a]	-
Rehydration			

WW	0.80±0.22[a]	0.65±0.05[a]	-
WD	0.45±0.09[a]	0.94±0.07[a]	-
LEAVES			
Maximum stress			
WW	1.05±0.47[a]	0.85±0.17[a]	0.10±0.01[a]
WD	0.52±0.09[a]	0.79±0.09[a]	0.23±0.05[a]
Rehydration			
WW	2.82±0.52[a]	1.33±0.12[a]	0.26±0.05[a]
WD	2.00±0.50[a]	1.58±0.05[a]	0.30±0.06[a]
LABELLED			
Maximum stress			
WW	0.50±0.18[a]	0.71±0.13[a]	0.07±0.01[a]
WD	0.28±0.05[a]	0.66±0.06[a]	0.08±0.01[a]
Rehydration			
WW	2.30±0.41[a]	1.43±0.15[a]	0.19±0.05[a]
WD	0.88±0.29[a]	1.73±0.09[a]	0.13±0.03[a]
TOP			
Maximum stress			
WW	1.68±0.40[a]	0.99±0.19[a]	0.17±0.03[a]
WD	0.21±0.02[a]	1.40±0.10[a]	0.36±0.03[b]
Rehydration			
WW	2.22±0.51[a]	1.36±0.13[a]	0.17±0.03[a]
WD	1.59±0.46[a]	1.41±0.22[a]	0.17±0.04[a]

3.5 Nitrogen metabolism

3.5.1 Total nitrogen

Roots exhibited the highest nitrogen accumulation across all conditions, with an increase under R with WD, reaching 362 g plant^{-1}. Stalk nitrogen content also increased during R under WD (146 g plant^{-1}) (Figure 13). The nitrogen content in leaves was higher under WW conditions during MS (193 g plant^{-1}) and decreased under WD (80 g plant^{-1}), indicating nitrogen remobilization from leaves to other tissues under stress. Leaf nitrogen content recovered after rehydration under WD, reaching 242 g plant^{-1}, the highest among all treatments. Nitrogen content in the labelled and top leaves had higher values during rehydration, particularly under water-deficit conditions.

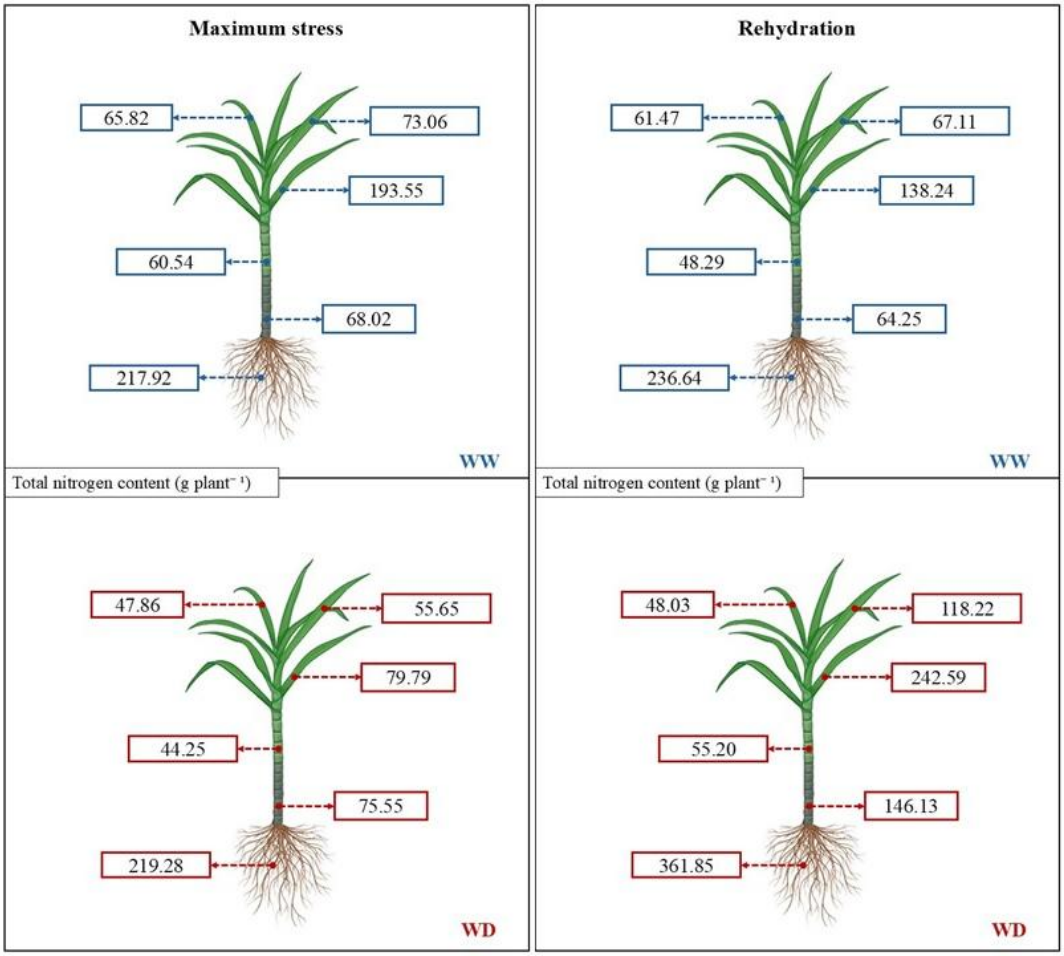

Figure 13. Total nitrogen content (g plant-[1]) in different plant parts under well-watered (WW, ~90%WHC) and water-deficit (WD, 20% WHC) conditions at Maximum Stress (MS, left panels) and Rehydration (R, right panels). Values in blue boxes represent WW plants, and values in red boxes represent WD plants. Plant parts include roots, stalks, sheath leaves, leaves, 15N-labelled leaves, and top leaves. Blue boxes represent WW plants, and red boxes represent WD plants.

3.5.2 Nitrogen use efficiency

At MS, WW plants exhibited the highest NUE in roots (1.65), followed by those under WD (1.52) (Figure 14). In contrast, leaf sheaths display an increase under WD during MS (0.92), compared to WW plants (0.80). After R, root NUE decreased in both treatments (1.41 under WD and 1.35 under WW) but remained relatively high. Leaf sheath maintained elevated NUE values after rehydration (0.86 in both treatments). Leaves and top leaves exhibited intermediate NUE values, with an increase under WD, particularly in top leaves after R (0.73). Stalks consistently showed the lowest NUE values across all treatments and time points,

especially under WD after R (0.42), suggesting a limited role in nitrogen remobilisation.

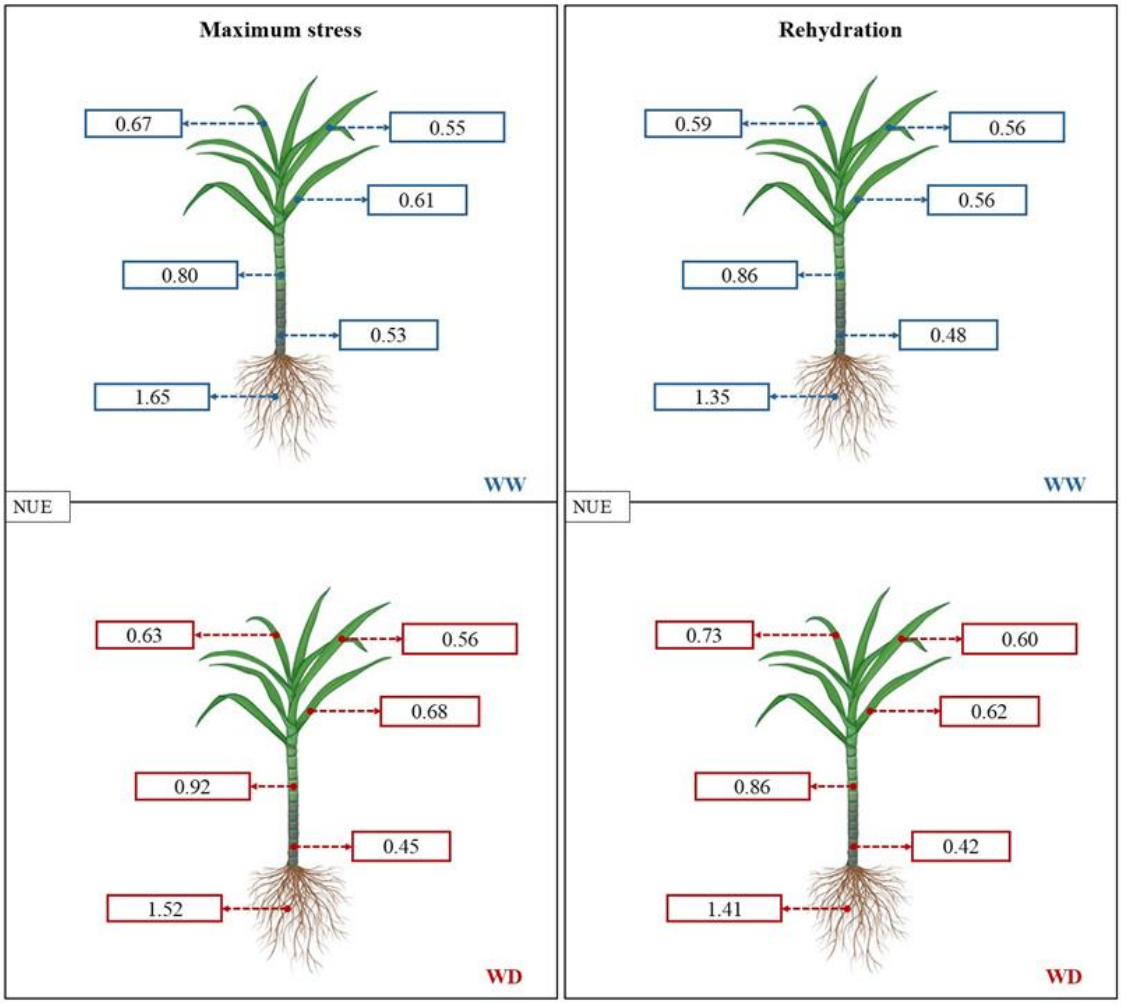

Figure 14. Nitrogen use efficiency (NUE) in different plant parts under well-watered (WW, ~90%WHC) and water-deficit (WD, 20% WHC) conditions at Maximum Stress (MS, left panels) and Rehydration (R, right panels). Values in blue boxes represent WW plants, and values in red boxes represent WD plants. Plant parts include roots, stalks, sheath leaves, leaves, 15N-labelled leaves, and top leaves. Blue boxes represent WW plants, and red boxes represent WD plants.

3.5.3 ^{15}N partition

The distribution of ^{15}N in plant tissues during MS and R revealed distinct nitrogen remobilisation patterns in WD and WW conditions (Figure 15). At MS, WD plants accumulated higher ^{15}N in leaf sheaths (12.1%) and stalks (6.0%) than in the WW plants, indicating remobilisation of nitrogen reserves to these tissues. Lower ^{15}N percentages were observed in the leaves (4.6%), top leaves (5.0%), and roots (1.9%), suggesting limited remobilisation in these organs during stress. Upon R, ^{15}N content increased in leaves (6.1%) and stalks (14.6%) under WD conditions. In contrast, top leaves (3.0%) and roots (1.9%) showed limited remobilisation.

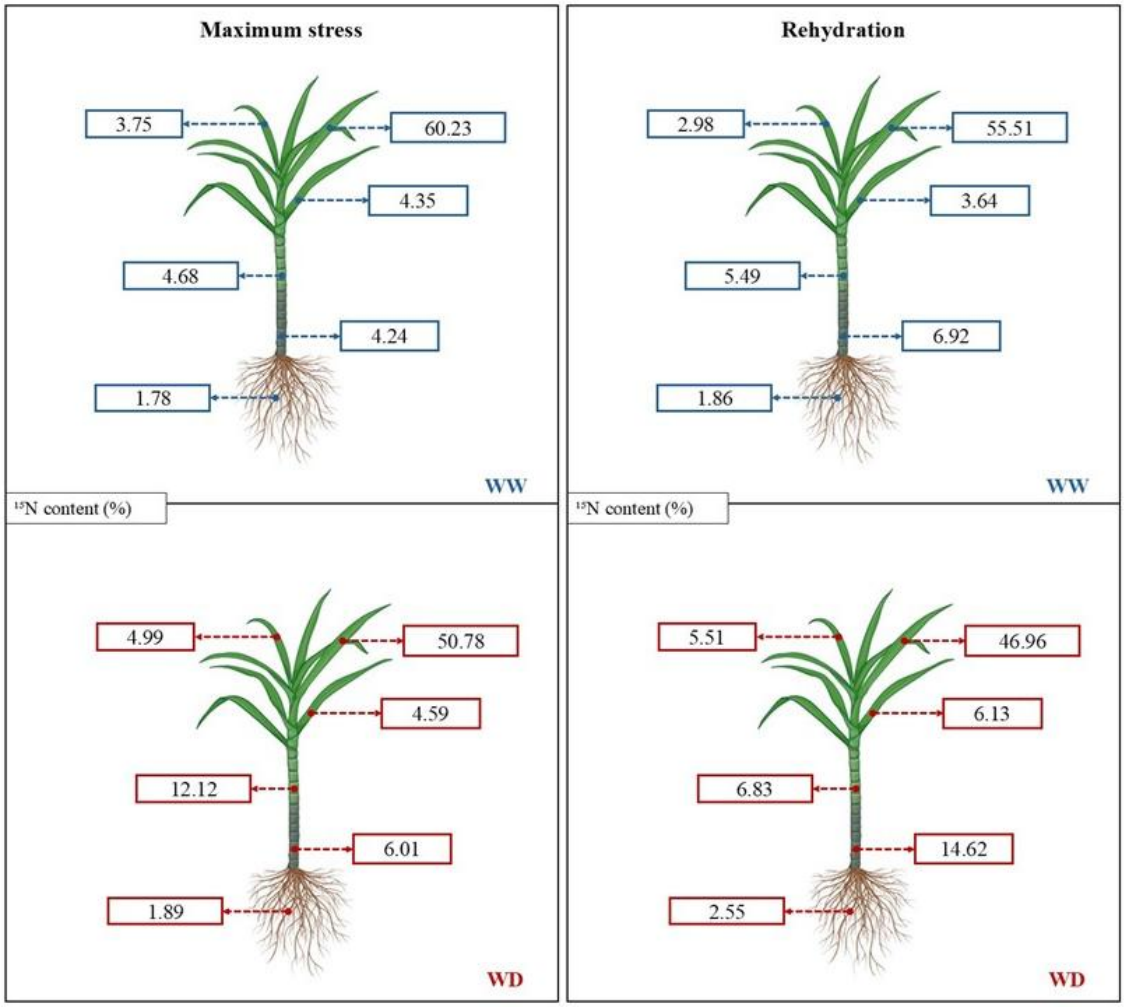

Figure 15. ^{15}N content (%) in different plant parts under well-watered (WW, ~90%WHC) and water-deficit (WD, 20% WHC) conditions at Maximum Stress (MS, left panels) and Rehydration (R, right panels). Values in blue boxes represent WW plants, and values in red boxes represent WD plants. Plant parts include roots, stalks, sheath leaves, leaves, 15N-labelled leaves, and top leaves. Blue boxes represent WW plants, and red boxes represent WD plants.

3.5.4 Nitrate, ammonium, and nitrogen enzymes

Under MS, nitrate concentrations decreased in the roots and leaf sheaths of WD plants when compared to WW (Table 2). Specifically, root nitrate content decreased from 3.51 mg NO_3- g^{-1} FW in WW plants to 2.53 mg NO_3- g^{-1} FW in WD plants (Table 2). Leaf sheath under WD was reduced from (0.59 mg NO_3- g^{-1} FW) to WW plants (1.54 mg NO_3- g^{-1} FW). After R, nitrate levels increased in the top leaves of WD plants (9.6 mg NO_3- g^{-1} FW). Ammonium content exhibited an opposite tendency. Under MS, WD plants accumulated higher ammonium levels in the roots (66.94 µg NH_4^+ g^{-1} FW) than WW plants (38.4 µg NH_4^+ g^{-1} FW). High ammonium concentrations were also observed in ^{15}N-labelled leaves and top leaves of WD plants. Following R, root ammonium content remained higher in WD plants than in WW, whilst an increase was also observed in the stalks of WD plants (35.8 µg NH_4^+ g^{-1} FW). Regarding

enzymatic activities, GOGAT remained stable across all tissues and treatments, with no differences between WD and WW plants, either under MS or after R. In the other hand, GS activity showed a response in WD plants. During MS, GS activity was higher in the top leaves of WD plants (15.1 µmol GGH g-¹ FW min-¹) compared to WW (12.6 µmol GGH g-¹ FW min-¹). After R, GS activity increased in the roots of WD plants (11.8 µmol GGH g-¹ FW min-¹). Nitrate reductase (NR) activity was reduced in WD plants under MS in roots and leaves. Root NR activity decreased from 1.7 µmol GGH g-¹ FW min-¹ in WW plants to 0.7 µmol GGH g-¹ FW min-¹ in WD plants. A similar trend was noted in the leaves, ^{15}N-labelled leaves, and top leaves. Upon R, NR activity in the roots of WD plants increased to 1.69 µmol GGH g-¹ FW min-¹ but did not differ from WW plants. NR activity in the leaves remained low across both treatments. Notably, NR activity was undetectable in leaf sheaths and stalks.

Table 2. Nitrate (NO_3-), ammonium (NH_4^+), glutamate synthase (GOGAT), glutamine synthetase (GS), and nitrate reductase (NR) in different plant tissues under well-watered (WW, ~90% WHC) and water-deficient (WD, 20% WHC) conditions at Maximum Stress and Rehydration. Plant tissues include roots, stalks, sheath leaves, leaves, 15N-labelled leaves, and top leaves. Data are mean ± SEM (n = 4). Different lowercase letters within the same tissue and time point indicate significant differences between treatments ($p \leq 0.05$, Tukey's test). A dash (-) indicates unavailable data.

Part/time/treatments	NITRATE (mg NO_3- g-¹ FW)	AMMONIUM (µg NH_4^+ g-¹ FW)	GOGAT (nmol NADH min^{-1} g^{-1} FW)	GS (µmol GGH g-¹ FW min-¹)	NR (nmol NADH min^{-1} g^{-1} FW)
ROOT					
Maximum stress					
WW	3.51±0.1^a	38.36±1.4^b	3.34±0.4^a	8.72±0.6^a	1.67±0.2^a
WD	2.53±0.3^b	66.94±5.2^a	3.26±0.5^a	8.11±0.2^a	0.73±0.1^b
Rehydration					
WW	2.84±0.5^a	32.73±2.6^b	3.17±0.4^a	7.56±0.4^b	0.89±0.5^a
WD	2.53±0.1^a	40.91±1.0^a	3.33±0.1^a	11.85±1.6^a	1.69±0.4^a
STALK					
Maximum stress					
WW	0.59±0.1^a	25.71±1.4^a	3.15±0.4^a	13.88±0.5^a	-
WD	0.44±0.2^a	31.63±3.1^a	3.53±0.3^a	16.90±2.2^a	-
Rehydration					
WW	0.61±0.1^a	26.19±0.8^b	3.39±0.3^a	12.52±1.1^a	-
WD	0.43±0.1^a	35.85±0.9^a	3.02±0.3^a	13.55±1.8^a	-
SHEATH					
Maximum stress					
WW	1.54±0.2^a	26.34±0.4^a	2.83±0.5^a	10.65±1.6^a	-

WD	0.59±0.1[b]	31.30±3.6[a]	3.58±0.5[a]	12.88±0.3[a]	-
Rehydration					
WW	4.01±0.2[a]	32.41±0.9[a]	2.86±0.1[a]	11.42±0.8[a]	-
WD	5.00±1.1[a]	38.53±5.2[a]	3.25±0.3[a]	11.89±2.3[a]	-
LEAVES					
Maximum stress					
WW	3.12±0.8[a]	25.97±0.4[a]	3.54±0.4[a]	17.97±1.8[a]	6.04±0.2[a]
WD	2.71±0.8[a]	31.97±2.6[a]	3.12±0.4[a]	23.33±2.5[a]	0.95±0.3[b]
Rehydration					
WW	4.18±0.3[a]	33.87±3.2[a]	3.32±0.3[a]	28.20±4.2[a]	1.48±0.7[a]
WD	3.11±0.5[a]	27.93±0.5[a]	3.32±0.6[a]	17.40±2.9[a]	1.66±0.5[a]
LABELLED					
Maximum stress					
WW	0.43±0.1[a]	23.74±1.1[b]	3.80±0.2[a]	14.14±1.9[a]	2.83±0.6[a]
WD	0.97±0.2[a]	38.28±1.1[a]	3.15±0.2[a]	15.54±0.6[a]	1.03±0.1[b]
Rehydration					
WW	3.22±0.4[a]	45.68±7.1[a]	3.55±0.5[a]	13.92±1.6[a]	0.51±0.1[a]
WD	4.08±0.2[a]	48.96±1.2[a]	3.48±0.3[a]	12.61±1.8[a]	0.42±0.1[a]
TOP					
Maximum stress					
WW	0.41±0.1[a]	29.19±0.7[b]	3.44±0.3[a]	12.64±0.6[b]	2.12±0.3[a]
WD	0.71±0.2[a]	40.10±2.4[a]	3.49±0.3[a]	15.07±0.2[a]	0.84±0.2[b]
Rehydration					
WW	3.39±0.7[a]	36.71±4.4[a]	3.71±0.3[a]	18.13±2.4[a]	1.59±0.7[a]
WD	9.63±1.8[a]	38.52±0.6[a]	3.40±0.6[a]	12.53±1.6[a]	1.40±0.5[a]

3.6 Carbon metabolism

3.6.1 Total carbon content

At MS, roots exhibited the highest carbon accumulation, particularly under WW conditions (5.4 g plant-^{1}), although a reduction was observed under WD (5.1 g plant-^{1}). On the contrary, following R, root carbon content increased under WD conditions (9.2 g plant-^{1}) (Figure 16). Stalk carbon content remained relatively stable across treatments and times, with values ranging from 0.6 to 1.2 g plant-^{1}. The leaf sheaths exhibited a higher carbon content under WW conditions at both time points but decreased under WD after R (0.4 g plant-^{1}). Leaf carbon content increased under WD, with the highest value observed during R (3.1 g plant-^{1}). The 15N-labelled leaves and top leaves presented little variation, with higher carbon content under WD at both stages.

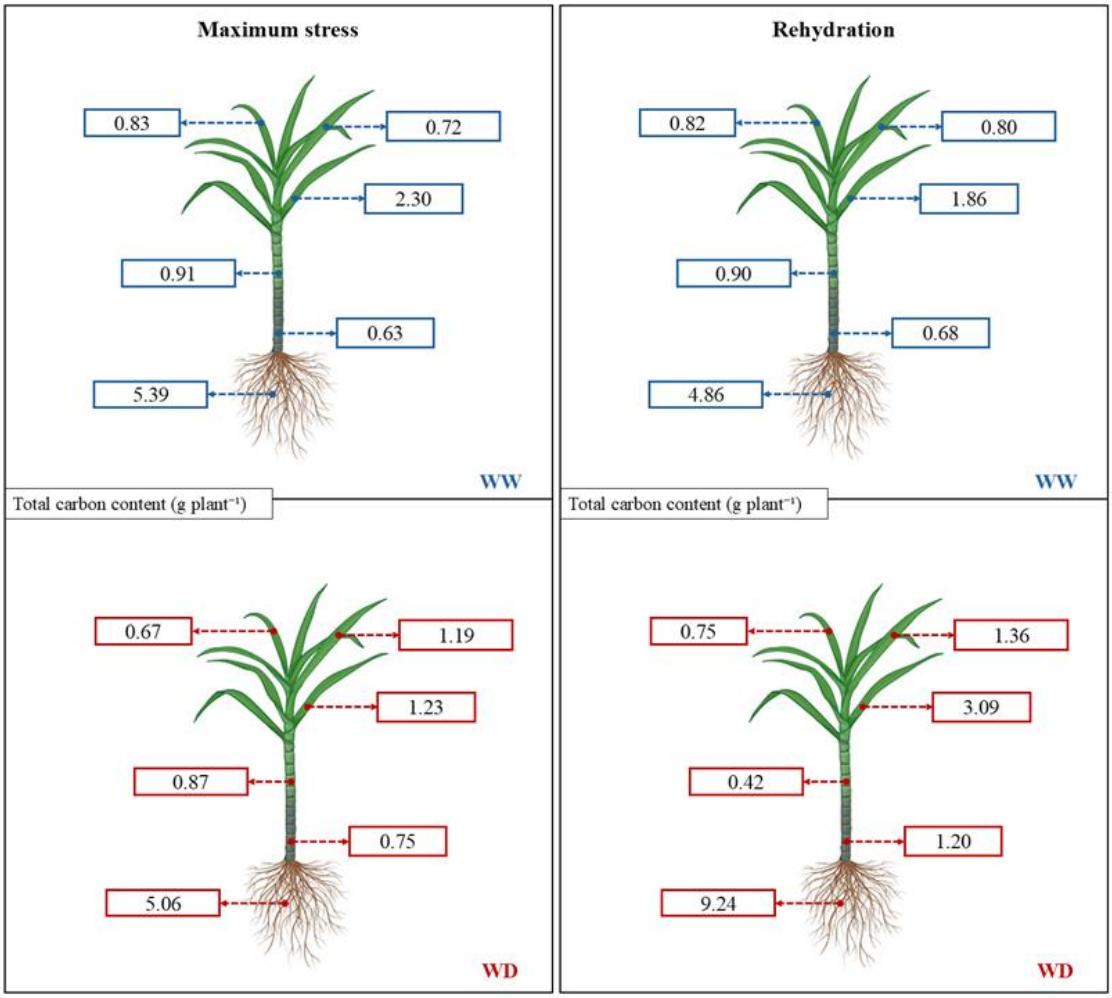

Figure 16. Total carbon content (g plant^{-1}) in different plant parts of Saccharum spp. cv. CTC9001bt under well-watered (WW, ~90% water holding capacity) and water-deficit (WD, 20% water holding capacity) conditions. Plant parts, shown from bottom to top, include roots, stalks, sheath leaves, leaves, 15N-labelled leaves, and top leaves. Measurements were performed at two sampling times: Maximum Stress (MS, left panels) and Rehydration (R, right panels). Values in blue boxes represent WW plants, and values in red boxes represent WD plants.

3.6.2 C:N ratio

Roots exhibited the highest C:N ratios across treatments, with the greatest value observed under R in WD conditions (25.5) (Figure 17). This indicates either an accumulation of carbon compounds or a reduction in nitrogen concentration in the roots. Stalks and leaves showed intermediate C:N ratios. In stalks, the lowest ratio was observed under MS with WD (8.2). The sheath showed differences between treatments, with the highest C:N ratio under MS with WD (19.6), indicating nitrogen export from these tissues under drought conditions. 15N-labelled leaves exhibited elevated C:N ratios under MS with WD (21.4), consistent with nitrogen depletion due to remobilisation. In contrast, top leaves maintained moderate C:N ratios across treatments, with an increase under R with WD (15.7), suggesting nitrogen was redirected to support new leaf growth during recovery.

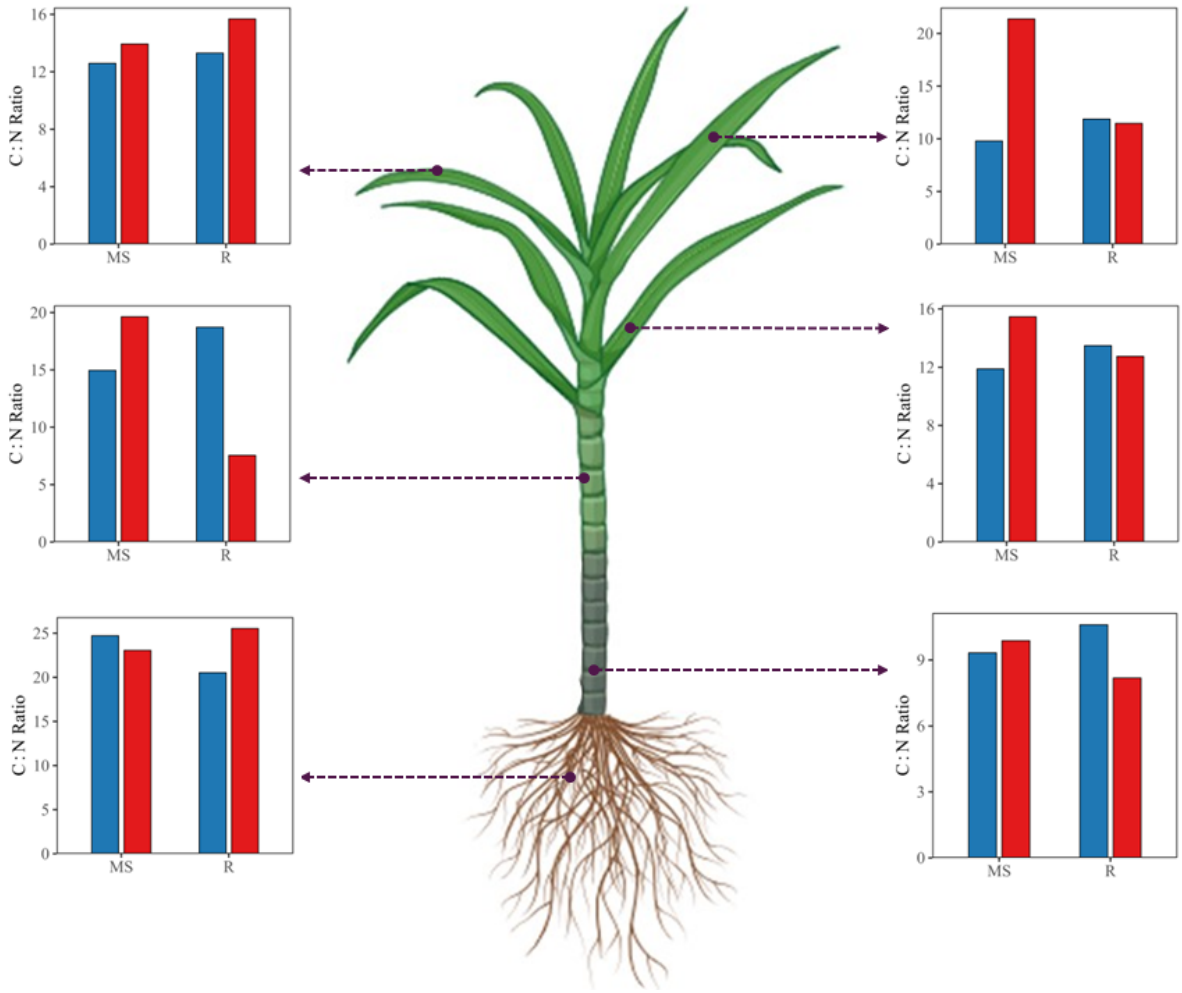

Figure 17. Carbon Nitrogen ratio (C:N) in different plant parts under well-watered (WW, ~90% WHC) and water-deficit (WD, 20%WHC) conditions at Maximum stress (MS) and Rehydration (R). Bar show C:N ratios for roots, stalks, sheath leaves, leaves, 15N-labelled leaves, and top leaves. Blue bars represent WW plants and red bars represent WD plants. Graphs are positioned adjacent to their corresponding plant parts.

3.6.3 Carbon isotope discrimination ($\Delta^{13}C$)

Under WD during MS, $\Delta^{13}C$ decreased in most tissues compared to WW plants. In roots, $\Delta^{13}C$ decreased from +7.1‰ in WW plants to +7.0‰ under WD. Similarly, the stalk exhibited an increase in $\Delta^{13}C$, from +4.5‰ to +4.8‰ (Figure 18). The leaves exhibited a decrease in $\Delta^{13}C$ under WD (from +4.2‰ to +4.9‰). During R, $\Delta^{13}C$ values in the roots and stalks were higher than during MS. 15N-labelled leaves under WD during MS showed the lowest $\Delta^{13}C$ (+3.1‰).

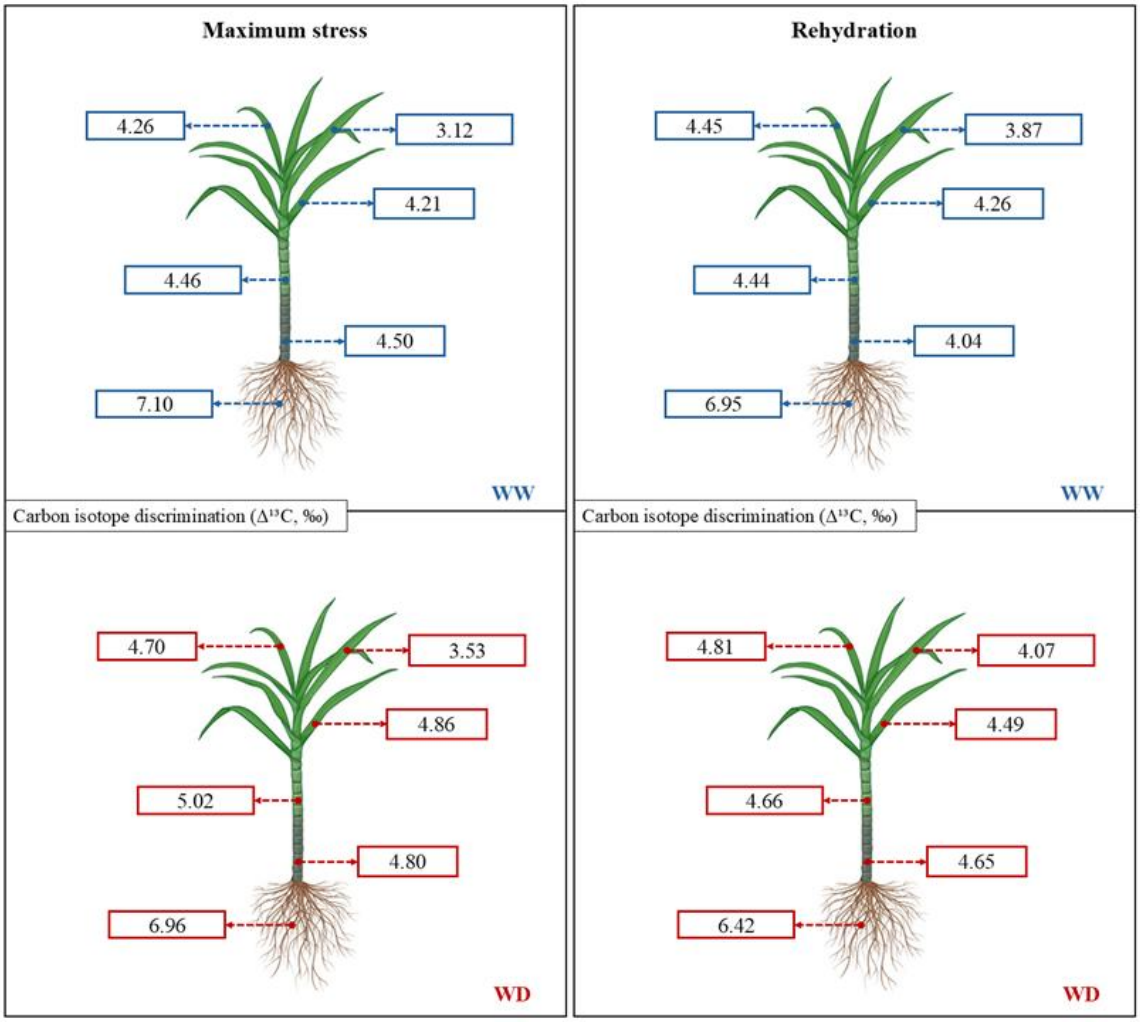

Figure 18. Carbon isotope discrimination ($\Delta^{13}C$, ‰) in different plant parts of Saccharum spp. cv. CTC9001bt under well-watered (WW, ~90% water holding capacity) and water-deficit (WD, 20% water holding capacity) conditions. Plant parts, shown from bottom to top, include roots, stalks, sheath leaves, leaves, 15N-labelled leaves, and top leaves. Measurements were performed at two sampling times: Maximum Stress (MS, left panels) and Rehydration (R, right panels). Values in blue boxes represent WW plants, and values in red boxes represent WD plants.

3.7 Osmotic adjustment compounds

Proline content was higher in WD roots (3.4 µmol g^{-1} DW) and stalks (3.6 µmol g^{-1} DW) compared to WW (1.6 and 0.8 µmol g^{-1} DW, respectively) (Table 3). However, in leaves, proline levels were lower under WD (1.5 µmol g^{-1} DW) than WW (3.0 µmol g^{-1} DW). Upon R, proline levels declined, but 15N-labelled leaves had elevated concentrations (1.0 µmol g^{-1} DW in WD vs. 0.8 µmol g^{-1} DW in WW), likely due to their exposure to ammonium sulphate, which may have prolonged nitrogen assimilation into proline. Sheath tissues displayed relatively stable proline concentrations between MS and R. Total soluble sugars increased under WD conditions in most tissues at MS, particularly in roots (36.7 µmol glucose g^{-1} DW) and stalks (40.31 µmol glucose g^{-1} DW) compared to WW (23.9 and 18.9 µmol glucose g^{-1} DW, respectively). Although sugar levels declined after R, sheath leaves maintained high

concentrations (25.0 µmol glucose g-¹ DW). Starch content followed a similar tendency, increasing in roots (40.92 µmol glucose g-¹ DW) and stalks (34.66 µmol glucose g-¹ DW) of WD plants at MS, while WW plants showed lower values (29.3 and 20.1 µmol glucose g-¹ DW, respectively). However, after R, starch reserves were better restored in WW conditions, whereas WD-treated 15N-labelled leaves and top leaves struggled to recover. Sucrose accumulation was higher in WD plants during MS, particularly in roots (23.3 µmol glucose g-¹ DW) and stalks (22.1 µmol glucose g-¹ DW). WD plants also exhibited elevated sucrose levels in top leaves (26.1 µmol glucose g-¹ DW) and sheath leaves (20.8 µmol glucose g-¹ DW). However, following R, sucrose concentrations dropped in WD roots (1.4 µmol glucose g-¹ DW) and leaves (10.1 µmol glucose g-¹ DW). Unlike WD plants, WW-treated plants maintained relatively stable sucrose concentrations across both conditions. Amino acid content was also elevated under WD conditions at MS across most tissues, with leaves showing a decline from 10.7 µmol g-¹ DW to 6.9 µmol g-¹ DW after R. Similarly, stalks showed a decline from 8.98 to 5.9 µmol g-¹ DW after R. Roots, however, showed an increase in amino acid content after R (5.4 µmol g-¹ DW) compared to MS (2.4 µmol g-¹ DW). In WW plants, amino acid levels remained more stable between MS and R. At MS, protein content in roots and stalks exhibited higher protein levels under WD than WW, with increases of 1.43 mg g-¹ DW and 1.0 mg g-¹ DW, respectively. However, sheath leaves and top leaves showed no significant differences. During R, the sheath exhibited the highest increase in protein content under WD (1.1 mg g-¹ DW). Top leaves also showed increased protein accumulation (0.8 mg g-¹ DW) under WD. Despite the increase in roots and leaves under WD, differences were not statistically significant. Finally, reducing sugars were elevated in WD roots (160.6 µmol glucose g-¹ FW), stalks (151.14 µmol glucose g-¹ FW), and top leaves (166.3 µmol glucose g-¹ FW) at MS compared to WW plants (95.18, 84.61, and 87.90 µmol glucose g-¹ FW, respectively). Following R, the reduction in sugar levels declined in WD plants, particularly in the roots and top leaves. 15N-labelled leaves, however, exposed a reduction in reducing sugar levels under WD conditions at MS (69.33 µmol glucose g-¹ FW) compared to WW (93.32 µmol glucose g-¹ FW).

Table 3. Concentrations of proline, total soluble sugars, starch, sucrose, amino acids, proteins, and reducing sugars in different plant tissues under well-watered (WW, ~90% WHC) and water-deficit (WD, 20% WHC) conditions at Maximum Stress (MS) and Rehydration (R). Plant tissues include roots, stalks, sheath leaves, leaves, 15N-labelled leaves, and top leaves. Data are mean ± SEM (n = 4). Letters within the same tissue and time point indicate significant differences between treatments ($p \leq 0.05$, Tukey's test).

Part/ time/ treatment	PROLINE (µmol g^{-1} DW)	TOTAL SOLUBLE SUGARS (µmol glucose g^{-1} DW)	STARCH (µmol glucose g^{-1} DW)	SUCROSE (µmol glucose g^{-1} DW)	AMINO ACIDS (µmol g^{-1} DW)	PROTEINS (mg g^{-1} DW)	REDUCING SUGAR (µmol glucose g^{-1} FW)
ROOT							
Maximum stress							
WW	1.6±0.5^{a}	23.9±3.5^{a}	29.3±1.8^{a}	13.4±1.6^{b}	2.4±0.4^{b}	2.0±0.1^{b}	95.2±2.1^{b}
WD	3.4±0.8^{a}	36.7±4.2^{a}	40.9±6.9^{a}	23.3±1.3^{a}	6.5±0.5^{a}	3.4±0.2^{a}	160.6±2.5^{a}
Rehydration							
WW	1.2±0.2^{a}	17.6±2.4^{a}	39.3±3.2^{a}	14.0±4.5^{a}	5.5±1.3^{a}	1.9±0.1^{b}	75.1±5.4^{a}
WD	1.6±0.3^{a}	7.6±1.10^{b}	25.8±4.8^{a}	1.4±0.3^{b}	7.2±0.4^{a}	2.5±0.1^{a}	79.1±7.3^{a}
STALK							
Maximum stress							
WW	0.8±0.1^{b}	18.9±2.6^{b}	20.0±2.6^{a}	5.0±1.2^{b}	4.1±0.7^{b}	1.6±0.2^{b}	84.6±3.3^{b}
WD	3.6±0.5^{a}	40.3±1.9^{a}	34.7±2.1^{b}	22.1±3.3^{a}	8.9±1.3^{a}	2.7±0.2^{a}	151.1±3.1^{a}
Rehydration							
WW	1.5±0.2^{a}	13.5±1.5^{b}	22.1±1.5^{a}	12.8±2.8^{a}	2.7±0.7^{b}	1.9±0.3^{a}	68.5±2.9^{a}
WD	1.8±0.3^{a}	21.3±0.3^{a}	28.3±2.6^{a}	7.1±1.3^{a}	5.9±0.3^{a}	1.6±0.1^{a}	78.2±7.6^{a}
SHEATH							
Maximum stress							
WW	1.5±0.4^{a}	22.6±2.6^{b}	24.4±1.3^{a}	14.8±0.6^{a}	3.9±0.2^{b}	2.4±0.1^{a}	116.4±5.0^{a}
WD	2.8±0.9^{a}	38.9±4.3^{a}	31.9±4.1^{a}	20.8±3.0^{a}	7.4±1.1^{a}	1.9±0.3^{a}	115.7±6.7^{a}
Rehydration							

WW	2.5±0.5[a]	10.1±0.7[b]	22.2±2.8[a]	7.9±3.4[a]	2.2±0.6[a]	1.1±0.1[b]	50.9±2.6[a]
WD	2.3±0.5[a]	25.0±5.2[a]	21.1±1.9[a]	6.4±1.6[a]	3.3±1.0[a]	2.3±0.2[a]	49.9±2.7[a]
LEAVES							
Maximum stress							
WW	2.9±0.5[a]	20.7±2.7[b]	24.7±1.4[a]	13.4±1.6[a]	2.9±0.4[b]	2.2±0.1[a]	152.8±5.4[a]
WD	1.5±0.2[b]	35.1±1.0[a]	24.1±1.1[a]	23.7±4.5[a]	10.6±0.4[a]	2.5±0.1[a]	143.7±2.7[a]
Rehydration							
WW	1.8±0.6[a]	19.1±1.0[a]	23.2±1.1[a]	14.4±1.9[a]	2.2±0.6[b]	1.5±0.1[b]	75.9±5.1[a]
WD	2.0±0.3[a]	22.4±4.5[a]	21.0±2.7[a]	10.1±1.5[a]	6.9±1.3[a]	2.1±0.1[a]	67.1±1.8[a]
LABELLED							
Maximum stress							
WW	1.8±0.7[a]	23.2±0.2[a]	30.3±2.4[a]	6.8±0.1[a]	1.4±0.1[a]	1.8±0.2[a]	93.3±4.9[a]
WD	1.8±0.9[a]	14.9±1.6[b]	15.8±1.7[b]	5.9±0.9[a]	2.6±0.5[a]	2.3±0.1[a]	69.3±0.9[b]
Rehydration							
WW	0.8±0.1[b]	6.9±2.3[a]	32.1±4.3[a]	9.9±3.31[a]	1.8±0.3[a]	1.1±0.1[b]	78.9±4.3[a]
WD	1.0±0.1[a]	12.1±2.9[a]	26.4±2.1[a]	5.4±0.90[a]	2.4±0.3[a]	1.9±0.3[a]	74.9±9.0[a]
TOP							
Maximum stress							
WW	1.8±0.3[a]	13.4±1.1[b]	39.6±1.2[a]	17.7±4.66[a]	5.9±0.2[b]	2.3±0.1[a]	87.9±3.7[b]
WD	1.4±0.4[a]	34.1±3.8[a]	28.2±1.1[b]	26.1±5.54[a]	10.1±0.9[a]	1.9±0.2[a]	166.3±5.1[a]
Rehydration							
WW	1.7±0.7[a]	13.5±0.9[a]	30.9±1.9[a]	8.6±2.07[a]	3.2±0.6[a]	1.5±0.1[b]	116.6±2.3[a]
WD	1.4±0.2[a]	19.0±2.7[a]	23.1±0.1[b]	10.4±2.85[a]	3.6±0.6[a]	2.3±0.3[a]	138.4±9.2[a]

3.8 Nutrient content

At MS, in roots, K levels were lower in WD plants (18.3 ± 0.8 g kg^{-1}) compared to WW plants (24.7 ± 3.9 g kg^{-1}), showing a reduced uptake of K under drought stress (Table 4). This decline was also observed in stalks, leaf sheaths, ^{15}N-labelled leaves, top leaves, and leaves, where K content was lower in WD plants. An increase in Fe content was verified in WD roots at MS, where Fe levels were higher (3776.6 ± 1958.2 mg kg^{-1}) than in WW roots (1247.3 ± 714.4 mg kg^{-1}). Mn levels in roots were lower in WD plants (105.1 ± 23.9 mg kg^{-1}) than in WW plants (211.7 ± 35.9 mg kg^{-1}). After R, N content in WD roots increased (361.8 g kg^{-1}) compared to WW roots (236.6 g kg^{-1}). P content in sheath leaves was lower in WD plants (1.9 ± 0.1 g kg^{-1}) than in WW plants (2.6 ± 0.1 g kg^{-1}). K reduction remained even after rehydration, with WD plants showing lower K content in their roots, stalks, leaf sheaths, labelled, tops, and other leaves compared to WW plants. Ca content in sheath leaves was higher in WD plants (3.91 ± 0.63 g kg^{-1}) than in WW plants (3.0 ± 0.3 g kg^{-1}). Mg content also increased in WD roots (7.8 ± 2.2 g kg^{-1}) compared to WW roots (4.4 ± 0.3 g kg^{-1}). Finally, Zn content in roots decreased under WD conditions at R (36.0 ± 6.0 mg kg^{-1}) compared to WW roots (55.8 ± 11.0 mg kg^{-1}). However, no differences in Zn content were observed in stalks or leaves.

Table 4. Nutrient content in sugarcane plants under well-watered and water-deficit conditions at MS: maximum stress and R: rehydration. Data are mean ± SEM (n = 4) of macronutrients (g kg^{-1})) and micronutrients (mg kg^{-1})) in roots, stalks, sheath leaves, leaves, 15N-labelled leaves, and top leaves subjected to well-watered (WW, ~90% WHC) and water-deficit (WD, 20% WHC) conditions. Samples were collected at maximum stress (MS) and after rehydration (R). Different letters indicate significant differences between treatments within each sampling time ($p \leq 0.05$, Tukey's test)

Part/ time/ treatment	NUTRIENT CONTENT (g kg^{-1})					NUTRIENT CONTENT (mg kg^{-1})			
	P	K	Ca	Mg	S	Cu	Fe	Mn	Zn
ROOT									
Maximum stress									
WW	2.5±0.3^{a}	24.7±3.9^{a}	4.4±0.2^{a}	7.7±1.7^{a}	5.7±1.6^{a}	24.6±6.3^{a}	1247.3±714.4^{a}	211.7±35.9^{a}	58.4±6.6^{a}
WD	2.2±0.1^{a}	18.3±0.8^{a}	4.5±0.2^{a}	5.9±0.3^{a}	5.4±1.4^{a}	16.8±2.3^{a}	3776.6±1958^{a}	105.1±23.9^{b}	65.1±16.1^{a}
Rehydration									
WW	1.7±0.2^{a}	18.2±3.1^{a}	4.3±1.2^{a}	4.4±0.3^{a}	4.1±1.9^{a}	17.8±4.8^{a}	425.7±27.3^{a}	225.6±49.8^{a}	55.7±11.1^{a}
WD	1.5±0.1^{a}	15.6±2.0^{a}	3.8±0.5^{a}	7.8±2.2^{a}	4.1±1.2^{a}	16.2±4.7^{a}	371.6±87.1^{a}	141.4±25.3^{a}	36.0±5.9^{a}
STALK									
Maximum stress									
WW	1.3±0.1^{a}	30.5±1.6^{a}	1.9±0.3^{a}	2.6±0.3^{a}	6.3±0.4^{a}	7.6±1.7^{a}	12.6±4.7^{b}	112.5±25.1^{a}	15.8±4.7^{a}
WD	1.3±0.1^{a}	20.4±1.7^{b}	2.3±0.2^{a}	3.1±0.5^{a}	5.8±0.8^{a}	13.6±4.4^{a}	66.0±18.3^{a}	94.6±14.4^{a}	23.8±5.8^{a}
Rehydration									
WW	2.2±0.2^{a}	31.1±1.1^{a}	2.1±0.2^{a}	2.3±0.2^{a}	7.2±0.4^{a}	6.3±0.4^{a}	41.0±30.6^{a}	162.1±16.2^{a}	21.0±9.2^{a}
WD	1.8±0.1^{a}	19.4±1.4^{b}	2.4±0.2^{a}	2.5±0.4^{a}	5.8±0.8^{a}	5.8±0.8^{a}	43.2±20.7^{a}	142.8±28.9^{a}	26.7±2.2^{a}
SHEATH									
Maximum stress									
WW	1.7±0.1^{a}	40.7±2.1^{a}	2.6±0.4^{a}	3.5±0.5^{a}	8.4±0.5^{a}	10.1±2.3^{a}	16.7±6.2^{b}	150.0±33.5^{a}	21.1±6.3^{a}
WD	1.8±0.2^{a}	31.4±2.6^{b}	3.6±0.3^{a}	4.7±0.7^{a}	8.9±1.3^{a}	20.9±6.8^{a}	101.5±28.2^{a}	145.5±22.1^{a}	36.7±8.9^{a}
Rehydration									
WW	2.6±0.1^{a}	41.5±1.5^{a}	2.8±0.3^{a}	3.0±0.3^{a}	9.6±0.5^{a}	11.9±5.2^{a}	54.7±40.8^{a}	216.2±21.6^{a}	28.0±12.2^{a}
WD	1.9±0.1^{b}	29.9±2.2^{b}	3.8±0.4^{a}	3.9±0.6^{a}	8.9±1.2^{a}	10.7±3.6^{a}	66.5±31.8^{a}	219.6±44.5^{a}	41.1±3.3^{a}
LEAVES									
Maximum stress									
WW	3.6±0.3^{a}	28.9±1.1^{a}	3.9±0.4^{a}	2.2±0.2^{b}	4.1±0.4^{a}	33.4±14.3^{a}	78.9±13.4^{a}	327.1±52.4^{a}	52.7±7.9^{a}
WD	2.1±0.2^{b}	30.7±0.6^{a}	4.6±0.1^{a}	2.8±0.1^{a}	3.8±0.1^{a}	7.3±2.0^{a}	12.5±4.8^{b}	135.4±24.9^{b}	13.2±4.8^{b}

Rehydration									
WW	3.6±0.2[a]	27.3±0.4[a]	5.8±0.4[a]	2.6±0.1[a]	4.9±0.1[a]	10.9±3.3[a]	106.9±44.3[a]	395.7±116.4[a]	47.2±10.9[a]
WD	3.4±0.2[a]	28.4±1.2[a]	3.9±0.4[b]	2.6±0.2[a]	4.0±0.4[b]	10.7±2.5[a]	62.4±12.7[a]	274.3±21.8[a]	37.9±7.3[a]
LABELLED									
Maximum stress									
WW	2.5±0.1[a]	38.1±2.3[a]	3.7±0.2[b]	3.1±0.2[a]	19.4±1.4[a]	8.4±1.7[b]	37.6±4.8[a]	300.4±15.1[a]	49.6±4.3[a]
WD	2.5±0.1[a]	35.9±1.3[a]	5.0±0.4[a]	3.6±0.1[a]	22.1±2.9[a]	20.6±4.6[a]	51.6±3.7[a]	255.4±30.6[a]	49.3±4.8[a]
Rehydration									
WW	3.1±0.1[a]	30.5±0.6[a]	5.1±0.3[a]	2.6±0.1[a]	6.4± 0.2[a]	19.4±1.4[a]	74.6±20.6[a]	385.7±56.3[a]	39.7±2.5[a]
WD	3.2±0.2[a]	29.7±1.2[a]	4.0±0.3[a]	2.7±0.2[a]	4.8±0.3[b]	22.1±2.9[a]	54.9±12.7[a]	329.6±29.4[a]	38.0±9.6[a]
TOP									
Maximum stress									
WW	2.1±0.2[a]	32.3±0.7[a]	3.2±0.4[b]	2.2±0.3[b]	5.7±0.4[a]	6.1±0.7[b]	23.7±4.1[a]	240.9±29.8[a]	28.7±5.1[a]
WD	2.6±0.1[a]	35.4±1.8[a]	4.8±0.2[a]	2.9±0.1[a]	5.7±0.3[a]	20.5±4.5[a]	60.0±19.2[a]	282.2±43.7[a]	53.7±10.3[a]
Rehydration									
WW	2.6±0.1[a]	33.7±0.8[a]	4.4±0.3[a]	2.7±0.1[a]	7.9±0.4[a]	12.0±3.9[a]	42.4±14.3[a]	375.7±44.3[a]	32.1±8.3[a]
WD	2.9±0.2[a]	32.5±0.6[a]	4.4±0.3[a]	2.9±0.3[a]	6.1±0.4[b]	44.9±24.5[a]	39.6±13.1[a]	408.1±72.4[a]	38.8±12.0[a]

3.9 Principal component analysis

3.9.1 Maximum stress

PCA explained 51.83% of the data variability, separating treatments along the principal components during MS (Figure 19). WW plants correlated with physiological parameters, including photosynthesis, transpiration, and stomatal conductance. In contrast, WD plants were associated with osmotic adjustment compounds and nitrogen remobilisation markers, such as ammonium, amino acids, and the C:N ratio.

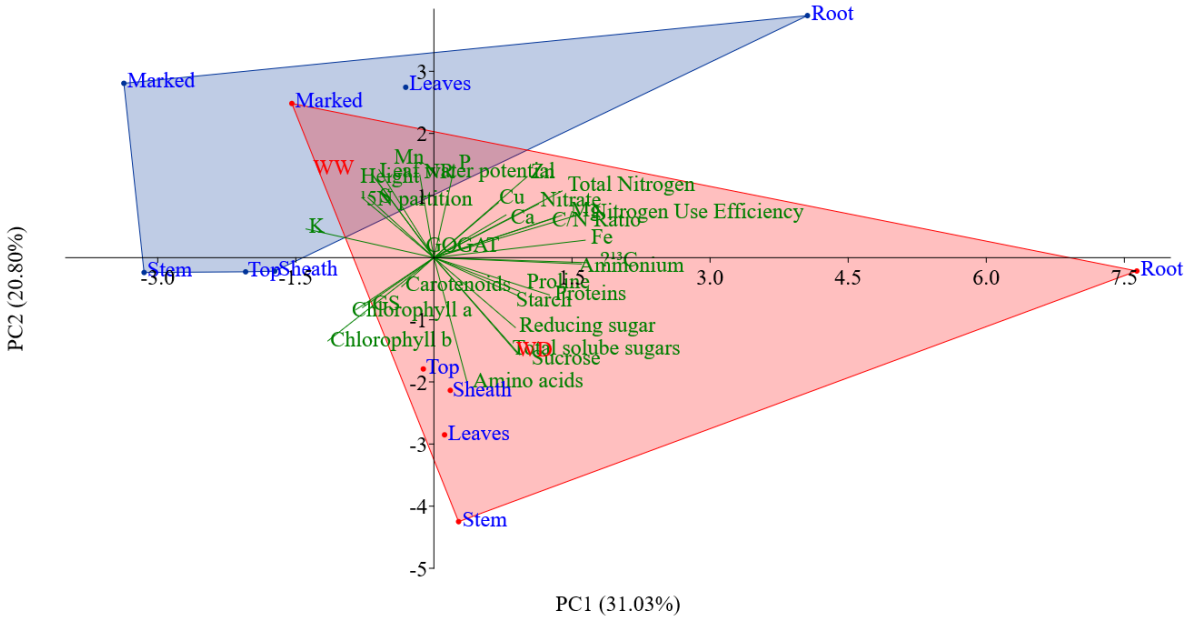

Figure 19. Principal component analysis (PCA) plants under water deficit (WD) and well-watered (WW) conditions at Maximum Stress. Dim1 and Dim2 explain 31.03% and 20.80% of the total variance, respectively. Symbols represent treatments: red circles for WD and blue triangles for WW. Arrows indicate the contribution and direction of each variable to the principal components.

3.9.2 Rehydration

During rehydration, PCA explained 50.6% of the variability in the data (Figure 20). WD plants were associated with nitrogen metabolism markers, such as total nitrogen and the C:N ratio, as well as carbon isotope discrimination. WW plants correlated with physiological parameters, including photosynthesis, transpiration, stomatal conductance, and leaf water potential.

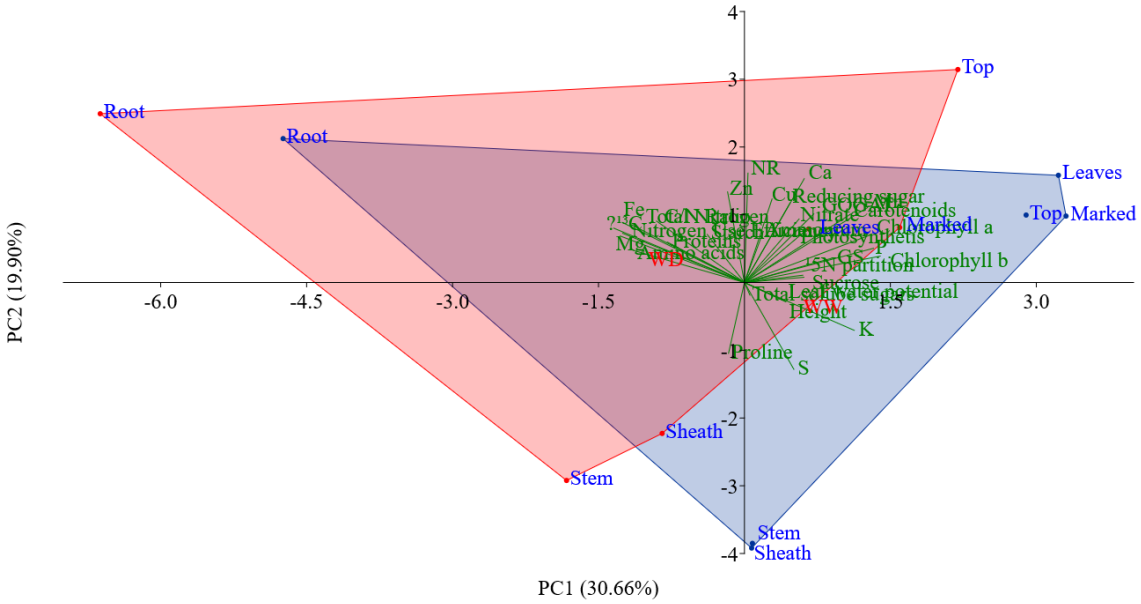

Figure 20, Principal component analysis (PCA) plants under water deficit (WD) and well-watered (WW) conditions at rehydration R. Dim1 and Dim2 explain 30.7% and 19.9% of the total variance, respectively. Symbols represent treatments: red circles for WD and blue triangles for WW. Arrows indicate the contribution and direction of each variable to the principal components.

4 DISCUSSION

4.1 Biometric traits

The observed reduction in plant height and biomass under water deficit (WD) conditions aligns with previous studies highlighting drought-induced limitations on cell elongation and expansion due to decreased cellular turgor pressure (DEVI et al., 2018; KHONGHINTAISONG et al., 2018). Although sugarcane plants showed partial recovery after rehydration, their failure to completely recover indicates persistent physiological constraints, possibly due to irreversible cellular damage or prolonged impairment of growth-related processes (DEVI et al., 2018; KHONGHINTAISONG et al., 2018). Reduced biomass, especially in roots and stalks after rehydration, suggests sustained impacts on resource absorption and translocation, thereby constraining recovery potential (MOHANRAJ et al., 2021). Despite rehydration, WD plants exhibited markedly lower RGR values in roots and stalks compared to WW plants, indicating minimal or negative biomass accumulation. In contrast, leaves in WD plants showed moderate RGR, suggesting a preferential allocation towards maintaining photosynthetic tissues. This indicates that the damage caused by drought could not be reversed after rehydration, and what grew under stress remained fundamentally limited (KHONGHINTAISONG et al., 2021).

4.2 Water relations

The lower leaf water potential (Ψw) observed in WD plants at MS indicates pronounced water stress, reflecting impaired root water uptake and hydraulic conductivity (BRODERSEN & MCELRONE, 2013; DOS SANTOS et al., 2015). The incomplete recovery of leaf Ψw following rehydration suggests residual hydraulic limitations. Similarly, significant reductions in transpiration and stomatal conductance under WD highlight adaptive stomatal regulation aimed at minimising water loss (SINGH et al., 2018). Partial recovery of these parameters after rehydration underscores the plant's cautious response to drought alleviation, potentially conserving water for sustained recovery (EKSTEEN et al., 2014).

4.3 Photosynthetic performance

Drought-induced reductions in photosynthesis primarily result from stomatal closure limiting intercellular CO_2 availability, which directly impacts carboxylation processes and biomass accumulation (BARBOSA et al., 2015; RIBEIRO et al., 2013). Differential responses across leaf sections suggest spatial variation in drought sensitivity, where basal leaf regions experienced more pronounced stress. The partial recovery of photosynthetic rates post-rehydration suggests lingering limitations, possibly from persisting stomatal dysfunction or

residual impairment of enzymatic activity (FLEXAS et al., 2004; MOHANRAJ et al., 2021). These limitations were consistent with the observed patterns in water use efficiency (WUE). WD plants initially exhibited higher WUE, indicating an improved balance between carbon assimilation and water loss. However, as drought progressed, WUE declined, reflecting reduced photosynthetic capacity and sustained stomatal closure. After rehydration, WUE values in WD plants converged with those of WW plants across all leaf regions, suggesting a partial restoration of gas exchange efficiency, although photosynthetic performance remained limited (SILVA et al., 2013). The significant decline in chlorophyll a under WD reflects impaired nitrogen availability and enhanced pigment degradation under drought stress (LIMA NETO et al., 2021). Conversely, the elevated chlorophyll b and carotenoid levels observed during stress indicate protective mechanisms against oxidative damage, maintaining photosynthetic integrity. Recovery in pigment concentrations post-rehydration, particularly chlorophyll a, suggests effective nitrogen remobilisation supporting pigment biosynthesis and photosynthetic reactivation (MASCLAUX-DAUBRESSE et al., 2010).

4.4 Nitrogen metabolism

Under drought, nitrogen accumulation shifted toward roots and sheath leaves, indicating prioritisation of nutrient storage in these tissues. Increased nitrogen remobilisation towards aerial tissues post-rehydration underscores a physiological strategy to restore photosynthetic capacity (HOANG et al., 2019; WANG et al., 2020). Elevated ammonium content under WD, coupled with increased glutamine synthetase (GS) activity, suggests active ammonium detoxification and nitrogen recycling during stress (MASCLAUX-DAUBRESSE et al., 2010; WANG et al., 2020). The reduction in nitrate reductase (NR) activity under drought reflects an energy conservation strategy and minimisation of toxic intermediate accumulation. Additionally, NR and GS/GOGAT may function as alternative electron sinks under stress, helping dissipate excess reducing power generated by restricted carbon assimilation (MASCLAUX-DAUBRESSE et al., 2010; MASCLAUX-DAUBRESSE, 2018). Partial recovery of NR activity post-rehydration signifies renewed nitrate assimilation capacity, closely linked to restored carbon metabolism (DINH et al., 2017; MASCLAUX-DAUBRESSE et al., 2010).

4.5 Carbon metabolism

Water-deficit induced shifts in carbon allocation, notably increasing carbon storage in roots and stalks as starch and soluble sugars, are indicative of osmotic adjustment strategies (GURRIERI et al., 2020; THALMANN & SANTELIA, 2017). Increased root carbon content

post-rehydration reflects compensatory allocation toward root repair and recovery. Elevated C:N ratios in WD roots indicate an imbalance favouring carbon accumulation, likely at the expense of nitrogen uptake due to impaired root function (YANG et al., 2023). Such metabolic rebalancing supports plant resilience during water stress and subsequent recovery.

Lower $\Delta^{13}C$ values under WD demonstrated decreased intercellular CO_2 concentrations resulting from stomatal closure. This response is typically associated with increased WUEi, as stomatal closure reduces water loss relative to carbon assimilation (SEIBT et al., 2008; KÖLLN et al., 2021). Following rehydration, increased $\Delta^{13}C$ values reflected restored stomatal conductance and improved CO_2 uptake, potentially resulting in lower WUEi due to increased transpiration (RAO et al., 2023). Although $\Delta^{13}C$ provides valuable insights into long-term gas exchange and water use strategies, its interpretation as a direct measure of WUEi requires caution, as mesophyll conductance and environmental conditions can influence this relationship (SEIBT et al., 2008).

4.6 Osmotic adjustment compounds

The increased accumulation of proline, soluble sugars, sucrose, amino acids, and reducing sugars under WD conditions highlights their critical role in osmotic adjustment, membrane stabilisation, and energy provision during drought (GANDONOU et al., 2011; GURRIERI et al., 2020). Declines in these osmolytes following rehydration suggest their utilisation for plant growth recovery and metabolic repair processes, reflecting dynamic metabolic adjustments during recovery phases (DIEN et al., 2019).

4.7 Nutrient content

Drought conditions reduced K uptake across plant tissues, reflecting diminished nutrient transport capacity and impaired stomatal function, essential for regulating water relations and photosynthesis (SILVA & CHIAIA, 2021; VASANTHA et al., 2017). Elevated Fe and decreased Mn in WD roots suggest altered micronutrient homeostasis, potentially related to oxidative stress management (SILVA & CHIAIA, 2021). Persistent imbalances in nutrients post-rehydration, particularly reduced potassium, likely limit complete physiological recovery.

4.8 Principal component analysis

Principal component analysis revealed distinct physiological and metabolic profiles under WD and WW conditions. At maximum stress, WD plants prioritised osmotic adjustment, nitrogen remobilisation, and carbon storage to sustain vital functions, contrasting with WW

plants' focus on maintaining growth and photosynthetic activity. During rehydration, WD plants continued to emphasise nitrogen use efficiency and carbon allocation, reflecting adaptive mechanisms that resume growth after stress alleviation. Conversely, WW plants maintained consistent physiological stability throughout (CHAVES et al., 2009; FLEXAS et al., 2004). Overall, these findings underscore adaptive mechanisms of sugarcane, emphasising resource reallocation, osmotic adjustment, and metabolic rebalancing as key responses to drought stress and recovery processes, with persistent physiological constraints evident even after rehydration.

5 CONCLUSIONS

This study demonstrated that drought stress in sugarcane significantly reduces root activity and the efficiency of nitrogen assimilating enzymes, thereby limiting nitrogen uptake from the soil and increasing the plant's reliance on internal nitrogen remobilisation. Under these conditions, nitrogen was preferentially reallocated to the roots and sheath leaves, supporting short-term adaptation through enhanced metabolic flexibility. Although rehydration partially restored physiological functions, osmoprotectants such as proline and soluble sugars were insufficient to fully reverse drought-induced limitations, indicating lasting metabolic constraints. These findings reinforce the importance of integrated nitrogen and carbon metabolism for sugarcane resilience under variable water availability. Agronomically, they emphasise the need to optimise irrigation and nitrogen fertilisation strategies to reduce dependence on internal remobilisation and prevent yield consequences during drought. The development of genotypes with enhanced nitrogen remobilisation efficiency may represent a valuable approach to improving drought tolerance and recovery in sugarcane. Also, future research should explore the molecular and metabolic pathways that regulate nitrogen-carbon interactions, thereby supporting long-term stress adaptation in this and other crop species.

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ANNEXES

ANEX A

Linear regression equations for chlorophyll a and b. This annex presents the linear regression equations fitted to chlorophyll a and b data during two distinct stages of the experiment: Maximum stress (08–12 December 2023) and Rehydration (13–16 December 2023). These stages correspond to the time intervals to fit separate linear models for each treatment and leaf section.

Chlorophyll	Treatment	Leaf section	Regression equation	R^2
a	WD	Base	y = +3.99x + -20.04	0.83
a	WD	Middle	y = +4.10x + -23.85	0.86
a	WD	Tip	y = +3.94x + -23.64	0.75
a	WW	Base	y = +0.17x + 37.98	0.01
a	WW	Middle	y = +0.14x + 37.16	0.02
a	WW	Tip	y = +0.19x + 34.91	0.03
a	WD	Base	y = -1.20x + 46.60	0.5
a	WD	Middle	y = -2.31x + 51.00	0.77
a	WD	Tip	y = -3.51x + 58.40	0.89
a	WW	Base	y = -0.03x + 40.60	0.0
a	WW	Middle	y = +0.07x + 37.60	0.0
a	WW	Tip	y = +0.28x + 34.20	0.05
b	WD	Base	y = +2.48x + -20.08	0.89
b	WD	Middle	y = +1.90x + -13.55	0.82
b	WD	Tip	y = +1.50x + -9.60	0.66
b	WW	Base	y = +0.27x + 14.88	0.05
b	WW	Middle	y = +0.29x + 13.16	0.08
b	WW	Tip	y = +0.44x + 9.66	0.24
b	WD	Base	y = -1.05x + 22.60	0.69
b	WD	Middle	y = -1.18x + 22.00	0.76
b	WD	Tip	y = -1.17x + 20.10	0.6
b	WW	Base	y = +0.58x + 12.10	0.61

b	WW	Middle	y = +0.82x + 9.00	0.62
b	WW	Tip	y = +1.09x + 5.90	0.63