Received: 2 August 2023 | Revised: 9 October 2023 | Accepted: 29 October 2023

DOI: 10.1111/ppl.14085

ORIGINAL RESEARCH

Physiologia Plantarum

Optimizing foliar N-fertilization in sugarcane depends on plant genotype and nitrogen concentration

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Funding information
Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Numbers: 2017/24516-7, 2017/25489-3

Edited by: Soren Husted

Abstract

Foliar N-fertilization (FNf) has emerged as a promising approach to synchronize plant nitrogen (N) demands and application timing, reducing the N losses to the environment associated with traditional soil-based fertilization methods. However, limited information exists regarding the effectiveness of FNf in sugarcane. This study aimed to optimize FNf in sugarcane by evaluating N-fertilizer recovery by the plant (NRP) and assessing potential toxicity effects. Four sugarcane genotypes were subjected to FNf using ^{15}N-urea at five nitrogen concentrations. NRP was assessed at five time points for roots, stalk, old leaves, ^{15}N-urea-fertilized leaves (^{15}NL), and unexpanded leaves (UEL). Leaf scorching, indicating FNf toxicity, was analyzed using morpho-anatomical and histochemical techniques. The results showed that FNf promoted high NRP, with an average recovery of 62.3%. Surprisingly, the redistribution of ^{15}N-urea did not follow the nitrogen uptake rate by sugarcane leaves, with an average of 41.3% of the total-NRP. The stalk emerged as the primary sink for ^{15}N-urea, followed by the UEL. Genotypes differed in the leaf scorching intensity, which increased with higher concentration of ^{15}N-urea. Genotypes also differed in the ^{15}N-urea uptake rate, down-regulated by the N content in the ^{15}NL. These findings emphasize that by carefully choosing the appropriate genotype and nitrogen concentration, FNf can significantly enhance N-fertilizer uptake, resulting in potential environmental and economic benefits.

KEYWORDS
labeled nitrogen, leaf scorching, nitrogen recovery, sugarcane varieties, urea

1 | INTRODUCTION

Nitrogen (N) is an important plant macronutrient, serving as a key constituent of proteins, hormones, enzymes, and chlorophyll. It also participates in several physiological processes, acting directly in the production of energy and carbohydrates for sugarcane development (Hawkesford et al. 2012; Robinson et al. 2013; Bassi et al. 2018). In sugarcane, nitrogen accounts for up to 2% of plant dry matter and is the second most required nutrient, with requirements ranging from 0.5 to 1.4 kg N Mg^{-1} of stalk (Robinson et al. 2013; Cherubin et al. 2019; Sanches and Otto 2022). Factors such as harvesting season length, plant varieties, soil type and management, and weather conditions contribute to variations in N requirements (Thorburn et al. 2017; Dias and Sentelhas 2018; de Castro et al. 2022).

Traditional soil-based N-fertilization methods result in low N-fertilizer recovery by sugarcane plants (NRP) at the end of the crop cycle (NRP is only 26% at harvest), with significant losses to the environment (27%) and substantial immobilization by the soil microbiota (32%; Otto et al. 2016; Quassi de Castro et al. 2021). On the other hand, N-fertilizers applied to the soil have a great contribution during

the early stages of sugarcane development, reaching up to 70% of total-N in the plant (Franco et al. 2011; Vieira-Megda et al. 2015; Quassi de Castro et al. 2021). Differences in NRP can be observed throughout the growing season, primarily influenced by factors such as root development, nitrogen uptake from various sources, e.g., mineralization of soil organic matter or sugarcane straw (Dourado-Neto et al. 2010; Trivelin et al. 2013), and by biological nitrogen fixation (Urquiaga et al. 2012; da Silva et al. 2017; Singh et al. 2020; Pereira et al. 2021). As a strategy to reduce the N-fertilizer rate applied to the soil and mitigate N-fertilizer losses to the environment, an emerging alternative is to employ foliar N-fertilization (FNf) in conjunction with traditional soil-based N-fertilization.

FNf presents several benefits over traditional soil N-fertilization. It can be directly applied to plant sink organs, offering a faster response (Eichert and Fernández 2012; Fernández et al. 2013), and mitigating issues such as nitrogen leaching, greenhouse gas emissions, i.e., nitrous oxide released from the soil during nitrate denitrification and ammonium nitrification processes, and ammonia (NH_3) losses via volatilization (Marschner 2012). Additionally, FNf has been shown to enhance the content of sugars in leaves, which are molecular signals for plant resilience to abiotic stresses like temperatures fluctuations and water deficit (Stoop et al. 1996; Seki et al. 2007; Cerqueira et al. 2019). Nonetheless, FNf can present negative effects, for example, leaf scorching, if the nutrient rate and application timing are not adequate (Johnson et al. 2001; Moore and Botha 2014). Leaf scorching is mainly promoted by ammonium accumulation (Krogmeier et al. 1989; Hachiya et al. 2021; Castro et al. 2022), at rates higher than 20 kg N ha^{-1} (Sangplung and Rosário 1978).

The effectiveness of FNf in sugarcane has not been extensively studied as limited information are available on varietal responses as well as detailed application protocols are missing. Consequently, this study aims to evaluate the uptake and redistribution of ^{15}N-urea, a commonly used foliar N-fertilizer, in four sugarcane varieties under controlled conditions. Urea has been chosen for FNf because it is a neutral molecule, rapidly absorbed by leaves through the cuticle and stomata (Bondada et al. 2001; Eichert and Burkhardt 2001; Wang et al. 2008; Fernández et al. 2021), reaching up to 85% of NRP when applied to the leaves (Wittwer and Teubner 1959; Humbert 1960; Trivelin et al. 1988).

Our results indicated that the rate of N-urea uptake by the leaves was influenced by both the leaf nitrogen content and the concentration of N-urea in the solution. Varietal differences in uptake efficiency were observed. Leaf scorching, a potential side effect of FNf, varied among sugarcane varieties and also increased with higher levels of N-urea concentration in the solution. These findings emphasize the significance of selection of sugarcane varieties and the optimization of N-fertilizer concentrations in FNf applications. This study provides a comprehensive overview of N-urea leaf uptake and redistribution across different sugarcane varieties. Overall, these information can be used for the implementation of customized FNf strategies to promote sustainable agricultural practices in the sugarcane industry.

2 | MATERIALS AND METHODS

2.1 | Experimental design and treatments

The experimental design was randomized blocks in a 4 x 5 x 4 factorial scheme with three replicates, leading to 80 treatments and a total of 240 experimental units in form of pots. The first factor was composed of four sugarcane varieties: CTC 9001 and RB96 6928 were chosen due to differences on their adaxial stomatal density (Supplementary Figure 1), and RB85 5156 and RB92 579 were chosen due to their responsiveness to traditional soil-based N-fertilization (Bassi et al. 2018; Castro et al. 2019). Moreover, those varieties are the most early harvesting sugarcane varieties cropped in the Southeast region of Brazil (Braga Junior et al. 2021). The second factor was represented by four concentrations of N-urea in aqueous solution (8, 16, 24, and 32% of N-urea), equivalent to the application of 5, 10, 15, and 20 kg N ha^{-1} using a volume of 60 l ha^{-1}, two N-rates that would not promote leaf scorching and two that would promote leaf scorching, plus a control treatment. The third factor corresponded to the evaluation times, performed at 0.5, 1.5, 5, and 20 days after FNf (DAFNf).

2.2 | Experimental units installation

The experiment was carried out in a greenhouse (coordinates 22°42' S 47°38' W) where the temperature and the relative humidity of the air (RHA %) were controlled and maintained below 26 °C (average of 22.1 °C) and above 70%.

Pre-sprouted sugarcane seedlings (PSS) were transplanted to pots filled with 10 kg of soil. The soil was previously air-dried, sieved through a 5 mm mesh sieve, and conditioned inside plastic packages, avoiding loss of water and nutrients through drainage. Soil texture and chemical attributes were determined (Gee and Bauder 1986; Raij et al. 2001). The soil was classified as medium-sandy texture, containing 815, 21, and 164 g kg^{-1} of total sand, silt, and clay respectively. The soil had a pH of 5.2, a sum of bases and cation exchange capacity of 16.9 and 35.9 $mmol_c$ dm^{-3}, a base and aluminum saturation percentage of 47% and 0%, and an organic matter content of 11.5 g dm^{-3}. Element contents were 4 mg P dm^{-3}, 8.5 mg S dm^{-3}, 0.9 $mmol_c$ K dm^{-3}, 12 $mmol_c$ Ca dm^{-3}, 4 $mmol_c$ Mg dm^{-3}, <0.02 $mmol_c$ Al dm^{-3}, 19 $mmol_c$ H + Al dm^{-3}, < 0.15 mg B dm^{-3}, 0.8 mg Cu dm^{-3}, 69 mg Fe dm^{-3}, 8.9 mg Mn dm^{-3}, and 1.3 mg Zn dm^{-3}. The soil water holding capacity (WHC) was determined, based on the methodology described by ISO (2003), aiming to maintain the soil moisture between 60 and 70% of WHC throughout the experimental period.

The PSS were grown in the greenhouse for 95 days. The experimental units were weighted daily and the water lost through evapotranspiration was replaced. Nutrients were applied to the soil throughout the experimental period (Supplementary Table 1). Nitrogen was applied only after PSS transplanting once we aimed to investigate the FNf as a complement to its application in the soil. The secondary tillers were removed every four days.

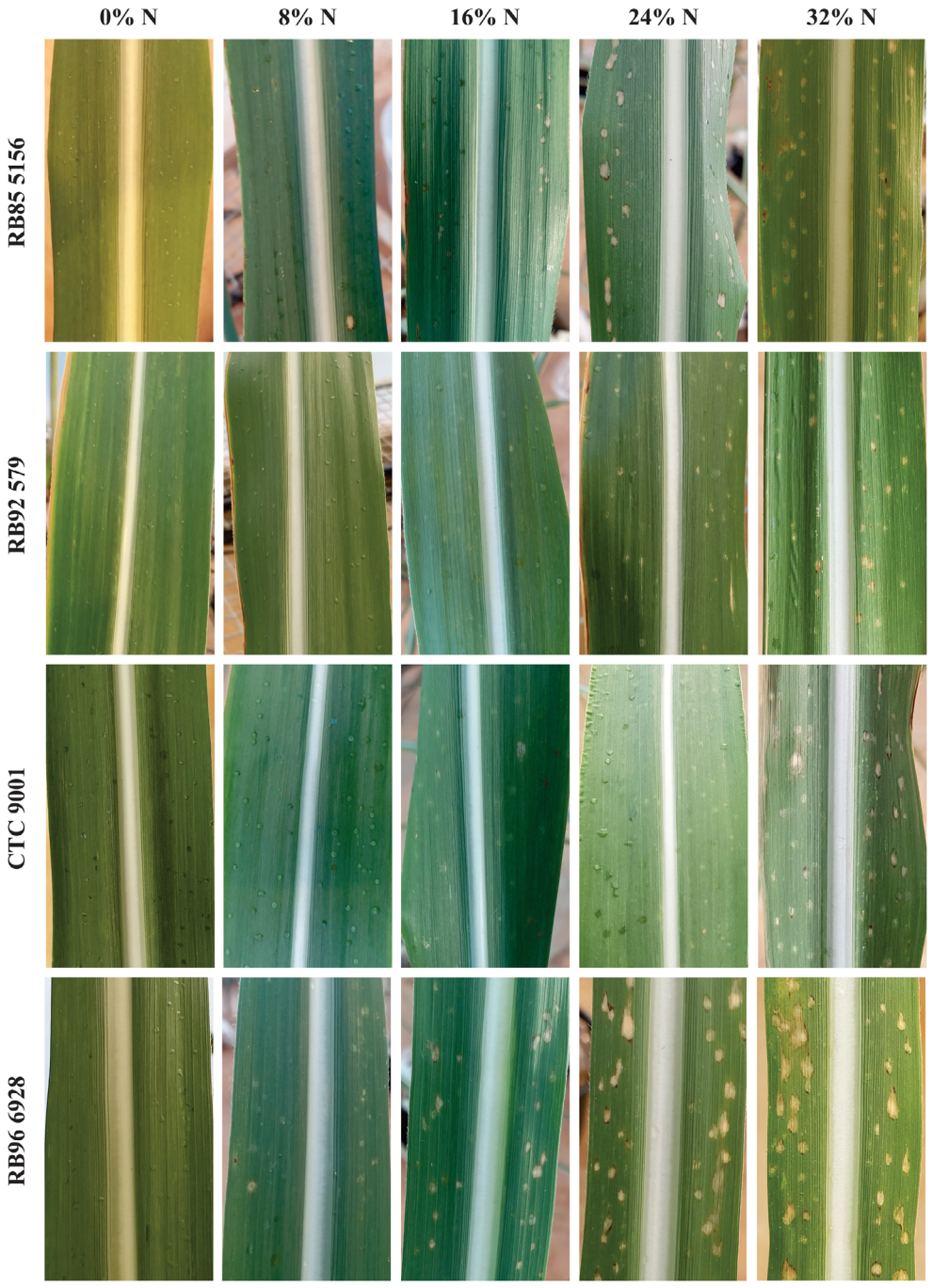

FIGURE 1 Leaf scorching in different sugarcane varieties (RB85 5156, RB92 579, CTC 9001, and RB96 6928) at five days after the application of a N-urea solution containing sucrose onto the leaf + 1. 1 μL droplets of N-urea solutions with different concentrations (from 0 to 32% of N-urea) were carefully pipetted onto the middle section of the adaxial surface of the three youngest fully expanded leaves.

2.3 | Foliar N-fertilization (FNf)

Prior to the FNf, the plastic packages were tied to the plant stalk preventing any N-urea droplets fall onto the soil. Labeled urea with an isotopic abundance of 1.26% of ^{15}N atoms ($CO[^{15}NH_2]_2$) was used to prepare the solutions. The N-urea solutions were applied as 1 μl droplets using a volume repeater pipette (Gilson, Distriman) with a 125 μl tip. 875 μl of N-urea solution was applied per plant, equivalent to the application of 60 l ha^{-1} in a sugarcane field with 1.5 m row spacing and a population of 10 plants per meter. Droplets were deposited onto the adaxial surface on the middle section of the three youngest fully expanded leaves (leaf + 1, +2, and + 3), according to the Kujiper system (Casagrande 1991). Sucrose was added to N-urea solutions (5% w:v; sucrose:N-urea solution) to increase the droplet adherence. The control treatment was composed of deionized water and sucrose (5% w:v). No adjuvant (e.g., spreader) was added to the solution as it may influence the nutrient uptake rate by the leaves leading to leaf toxicity which makes it difficult to distinguish leaf scorching between the N-urea concentration in the solution and fast uptake rate. At the dawn of each day, leaves were re-moisturized with deionized water spraying microdroplets (Trivelin et al. 1988; Leite et al. 2020).

Experimental units were evaluated at 0.5, 1, 5, and 20 DAFNf and aboveground and belowground tissues were sampled. The aboveground samples were divided in four plant tissues in the first three evaluation times: (i) ^{15}N-urea fertilized leaves (^{15}NL), (ii) old leaves (OL), i.e., green leaves with leaf sheath insertion below leaf + 3, (iii) unexpanded leaves (UEL), i.e., leaves that were not fully expanded when FNf was performed, and (iv) stalk. At 20 DAFNf, the plants presented up to three young fully expanded leaves above ^{15}NL (YFEL), that is, they expanded after FNf and were sampled apart. The ^{15}NL were washed in deionized water after sampling to remove the ^{15}N-urea that had not been absorbed (Trivelin et al. 1988). The belowground samples in form of roots were not partitioned but first washed in tap water and then in deionized water.

2.4 | Morphoanatomical analyzes of the leaf + 1

The leaf+1 was sampled at 5 DAFNf. Sampling was performed in the middle third of the leaf where N-urea droplets were applied, excluding the midrib.

The stomatal density was evaluated in six epidermal impression points by the enamel method (Rodella et al. 1983). Two images of each impression were captured, and used to calculate stomatal density, i.e., number of stomata in 0.4 mm^2 (Cutter 1978). Images were obtained at a 100X magnification using a light microscope (Carl Zeiss Axioskop 40).

The morphological characterization of the adaxial and abaxial leaf + 1 surface and the leaf scorching, i.e., the damage promoted to the leaf cuticle due to FNf, were carried out by scanning electron microscopy (SEM). Leaf + 1 fragments were immediately fixed in a 4% paraformaldehyde solution for 72 hours. Sample dehydration was performed in increasing ethyl series (20, 30, 40, 50, 60, 70, 80, 90, and 100%) for 30 minutes each series. Samples were dried to the critical point using liquid CO_2 (Leica, EM CPD 300), and metallized with gold for 180 seconds (Leica, EM ACE 600). Images were obtained using a SEM (JEOL, JSM-IT300 LV) operating at 20 kV.

Anatomical evaluations were performed by two histochemical tests: the periodic acid Schiff test to identify fiber, pectin, mucilage, polysaccharides, and starch grains (O'Brien and McCully 1981), and the ponceau xylidine test to locate protein bodies (De Campos Vidal 1977). Leaf + 1 fragments were fixed and dehydrated as described above. Samples were then transferred to 100% propanol followed by 100% butanol and infiltrated with hydroxyethil methacrylate (HistoResin kit, Leica) at 4 °C. Polymerization was carried out at room temperature for 48 hours. 4 µm sections were obtained (Leica Microtome, RM 2155), deposited on microscope slides and mounted with Entellan® (Merck, Millipore). Images were obtained using a transmitted light microscope (Carl Zeiss, Axioskop 40).

2.5 | Plant tissue dry mass

The plant tissues were weighted after sampling (fresh mass), placed in paper bags, and dried in an oven at 65 °C until achieving constant weight in form of dry mass.

2.6 | Total-N content and N-fertilizer recovery by each plant tissue

The dried plant tissues were ground in a Wiley mill with a 0.5 mm (35 mesh) sieve, followed by cleaning with deionized water and alcohol to prevent cross-contaminations. 8 to 10 mg of aboveground plant tissues and 10 to 12 mg of belowground plant tissue were weighted on an analytical scale and packed in tin capsules. In order to obtain the isotopic abundance of the ^{15}N atoms in % and the total-N (g kg^{-1}), samples were analyzed in an isotope ratio mass spectrometer (Sercon, Hydra 20/20) interfaced to an automatic N analyzer (Sercon, Anca GSL). The N in each plant tissue derived from fertilizer (NPDFF, %, and ANPDFF, g kg^{-1}) and the N-fertilizer recovery by each plant tissue (NRP, %) were calculated, according to the following equations. Total N-fertilizer recovery by the plant (total-NRP) is the sum of the NRP of each plant tissue.

$$\mathrm{NPDFF}\,(\%) = [(a-b) \div (c-b)]\,x\,100 \qquad (Eq1)$$

where a is the ^{15}N isotopic abundance of each plant tissue, b is the ^{15}N isotopic natural abundance, and c is the ^{15}N isotopic abundance of the fertilizer;

$$\mathrm{NRP}\,(\%) = \{[(\mathrm{NPDFF} \div 100)\,x\,\mathrm{total}-\mathrm{N}] \div \mathrm{N}-\mathrm{rate}\}\,x\,100 \qquad (Eq2)$$

where total-N is the nitrogen content in each plant tissue, and N-rate is the N-fertilizer rate applied to the leaves (g plant^{-1}).

2.7 | Statistical analysis

Considering the plant dry biomass, total-N, total-N amount (g N plant^{-1}), NPDFF, and total-NRP, principal component analyzes were performed at each evaluation time, assuming the combinations of N-urea concentration and sugarcane variety. The other variables were not used due to the high correlation with those presented above. At 5 DAFNf, multivariate analysis was carried out within N-urea concentrations considering adaxial stomatal density.

The model $y_{ijklmno}$ was fitted for each variable, except for stomatal density of leaf + 1.

$$\begin{aligned} y_{ijklmno} = {} & \mu + a_i + b_{j(i)} + c_{k(ij)} + \alpha_l + \beta_m + \gamma_n + \delta_o + (\alpha\beta)_{lm} + (\alpha\gamma)_{ln} + (\alpha\delta)_{lo} \\ & + (\beta\gamma)_{mn} + (\beta\delta)_{mo} + (\gamma\delta)_{no} + (\alpha\beta\gamma)_{lmn} + (\alpha\beta\delta)_{lmo} + (\alpha\gamma\delta)_{lno} \\ & + (\beta\gamma\delta)_{mno} + (\alpha\beta\gamma\delta)_{lmno} + e_{ijklmno}, \end{aligned}$$

where, μ, is a constant, a_i is the effect of the *i-th* block, so $a_i \sim N(0,\sigma_a^2)$, $b_{j(i)}$ is the effect of *j-th* greenhouse table into the *i-th* block, so $b_{j(i)} \sim N(0,\sigma_b^2)$, c_k is the effect of *k-th* experimental unit into *j-th* greenhouse table of the *i-th* experimental unit, so $c_{k(ij)} \sim N(0,\sigma_c^2)$, α_l is the effect of *l-th* sugarcane variety, β_m is the effect of *m-th* N-urea concentration, γ_n is the effect of *n-th* evaluation time, δ_o is the effect of *o-th* plant tissue, $(\alpha\beta)_{lm}$ is the interaction effect between *l-th* sugarcane variety and *m-th* N-urea concentration, $(\alpha\gamma)_{ln}$ is the

interaction effect between *l-th* sugarcane variety and *n-th* evaluation time, $(\alpha\delta)_{lo}$ is the interaction effect between *l-th* sugarcane variety and *o-th* plant tissue, $(\beta\gamma)_{mn}$ is the interaction effect between *m-th* N-urea concentration and *n-th* evaluation time, $(\beta\delta)_{mo}$ is the interaction effect between *m-th* N-urea concentration and *o-th* plant tissue, $(\alpha\beta\gamma)_{lmn}$ is the interaction effect between *l-th* sugarcane variety, *m-th* N-urea concentration and *n-th* evaluation time, $(\alpha\beta\delta)_{lmo}$ is the interaction effect between *l-th* sugarcane variety, a *m-th* N-urea concentration and *o-th* plant tissue, $(\alpha\gamma\delta)_{lno}$ is the interaction effect between *l-th* sugarcane variety, *n-th* evaluation time and *o-th* plant tissue, $(\beta\gamma\delta)_{mno}$ is the interaction effect between a *m-th* N-urea concentration, *n-th* evaluation time and *o-th* plant tissue, $(\alpha\beta\gamma\delta)_{lmno}$ is the interaction effect between *l-th* sugarcane variety, *m-th* N-urea concentration, *n-th* evaluation time and *o-th* plant tissue and $e_{ijklmno}$ is the error, so $e_{ijklmno} \sim N(0, \sigma_o^2)$, i.e., error variances are different for each plant tissue.

The estimation method used was the residual maximum likelihood method and the model was chosen by the likelihood ratio test when these were nested models, otherwise, the Akaike Information Criterion was used. Wald-F test was used to evaluate the fixed effects. For mean comparison the post-hoc Fisher's LSD test ($\alpha = 5\%$) was used. Analyses were done in R (R Core Team 2021) using the library asreml (Butler 2021) and asremlPlus (Brien 2020).

Stomatal density was analyzed as a two-factor randomized block design (sugarcane varieties and N-urea concentration) with three biological replicates. The average of stomatal density in each leaf surface was analyzed by F test ($p < 0.05$), significant difference was compared by Tukey's post-hoc test ($p < 0.05$) and analyses were also performed in R using the ExpDes library (Ferreira et al. 2014).

3 | RESULTS

3.1 | Leaf scorching in sugarcane differs depending on both, the sugarcane variety and the concentration of N-urea in the solution

No leaf scorching was observed in the ^{15}N-fertilized leaves (^{15}NL) at 0.5 day after foliar N-fertilization (DAFNf). However, at 1.5 DAFNf, leaf scorching started to be observed underneath the droplets in the RB96 6928, specifically at N-urea concentrations of 16, 24, and 32% (Supplementary Figure 2). The other sugarcane varieties did not exhibit any visible leaf scorching at this stage.

At 5 DAFNf, leaf scorching was observed in the ^{15}NL across all sugarcane varieties, with variations dependent on the concentration of N-urea in the solution and the specific sugarcane variety (Figure 1). RB96 6928 exhibited a range of leaf scorching intensities, from mild at 8% of N-urea, to very severe at both 24 and 32% of N-urea (Figure 1). Similar patterns were observed for RB85 5156, although with slightly lower severity of leaf scorching (Figure 1). RB92 579 displayed severe leaf scorching at 32% of N-urea, and mild leaf scorching at 24% of N-urea (Figure 1). CTC 9001 displayed severe leaf scorching at 32% of N-urea, and mild leaf scorching at both 16 and 24% of N-urea (Figure 1). No leaf scorching was observed in RB85 5156, RB92 579, and CTC 9001 at 8% of N-urea. At 20 DAFNf, leaf scorching patterns were similar to those observed at 5 DAFNf, except for RB85 5156 at 32% of N-urea and for RB96 6928 at 16, 24, and 32% of N-urea, in which the severity of leaf scorching increased.

The above observations suggest that concentrations higher than 24% of N-urea lead to severe leaf scorching in sugarcane. Scorching patterns clearly indicate that the extent of leaf damage is dependent on the concentration of N-urea and the sugarcane variety. The tolerance to concentrations of N-urea was lowest for RB96 6928 and highest for RB92 579.

3.2 | Characterization of sugarcane leaf morpho-anatomy and leaf scorching

The adaxial and abaxial stomatal density of the leaf + 1 exhibited variations among sugarcane varieties (Table 1). Adaxial stomatal density was lowest in RB96 6928, approximately 20% lower than that of CTC 9001 and RB92 579, both of which exhibited similar densities. Abaxial stomatal density was lowest in RB96 6928 and highest in RB85 5156. Based on stomatal density results, the sugarcane varieties used in the present study can be classified as hypostomatic, with the number of stomata being double on the abaxial leaf surface relative to the adaxial leaf surface.

To characterize the leaf morphology and the degradation of the cuticle underneath the droplet (i.e., leaf scorching caused by FNf) scanning electron microscopy was employed. The number of trichomes was highest on the abaxial leaf surface (Supplementary Figure 3). Notably, there was a pronounced deposition of epicuticular wax around the guard cells of the stomata, while other regions of the leaf blade showed comparatively lower levels of wax deposition (Figure 2 and 3). Disruption of the cuticle was evident on both surfaces of the leaf + 1 where the N-urea droplet was applied, with the severity of disruption being more prominent on the adaxial surface (Figure 2) than on the abaxial surface (Figure 3).

Protein bodies (Supplementary Figure 4) and polysaccharides (Supplementary Figure 5) were observed within the chloroplasts of vascular bundle sheath cells and adjacent mesophyll cells. Additionally, mucilages and a few starch grains were detected in the adjacent mesophyll cells (Supplementary Figure 5). Lignin deposition was identified in the cuticle, particularly on the adaxial leaf surface, as well as in the vascular bundle cells adjacent to the phloem (Supplementary Figure 5). Notably, FNf only influenced the presence of protein bodies, which were more prominent at higher concentrations of N-urea across all sugarcane varieties.

3.3 | Influence of sugarcane varieties and N-urea concentration on the N-fertilizer uptake rate

The uptake rate of the N-urea applied onto sugarcane leaves is influenced by both the sugarcane variety and the concentration of N-urea

TABLE 1 Adaxial and abaxial leaf + 1 stomatal density, i.e., number of stomata quantified in 0.4 mm^2, of the sugarcane varieties CTC 9001, RB85 5156, RB92 579, and RB96 6928, at five days after the application of N-urea solutions with the concentration of 0, 8, 16, 24, and 32% of N

Variety	0% of N			8% of N			16% of N			24% of N			32% of N			Mean
	---------- Adaxial ----------															
CTC 9001	36	a		35	a		37	a		36	a		35	a		36
RB85 5156	29	b	B	30	b	AB	32	b	AB	33	b	A	34	a	A	32
RB92 579	36	a		34	a		37	a		36	ab		36	a		36
RB96 6928	25	c	C	26	c	BC	30	b	A	28	c	AB	30	b	AB	28
Mean	32			31			34			33			34			33
	---------- Abaxial ----------															
CTC 9001	61	a		61	a		62	a		64	b		62	ab		62
RB85 5156	63	a	B	62	a	B	63	a	B	68	a	A	66	a	AB	64
RB92 579	62	a	A	63	a	A	64	a	A	63	bc	A	57	c	B	62
RB96 6928	58	a	AB	55	b	B	57	b	AB	59	c	AB	59	bc	A	58
Mean	61			60			62			63			61			61

*Lowercase letters indicate differences among sugarcane varieties at each N-urea concentration in the solution. Capital letters indicate differences among N-urea concentrations in the solution at each sugarcane variety. The absence of capital letters indicates no significant difference among N-urea concentrations in the solution at each sugarcane variety. These comparisons were determined using Tukey's post-hoc test with a significance level of $p < 0.05$.

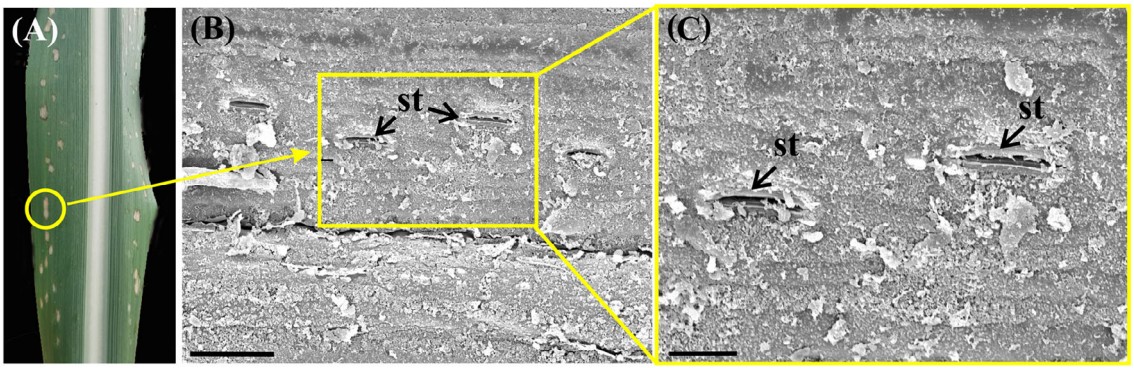

FIGURE 2 Occurrence of leaf scorching on the upper surface (adaxial surface) of leaf + 1 (A) resulting from the application of a N-urea solution with a concentration of 24% of nitrogen (N) on the RB85 5156 sugarcane variety at five days after foliar N-fertilization (DAFNf). Leaf scorching underneath the N-urea droplet observed with scanning electron microscopy (B, C). The accompanying caption indicates the presence of stomata (st) on the leaf surface. Scale bars = 50 μm (B) and 20 μm (C).

in the solution (Figure 4). Contrary to expectations based on a previous study (Trivelin et al. 1988), there was no significant uptake of N-urea by sugarcane leaves within the first 12 hours after foliar N-fertilization (0.5 DAFNf; Figure 4). Instead, we observed a linear increase in the total-NRP up to 5 DAFNf (Supplementary Figure 6). The mean total-NRP at 0.5 DAFNf for N-urea concentrations of 8, 16, 24, and 32% was 12.5, 10.1, 7.0, and 4.8%, respectively. At 1.5 DAFNf, the mean total-NRP increased to 36.7, 27.0, 21.9, and 20.3% for the corresponding N-urea concentrations. At 5 DAFNf, the mean total-NRP rose to 66.5, 61.7, 52.2, and 48.3%, and at 20 DAFNf, it reached 74.5, 61.8, 58.7, and 54.3%, respectively for N-urea concentrations of 8, 16, 24, and 32%.

The main differences in total-NRP among the sugarcane varieties were observed at 0.5 and 1.5 DAFNf (Figure 4). At 0.5 DAFNf, total-NRP was lowest in CTC 9001 and highest in RB96 6928 at 8, 16, and 24% of N-urea. Conversely, RB96 6928 displayed the lowest and RB92 579 exhibited the highest total-NRP at 32% of N-urea. At 1.5 DAFNf, sugarcane varieties only differed at 16 and 32% of N-urea. Among these, RB92 579 and RB96 6928 varieties displayed the highest total-NRP. At 5 and 20 DAFNf, the total-NRP did not differ among sugarcane varieties within the concentration of N-urea, except at 5 DAFNf with 32% of N-urea and at 20 DAFNf with 24% of N-urea.

The leaf scorching was up-regulated by the concentration of N-urea in the solution (Figure 1). Moreover, a noticeable variation in N-urea uptake was observed among the different sugarcane varieties, highlighting the necessity to evaluate the impact of N-urea concentration on total-NRP (Figure 4). The concentration of N-urea in the solution inversely influenced total-NRP whereby the total-NRP at 8% of N-urea was higher than that at 32% of N-urea.

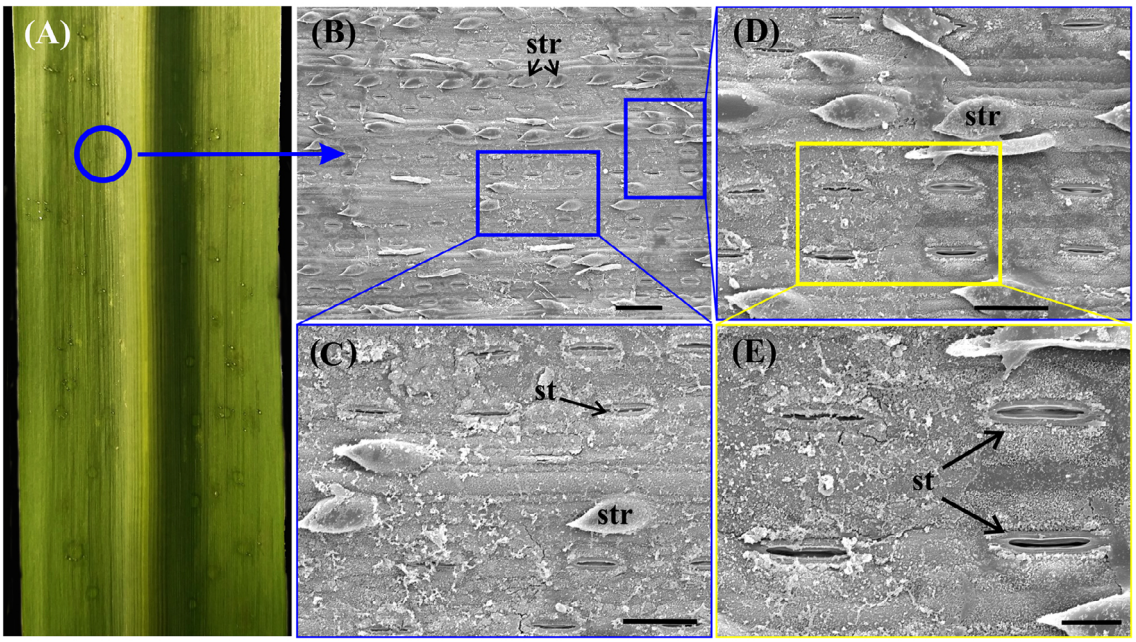

FIGURE 3 Occurrence of leaf scorching on the underside (abaxial surface) of leaf + 1 (A) as a result of applying a N-urea solution with a concentration of 24% of nitrogen (N) on the RB85 5156 sugarcane variety at five days after foliar N-fertilization (DAFNf). Leaf scorching underneath the N-urea droplet (B, C) and at the border of the droplet, i.e., transition from scorched to non-scorched region (D, E), by scanning electron microscopy. The caption indicates the presence of stomata (st) and spiniform trichomes (str) on the leaf surface. Scale bars = 100 μm (B), 50 μm (C, D) and 20 μm (E).

To assess the multivariate effects of N-urea concentration in the solution and sugarcane varieties on the variables of interest, we performed principal component analysis (PCA) for each evaluation time (Supplementary Figure 7). The PCA consistently accounted for over 90% of the data variance across all evaluation times. Among the variables of interest, total-N exhibited the most significant variation among sugarcane varieties, while total-NRP showed a negative correlation with the N-urea concentration in the solution (Supplementary Figure 7). At five days after foliar N-fertilization (DAFNf), total-NRP reached a stable state, leading to the implementation of PCA exclusively within the N-urea concentration in the solution (Figure 5 and Supplementary Figure 8). In treatments with 8 and 16% of N-urea in the solution, in which leaf scorching was mild or absent (Figure 1), total-NRP displayed an inverse relationship with total-N and stomatal density as depicted by the main PCA component (Can1; Figure 5). Interestingly, sugarcane varieties with lower stomatal adaxial density of the ^{15}NL exhibited higher total-NRP, which contrasts the anticipated expectations. Conversely, sugarcane varieties with lower total-N content in the ^{15}NL demonstrated greater total-NRP. Additionally, it is noteworthy that total-N was down-regulated by the dry mass of the plant, indicating that an increase in dry mass leads to a reduction in total-N levels.

3.4 | The stalk and unexpanded leaves of the sugarcane plant serve as major nitrogen sink for N-urea applied to the leaves

The understanding of N-fertilizer redistribution rates and fate within the sugarcane plant after FNf is important and remains unexplored. To address this knowledge gap, sugarcane plants were divided into five plant tissues for evaluations at 0.5, 1.5, and 5 days after foliar N-fertilization (DAFNf), and in six plant tissues at 20 DAFNf.

Among the plant tissues, roots consistently exhibited the highest dry mass across all sugarcane varieties, while the stalk exhibited the greatest increase in dry mass throughout the evaluation times (Supplementary Figure 9). Notably, the concentration of N-urea in the solution did not have a significant influence on the dry mass of the plant tissues.

The total-N content in the plant tissues generally decreased over the evaluation times (Supplementary Table 2), except for the ^{15}NL. In terms of the ^{15}NL (Figure 6), CTC 9001 exhibited the highest total-N content, while RB96 6928 and RB92 579 displayed the lowest. The total-N content of the ^{15}NL showed an increase up to 5 DAFNf and was up-regulated by the concentration of N-urea in the solution. At 20 DAFNf, the total-N content of the ^{15}NL at varying N-urea concentrations (0, 8, 16, 24, and 32% of N-urea) was, on average, 10.7, 10.8, 11.6, 13.5, and 14.4 g kg^{-1}, respectively, in the RB92 579, RB85 5156 and RB96 6928 varieties. In the CTC 9001 variety, the corresponding values were 14.6, 15.2, 16.2, 21.3, and 21.2 g kg^{-1}. The total-N content of the other plant tissues was minimally influenced by the concentration of N-urea in the solution (Supplementary Table 2).

The nitrogen in each plant tissue derived from the fertilizer (NPDFF, in percentage) increased in all plant tissues throughout the evaluation times, except for ^{15}NL and OL tissues at 5 and 20 DAFNf (Supplementary Table 3). The ^{15}NL exhibited the highest NPDFF at all evaluation times, up-regulated by N-urea concentration in the solution. The average of the NPDFF in the ^{15}NL at 0.5 DAFNf was 2.9, 4.5, 4.9,

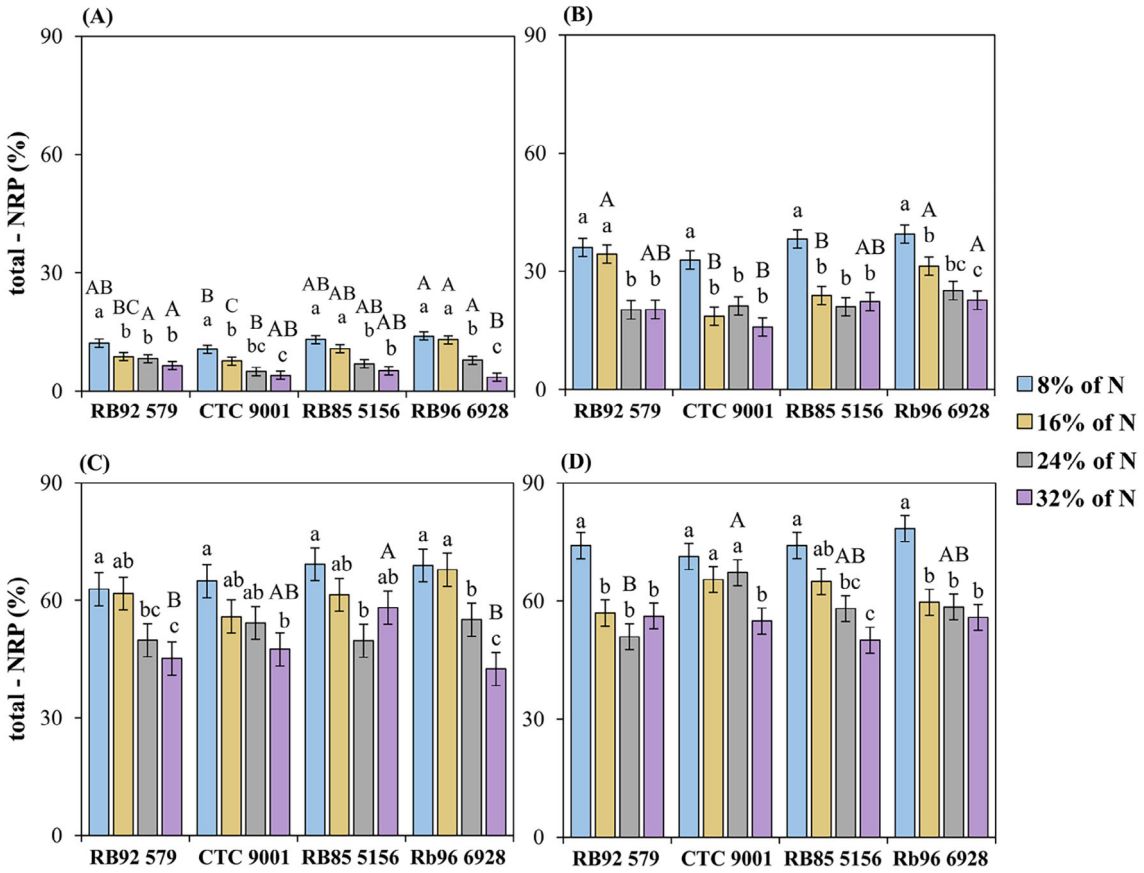

FIGURE 4 Total N-fertilizer recovery by the plant (total-NRP, in percentage) of sugarcane varieties RB92 579, CTC 9001, RB85 5156, and RB96 6928. The plants were fertilized using a N-urea solution which was applied onto the three youngest fully expanded leaves at different N concentrations (8, 16, 24, and 32% of N-urea). The evaluation times were 0.5 (A), 1.5 (B), 5 (C), and 20 (D) days after foliar nitrogen fertilization. The data represent the average values obtained from three independent biological replicates and the error bars indicate the standard error. Lowercase letters indicate differences among N-urea concentration in the solution within each fixed interaction (evaluation time and sugarcane variety). Capital letters indicate differences among sugarcane varieties within each fixed interaction (evaluation time and N-urea concentration). The absence of capital letters indicates no significant difference among sugarcane varieties for a given fixed interaction. These comparisons were determined using Fisher's LSD post-hoc test with a significance level of $p < 0.05$.

and 4.3% in 8, 16, 24, and 32% of N-urea in the solution, respectively, and at 5 DAFNf reached 11.4, 21.3, 26.3, and 29.9%, respectively. Sugarcane varieties also differed regarding the NPDFF in the ^{15}NL at 0.5 and 1.5 DAFNf, in which RB92 579 and RB96 6928 varieties showed the highest NPDFF. The other plant tissues differed in NPDFF mainly at 20 DAFNf. Stalk and UEL were the drains of N-fertilizer, up-regulated by the N-urea concentration. The NPDFF of UEL differed among the sugarcane varieties only at 20 DAFNf and in the Stalk it differed at 5 and 20 DAFNf.

The ^{15}N-urea redistribution (Supplementary Table 4), i.e., the transport of ^{15}N-urea from ^{15}NL to other plant tissues, was slower than the N-fertilizer uptake rate by sugarcane leaves. The ^{15}N-urea redistribution was mainly observed at 20 DAFNf (Table 2). In this evaluation time, the average of N-fertilizer redistribution was 54.4, 44.1, 35.8, and 30.9% of the total-NRP respectively to 8, 16, 24, and 32% of N-urea in the solution, regardless of the sugarcane variety. The NRP of each plant tissue increased throughout the experiment, except for ^{15}NL, which NRP decreased between 5 and 20 DAFNf (Supplementary Table 4 and Table 2). The ^{15}NL tissue exhibited the highest NRP in all evaluation time and sugarcane varieties. The average of NRP of ^{15}NL was 86.3, 92.0, 85.7, and 58.7% of the total-NRP respectively, regardless of the sugarcane variety and the N-urea concentration in the solution. At 20 DAFNf the main drain of ^{15}N-urea applied to the sugarcane leaves was the stalk. The average of NRP of stalk was 17, 24, 19, and 23% of the total-NRP in CTC 9001, RB92 579, RB85 5156, and RB96 6928, respectively. The UEL and roots tissues were, respectively, the second and third ^{15}N-urea drain plant tissue, while the OL tissue had an NRP lower than 1%, not being a drain of foliar-applied N.

4 | DISCUSSION

4.1 | Varietal differences in N-urea uptake rate by sugarcane leaves are dependent on the total-N content

The understanding of varietal variations in N-fertilizer use efficiency (NUE) under soil N-fertilization in sugarcane is limited and has been

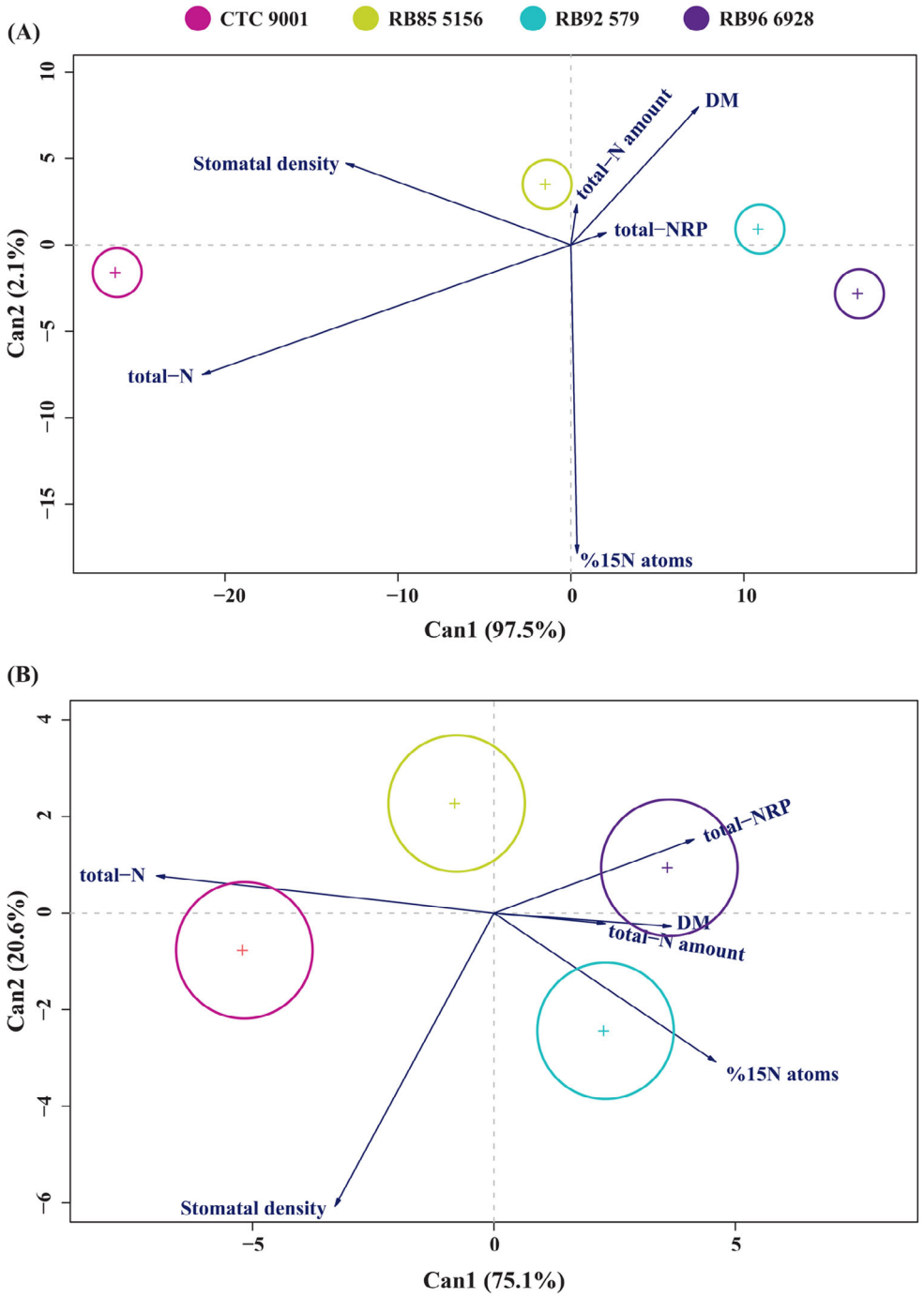

FIGURE 5 Principal component analysis (PCA) conducted on variables of interest, including the isotopic abundance (%15 N atoms), total-N and adaxial stomatal density of the ^{15}NL, plant total dry mass (DM), total nitrogen amount in the plant (total-N amount), and total N-fertilizer recovery by the plant (total-NRP). The PCA was performed separately for 8% (A) and 16% (B) of N-urea in the solution. The components Can1 and Can2 represent the first and the second main components of the PCA, respectively. The size of the circles in the plot represents the confidence ellipse of each sugarcane variety at 95% confidence level.

explored in few studies (Bassi et al. 2018; Yang et al. 2019). Moreover, no previous research has investigated the N-fertilizer recovery by the plant (NRP) or NUE in the context of foliar N-fertilization (FNf) for sugarcane.

The absorption of solutions by plant leaves occurs through several pathways such as for instance, through stomata openings (Eichert and Burkhardt 2001; Eichert and Goldbach 2008; Fernández et al. 2017, 2021). In this study, special care was taken to maintain the relative humidity of the air (RHA) within the greenhouse higher than 70%, i.e., above the point of efflorescence of urea (50%), to prevent solution crystallization (Casali et al. 2019). Interestingly, the sugarcane variety with the highest total-NRP at 0.5 and 1.5 days after foliar N-fertilization (DAFNf) exhibited the lowest stomatal density on the adaxial leaf surface (Table 1 and Figure 4). Although stomata need to be hydraulically activated for solution uptake, typically only a small portion, approximately 10–20% of stomata are activated (Eichert et al. 2008; Burkhardt et al. 2012; Eichert and Fernández 2012). This suggests the existence of alternative uptake pathways for NRP.

Models of the solution uptake through the cuticle, known as cuticular diffusion, have been proposed in the scientific literature (Schönherr 2006; Fernández et al. 2017). The rate of solution diffusion is influenced by the concentration gradient (Riederer and Friedmann 2006;

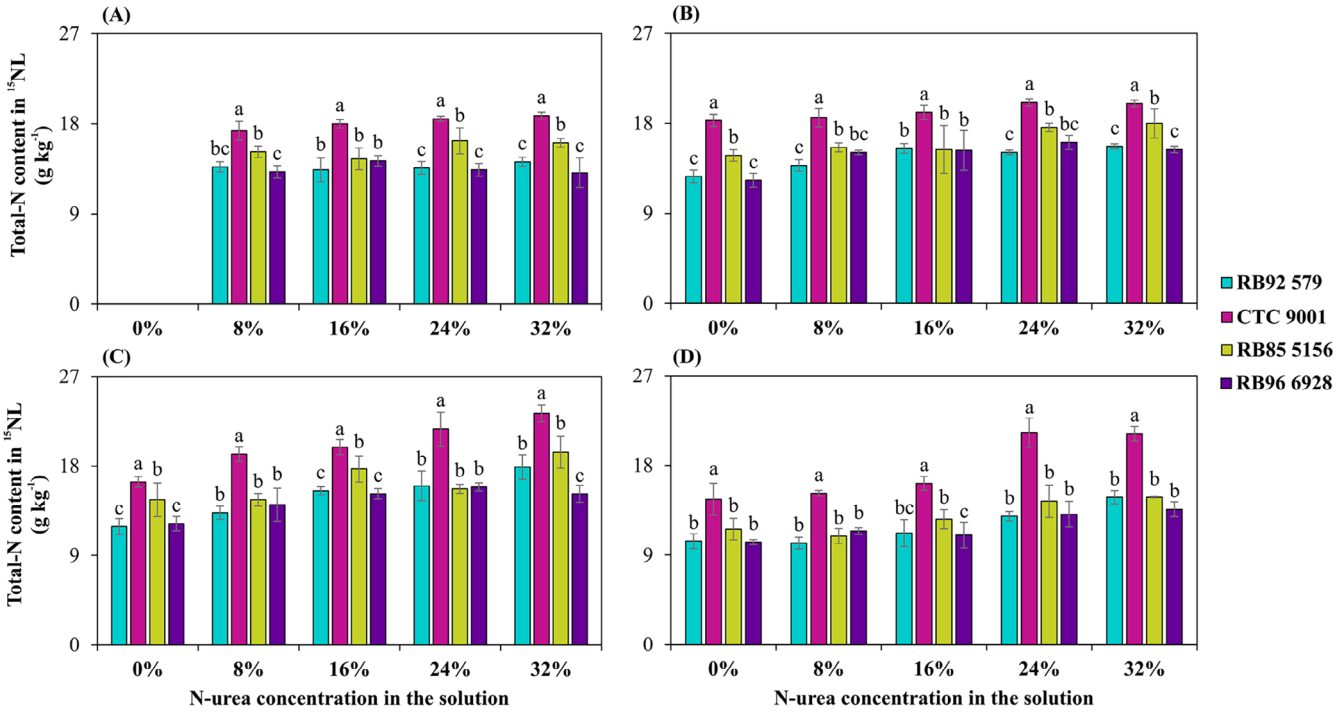

FIGURE 6 Total-N content (g kg^{-1}) in the ^{15}N-urea fertilized leaves (^{15}NL) of the sugarcane varieties RB92 579, CTC 9001, RB85 5156, and RB96 6928 at 0.5 (A), 1.5 (B), 5 (C), and 20 (D) days after foliar N-fertilization with N-urea solutions containing 0 (control), 8, 16, 24, and 32% of N-urea. The data represent the average values obtained from three independent biological replicates and the error bars indicate the standard error. ^{15}NL was not sampled in the control (0%) at 0.5 day after foliar N-fertilization. Lowercase letters indicate differences among sugarcane varieties within each combination of evaluation time, and N-urea concentration in the solution according to Fisher's LSD post-hoc test ($p < 0.05$).

Bi and Scagel 2008; Fernández and Brown 2013) and can be affected by various environmental factors (Fernández et al. 2017). The observed results in our experiment could potentially be explained by the cuticular uptake pathway, as sugarcane varieties with lower nitrogen (N) content in the ^{15}N-urea fertilized leaves (^{15}NL) exhibited higher total-NRP at 0.5 and 1.5 DAFNf, indicating that the total-NRP is down-regulated by the total-N content in ^{15}NL (Figure 4 and 6, and Supplementary Figure 7). A similar pattern was observed at 5 DAFNf, when maximum uptake of N-urea occurred, using N-urea concentrations of 8 and 16% of N in the solution (Figure 5). Similar results were obtained by Uscola et al. (2014) in a study involving foliar N-fertilization of Mediterranean tree seedlings with different nitrogen sources like urea, ammonium sulfate, and glycine. However, at 20 DAFNf, different patterns of total-NRP were observed in relation to the analyzed variables of interest (Supplementary Figure 7), indicating that alternative leaf uptake pathways, as well as other factors such as cuticle structure and bulliform cells, need to be considered in the long-term (Bondada et al. 2001; Ferreira et al. 2007; Wuyts et al. 2015). Consequently, future experiments should include the evaluation of hydration and characterization of the cuticle and bulliform cells to enhance our understanding of the N-urea uptake pathway in sugarcane leaves.

The absorption of nutrients through the leaves can also occur via trichomes, as described in previous studies (Winkler and Zotz 2010; Li et al. 2018; Arsic et al. 2022). However, in the present study, it is important to note that trichomes are primarily located on the abaxial surface of sugarcane leaves, while the N-urea solution was applied to the adaxial leaf surface. Therefore, it is likely that the uptake through trichomes had no effect on the results obtained in this study.

4.2 | Stalk and unexpanded leaves are the sinks of N-urea applied to the sugarcane leaves

Urea, once absorbed by the leaves, undergoes hydrolysis to NH_3 and carbon dioxide catalyzed by the enzyme urease (Witte 2011). A fraction of the urea applied to the leaves is hydrolyzed on the leaf surface (Bowman and Paul 1990; Smith et al. 1991; Wang et al. 2008), explaining why total-NRP was not 100% (Figure 4 and Supplementary Figure 6). Following hydrolyses, ammonium is produced and assimilated into other compounds, such as amino acids and proteins, within the leaf (Supplementary Figure 4; Hawkesford et al. 2012; Zhang et al. 2017). These compounds are then transported to other plant tissues based on the source-sink strength and the mobility of nutrients within the phloem (Tegeder and Masclaux-Daubresse 2018). Nitrogen is known to be mobile within the phloem. However, the redistribution rate of N-urea within the plant was unexpectedly slow, with a more pronounced effect observed at 20 days after foliar N-fertilization (DAFNf), ranging from 22.3 to 57% of total-NRP and being influenced by the concentration of N-urea in the solution.

TABLE 2 N-fertilizer recovery by each plant tissue (NRP, in percentage [%]) of the sugarcane varieties RB92 579, CTC 9001, RB85 5156, and RB96 6928 at 20 days after foliar N-fertilization with N-urea solutions containing 8, 16, 24, and 32% of N-urea

N-urea concentration in the solution (%)	Plant tissue	RB92 579		CTC 9001		RB85 5156		RB96 6928	
8%	^{15}NL	31.8	a	32.6	a	35.5	a	35.8	a
	Stalk	22.3	b	18.9	b	16.8	b	23.5	b
	UEL	10.8	c	10.2	c	11.3	c	9.3	c
	Roots	6	d	7.1	c	7.1	c	7.3	c
	OL	0.8	f	0.6	e	1.2	d	0.7	e
	YFEL	2.3	e	1.8	d	2.3	d	1.8	d
16%	^{15}NL	29	a B	42.8	a A	34	a AB	32.8	a B
	Stalk	13.7	b	11.4	b	12.1	b	15.2	b
	UEL	8.2	c AB	6.1	c B	9.1	b A	6.1	c B
	Roots	3.7	d AB	3.3	d B	7.2	b A	4.1	c AB
	OL	0.6	f	0.6	e	0.8	c	0.4	e
	YFEL	1.7	e	1.3	e	1.6	c	1.1	d
24%	^{15}NL	28.8	a B	52.3	a A	34.8	a B	36.5	a B
	Stalk	11	b	7.9	b	9.2	b	11.6	b
	UEL	6	c AB	3.7	c C	6.8	b A	4.7	c BC
	Roots	3.7	d A	1.9	d B	5.8	b A	4.3	c A
	OL	0.4	f AB	0.3	e B	0.3	d AB	0.6	d A
	YFEL	1	e	1.2	d	1.1	c	0.7	d
32%	^{15}NL	38	a AB	43.1	a A	29.9	a B	39.6	a AB
	Stalk	10.7	b A	6.5	b B	9	b AB	9.2	b AB
	UEL	4	c B	3	c B	5.9	c A	3.3	c B
	Roots	2.1	d BC	1.3	d C	4.4	c A	2.8	c AB
	OL	0.3	e	0.3	e	0.5	d	0.4	d
	YFEL	1.1	d A	0.8	d AB	0.5	d B	0.6	d AB

*Lowercase letters indicate differences among plant tissues within each fixed combination of variety, and N-urea concentration. Capital letters indicate differences among sugarcane varieties within each fixed combination of plant tissue, and N-urea concentration. The absence of capital letters indicates lack of significant differences among the sugarcane varieties within that specific combination according to Fisher's LSD post-hoc test ($p < 0.05$).

FNf was conducted during the peak growth phase of the sugarcane plants when nitrogen demand is high and new plant tissues act as major sinks for nitrogen (Trivelin 2000; Leite et al. 2016). The application of N-urea was targeted specifically to the ^{15}NL and it was initially expected that unexpanded leaves (UEL) would serve as the primary sink for the absorbed nitrogen. However, our findings revealed that a significant portion of total-NRP was transported to the stalk regardless of the sugarcane varieties studied (Table 2). The Stalk serves as the primary storage organ and sink of nutrients throughout the sugarcane growing season. It represents the major biomass of the sugarcane plants and exhibits greater sink strength compared to the UEL (McCormick et al. 2008; Wang et al. 2013; Dantas et al. 2020). A low NRP in the roots using FNf (Table 2) was expected as the soil had been fertilized with nitrogen (Trivelin et al. 1984, 1988). NRP in the roots is high (~15%) only when N-fertilizer applied to the leaves runs off to the soil (Trivelin et al. 1985).

4.3 | FNf toxicity tolerance differs between sugarcane varieties

The main disadvantage associated with the application of N-fertilizer to leaves is the occurrence of leaf scorching (Sangplung and Rosário 1978; Dick et al. 2016; Ferrari et al. 2021). Leaf scorching is influenced by several factors, including the source, rate, and timing of N-fertilizer application, as well as prevailing weather conditions (Clapp and Parham 1991; Widders 1991; Phillips and Mullins 2004; Fageria et al. 2009; Fernández et al. 2020). In the present study, these factors were controlled, except for the N-rate, as N-urea concentration in the solution, which was the specific factor under evaluation.

High concentrations of ammonium and urea, which are components of N-urea fertilizers, have been shown to be toxic to plants in prior studies (Krogmeier et al. 1989; Tan et al. 1999; Britto and Kronzucker 2002; Esteban et al. 2016). Here the highest concentration of N-urea in the solution resulted in the highest total-N content

in ^{15}NL of all sugarcane varieties studied (Figure 6). This might leads to a probable increase in the ammonium content, which is the primary product of urea hydrolysis (Witte 2011; Castro et al. 2022). The assimilation of ammonium into glutamine and glutamate contributes to the accumulation of protons, leading to abiotic stress in plants (Britto and Kronzucker 2005; Masclaux-Daubresse et al. 2010; Hachiya et al. 2021). Consequently, this justifies the observed increase in leaf scorching in response to higher concentrations of N-urea in the solution (Figure 1, and Supplementary Figure 2).

Surprisingly, there were differences in the intensities of leaf scorching among sugarcane varieties (Figure 1). This was not expected since ammonium toxicity had not been reported in sugarcane grown in ammonium solution (Boschiero et al. 2019). Bassi et al. (2018) classified the sugarcane varieties RB96 6828 and RB92 579 as responsive and non-responsive to NUE, respectively, differing in the content of glutamine and glutamate in the leaves. These results, together with those obtained in the present study, allow us to hypothesize that the difference among the sugarcane varieties in the intensity of leaf scorching can be explained by their difference in the rate of N assimilation and mechanisms, as well as in their antioxidant metabolism (Domínguez-Valdivia et al. 2008; Podgórska et al. 2013; Bittsánszky et al. 2015; Jian et al. 2018).

4.4 | FNf and the importance for sugarcane in reducing GHG emissions and expenses with fertilizers

Sugarcane, a high N demanding and a semi-perennial crop, is usually grown in soil with low content of organic matter, requiring careful fertilization management to ensure optimal yield and longevity. Restoring exported nitrogen from harvested stalks and supplementing the ratoons are crucial for maintaining productive sugarcane fields (Dourado-Neto et al. 2010; Sanches and Otto 2022). N-fertilization has been shown to mitigate the decline in stalk yield over multiple growing seasons (Quassi de Castro et al. 2018; Tenelli et al. 2019; Boschiero et al. 2020). However, it is essential to consider both, environmental conditions (Thorburn et al. 2017; Dias and Sentelhas 2018; Castro et al. 2019) and the N requirement of the plant (de Oliveira et al. 2013; Leite et al. 2016; Mariano et al. 2016) to promote great NUE and NRP, minimizing nitrogen losses, and supporting sustainable agricultural practices (Zhang et al. 2015; Bowles et al. 2018). Adjusting the timing of N-fertilizer application (Lofton and Tubaña 2015; de Castro et al. 2022) and employing appropriate application methods (Borges et al. 2019) have been found to reduce greenhouse gas (GHG) emissions and improve NUE in sugarcane. However, these actions alone are insufficient as a significant proportion of nitrogen becomes immobilized in the soil (Otto et al. 2016; Quassi de Castro et al. 2021; Sanches and Otto 2022).

FNf is an agricultural management practice that offers high NRP, reaching up to 78% at 20 DAFNf (Figure 4 and Supplementary Figure 6; Wittwer and Teubner 1959; Humbert 1960; Trivelin et al. 1988). This enhanced NRP is primarily attributed to the direct application of nutrients onto the photosynthetic plant tissue, which might decrease the N-fertilizer requirements by the sugarcane plant and the GHG emissions, an important consideration in alignment with the goals of the Paris Agreement (UNFCC 2015). Apart from its environmental benefits for sugarcane-producing countries, such as Brazil, India, China, Thailand and Pakistan (FAO, 2018), FNf implementation is also expected to yield economic advantages, particularly in Brazil, which imports approximately 88% of its N-fertilizer (Polidoro and Perez 2022). In the current global scenario, characterized by high fertilizer prices and limited supply, due to geopolitical concerns, the reduced demand for N-fertilizer resulting from increased NRP provided by FNf holds the potential to enable sustainable and environmental-friendly production practices.

5 | CONCLUSION

The findings of this study demonstrate that the N-urea uptake by sugarcane leaves starts just after foliar N-fertilization (FNf), resulting in a high N-fertilizer recovery by the plant, referred to as total-NRP, within the following five days (average of 62.3%). Subsequently, the N-fertilizer absorbed by sugarcane leaves is redistributed to other plant tissues at an average of 41.3%, with the stalk and unexpanded leaves serving as the main storage plant tissues. Notably, a higher concentration of N in the solution suppresses the overall total-NRP, and increases the occurrence of leaf scorching. Moreover, the uptake rate of N-urea by sugarcane leaves is influenced by the specific variety, depending on the initial total-N content of the fertilized leaves. Furthermore, variations among sugarcane varieties are observed in terms of the intensity of leaf scorching induced by FNf, suggesting that certain varieties may be better suited for N-urea application onto the leaves than others.

AUTHOR CONTRIBUTIONS

Conceptualization of the study was done by S.A.Q.C., and P.C.O. The methodology was performed by S.A.Q.C., P.C.O., F.S.L., and M.L.R.; The investigation within the greenhouse was done by S.A.Q.C., and R.R.L.C.; The laboratory investigations were done by S.A.Q.C., M.L.R., and R.R.L.C. Statistical analyses and data interpretation were performed by R.A.S. and S.A.Q.C. The original draft was written by S.A.Q.C. The manuscript reviewing and editing was done by all authors. The supervision was executed by P.C.O. The funding acquisition was done by P.C.O., and S.A.Q.C. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGEMENTS

This research was supported by the São Paulo Research Foundation (FAPESP), grant #2017/24516-7 to PCOT. SAQC received a Ph.D. scholarship from FAPESP, grant #2017/25489-3. The authors thank the technicians (Ana Paula G. de M. Duarte and Hugo H. Batagello) and the internships of the Stable Isotope Laboratory from the Center of Nuclear Energy in Agriculture (LIE/DVTEC/CENA/USP) for helping with the laboratory analyses, Prof. Adriana Pinheiro Martinelli (DVPROD/CENA/USP) for allowing the use of the microscope and Prof. Elliot Kitajima (LME/ESALQ/USP) for support access to the scanning electron microscope. SAQC thanks the FAPESP for the financial support during the Ph.D., the University of São Paulo for

the greenhouse structure, Prof. Marcílio de Almeida and Dra. Erika Mendes Graner for the teachings and discussions throughout the plant anatomy course, and Dra. Nicole C. Cheng and the bachelor's students (Régis R. L. Vieira and Eluisa R. Elias) for their help at each time of plant sampling.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Quassi de Castro, S.A., Sermarini, R.A., Rossi, M.L., Linhares de Castro, R.R., Trivelin, P.C.O. & Linhares, F.S. (2023) Optimizing foliar N-fertilization in sugarcane depends on plant genotype and nitrogen concentration. *Physiologia Plantarum*, 175(6), e14085. Available from: https://doi.org/10.1111/ppl.14085