Plant Physiology and Biochemistry 139 (2019) 246–255

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Influence of nitrate - ammonium ratio on the growth, nutrition, and metabolism of sugarcane

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ARTICLE INFO

Keywords:
Saccharum spp.
Nitrogen uptake
Nitrogen use efficiency
Photosynthesis
Oxidative stress

ABSTRACT

Although ammonium (NH_4^+) has been claimed as the preferential N source for sugarcane (*Saccharum* spp.), the intense uptake of this mineral form by plants can impair metabolic processes and crop yield. We aimed to assess the growth, nutrition, and metabolic responses of sugarcane grown under different amounts of nitrate (NO_3^-) and NH_4^+. Sugarcane setts were grown in nutrient solution at a total concentration of 15 mM N using different NO_3^-/NH_4^+ ratios (100/0, 75/25, 50/50, 25/75, and 0/100, respectively) for 163 d under controlled conditions. The pH of the medium was daily adjusted to 5.8 ± 0.1, with replacement of the hydroponic solution every 10 d. NH_4^+–only fed plants showed lower dry biomass yield, nutrient content, leaf surface area, and leaf gas exchange than those under sole NO_3^- supply, in addition to favoring the development of brown rust (*Puccinia melanocephala*). However, there was no indication that NH_4^+ is directly related to oxidative stress in sugarcane. On the other hand, the highest N utilization efficiency was obtained with NO_3^-–only fed plants, which also resulted in the highest biomass yield, leaf surface area, nutrient content, leaf gas exchange, and root growth. Since NO_3^- was not stored in plant tissues, we therefore suggested that most of this N form is assimilated following its uptake. Despite the well–known preference of the crop for NH_4^+, the optimal growth response of sugarcane plants to NO_3^-/NH_4^+ ratios was observed under NO_3^- supply.

1. Introduction

The main nitrogen (N) forms taken up by plants are nitrate (NO_3^-) and ammonium (NH_4^+), and the uptake and assimilation of these forms depends on several factors, such as their availability in the soil, plant species, physiological status, CO_2 concentration, pH, temperature, and light intensity (Andrews et al., 2013, 2009; Esteban et al., 2016a; Lea and Azevedo, 2006; Mengel et al., 2001). Better knowledge and understanding of the mineral N uptake by plants is critical as NO_3^- and NH_4^+ act differently in metabolic and physiological plant processes, affecting not only the N assimilation but also the root respiration, water relations, photosynthesis, and plant secondary metabolism (Britto and Kronzucker, 2013; Cramer and Lewis, 1993; Guo et al., 2007; Lopes et al., 2004; Lopes and Araus, 2006; Lu et al., 2009; Nakamura et al., 2010). Plants with preference for NO_3^- can exhibit NH_4^+ toxicity symptoms, while those preferring NH_4^+ may have an atrophied NO_3^- uptake system (Britto and Kronzucker, 2002; Kronzucker et al., 1997).

The majority of plant species prefer NO_3^- to NH_4^+, even though the energetic cost for NO_3^- uptake and assimilation is considerably higher than that for NH_4^+ (Britto and Kronzucker, 2005). This is because high concentrations (> 0.5 mM) of NH_4^+ in the solution (especially at low pH values) may be toxic to plants, thus decreasing the biomass production (Magalhães and Huber, 1989; Sarasketa et al., 2016). Conversely, it has been reported the sugarcane (*Saccharum* spp.) preference for NH_4^+, essentially when exposed to high N concentrations (10 mM; Robinson et al., 2011). In contrast, de Armas et al. (1992) concluded that both N forms (NH_4^+ and NO_3^-) could be used efficiently by the crop, and although NH_4^+–fed plants exhibited higher dry biomass

Abbreviations: A, CO_2 assimilation rate; C_i, capacity of internal carbon use; CAT, catalase; DAT, days after transplantation; *E*, transpiration; FW, fresh weight; F + 1, first leaf with visible dewlap from the shoot apex; N, nitrogen; NR, nitrate reductase; NUpE, nitrogen uptake efficiency; NUtE, nitrogen utilization efficiency; ROS, reactive oxygen species; GR, glutathione reductase; *gs*, stomatal conductance; MDA, malondialdehyde; SOD, superoxide dismutase

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https://doi.org/10.1016/j.plaphy.2019.03.024
Received 10 December 2018; Received in revised form 13 March 2019; Accepted 13 March 2019
Available online 18 March 2019

compared to sole NO_3^- supply, no differences between the two N forms were detected relative to photosynthetic parameters. Brazilian varieties of sugarcane exhibited a preference for NH_4^+ uptake during the first 3 d after fertilization (Kölln, 2016). However, for a longer period (~365 d after fertilization, which represents a ratoon growth season), NH_4^+ supply did not increase the ^{15}N–fertilizer recovery (NRec; ratio between the amount of fertilizer N taken up by the crop and amount of fertilizer N applied) by sugarcane compared to NO_3^- supply (Boschiero et al., 2018). In the same study, but in hydroponic conditions, NO_3^- had a slower uptake rate than NH_4^+, but NO_3^-–only fed plants showed a higher ^{15}N–fertilizer recovery than those exposed to NH_4^+ (Boschiero et al., 2018). Due to the several transformations that N undergo in the soil, pot experiments using hydroponic solution are useful to assess the effect of N forms in morphological, physiological, and metabolic plant properties, as well as on proteomic analysis (Beatty et al., 2009; de Armas et al., 1992; Hajari et al., 2017; Helali et al., 2010). In addition, pot experiments are valuable for evaluation of shoot and root tissues (mainly for the root system, where sampling process is laborious under field conditions), and have already been used to assess the effect of N forms on N recovery by sugarcane (Hajari et al., 2017; Robinson et al., 2011). This information is fundamental, for example, to estimate the N use efficiency derived from each mineral N form (e.g., NH_4^+ and NO_3^-), which is virtually impossible in the field due to the N dynamics in the soil–plant system.

Excess NH_4^+ may alter plant metabolic processes, such as photosynthetic activity (Cramer and Lewis, 1993; Lopes et al., 2004; Lopes and Araus, 2006). However, the role of NH_4^+ in the oxidative stress of plants has not yet been elucidated. The NH_4^+ nutrition can modify the redox state of several reactive oxygen species (ROS), thus altering ROS homeostasis (Patterson et al., 2010). Increases in mitochondrial ROS levels in *Arabidopsis* leaves were associated with NH_4^+ stress (Podgórska et al., 2013). Other studies also reported increases in the proline content and in the activities of antioxidant enzymes induced by the presence of NH_4^+ in maize roots (Vuletić et al., 2010). However, the stress promoted by NH_4^+ supply in pea (*Pisum sativum* L.) and spinach (*Spinacia oleracea* L.), considered tolerant and sensitive to NH_4^+ toxicity, respectively, was not associated with enzymes of the oxidative stress (Domínguez-Valdivia et al., 2008). Thus, the relationship between NH_4^+ and oxidative stress in plants remains unclear (Bittsánszky et al., 2015; Esteban et al., 2016a), and, to our knowledge, there are no studies associating NH_4^+ supply with oxidative stress in sugarcane.

Despite the reported preferential NH_4^+ uptake by sugarcane under N–replete conditions, our hypothesis is that the sole NH_4^+ supply will result in lower growth and some plant stress compared to other NO_3^-/NH_4^+ ratios, and the best growth of sugarcane will occur when the two forms of N (NO_3^- and NH_4^+) are provided in the nutrient solution. Our objective was to evaluate the growth, production, metabolism, and nutrition of sugarcane supplied with different NO_3^-/NH_4^+ ratios using hydroponic solution.

2. Materials and methods

2.1. Growth conditions and experimental setup

Sugarcane setts (~3 cm in length) with one apical gem (cultivar CTC15, released by Sugarcane Technology Center, São Paulo, Brazil) were obtained from part of a stalk and planted in plastic trays containing washed sand as a substrate. The setts were watered with deionized water and transplanted 22 d after planting to pots containing 5.5 L of nutrient solution (Table 1) in an aerated medium. Five NO_3^-/NH_4^+ ratios (100/0, 75/25, 50/50, 25/75, and 0/100) with 15 mM N concentration were used, since previous studies indicated no differences between the NO_3^- and NH_4^+ for biomass production, photosynthesis and N recovery by sugarcane at low N concentrations (de Armas et al., 1992; Robinson et al., 2011). The plants were cultivated in nutrient solution with 25% ionic strength for the first 10 d after transplantation (DAT), and later, the complete (100% ionic strength) Hoagland and Arnon (1950) nutrient solution was used (Table 1). Although we have used a nutrient solution where the presence of nitrifiers is negligible, or even null, a synthetic nitrification inhibitor (dicyandiamide – DCD, 7 μM) was added to prevent NH_4^+ oxidation (Song et al., 2011). At 47 DAT, the plants with the lowest NO_3^-/NH_4^+ ratios (0/100 and 25/75) showed visual symptoms of K deficiency. To treat this nutrient deficiency, K concentration was increased from 6 to 9 mM through K_2SO_4, with consequent increase of S concentration from 2 to 3.5 mM in all treatments.

The experiment was performed in a controlled greenhouse. The mean air temperature was 26.7 °C during the day and 19.5 °C at night. The pots were distributed in a randomized complete block design with five replicates. The pH of the nutrient solution of each pot was recorded daily to monitor its temporal variation throughout the experimental period (Fig. 1). Subsequently, the pH was adjusted to 5.8 ± 0.5 (mean ± SEM) with 0.5 M HCl or 0.5 M NaOH. The nutrient solution was replaced every 10 d. At 140 DAT, the 5.5 L pots were replaced by larger ones (10.0 L), to avoid limiting the growth of the root system.

2.2. Biomass content, leaf surface area and tissue nutrient analysis

At 163 DAT, the plants were harvested and divided into dry leaves, green leaves, stalks, and roots. The leaf surface area of the green leaves was measured using a leaf area integrator (model LAI–3100, LI–COR Inc., Lincoln, NE, USA) immediately after harvest. Fresh samples from the (i) first leaf with visible dewlap from the shoot apex (F + 1; central part, excluding mid–ribs); (ii) stalks (central part); and (iii) roots were sampled for enzymatic analysis. Plant tissues were cryopreserved using liquid N and stored at −80 °C. Roots were sampled and rinsed in 10 mM KCl followed by deionized water to avoid contamination of nutrients from the hydroponic solution. Approximately 10% of the fresh biomass was separated for morphological evaluation. The plant fractions were oven–dried at 65 °C to a constant weight and ground in a Wiley mill, passing through a 0.5–mm sieve. The biomass content in the shoots was estimated by the summation of dry leaves, green leaves, and stalks.

The total N concentration was determined using an isotope ratio mass spectrometer (Hydra 20–20, Sercon Ltd., Crewe, UK) interfaced to an automatic N analyzer (ANCA–GSL, Sercon Ltd., Crewe, UK; Barrie and Prosser, 1996). The mineral N concentration in plant tissues was determined following Tedesco et al. (1985). Briefly, 1 g of plant tissue was extracted with 1 M KCl (ratio of 1:15, plant tissue: solution, w/v), distilled with MgO and Devarda's alloy and titrated with 2.5 mM H_2SO_4. The other nutrients were extracted by nitric–perchloric digestion (wet extraction) and quantified via colorimetry (P), atomic spectroscopy absorption (K, Ca, and Mg), and turbidimetry (S) methods following van Raij et al. (2001). The nutrient content was calculated by multiplying the dry biomass content by its nutrient concentration. Plant dry biomass and N content were estimated by summation of the dry biomass yield and N accumulation from all plant fractions, respectively.

The following N efficiency indexes were then calculated: (i) N uptake efficiency based on dry biomass (NUpE, mg g^{-1}) = [(plant N content, mg)/root dry biomass, g] (Swiader et al., 1994); (ii) N uptake efficiency based on root length (NUpE, mg m^{-1}) = [(plant N content, mg)/root length, m] (Rosolem et al., 2000); (iii) harvest index or N transport efficiency (Harvest index, %) = [N content in the shoots, mg/plant N content, mg) × 100] (Li et al., 1991); and (iv) N utilization efficiency (NUtE, g mg^{-1}) = (plant dry biomass, g/plant N content, mg) (Siddiqi and Glass, 1981).

2.3. Leaf gas exchange

Evaluations of gas exchange were measured between 10:00 a.m. and midday using a portable gas exchange device (Infra–Red Gas Analyzer –

Table 1
Composition of the nutrient solution as affected by NO_3^-/NH_4^+ ratios.

NO_3^-/NH_4^+	Element concentration (mM)								
	pH	NO_3^-–N	NH_4^+-N	$H_2PO_4^-$–P	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}–S	Cl^-
100/0	4.8	15.0	0.0	1.0	9.0	5.0	2.0	3.5	–
75/25	4.9	11.2	3.7	1.0	9.0	5.0	2.0	3.5	7.5
50/50	4.9	7.5	7.5	1.0	9.0	5.0	2.0	3.5	15.0
25/75	4.9	3.7	11.2	1.0	9.0	5.0	2.0	3.5	17.5
0/100	4.9	0.0	15.0	1.0	9.0	5.0	2.0	3.5	25.0

Micronutrients were supplied at the following concentrations: 5 mg L^{-1} Fe; 0.05 mg L^{-1} B, Mn, and Zn; 0.01 mg L^{-1} Mo.
After preparation, nutrient solution pH was brought to 5.8 ± 0.1 (mean ± SEM), regardless of the NO_3^-/NH_4^+ ratio.

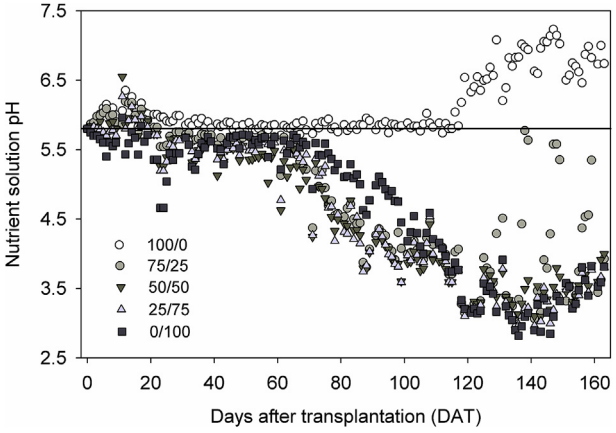

Fig. 1. Temporal variation of the nutrient solution pH during the experimental period before its daily correction to 5.8 ± 0.1 (n = 5), which is indicated by the continuous black line.

IRGA, model LI–6400, LI–COR Inc., Lincoln, NE, USA) one day before harvesting. The initial conditions utilized for measurements were: 1500 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) provided by LED lamps and chamber maintained between 20 and 25 °C and 380 µmol mol^{-1} of CO_2 (McCormick et al., 2008). The parameters CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}), internal CO_2 concentration in the substomatal chamber (Ci, µmol mol^{-1}), and transpiration (E, mmol H_2O m^{-2} s^{-1}) were measured in the central part of the F + 1 leaf.

2.4. Morphological analysis of the root system

The root system was sampled at harvest to assess morphological evaluations. Primary and secondary roots were gently collected. The root biomass was dried in towel paper and weighed; the roots were cut into eight longitudinal parts and one part, representing ~10% of entire the root system was separated, weight, and stored in 50% (v/v) ethanol solution at 4 °C until analyses. The WinRhizo software (version 2016a, Regent Instruments Inc., Quebec, Canada) was coupled to a professional scanner (model 10000XL, Seiko Epson Corp., Suwa, Japan) following Bouma et al. (2000), with adaptations. Roots were not stained. To obtain a scanned image (400 DPI resolution), the roots were gently placed in acrylic vats containing deionized water. The following parameters were determined: root length, surface area, average diameter, and root volume. The specific length was obtained from the ratio of the total length to the dry biomass of roots (Liu, 2009). The results were extrapolated for the whole root system.

2.5. Nitrate reductase activity (EC 1.7.1.1)

The *in vivo* nitrate reductase (NR) activity was determined as described by (Hageman and Reed, 1980) with modifications. Tissue samples from the central part of F + 1 leaves (excluding mid–ribs) and root samples were collected at 159 DAT (2 d after replacement of the nutrient solution). The fresh tissue was cut into small segments, weighed (200 mg) and transferred to assay tubes containing 4 mL of phosphate buffer solution at pH 7.4 (50 mM Na–phosphate buffer + 250 mM KNO_3). The assay tubes, wrapped in Al foil to protect them from light, were incubated in a water bath at 35 °C for 2 h. Subsequently, 1 mL of the extract was added to 50 mL–volumetric flasks containing ~30 mL of deionized water, and the reaction was stopped with the addition of 1 mL of sulfanilic acid (33.5 mM) in HCl 20% solution, followed by 1 mL of alpha–naphthylamine indicator (83.8 mM) and 1 mL of sodium acetate buffer (2 M). The volume of each flask was brought to 50 mL with deionized water. The nitrite (NO_2^-) produced was measured in a spectrophotometer at 560 nm using a nitrite standard calibration curve. The enzyme activity was expressed in µmol NNO_2^- g^{-1} h^{-1} fresh weight (FW).

2.6. Malondialdehyde content

Lipid peroxidation was determined by estimating the content of reactive substances to 2–thiobarbituric acid (TBA) and expressed as malondialdehyde (MDA; (Buege and Aust, 1978; Heath and Packer, 1968). Samples of 200 mg of fresh tissue were homogenized with liquid N in a mortar with 2 mL of 0.1% (v/v) trichloroacetic acid (TCA) and 20% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 12100 *g* for 10 min at 4 °C. An aliquot (250 µL) of the supernatant was mixed with 1 mL of 20% (v/v) TCA and 0.5% (v/v) TBA, and incubated in a dry bath at 95 °C for 30 min. An ice bath was used to stop the reaction. The absorbance was measured at 535 and 600 nm with a dual–wavelength spectrophotometer. The MDA concentration was expressed as µmol MDA g FW^{-1}.

2.7. Hydrogen peroxide content

The H peroxide (H_2O_2) content was determined following Alexieva et al. (2001). Fresh plant tissues were subjected to the same initial protocol used for lipid peroxidation determination. Two hundred microliters of supernatant were added to 200 µL of 100 mM potassium phosphate buffer at pH 7.5 and 800 µL of 1 M KI. The assay tubes were placed on ice and kept in the dark for 1 h, and left to stand at room temperature for additional 20 min. The absorbance was read at 390 nm. The amount of H_2O_2 was expressed as µmol g FW^{-1}.

2.8. Extraction and determinations of antioxidative enzymes and proteins

The fresh plant material was macerated in a mortar containing liquid N. The protein extracts were obtained from 1 g of fresh material in 100 mM potassium phosphate buffer at pH 7.5, 1 mM EDTA and 3 mM DDT (dithiothreitol). In addition to the buffer, 20% (w/v) PVPP was added. The homogenate extract was centrifuged at 12100 *g* for 30 min at 4 °C. The supernatant was stored in 1.5–mL microcentrifuge tubes, frozen in liquid N and stored at −80 °C. The total soluble protein

was determined using the method of Bradford (1976) with bovine serum albumin as a standard. Aliquots of 100 μL of the extract were mixed in 5 mL of Bradford reagent with three replications. The quantification was performed in a spectrophotometer at 595 nm. The results were used to calculate the antioxidative enzyme concentration.

Catalase (CAT) activity (EC 1.11.1.6) was measured by monitoring the degradation of H_2O_2 at 25 °C in a spectrophotometer as described by Kraus et al. (1995) with the modifications of Azevedo et al. (1998). The reaction was initiated by the addition of 25 μL of protein extract to 1 mL of 100 mM potassium phosphate buffer at pH 7.5 and 2.5 μL of H_2O_2 (30% solution). The activity was determined using the decomposition of H_2O_2 at 240 nm over the course of 1 min. Catalase activity was expressed as μmol min^{-1} mg^{-1} protein.

The superoxide dismutase (SOD) activity (EC 1.15.1.1) was determined as described by Giannopolitis and Ries (1977) using a spectrophotometer. Fifteen microliters of protein extract were added to 1.48 μL of reaction medium consisting of 50 mM potassium phosphate buffer at pH 7.8, 50 mM methionine, 1 mM nitroblue tetrazolium chloride (NBT), 10 mM EDTA and 0.1 mM riboflavin. The reaction was conducted in a reaction chamber (box) under the illumination of a 15 W fluorescent light bulb at 25 °C. After a 5–min exposure to light, the illumination was interrupted, and the blue formazan compound produced was measured at 560 nm. Two additional test tubes were used blanks for each sample. One was exposed to light for 5 min, indicating the maximum production potential of the blue formazan, while the other was kept in the dark for 5 min to zero the spectrophotometer. The results were expressed in U SOD mg^{-1} protein.

Glutathione reductase (GR) activity (EC 1.6.4.2) was determined as described by Gomes-Junior et al. (2006) with modifications. The reaction medium consisted of 1 mL of 100 mM potassium phosphate buffer at pH 7.5 with 1 mM 2–nitrobenzoic acid, 1 mM oxidized glutathione and 0.1 mM NADPH at 30 °C. The reaction was initiated with 50 μL of protein extract. GR activity was estimated by the reduction of oxidized glutathione with a spectrophotometer at 412 nm during 1 min. The GR activity was expressed as μmol min^{-1} mg^{-1} protein.

2.9. Statistical analysis

The results were subjected to an analysis of variance (ANOVA) with Statistical Analysis System (SAS) software (version 9.3, SAS Institute, Inc., Cary, NC, USA) using the GLM procedure. The F test was used followed by the Fisher's protected LSD test for pairwise post–hoc comparison ($P \leq 0.05$).

3. Results

3.1. Nutrient solution pH

The sole NO_3^- supply to sugarcane plants promoted the alkalization of the nutrient solution, while the supply with either NO_3^- and NH_4^+ or sole NH_4^+ promoted acidification (Fig. 1). Variations in pH (alkalization or acidification) increased as the plants developed and intensified the uptake of nutrients, which occurred primarily after 115 DAT, where pH decreased from 5.8 to 2.8 in a 24–h period for the sole NH_4^+.

3.2. Growth and development of shoot and roots

After 163 DAT, no symptoms of NH_4^+ toxicity were detected in sugarcane plants. However, plants under NO_3^-/NH_4^+ ratios lower than 1 (0/100 and 25/75) displayed symptoms of brown rust (*Puccinia melanocephala*) in the leaves. The dry biomass yield was affected by the NO_3^-/NH_4^+ ratios. The NH_4^+–only fed plants showed a shoot and root dry biomass 20% and 38% lower than those supplied with NO_3^- as sole N source, respectively (Fig. 2a). Similarly, the leaf surface area of NH_4^+–only fed plants was 32% lower than that under sole NO_3^- supply (Fig. 2b). The sole NH_4^+ supply in the nutrient solution also decreased the root length, root surface area, and root volume by 50%, 44%, and 44%, respectively, relative to those supplied with the largest NO_3^-/NH_4^+ ratios (100/0 and 75/25; Table 2). In addition, the sole NH_4^+ supply also resulted in roots with an average diameter 18% larger and specific length 22% lower than plants supplied solely with NO_3^- (Table 2).

3.3. Nutrient content in plant fractions

The NO_3^-/NH_4^+ ratios in the nutrient solution changed the P, K, Ca, Mg, and S content in the shoots and roots, as well as the N content in the roots (Table 3). The N supply through NH_4^+ as sole source decreased the content of nutrients than that of sole NO_3^- supply. The shoots showed a reduction of 16%, 27%, 30%, 20%, and 27% in the P, K, Ca, Mg, and S content, respectively, in NH_4^+–only fed plants compared to those supplied with NO_3^-. This negative effect of NH_4^+ was even more remarkable in the roots, where the content of N, P, and K were decreased by 20–40%, in addition to 71% and 63% for Ca and Mg, respectively, compared to NO_3^-–only fed plants.

The higher NH_4^+-N content was detected in the stalks and roots of sugarcane, and as expected, low NO_3^-/NH_4^+ ratios in the nutrient solution generally resulted in greater plant NH_4^+-N content (Fig. 3). The amount of NH_4^+-N in the root system was 180%–400% higher than that found in the green leaves, depending on the treatment (Fig. 3bd). However, the NO_3^--N content was not affected by the N form and showed very low values: 3.7, 2.3, 1.6, and 0.9 mg $plant^{-1}$ (average of treatments) in the stem, green leaves, roots, and dry leaves, respectively.

3.4. Nitrogen use efficiency indexes

The sole NH_4^+ supply resulted in 54% and 80% higher NUpE than NO_3^-–only supply based on the root dry biomass and root length, respectively (Fig. 4ab). The harvest index of NH_4^+–fed plants was 12% higher than the rest of NO_3^-/NH_4^+ ratios (Fig. 4c). On the other hand, the NUtE was 20% higher for NO_3^-–fed plants compared to those treated solely with NH_4^+, and as the amount of NH_4^+ supplied increased, the NUtE value decreased (Fig. 4d).

3.5. Metabolic changes in enzymatic activity and photosynthesis

Higher NR activity was found in the leaf than in the root (Fig. 5). The plants under sole NO_3^- supply had a higher NR activity in the roots and leaves than those exposed solely to NH_4^+. The gas exchange results indicated that the sole NH_4^+ supply resulted in a decrease of 41%, 57%, 50%, and 44% in the *A*, *gs*, *Ci*, and *E* parameters, respectively, compared to the NO_3^- supply (Fig. 6).

3.6. Oxidative stress

The MDA content in the stalk was highest for the 75/25 and 50/50 NO_3^-/NH_4^+ ratios (Fig. 7a). Approximately 90% of the H_2O_2 content was produced in the leaf, regardless of NO_3^-/NH_4^+ ratio (Fig. 7b). In the roots, the greatest amount of NH_4^+ (0/100 and 25/75) in solution decreased the H_2O_2 content by 54% compared to the 75/25 NO_3^-/NH_4^+ ratio. The CAT activity was affected by the NO_3^-/NH_4^+ ratios in the stalk and the leaf (Fig. 7c). The higher CAT activity occurred in the stalk (120–150 μmol min^{-1} mg^{-1} protein), and the sole NO_3^-–fed plants had 19%–28% higher activity than the plants treated with the 0/100 and 25/75 NO_3^-/NH_4^+ ratios. The opposite occurred for the CAT activity in the leaves, where NH_4^+–fed plants showed 35% higher CAT activity than those under the sole NO_3^- exposure (Fig. 7c). The highest SOD activity was found in the stalk (50–60%), followed by the root (30–40%) and leaf (~10%). However, only the SOD activity in the roots was affected by the NO_3^-/NH_4^+ ratios, where NO_3^-–only fed

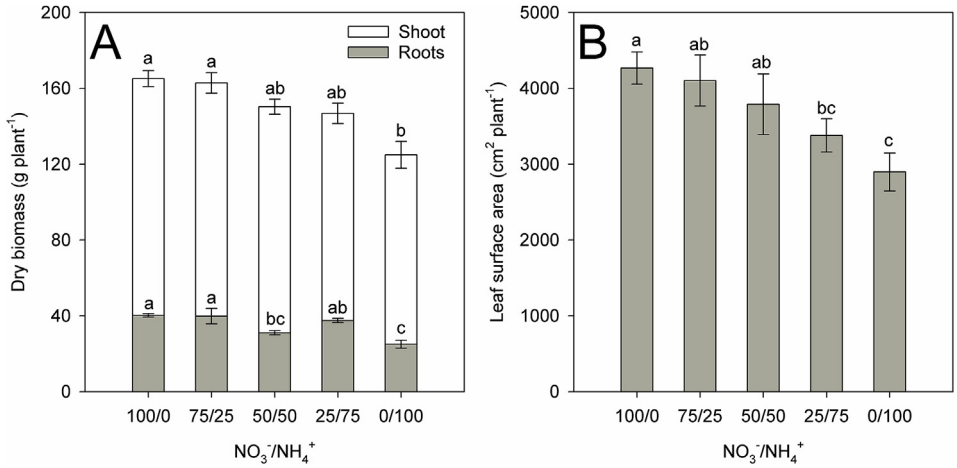

Fig. 2. Dry biomass yield in the shoot and roots (a) and leaf surface area (b) of sugarcane plants supplied with different NO_3^-/NH_4^+ ratios following 163 d. The error bars indicate the SEM (n = 5). Common lowercase letters not indicate differences between treatments, according to Fisher's protected LSD test ($P \leq 0.05$).

Table 2
Root morphological parameters of sugarcane plants supplied with different NO_3^-/NH_4^+ ratios following 163 d. Values represent means ± SEM (n = 5).

NO_3^-/NH_4^+	Length	Surface area	Volume	Average diameter	Specific length
	m	m^2	cm^3	mm	m g^{-1}
100/0	4328 ± 288a	4.2 ± 0.4a	327 ± 11a	0.298 ± 0.006b	101 ± 6a
75/25	4306 ± 474a	4.0 ± 0.4a	309 ± 25 ab	0.308 ± 0.004b	101 ± 3a
50/50	3841 ± 757 ab	3.7 ± 0.7a	282 ± 37 ab	0.314 ± 0.004b	96 ± 4a
25/75	2682 ± 168bc	3.1 ± 0.2 ab	254 ± 16b	0.350 ± 0.009a	68 ± 5b
0/100	2137 ± 200c	2.3 ± 0.2b	177 ± 14c	0.352 ± 0.010a	79 ± 6b
P value	0.012	0.028	0.003	< 0.001	0.001

Common lowercase letters within a column not indicate differences between treatments, according to Fisher's protected LSD test ($P \leq 0.05$).

Table 3
Nutrient content in the shoot and roots of sugarcane plants supplied with different NO_3^-/NH_4^+ ratios following 163 d. Values represent means ± SEM (n = 5).

NO_3^-/NH_4^+	Nutrient content (mg $plant^{-1}$)					
	N	P	K	Ca	Mg	S
Shoot						
100/0	2112 ± 43	751 ± 17a	3104 ± 117a	508 ± 11a	275 ± 8a	589 ± 21a
75/25	2333 ± 115	773 ± 17a	2891 ± 82a	469 ± 19a	291 ± 13a	597 ± 28a
50/50	2280 ± 92	703 ± 20 ab	2502 ± 79b	420 ± 32b	262 ± 17 ab	500 ± 40 ab
25/75	2391 ± 115	690 ± 37 ab	2468 ± 115b	410 ± 11b	252 ± 17 ab	514 ± 35 ab
0/100	2240 ± 117	630 ± 45b	2264 ± 113b	358 ± 13c	221 ± 18b	430 ± 28b
P value	0.344	0.034	< 0.001	< 0.001	0.037	0.015
Roots						
100/0	803 ± 2 ab	285 ± 8a	195 ± 7a	125 ± 4a	71 ± 3a	252 ± 14a
75/25	868 ± 65a	285 ± 27a	185 ± 4 ab	83 ± 11b	60 ± 5b	249 ± 20a
50/50	726 ± 14b	227 ± 22bc	170 ± 6bc	58 ± 7c	42 ± 4c	199 ± 26 ab
25/75	828 ± 68 ab	267 ± 8 ab	171 ± 3b	60 ± 7c	46 ± 4c	244 ± 14a
0/100	535 ± 40c	180 ± 16c	152 ± 7c	37 ± 2d	27 ± 1d	160 ± 16b
P value	0.001	0.004	0.001	< 0.001	< 0.001	0.008

Common lowercase letters within a column and plant fraction not indicate differences between treatments, according to Fisher's protected LSD test ($P \leq 0.05$).

plants had 72% higher SOD activity than those under NH_4^+ as sole N source (Fig. 7d). The GR activity in the roots represented 50–60% of the total activity in the plant and, on average, the highest NO_3^-/NH_4^+ ratio increased the GR activity by 30% compared to the other treatments. In contrast, the sole NH_4^+ supply increased the GR activity by 52% and 61% compared to plants supplied with 75/25 and 50/50 NO_3^-/NH_4^+ ratios in the leaf, respectively (Fig. 7e).

4. Discussion

As nutrient solutions have low buffering capacity (Dickson et al., 2016), a wide change in pH values was observed: pH increased under sole NO_3^- supply and decreased with some amount of NH_4^+ in solution, and this trend increased with the plant growth. Changes in nutrient solution pH are caused by the imbalance uptake of cations and anions and the N assimilation by plants. Net cation uptake is compensated by the H^+ efflux from roots, whereas net anion uptake is balanced by OH^-/HCO_3^- efflux (Dickson et al., 2016; Kirkby and Knight, 1977; Lea-Cox et al., 1996; Marschner, 2012). Nitrogen uptake plays a fundamental role in the cation/anion balance of the plant. Approximately 70–80% of the uptake of nutrients by plants is influenced by the NH_4^+ and NO_3^- uptake (Lea-Cox et al., 1996; Marschner, 2012). In addition, assimilation of mineral nutrients into organic compounds also changes the external pH. For N, one mol of NH_4^+ results in 1.2 mol H^+; the

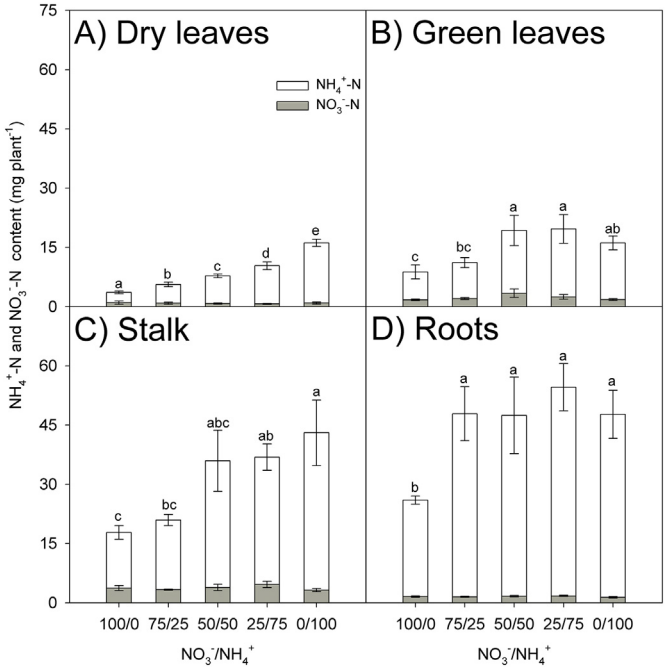

Fig. 3. Content of free NH_4^+-N and NO_3^-–N in dry leaves (a), green leaves (b), stalk (c), and roots (d) of sugarcane of sugarcane plants supplied with different NO_3^-/NH_4^+ ratios following 163 d. The error bars indicate the SEM ($n = 5$). Common lowercase letters not indicate differences between treatments, according to Fisher's protected LSD test ($P \leq 0.05$).

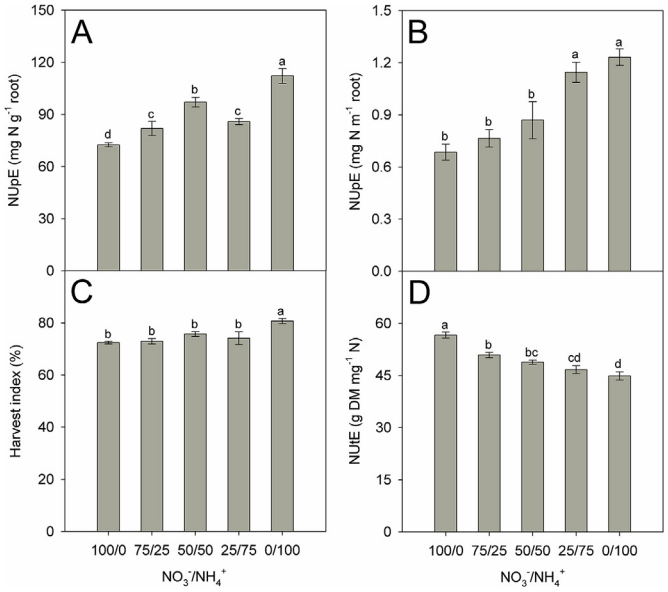

Fig. 4. Nitrogen uptake efficiency (NUpE) based on dry biomass (a) and root length (b), harvest index (c), and N utilization efficiency (NUtE; d) of sugarcane of sugarcane plants supplied with different NO_3^-/NH_4^+ ratios following 163 d. The error bars indicate the SEM ($n = 5$). Common lowercase letters not indicate differences between treatments, according to Fisher's protected LSD test ($P \leq 0.05$).

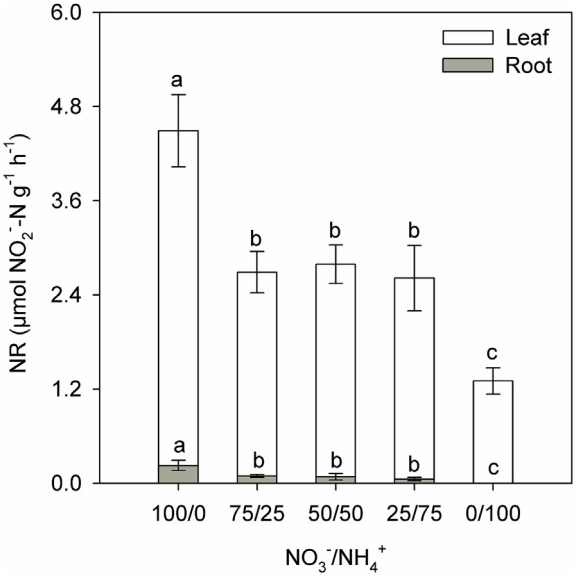

Fig. 5. Nitrate reductase activity (NR) in leaf and root of sugarcane plants supplied with different NO_3^-/NH_4^+ ratios following 163 d. The error bars indicate the SEM ($n = 5$). Common lowercase letters not indicate differences between treatments, according to Fisher's protected LSD test ($P \leq 0.05$).

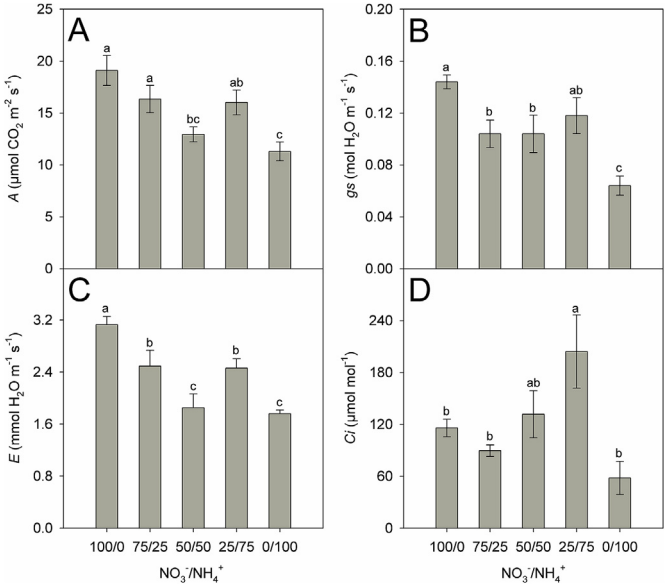

Fig. 6. Net photosynthetic rate (*A*; a), stomatal conductance (*gs*; b), transpiration rate (*E*; c), and substomatal CO_2 concentration (*Ci*; d) of sugarcane plants supplied with different NO_3^-/NH_4^+ ratios following 163 d. The error bars indicate the SEM ($n = 5$). Common lowercase letters not indicate differences between treatments, according to Fisher's protected LSD test ($P \leq 0.05$).

greatest amount extruded to the soil (Andrews et al., 2004; Raven, 1988). In turn, one mol of NO_3^- results in 0.78 mol OH^-; the highest amount is extruded to the soil if assimilated in the roots, while NO_3^- assimilation in the shoots is neutralized by the synthesis of organic acids (Andrews et al., 2004; Raven, 1988). In sugarcane plantations, the rapid nitrification rate and subsequent release of protons into the solution must also be considered when the influence of NO_3^-/NH_4^+ ratios on rhizosphere pH is evaluated (Argo and Biernbaum, 1997; Lang and Elliott, 1991). In the conditions above, NH_4^+ – fed plants take up a higher amount of cations than anions, leading to lower pH in the rhizosphere relative to the bulk soil, while the opposite is observed for NO_3^- – fed plants (Tang and Rengel, 2003; Jing et al., 2012; Ma et al., 2014; Rengel, 2015).

NH_4^+ has been reported as the preferred N source by sugarcane in high – N input environments (Robinson et al., 2011; Hajari et al., 2017; Boschiero et al., 2018). The so – called 'preference' refers to the faster uptake of NH_4^+ than NO_3^-. However, the results of this study indicated that the long – term supply of NH_4^+ as N source might impair the growth and metabolism of sugarcane due to its toxicity. Thus, it is

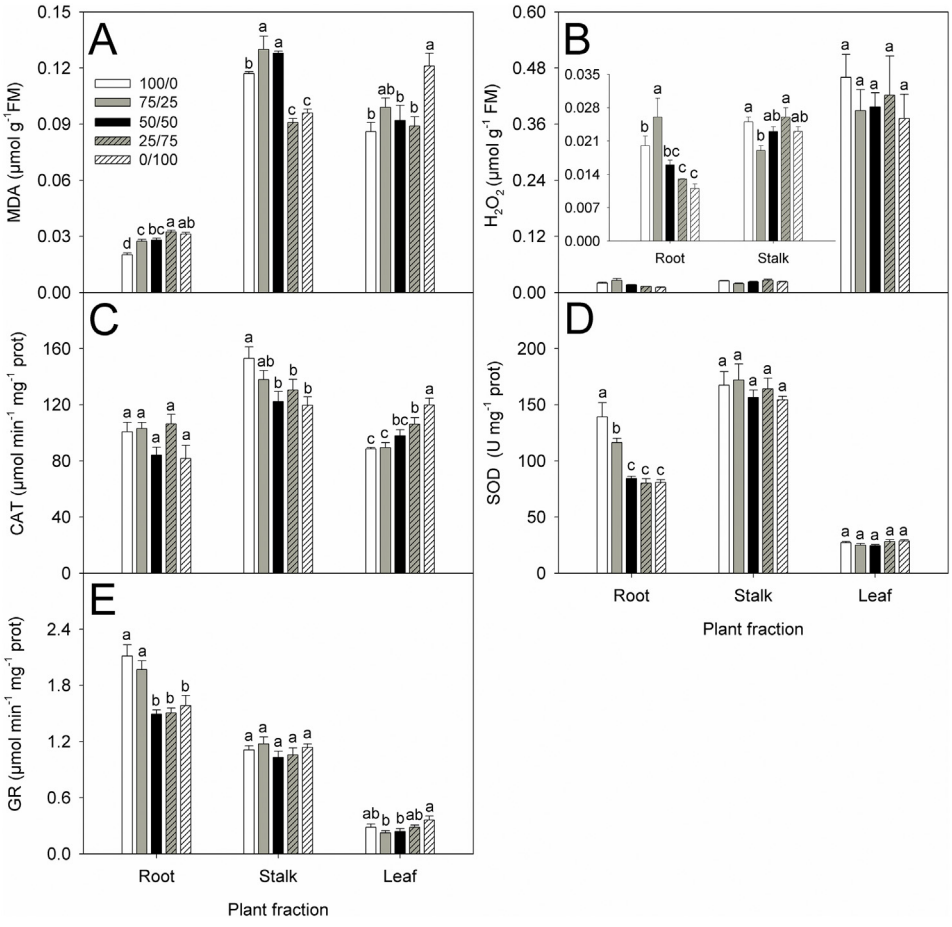

Fig. 7. Lipid peroxidation, expressed by the malondialdehyde (MDA) content (a), H peroxide (H_2O_2) content (b), and antioxidant activities of catalase (CAT; c), superoxide dismutase (SOD; d) and glutathione reductase (GR; e) of sugarcane plants supplied with different NO_3^-/NH_4^+ ratios following 163 d. The error bars indicate the SEM ($n = 5$). Common lowercase letters not indicate differences between treatments, according to Fisher's protected LSD test ($P \leq 0.05$).

possible that a change in the N−form preference occurred throughout the sugarcane growth. Thereby, in spite of NH_4^+ preference, the best sugarcane growth and nutrition was observed with the sole NO_3^- supply. The lowest biomass yield in NH_4^+−only fed plants can be explained by the increase in respiration rate by the roots when a large amount of NH_4^+ was taken up, as most of this N form is assimilated in the same plant organ (Kronzucker et al., 1998; Nakamura et al., 2010). Thus, the high NH_4^+ uptake requires a large amount of C skeletons for N assimilation (Britto and Kronzucker, 2013). The intense translocation of carbohydrates to the roots decreases the availability of photoassimilates available for vegetative growth. The uptake and assimilation of NH_4^+ by plants can favoring metabolic energy savings; however, for many species NH_4^+ decreases dry biomass yield than NO_3^- supply and the best growth occurs with both N forms are supplied together (Hajari et al., 2015; Helali et al., 2010; Marschner, 2012; Walch-Liu et al., 2000). The alleviation of NH_4^+ toxicity by NO_3^- still remains unclear, but appears to be closely related to the processes of uptake, reduction and signaling promoted by this anion (Hachiya et al., 2012).

No visual symptoms of NH_4^+ toxicity affecting sugarcane plants were observed in this study, unlike previous reports for other species (Britto and Kronzucker, 2002). However, NH_4^+−only fed plants could be distinguished visually from the other treatments because they displayed symptoms of brown rust (*Puccinia melanocephala*) infection, while plants receiving the highest NO_3^-/NH_4^+ ratio were healthier. However, under field conditions, uptake of NH_4^+ likely causes a different effect than our findings. The NH_4^+ uptake and assimilation by plants decreases the rhizosphere pH, increasing the solubilization and uptake of Si, therefore reducing the incidence of plant diseases (Borges et al., 2016; Keeping et al., 2015).

The K deficiency symptoms at high NH_4^+ supply is likely explained by an uptake competition between K^+ and NH_4^+ (Hoopen et al., 2010; Roosta and Schjoerring, 2008), as K−specific channels can mediate NH_4^+ uptake into the root cells (Balkos et al., 2010; Coskun et al., 2013). Consequently, high K^+ concentration in the nutrient solution might have mitigated the NH_4^+ toxicity, as the high K^+ supply likely suppress the NH_3/NH_4^+ influx and efflux in the short term (Balkos et al., 2010; Coskun et al., 2013; Szczerba et al., 2008). Therefore, K^+ can decrease the NH_4^+ toxicity by (i) increasing the NH_4^+ incorporation into organic compounds by enzymes such as glutamine synthetase and glutamine dehydrogenase; (ii) inhibiting the NH_4^+ acquisition by low affinity transporter systems; and (iii) interfering with the NH_3 transport, as K^+ regulates the aquaporin activity (suggested NH_3 transporters into the cell) and the plant water balance (Balkos et al., 2010; Coskun et al., 2016; Sarasketa et al., 2016; Szczerba et al., 2008).

The competition of NH_4^+ and other cations (K^+, Ca^{2+}, and Mg^{2+}) for plant acquisition, which showed decreased content in NH_4^+–only fed plants can be attributed to the lower synthesis of organic acids in this treatment, in contrast to plants supplied with NO_3^- (Salsac et al., 1987; Von Wirén et al., 2001). The low synthesis of organic acids ceases to provide anionic charge to accompany the cation accumulation in plants (Salsac et al., 1987). Organic acids have an important role in the regulation of cellular turgor, and NH_4^+–fed plants appear to have difficulty maintaining turgor pressure (Von Wirén et al., 2001). According to de Armas et al. (1992), sugarcane has the ability to preserve the synthesis of organic acids independently of the NO_3^- reduction, having an equivalent or higher growth when supplied with NH_4^+. The low synthesis of organic acids in the lack of NO_3^- supply is overcome with other ions that regulate the osmotic potential (such as Cl^-), with the decoupling of PEP carboxylase activity and NR activity in C_4 plants, such as sugarcane (Von Wirén et al., 2001).

The lowest content of plant nutrients under sole NH_4^+ supply (NO_3^-/NH_4^+ ratio of 0/100) likely contributed to the lowest allocation

of dry biomass in the roots and the lowest length, surface area, and volume of this plant fraction. Studies that evaluated the effect of NO_3^- on the root architecture of *Arabidopsis* suggest that the signal for root growth regulation based on the N content is hormonal, through auxin and/or abscisic acid (Signora et al., 2001; Zhang and Forde, 2000). It has been exanimated that both the NO_3^- transporter NRT1.1 and NO_3^- itself are involved in the regulation of endogenous auxin uptake in root cells, thus stimulating lateral root development (Esteban et al., 2016a). Similarly, *Medicago truncatula* plants receiving NH_4^+ or urea decreased the main elongation rate and lateral root development than plants exposed to NO_3^-, and the total root length was positively correlated with the auxin content in the plant (Esteban et al., 2016b).

Most plant species grown in high-N input environments (10–20 mM) can store large amounts of NO_3^- in the vacuole (Cruz et al., 2006; Marschner, 2012). However, sugarcane is an exception, as indicated by the low amount of free NO_3^- in plant tissues, even with the supply of 15 mM NO_3^-–N. This finding is congruent with Robinson et al. (2011), which postulated that sugarcane and some of its ancestors have a low capacity to store NO_3^-. In contrast, sugarcane accumulated high amounts of free NH_4^+ in the cell, primarily in the roots. The NH_4^+-N content in this organ was 50 mg plant^{-1} with the supply of 15 mM NH_4^+-N, which is equivalent to 121 μmol g^{-1} dry matter (DM). In susceptible plant species, the NH_4^+ concentration in the roots ranged from 13 to 77 μmol g^{-1} of DM, whereas tolerant species had values ranging from 300 to 370 μmol g^{-1} of DM (Cruz et al., 2006). The NH_4^+ accumulation in the roots prevents translocation to the shoots, which is more sensitive to high NH_4^+ concentration (Schjoerring et al., 2002), and probably explain the tolerance of certain species to NH_4^+ toxicity.

Although the NUpE and harvest index were highest in NH_4^+–only fed plants, the NO_3^- supply led to the highest NUtE. Previous study suggested an inverse correlation between the V_{max} (i.e., the ion uptake preference) and NUtE (for NH_4^+ and NO_3^-) in sugarcane, as well as a positive correlation between the K_m (which indicates the ion transport affinity) and NUtE (Hajari et al., 2014). Thus, although the NH_4^+ preference by the crop, as demonstrated by the highest NUpE and harvest index, the sole NO_3^- supply resulted in higher biomass production per unit of N taken up (i.e., NUtE). This finding is clear evidence that the term 'preference' should be revisited when the biomass production or crop yield is taken into consideration.

The gas exchange in sugarcane was impaired by the NH_4^+ supply, and the main limiting factor for *A* was the stomatal closure, which in turn causes the reduction of *E* (Lopes et al., 2004; Lopes and Araus, 2006). The lower *gs* in NH_4^+–only fed plants can be explained by the lower uptake of cations a well as the lack of NO_3^- in the solution, which has an important osmotic role and is essential for the transport of cations through the xylem, resulting in poor osmotic regulation (Lopes and Araus, 2006). Conversely, the lowest K content in the shoots of NH_4^+–only fed plants may also affect the stomatal function (Laporte et al., 2002).

The effect of NH_4^+ and NO_3^- on oxidative stress reported in previous studies is heterogeneous: while some NH_4^+–sensitive plants had a decrease of oxidative stress under NH_4^+ supply, the opposite was observed in other species (Domínguez-Valdivia et al., 2008; Podgórska et al., 2013; Polesskaya et al., 2004). Although it has been postulated that NO_3^- reduction can consume the excess reductive equivalents and thereby lowering the oxidative stress, it remains unclear to what extent the alleviation of NH_4^+ toxicity in the presence of NO_3^- is affecting the oxidative stress (Bloom et al., 1992; Escobar et al., 2006; Hachiya et al., 2012). Generally, we observed that NO_3^-–only fed plants had higher MDA content in the stalk and H_2O_2 content in the roots, as well as higher SOD and GR activity in the roots and CAT activity in the stalk than NH_4^+–only fed plants. Based on these results, there is no evidence of NH_4^+ influence on oxidative stress in sugarcane. Lastly, as the enzymes analyzed are known to be present in most plants as isoenzymes and found in distinct cell compartments (Azevedo et al., 1998), future research should evaluate specific isoenzymes. The oxidative stress can be better understood if correlated or not with specific isoenzymes.

5. Conclusions

Our results indicate that NH_4^+ uptake and its transport efficiency in sugarcane are higher than those under NO_3^- supply. In addition, NH_4^+–only fed plants present the lowest leaf surface area, dry biomass yield, nutrient content, and photosynthetic activity among all treatment ratios. Therefore, the sugarcane preference for NH_4^+ over NO_3^-, which essentially represents a faster uptake of this cation, does not provide higher utilization efficiency (biomass produced per unit of N taken up by the crop). In contrast, sugarcane plants exposed to NO_3^- as sole N source show higher biomass yield, leaf surface area, root growth, nutrient content, and leaf gas exchange, thus improving the N utilization efficiency. Finally, under the experiment conditions, NH_4^+ uptake by sugarcane does not exacerbate the activity of oxidizing enzymes, showing little influence on oxidative stress in sugarcane.

Conflicts of interest

The authors declare that they have no competing interests.

Author contributions

BNB and PCOT conceived and designed the experiment; BNB and EM performed the research; BNB performed all the laboratory analyses; BNB, EM, RAA, and PCOT analyzed the data and wrote the manuscript. All authors reviewed and approved the final manuscript.

Acknowledgments

BNB received a scholarship from the FAPESP–São Paulo Research Foundation [grant number 2013/17278–1]. We would like to thank Carolina Z. Caon and Dra. Salete A. Gaziola for their assistance with the crop management and laboratory work (assessment of tissue oxidative stress), respectively.

References

Domínguez–Valdivia, M.D., Aparicio–Tejo, P.M., Lamsfus, C., Cruz, C., Martins–Loução, M.A., Moran, J.F., 2008. Nitrogen nutrition and antioxidant metabolism in ammonium –tolerant and –sensitive plants. Physiol. Plantarum 132, 359–369. https://doi.org/10.1111/j.1399-3054.2007.01022.x.

Gomes–Junior, R.A., Moldes, C.A., Delite, F.S., Pompeu, G.B., Gratão, P.L., Mazzafera, P., Lea, P.J., Azevedo, R.A., 2006. Antioxidant metabolism of coffee cell suspension cultures in response to cadmium. Chemosphere 65, 1330–1337. https://doi.org/10.1016/j.chemosphere.2006.04.056.

Walch–Liu, P., Neumann, G., Bangerth, F., Engels, C., 2000. Rapid effects of nitrogen form on leaf morphogenesis in tobacco. J. Exp. Bot. 51, 227–237. https://doi.org/10.1093/jexbot/51.343.227.

Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E., 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ. 24, 1337–1344. https://doi.org/10.1046/j.1365–3040.2001.00778.x.

Andrews, M., Lea, P.J., Raven, J.A., Lindsey, K., 2004. Can genetic manipulation of plant nitrogen assimilation enzymes result in increased crop yield and greater N-use efficiency? An assessment. Ann. Appl. Biol. 145, 25–40. https://doi.org/10.1111/j.1744-7348.2004.tb00356.x.

Andrews, M., Lea, P.J., Raven, J.A., Azevedo, R.A., 2009. Nitrogen use efficiency. 3. Nitrogen fixation: genes and costs. Ann. Appl. Biol. 155, 1–13. https://doi.org/10.1111/j.1744–7348.2009.00338.x.

Andrews, M., Raven, J.A., Lea, P.J., 2013. Do plants need nitrate? The mechanisms by which nitrogen form affects plants. Ann. Appl. Biol. 163, 174–199. https://doi.org/10.1111/aab.12045.

Argo, W.R., Biernbaum, J.A., 1997. Lime, water source, and fertilizer nitrogen form affect substrate-pH and nitrogen accumulation and uptake. Hortscience 32, 71–74.

Azevedo, R.A., Alas, R.M., Smith, R.J., Lea, P.J., 1998. Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild–type and a catalase–deficient mutant of barley. Physiol. Plantarum 104, 280–292. https://doi.org/10.1034/j.1399–3054.1998.1040217.x.

Balkos, K.D., Britto, D.T., Kronzucker, H.J., 2010. Optimization of ammonium acquisition and metabolism by potassium in rice *Oryza sativa* L. cv. IR–72). Plant Cell Environ. 33, 23–34. https://doi.org/10.1111/j.1365-3040.2009.02046.x.

Barrie, A., Prosser, S.J., 1996. Automated analysis of light–element stable isotopes by isotope ratio mass spectrometry. In: Boutton, T.W., Yamasaki, S. (Eds.), Mass

Spectrometry of Soils. Marcel Dekker, New York, pp. 1–46.
Beatty, P.H., Shrawat, A.K., Carroll, R.T., Zhu, T., Good, A.G., 2009. Transcriptome analysis of nitrogen–efficient rice over–expressing alanine aminotransferase. Plant Biotechnol. J. 7, 562–576. https://doi.org/10.1111/j.1467–7652.2009.00424.x.
Bittsánszky, A., Pilinszky, K., Gyulai, G., Komives, T., 2015. Overcoming ammonium toxicity. Plant Sci. 231, 184–190. https://doi.org/10.1016/j.plantsci.2014.12.005.
Bloom, A.J., Sukrapanna, S.S., Warner, R.L., 1992. Root respiration associated with ammonium and nitrate absorption and assimilation by barley. Plant Physiol. 99, 1294–1301. https://doi.org/10.1104/pp.99.4.1294.
Borges, B.M.M.N., Almeida, T.B.F., Prado, R.M., 2016. Response of sugarcane ratoon to nitrogen without and with the application of silicon. J. Plant Nutr. 39, 793–803. https://doi.org/10.1080/01904167.2015.1109101.
Boschiero, B.N., Mariano, E., Trivelin, P.C.O., 2018. "Preferential" ammonium uptake by sugarcane does not increase the ^{15}N recovery of fertilizer sources. Plant Soil 429, 253–269. https://doi.org/10.1007/s11104–018–3672–z.
Bouma, T.J., Nielsen, K.L., Koutstaal, B., 2000. Sample preparation and scanning protocol for computerised analysis of root length and diameter. Plant Soil 218, 185–196. https://doi.org/10.1023/A:1014905104017.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003–2697(76)90527–3.
Britto, D.T., Kronzucker, H.J., 2002. NH_4^+ toxicity in higher plants: a critical review. J. Plant Physiol. 159, 567–584. https://doi.org/https://doi.org/10.1078/0176–1617–0774.
Britto, D.T., Kronzucker, H.J., 2005. Nitrogen acquisition, PEP carboxylase, and cellular pH homeostasis: new views on old paradigms. Plant Cell Environ. 28, 1396–1409. https://doi.org/10.1111/j.1365–3040.2005.01372.x.
Britto, D.T., Kronzucker, H.J., 2013. Ecological significance and complexity of N–source preference in plants. Ann. Bot. 112, 957–963. https://doi.org/10.1093/aob/mct157.
Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310. https://doi.org/10.1016/S0076–6879(78)52032–6.
Coskun, D., Britto, D.T., Li, M., Becker, A., Kronzucker, H.J., 2013. Rapid ammonia gas transport accounts for futile transmembrane cycling under NH_3/NH_4^+ toxicity in plant roots. Plant Physiol. 163, 1859–1867. https://doi.org/10.1104/pp.113.225961.
Coskun, D., Britto, D.T., Kronzucker, H.J., 2016. The nitrogen–potassium intersection: membranes, metabolism, and mechanism. Plant Cell Environ. 100, 1–13. https://doi.org/10.1111/pce.12671.
Cramer, M.D., Lewis, O.A.M., 1993. The influence of NO_3^- and NH_4^+ nutrition on the carbon and nitrogen partitioning characteristics of wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) plants. Plant Soil 154, 289–300. https://doi.org/10.1007/BF00012534.
Cruz, C., Bio, A.F.M., Domínguez–Valdivia, M.D., Aparicio–Tejo, P.M., Lamsfus, C., Martins–Loução, M.A., 2006. How does glutamine synthetase activity determine plant tolerance to ammonium? Planta 223, 1068–1080. https://doi.org/10.1007/s00425-005-0155-2.
de Armas, R., Valadier, M.H., Champigny, M.L., Lamaze, T., 1992. Influence of ammonium and nitrate on the growth and photosynthesis of sugarcane. J. Plant Physiol. 140, 531–535. https://doi.org/https://doi.org/10.1016/S0176–1617(11)80783–2.
Dickson, R.W., Fisher, P.R., Argo, W.R., Jacques, D.J., Sartain, J.B., Trenholm, L.E., Yeager, T.H., 2016. Solution Ammonium: nitrate ratio and cation/anion uptake affect acidity or basicity with floriculture species in hydroponics. Sci. Hortic. (Amst.) 200, 36–44. https://doi.org/10.1016/j.scienta.2015.12.034.
Escobar, M.A., Geisler, D.A., Rasmusson, A.G., 2006. Reorganization of the alternative pathways of the Arabidopsis respiratory chain by nitrogen supply: opposing effects of ammonium and nitrate. Plant J. 45, 775–788. https://doi.org/10.1111/j.1365-313X.2005.02640.x.
Esteban, R., Ariz, I., Cruz, C., Moran, J.F., 2016a. Review: mechanisms of ammonium toxicity and the quest for tolerance. Plant Sci. 248, 92–101. https://doi.org/10.1016/j.plantsci.2016.04.008.
Esteban, R., Royo, B., Urarte, E., Zamarreño, Á.M., Garcia–Mina, J.M., Moran, J.F., 2016b. Both free indole–3–acetic acid and photosynthetic performance are important players in the response of *Medicago truncatula* to urea and ammonium nutrition under axenic conditions. Front. Plant Sci. 7 article 140. https://doi.org/10.3389/fpls.2016.00140.
Giannopolitis, C.N., Ries, S.K., 1977. Superoxide Dismutases. I. Occurrence in higher plants. Plant Physiol. 59, 309–314. https://doi.org/10.1146/annurev.bi.44.070175.001051.
Guo, S., Zhou, Y., Shen, Q., Zhang, F., 2007. Effect of ammonium and nitrate nutrition on some physiological processes in higher plants – growth, photosynthesis, photorespiration, and water relations. Plant Biol. 9, 21–29. https://doi.org/10.1055/s–2006–924541.
Hachiya, T., Watanabe, C.K., Fujimoto, M., Ishikawa, T., Takahara, K., Kawai–Yamada, M., Uchimiya, H., Uesono, Y., Terashima, I., Noguchi, K., 2012. Nitrate addition alleviates ammonium toxicity without lessening ammonium accumulation, organic acid depletion and inorganic cation depletion in *Arabidopsis thaliana* shoots. Plant Cell Physiol. 53, 577–591. https://doi.org/10.1093/pcp/pcs012.
Hageman, R.H., Reed, A.J., 1980. Nitrate reductase from higher plants. In: Methods in Enzymology. Academic Press, San Diego, pp. 270–280. https://doi.org/10.1016/S0076–6879(80)69026–0.
Hajari, E., Snyman, S.J., Watt, M.P., 2014. Inorganic nitrogen uptake kinetics of sugarcane (*Saccharum* spp.) varieties under in vitro conditions with varying N supply. Plant Cell Tissue Organ Cult. 117, 361–371. https://doi.org/10.1007/s11240–014–0445–0.
Hajari, E., Snyman, S.J., Watt, M.P., 2015. Nitrogen use efficiency of sugarcane (*Saccharum* spp.) varieties under in vitro conditions with varied N supply. Plant Cell Tissue Organ Cult. 122, 21–29. https://doi.org/10.1007/s11240–015–0746–y.
Hajari, E., Snyman, S.J., Watt, M.P., 2017. The effect of form and level of inorganic N on nitrogen use efficiency of sugarcane grown in pots. J. Plant Nutr. 40, 248–257. https://doi.org/10.1080/01904167.2016.1237648.
Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. Arch. Biochem. Biophys. 125, 189–198. https://doi.org/10.1016/0003–9861(68)90654–1.
Helali, S.M., Nebli, H., Kaddour, R., Mahmoudi, H., Lachaâl, M., Ouerghi, Z., 2010. Influence of nitrate–ammonium ratio on growth and nutrition of *Arabidopsis thaliana*. Plant Soil 336, 65–74. https://doi.org/10.1007/s11104–010–0445–8.
Hoagland, D.R., Arnon, D.I., 1950. The water culture method for growing plants without soil. In: Circular (Ed.), California Agriculture Experimental Stationon: Circular No. 347, Berkeley.
Hoopen, F. Ten, Cuin, T.A., Pedas, P., Hegelund, J.N., Shabala, S., Schjoerring, J.K., Jahn, T.P., 2010. Competition between uptake of ammonium and potassium in barley and *Arabidopsis* roots: molecular mechanisms and physiological consequences. J. Exp. Bot. 61, 2303–2315. https://doi.org/10.1093/jxb/erq057.
Jing, J., Zhang, F., Rengel, Z., Shen, J., 2012. Localized fertilization with P plus N elicits an ammonium-dependent enhancement of maize root growth and nutrient uptake. Field Crop. Res. 133, 176–185. https://doi.org/10.1016/j.fcr.2012.04.009.
Keeping, M.G., Rutherford, R.S., Sewpersad, C., Miles, N., 2015. Provision of nitrogen as ammonium rather than nitrate increases silicon uptake in sugarcane. AoB Plants 7, plu080. https://doi.org/10.1093/aobpla/plu080.
Kirkby, E.A., Knight, A.H., 1977. Influence of the level of nitrate nutrition on ion uptake and assimilation, organic acid accumulation, and cation–anion balance in whole tomato plants. Plant Physiol. 60, 349–353. https://doi.org/10.1104/pp.60.3.349.
Kölln, O.T., 2016. Eficiência de uso de nitrogênio pela cana–de–açúcar: diferenças genotípicas, preferência por amônio e emissão de N2O. University of São Paulo (Center for Nuclear Energy in Agriculture).
Kraus, T.E., McKersie, B.D., Fletcher, R.A., 1995. Paclobutrazol–induced tolerance of wheat leaves to paraquat may involve increased antioxidant enzyme activity. J. Plant Physiol. 145, 570–576. https://doi.org/10.1016/S0176-1617(11)81790-6.
Kronzucker, H.J., Siddiqi, M.Y., Glass, A.D.M., 1997. Conifer root discrimination against soil nitrate and the ecology of forest succession. Nature 385, 59–61. https://doi.org/10.1038/385059a0.
Kronzucker, H.J., Schjoerring, J.K., Erner, Y., Kirk, G.J.D., Siddiqi, M.Y., Glass, A.D.M., 1998. Dynamic interactions between root NH_4^+ influx and long–distance N translocation in rice: insights into feedback process. Plant Cell Physiol. 39, 1287–1293. https://doi.org/10.1093/oxfordjournals.pcp.a029332.
Lang, H.J., Elliott, G.C., 1991. Influence of ammonium : nitrate ratio and nitrogen concentration on nitrification activity in soilless potting media. J. Am. Soc. Hortic. Sci. 16, 642–645.
Laporte, M.M., Shen, B., Tarczynski, M.C., 2002. Engineering for drought avoidance: expression of maize NADP–malic enzyme in tobacco results in altered stomatal function. J. Exp. Bot. 53, 699–705. https://doi.org/10.1093/jexbot/53.369.699.
Lea, P.J., Azevedo, R.A., 2006. Nitrogen use efficiency. 1. Uptake of nitrogen from the soil. Ann. Appl. Biol. 149, 243–247. https://doi.org/10.1111/j.1744–7348.2006.00101.x.
Lea-Cox, J.D., Stutte, G.W., Berry, W.L., Wheeler, R.M., 1996. Charge balance - a theoretical basis for modulating pH fluctuations in plant nutrient delivery systems. Life Support Biosph. Sci. 3, 53–59.
Li, B., McKeand, S.E., Allen, H.L., 1991. Genetic variation in nitrogen use efficiency of loblolly pine seedlings. For. Sci. 37, 613–626.
Liu, W., 2009. Correlation between specific fine root length and mycorrhizal colonization of maize in different soil types. Front. Agric. China 3, 13–15. https://doi.org/10.1007/s11703–009–0004–3.
Lopes, M.S., Araus, J.L., 2006. Nitrogen source and water regime effects on durum wheat photosynthesis and stable carbon and nitrogen isotope composition. Physiol. Plantarum 126, 435–445. https://doi.org/10.1111/j.1399–3054.2006.00595.x.
Lopes, M.S., Nogués, S., Araus, J.L., 2004. Nitrogen source and water regime effects on barley photosynthesis and isotope signature. Funct. Plant Biol. 31, 995. https://doi.org/10.1071/FP04031.
Lu, Y.L., Xu, Y.C., Shen, Q.R., Dong, C.X., 2009. Effects of different nitrogen forms on the growth and cytokinin content in xylem sap of tomato (*Lycopersicon esculentum* Mill.) seedlings. Plant Soil 315, 67–77. https://doi.org/10.1007/s11104–008–9733–y.
Ma, Q., Wang, X., Li, H., Li, H., Cheng, L., Zhang, F., Rengel, Z., Shen, J., 2014. Localized application of NH_4^+-N plus P enhances zinc and iron accumulation in maize via modifying root traits and rhizosphere processes. Field Crop. Res. 164, 107–116. https://doi.org/10.1016/j.fcr.2014.05.017.
Magalhães, J.R., Huber, D.M., 1989. Ammonium assimilation in different plant species as affected by nitrogen form and pH control in solution culture. Fert. Res. 21, 1–6. https://doi.org/10.1007/BF01054728.
Marschner, P., 2012. Marschner's Mineral Nutrition of Higher Plants, third ed. Academic Press, London.
McCormick, A.J., Cramer, M.D., Watt, D.A., 2008. Changes in photosynthetic rates and gene expression of leaves during a source–sink perturbation in sugarcane. Ann. Bot. 101, 89–102. https://doi.org/10.1093/aob/mcm258.
Mengel, K., Kirkby, E.A., Kosegarten, H., Appel, T., 2001. Principles of Plant Nutrition, fifth ed. Kluver Academic, Dordrecht.
Nakamura, M., Nakamura, T., Tsuchiya, T., 2010. Advantages of NH_4^+ on growth, nitrogen uptake and root respiration of Phragmites australis. Plant Soil 331, 463–470. https://doi.org/10.1007/s11104–009–0267–8.
Patterson, K., Cakmak, T., Cooper, A., Lager, I., Rasmusson, A.G., Escobar, M.A., 2010. Distinct signalling pathways and transcriptome response signatures differentiate ammonium– and nitrate–supplied plants. Plant Cell Environ. 33, 1486–1501. https://doi.org/10.1111/j.1365–3040.2010.02158.x.
Podgórska, A., Gieczewska, K., Łukawska-Kuźma, K., Rasmusson, A.G., Gardeström, P., Szal, B., 2013. Long–term ammonium nutrition of Arabidopsis increases the

extrachloroplastic NAD(P)H/NAD(P)+ ratio and mitochondrial reactive oxygen species level in leaves but does not impair photosynthetic capacity. Plant Cell Environ. 36, 2034–2045. https://doi.org/10.1111/pce.12113.

Polesskaya, O.G., Kashirina, E.I., Alekhina, N.D., 2004. Changes in the activity of antioxidant enzymes in wheat leaves and roots as a function of nitrogen source and supply. Russ. J. Plant Physiol. 51, 615–620. https://doi.org/10.1023/B:RUPP.0000040746.66725.77.

Raven, J.A., 1988. Acquisition of nitrogen by the shoots of land plants: its occurrence and implications for acid-base regulation. New Phytol. 109, 1–20. https://doi.org/10.1111/j.1469-8137.1988.tb00212.x.

Rengel, Z., 2015. Availability of Mn, Zn and Fe in the rhizosphere. J. Soil Sci. Plant Nutr. 15, 397–409. https://doi.org/10.4067/S0718-95162015005000036.

Robinson, N., Brackin, R., Vinall, K., Soper, F., Holst, J., Gamage, H., Paungfoo–Lonhienne, C., Rennenberg, H., Lakshmanan, P., Schmidt, S., 2011. Nitrate paradigm does not hold up for sugarcane. PLoS One 6, 1–9. e19045. https://doi.org/10.1371/journal.pone.0019045.

Roosta, H.R., Schjoerring, J.K., 2008. Effects of nitrate and potassium on ammonium toxicity in cucumber plants. J. Plant Nutr. 31, 1270–1283. https://doi.org/10.1080/01904160802135050.

Rosolem, C.A., Giommo, G.S., Laurenti, R.L.B., 2000. Crescimento radicular e nutrição de cultivares de algodoeiro em resposta à calagem. Pesqui. Agropecuária Bras. 35, 827–833. https://doi.org/10.1590/S0100-204X2000000400021.

Salsac, L., Chaillou, S., Morot–Gaudry, J., Lesaint, C., Jolivet, E., 1987. Nitrate and ammonium nutrition in plants. Plant Physiol. Biochem. 25, 805–812.

Sarasketa, A., González-Moro, M.B., González-Murua, C., Marino, D., 2016. Nitrogen source and external medium pH interaction differentially affects root and shoot metabolism in Arabidopsis. Front. Plant Sci. 7, 1–12. https://doi.org/10.3389/fpls.2016.00029.

Schjoerring, J.K., Husted, S., Mäck, G., Mattsson, M., 2002. The regulation of ammonium translocation in plants. J. Exp. Bot. 53, 883–890. https://doi.org/10.1093/jexbot/53.370.883.

Siddiqi, M.Y., Glass, A.D.M., 1981. Utilization index: a modified approach to the estimation and comparison of nutrient utilization efficiency in plants. J. Plant Nutr. 4, 289–302. https://doi.org/10.1080/01904168109362919.

Signora, L., De Smet, I., Foyer, C.H., Zhang, H., 2001. ABA plays a central role in mediating the regulatory effects of nitrate on root branching in *Arabidopsis*. Plant J. 28, 655–662. https://doi.org/10.1046/j.1365–313x.2001.01185.x.

Song, W., Makeen, K., Wang, D., Zhang, C., Xu, Y., Zhao, H., Tu, E., Zhang, Y., Shen, Q., Xu, G., 2011. Nitrate supply affects root growth differentially in two rice cultivars differing in nitrogen use efficiency. Plant Soil 343, 357–368. https://doi.org/10.1007/s11104–01–0723–0.

Swiader, J.M., Chyan, Y., Freiji, F.G., 1994. Genotypic differences in nitrate uptake and utilization efficiency in pumpkin hybrids. J. Plant Nutr. 17, 1687–1699. https://doi.org/10.1080/01904169409364840.

Szczerba, M.W., Britto, D.T., Balkos, K.D., Kronzucker, H.J., 2008. Alleviation of rapid, futile ammonium cycling at the plasma membrane by potassium reveals K^+–sensitive and –insensitive components of NH_4^+ transport. J. Exp. Bot. 59, 303–313. https://doi.org/10.1093/jxb/erm309.

Tang, C., Rengel, Z., 2003. Role of plant cation/anion uptake ratio in soil acidification. In: Rengel, Z. (Ed.), Handbook of Soil Acidity. Marcel Dekker, New York, pp. 57–81.

Tedesco, M.J., Volkweiss, S.J., Bohnen, H., 1985. Análise de solo, plantas e outros materiais (Boletim Técnico n. 5). Universidade Federal do Rio Grande do Sul, Porto Alegre.

van Raij, B., Andrade, J.C., Cantarella, H., Quaggio, J.A., 2001. Análise química para avaliação da fertilidade de solos tropicais. Instituto Agronômico, Campinas.

Von Wirén, N., Gojon, A., Chaillou, S., Raper, D., 2001. Mechanism and regulation of ammonium uptake in higher plants. In: Lea, P.J., Morot–Gaudry, J.F. (Eds.), Plant Nitrogen. Springer Verlag, Berlin; London, pp. 61–77.

Vuletić, M., Šukalović, V.H., Marković, K., Maksimović, J.D., 2010. Antioxidative system in maize roots as affected by osmotic stress and different nitrogen sources. Biol. Plant. 54, 530–534. https://doi.org/10.1007/s10535–010–009–0.

Zhang, H., Forde, B.G., 2000. Regulation of *Arabidopsis* root development by nitrate availability. J. Exp. Bot. 51, 51–59. https://doi.org/10.1093/jexbot/51.342.51.