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REGULAR ARTICLE

Importance of the nitrogen source in the grass species *Brachiaria brizantha* responses to sulfur limitation

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Abstract
Background and aims Plant responses to S supply are highly dependent on N nutrition. We investigated the effect of S status on metabolic, nutritional, and production variables in *Brachiaria brizantha* treated with different N forms. Additionally, ^{15}N and ^{34}S root influx were determined in plants under short- and long-term S deprivation.
Methods Plants were submitted to soil fertilization treatments consisted of combinations of N forms [without N, ammonium (NH_4^+), nitrate (NO_3^-) or NH_4^++ NO_3^-] at S rates (0, 15, 30, or 45 mg dm^{-3}). N and S influx capacity was determined in hydroponically-grown plants.
Results Shoot production due to S supply increased 53, 145 and 196 % with NH_4^+, NH_4^++NO_3^- and NO_3^- treatments, respectively. No or low S impaired protein synthesis and led to high accumulation of N-NO_3^- and asparagine in NO_3^--fed plants, both alone and with NH_4^+. Proline accumulation was observed in NH_4^+-fed plants. Short- and long-term S deprivation did not promote considerable changes in ^{15}N influx. ^{34}S absorption decreased depending on the N form provided: NH_4^++ NO_3^- > only NH_4^+ > only NO_3^- > low N.
Conclusions Including both NH_4^+ and NO_3^- forms in fertilizer increases N and S intake potential and thereby enhances plant growth, nutritional value and production.

Keywords Amino acids · Ammonium nitrate · Nitrogen and sulfate root influx · Soil fertilization · Sulfur deficiency

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Introduction

Reduced plant production due to sulfur (S) limitation in soils is an increasingly common phenomenon worldwide (Scherer 2001). In addition to the intrinsic negative effects of S deficiency, impaired plant growth and development are strongly attributed to diminished nitrogen (N) fertilizer use efficiency by crops growing under low S availability (Ceccotti 1996). The importance of balanced N and S nutrition for improving plant production has been documented for several species, including *Brachiaria brizantha* (De Bona and Monteiro 2010a), *Brassica napus* (oilseed rape) (Dubousset et al. 2009), *Trifolium repens* (white clover) (Varin et al. 2010), *Triticum aestivum* (wheat) (Salvagiotti et al. 2009), and *Lolium perenne* (ryegrass) (Millard et al. 1985). In a classic work, Dijkshoorn and van Wijk (1967) showed that plants require optimum quantities and proportions of N and S in order to grow satisfactorily. Since then, correlated studies have reported that plant response to N and S fertilization is not only dependent on the

availability of these nutrients, but also on the form of N [ammonium (NH_4^+) and/or nitrate (NO_3^-)] supplied (Migge et al. 2000; Prosser et al. 2001; De Bona et al. 2011). Differences in physiological and metabolic pathways and energy costs related to the assimilation and allocation of N forms (Crawford et al. 2000) may alter the N and S connected metabolism in the plant system. Previous studies have noted a decline in NO_3^- reduction and assimilation in S-deficient plants of *Nicotiana tabacum* (tobacco), *Lycopersicum esculentum* (tomato), and *Brassica rapa* (rapeseed) grown in hydroponic solutions (Migge et al. 2000; Prosser et al. 2001; Kaur et al. 2011).

The accumulation of N-rich amino acids and the impaired protein synthesis observed in S-starved plants (Karmoker et al. 1991; Nikiforova et al. 2006; Kaur et al. 2011) seem to be linked to the N forms provided in the nutrition medium. In an earlier study, De Bona et al. (2011) found a higher accumulation of the free amino acid asparagine in the shoot tissues of barley fed with NO_3^- as the predominant N form in the soil solution than in those supplied with NH_4^+. As higher plants differ in NO_3^- and NH_4^+ uptake potential (Von Wirén et al. 1997) and NH_4^+ tolerance (Britto and Kronzucker 2002), it is reasonable to suppose that the metabolic interconnection of N and S in response to S deprivation and N forms also varies between plant species.

Lower N acquisition by S-deficient plants is commonly associated with a decrease in N uptake by roots (Clarkson et al. 1989; Karmoker et al. 1991). This N and S regulation at the ion absorption level might be influenced by the available N form in the medium. Clarkson et al. (1989) reported that limiting S supply to *Hordeum vulgare* (barley) plants severely depressed NO_3^- uptake, while NH_4^+ uptake was much less affected. It is also known that N status regulates S-sulfate ($S\text{-}SO_4^{2-}$) uptake and assimilation by plants (Brunold and Suter 1984; Clarkson et al. 1999; Koprivova et al. 2000). Few studies, however, have examined in detail how NO_3^-, NH_4^+, or both forms together as an N source affect S absorption by roots (Kruse et al. 2007).

The objective of this study was to investigate the effect of S status on some metabolic, nutritional, and production variables in the tropical forage grass *Brachiaria brizantha* in soil treated with varying forms of N. Complementary studies to evaluate N and S uptake in *B. brizantha* plants supplied with different forms of N and subjected to short- or long-term S deprivation were performed in a controlled environment through stable isotope techniques.

Material and methods

Plant material, soil substrate, and growth conditions

The experiment was carried out in a greenhouse using 3.6-L pots (height: 17.0 cm; diameter: 18.1 cm) filled with 5.5 kg of dried soil planted with the tropical forage grass *Brachiaria brizantha* cv. Marandu (five plants per pot). Average air temperature in the greenhouse was 28/22 °C during the day/night cycle. The substrate was collected from the surface (0–20 cm) layer of a sandy Entisol located near Piracicaba, São Paulo state, Brazil (22° 43′ S, 47° 38′ W). Collected soils were dried and sieved and organic residues >2.0 mm removed. The main chemical attributes of the soil substrate before the N and S fertilization treatments were: pH ($CaCl_2$): 4.6, organic carbon (OC): 10.4 g kg^{-1}; phosphorus (P) extracted by resin: 6.0 mg dm^{-3}; potassium (K): 109.5 mg kg^{-1}; calcium (Ca): 440.8 mg kg^{-1}; magnesium (Mg): 145.8 mg kg^{-1}; total N: 590.4 mg kg^{-1}; $N\text{-}NO_3^-$: 31.5 mg kg^{-1} (2.25 mol kg^{-1}); $N\text{-}NH_4^+$: 12.0 mg kg^{-1} (0.86 mol kg^{-1}); total S: 150.2 mg kg^{-1}; SO_4^{2-}-S: 3.0 mg kg^{-1} (0.09 mol kg^{-1}) and base saturation (V): 40 %. To satisfy the nutritional requirements of *B. brizantha*, $CaCO_3$ and $MgCO_3$ salts were added to the substrate to increase its base saturation to 50 %. After carbonates were added, the soil was incubated with 950 mL of water for 30 day.

Soil fertilization treatments consisted of four combinations of N forms [none (without N), NO_3^-, NH_4^+ or NH_4^++NO_3^-] at four S rates [none (0), 15, 30, or 45 mg dm^{-3} (mg kg^{-1})]. Thus, the 16 combinations consisted of: without N – 0S; without N – 15S; without N – 30S; without N – 45S; NO_3^- – 0S; NO_3^- – 15S; NO_3^- – 30S; NO_3^- – 45S; NH_4^+ – 0S; NH_4^+ – 15S; NH_4^+ – 30S; NH_4^+ – 45S; NH_4^++NO_3^- – 0S; NH_4^++NO_3^- – 15S; NH_4^++NO_3^- – 30S; and NH_4^++NO_3^- – 45S. N forms were applied at a single rate of 300 mg kg^{-1}. Nitrogen and S were provided via the following analytical reagents: $Ca(NO_3)_2$; NH_4Cl; and $CaSO_4.2H_2O$. The NH_4^+-N and NO_3^--N ratio in the NH_4^++NO_3^- treatment was 30:70 % (Santos 2003). Ammonium-containing treatments received 20 % of

total N through the nitrification inhibitor dicyandiamide (DCD). The treatments were arranged in a randomized complete block design with four replications.

Basal fertilization with macronutrients for the establishment of *B. brizantha* was as follows: P: 200 mg kg^{-1}; K: 250 mg kg^{-1}; Mg: 160 mg kg^{-1}, provided through the salts KH_2PO_4 and $MgCl_2.6H_2O$. Additional $CaCl_2.6H_2O$ was applied to balance Ca (485 mg kg^{-1}) supplied by the NO_3^- and SO_4^{2-} sources. Micronutrients were added as follows: H_3BO_3: 1.5 mg kg^{-1}; $CuCl_2.2H_2O$: 2.5 mg kg^{-1}; $ZnCl_2$: 2.0 mg kg^{-1}; and $Na_2MoO_4.2H_2O$: 0.25 mg kg^{-1}. All nutrients were dissolved in water and applied as an aqueous solution to the soil in the pots before grass seedlings were transplanted.

B. brizantha seeds were germinated in a sterile sand medium. After 10 day seedlings were transplanted to the soil substrate at a density of five plants per pot. Soils were irrigated daily to maintain soil water content at approximately 80 % of field capacity. In order to eliminate the effects of the establishment phase, all plants were evenly cut up to 4 cm above the soil 33 day after transplanting. Plant measurements reported in this paper thus refer to the first regrowth cycle of the grass. Nitrogen and S fertilization treatments were repeated for grass regrowth. Potassium was also re-supplied after the first harvest using the salt KCl.

Plant and soil measurements

Plant shoots were harvested 28 day after cutting (DAC) and the shoot tissue dried at 65 °C to constant weight. At harvest, tillers and leaves were counted. Additionally, chlorophyll measurements were performed on newly expanded leaves using a chlorophyll meter (SPAD-502, Minolta).

Concentrations of N, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, S, and $S\text{-}SO_4^{2-}$ were determined in ground (<1-mm particle) dry plant material of newly expanded leaf tissue or diagnostic leaves (Melo et al. 2010) sampled at harvesting. Nitrogen content in the plant tissue was determined according to Nelson and Sommers (1973). Sulfur content in the plant material was determined by wet digestion with HNO_3 and $HClO_4$ (Blanchar et al. 1965) and quantified by turbidimetry (Lisle et al. 1994). $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ contents in plant tissue were measured following the distillation methodology described by Bremner and Keeney (1966). Extractable plant $S\text{-}SO_4^{2-}$ content was determined according to the procedure of Sinclair (1974) and Johnson and Nishita (1952).

To analyze protein content and free amino acids in the grass tissue, shoots were sampled at 21 DAC. The plant material was harvested, frozen in liquid N_2, and stored at −80 °C. Protein content in shoot tissue was determined via the Dumas method (AOAC 1995). For the free amino acids analysis, 500 mg of plant material were mixed with 10 mL extraction solution (10 m*M* CH_2O_2) and homogenized using an Ultra-Turrax (T 18 basic, Ika). Plant extracts were subsequently vortexed for 10 min and centrifuged at 4500 rpm for 10 min to separate phases. The supernatant was centrifuged at 13200 rpm for 30 min and 1.5 mL of the aqueous phase were recovered. Prior to amino acid determination, 1.5 mL aliquots of the supernatant were diluted in distilled water (1:10). Norvaline was used as an internal standard. Amino acids were assayed after pre-column derivatization with o-phthaldialdehyde (OPA) followed by high-performance liquid chromatography (Shimadzu) using a sodium buffer gradient. The system included a C-18 reversed phase column (Spherisorb ODS 2) and fluorometric detection with excitation at 340 nm and emission at 455 nm. Peak areas and amino acid concentrations were calculated by using an area integrator (Spectra Physics SP 4270).

Nitrate and nitrite reductase enzyme activities were assessed in the newly expanded *B. brizantha* leaves collected at 20 DAC. Nitrate reductase activity was determined in vivo following the method reported by Nicholas et al. (1976). Leaf tissue was cut into 0.2–0.5 cm pieces (1 g), placed in 40 mL of incubation medium (0.1*M* KNO_3, 0.1*M* K_2HPO_4/KH_2PO_4 at pH 7.5, 2 % n-propanol and 0.01*M* Na_2CO_3), vacuum-infiltrated twice (for 2 min each time) and incubated in a shaker (60 rpm) under light (400 μmol photons m^{-2} s^{-1}) at 25 °C. Aliquots of 0.5 mL of the supernatant were removed at 30 and 60 min. The reaction was stopped by adding 0.5 mL 1 % sulfanilamide in 3*M* HCl and 0.02 % naphthyl ethylenediamine dihydrochloride to 0.5 mL of the supernatant and absorbance at 540 nm was determined (Genesys 10, Thermo Scientific).

In vivo nitrite reductase activity was determined using the methods described by Srivastava et al. (1979) combined with a method to quantify the disappearance of nitrite from the assay medium (Aslam and Huffaker 1989). Freshly-cut pieces of grass leaf tissue

were incubated in 20 mL of incubation medium containing 6 m*M* KNO_2, 0.1 M K_2HPO_4/KH_2PO_4 at pH 7.5, 20 m*M* methyl viologen, and 16 mL of distilled water. Assays were conducted under light (400 μmol photons m^{-2} s^{-1}) without shaking at 25 °C. The reaction was started by adding 4 mL $Na_2S_2O_4$ (150 mg mL^{-1}) in 0.3*M* $NaHCO_3$ and stopped after 40 min by adding 1 mL of n-propanol to the assay medium. Subsequently 1 mL of supernatant was removed and vortexed until the methyl viologen was completely oxidized (15–20 s). An 1 mL aliquot of supernatant was diluted in 9 mL distilled water and mixed with 1 mL 1 % sulfanilamide in 3*M* HCl and 1 mL 0.02 % naphthyl ethylenediamine dihydrochloride before determining absorbance at 540 nm to quantify the disappearance (reduction) of nitrite from the incubation medium.

Soils were sampled at the *B. brizantha* harvest (28 DAC) with a soil probe (length: 20 cm; diameter: 1.5 cm) that extracted samples from the top to the bottom of the pots. Soil samples for mineral N analysis were stored in a freezer at -20 °C until analysis. N-NH_4^+ and N-NO_3^- contents were determined in wet soil samples immediately after thawing. After extraction with 1*M* KCl, N-NH_4^+ and N-NO_3^- contents in soil were determined by distillation and titration according to the method developed by Bremner and Keeney (1966). The soil samples used to determine inorganic SO_4^{2-}-S were air-dried, milled, and sieved through 2-mm mesh. Inorganic SO_4^{2-}-S was extracted with 0.01*M* $Ca(H_2PO_4)_2$ solution and quantified by turbidimetry (Tabatabai and Bremner 1970). Soil pH was determined in a 0.01*M* $CaCl_2$ solution by potentiometry (Thomas 1996).

Nitrogen and S influx assays using the ^{15}N and ^{34}S stable isotope technique

Four experiments were carried out in a growth chamber to evaluate the N and S influx capacity of *B. brizantha* plants grown under strong S limitation (S-deficient) and/or subjected to S shortage (S-deprived), and fed with N forms in nutrient solution. Grass seeds were germinated in the dark between filter papers soaked with deionized water for 7 day and transferred to 2.7 L pots (three seedlings per pot) containing nutrient solution. Plants were grown hydroponically under aerated conditions in a growth chamber under the following conditions: 16/8 h light/dark; light intensity 250 μmol photons m^{-2} s^{-1}, and temperature 30/20 °C at 60 % humidity. The composition of the nutrient solution was adjusted to vary the N form and S supply [low S: 0.1 m*M* ("-S") and high S: 1.2 m*M* ("+S")] combination treatments (Low N – -S; low N – + S; NO_3^- – -S; NO_3^- – + S; NH_4^+ – -S; NH_4^+ – + S; NH_4^++NO_3^- – -S; and NH_4^++NO_3^- – + S). Thus, the nutrient solution with low S contained the follow macronutrient composition according to N form: low N (0.5 m*M* $MgCl_2.6H_2O$, 1.3 m*M* KCl, 0.1 m*M* KH_2PO_4, 2 m*M* $CaCl_2.2H_2O$, 0.1 m*M* K_2SO_4 and 0.1 m*M* NH_4NO_3); NO_3^- [0.5 m*M* $MgCl_2.6H_2O$, 1.3 m*M* KCl, 0.1 m*M* KH_2PO_4, 1 m*M* $CaCl_2.2H_2O$, 0.1 m*M* K_2SO_4 and 1 m*M* $Ca(NO_3)_2.4H_2O$]; NH_4^+ (0.5 m*M* $MgCl_2.6H_2O$, 1.3 m*M* KCl, 0.1 m*M* KH_2PO_4, 2 m*M* $CaCl_2$, 0.1 m*M* K_2SO_4 and 2 m*M* NH_4Cl); and NH_4^++NO_3^- [0.5 m*M* $MgCl_2.6H_2O$, 1.3 m*M* KCl, 0.1 m*M* KH_2PO_4, 1.3 m*M* $CaCl_2.2H_2O$, 0.1 m*M* K_2SO_4 0.7 m*M* $Ca(NO_3)_2.4H_2O$ and 0.6 m*M* NH_4Cl]. To obtain the nutrient solution with high S the $MgCl_2.6H_2O$ salt was replaced by $MgSO_4.7H_2O$ (0.5 m*M*) and the KCl and K_2SO_4 adjusted to 0.1 and 0.7 m*M*, respectively. Micronutrients were supplemented in the nutrient solution as follows: 1 μ*M* H_3BO_3, 0.5 μM $MnCl_2.2H_2O$, 0.5 μ*M* $ZnCl_2$, 0.2 μ*M* $CuCl_2.2H_2O$, 0.007 μ*M* $Na_2MoO_4.2H_2O$, and 300 μ*M* Fe(III)-EDTA. The nutrient solution was pH-buffered (around 7) with bicarbonate salt and renewed every 3 day.

Long-term S limitation experiments in order to obtain S-deficient plants were performed by growing *B. brizantha* with varying N forms (Low N; NO_3^-; NH_4^+; and NH_4^++NO_3^-) combined with low and high S supply in nutrient solution (as described above) during 15 day before the ^{15}N or ^{34}S influx assays.

Short-term S deprivation experiments were performed by growing grass plants with varying N forms (Low N; NO_3^-; NH_4^+; and NH_4^++NO_3^-) supplied with high S in the nutrient solution (as described above). After 11 day, all S was removed from the nutrient solution (Mg and K contents were adjusted using the salts $MgCl_2.6H_2O$ and KCl, respectively) of half the plants of each treatment for 4 day before the ^{15}N or ^{34}S influx assays.

For ^{15}N influx assays, plants attached to frames were taken from the N forms and S supply pretreatments and placed in a 2.5 L labeled deionized water-based medium containing 2 m*M* $^{15}NHCl$ (10.6 atoms% ^{15}N), 2 m*M* $K^{15}NO_3$ (12.15 atoms% ^{15}N), or 0.6 m*M* $^{15}NHCl$ + 1.4 m*M* $K^{15}NO_3$, which was aerated. Plant labeling continued for 3 h, after which the

roots were washed during 2 min with deionized water to remove from the free space labeled solution and rapidly exchangeable NH_4^+ and/or NO_3^-. Roots were separated from shoots. Plant material was dried in a ventilated oven at 65 °C for 48 h. Dried material was ground to a fine powder using a coffee grinder (Model MDR 301, Cadence, Brazil) and dry ice. Aliquots of 3–4 mg of the powder were weighed and placed in small tin cups to measure ^{15}N in plant tissue via a mass spectrometer with an automated N analyzer (ANCA-GSL model 20–20, Sercon, Crewe, United Kingdom) (Barrie and Prosser 1996).

For ^{34}S influx assays, intact plants pre-treated with varying N forms and S supplies were placed in a 2.5 L labeled deionized water-based medium containing 2 m*M* $K_2{}^{34}SO_4$ (11.5 atoms% ^{34}S), which was aerated. Plant labeling continued for 2 h, after which roots were washed during 2 min with deionized water and separated from shoots. The plant material was dried and ground following the procedure described for the ^{15}N assays. Aliquots of 500 mg of ground plant material were used for ^{34}S isotopic measurements. Determination of ^{34}S atoms% in plant tissue was performed using a mass spectrometer (ATLAS MAT, model CH-4, Berlin, Germany) (Bendassolli et al. 1997).

All N and S influx assays were performed using three replicates for each N form and S supply pre-treatment combination. The N or S in plant tissue derived from nutrient solution containing ^{15}N (as distinct N forms) or ^{34}S (as SO_4^{2-}) stable isotopes was calculated following the equation:

$$\text{N or S absorbed} = \left[\left(^{15}\text{N or}^{34}\text{S atom\% excess in plant material}\right) / \left(^{15}\text{N or}^{34}\text{S atom\% excess of nutrient solution}\right)\right] \times (\text{N or S content in plant material})$$

Nitrogen or S mean influxes were estimated by dividing the N or S absorbed by the respective plant labeling duration.

Statistical analysis

The statistical software SAS version 9.01 (SAS Institute Inc., Cary, USA) was used to perform analysis of variance. Means were compared via Tukey's test. All statistical tests were conducted at the 5 % significance level.

Results

Soil conditions, plant growth performance, nutritional status, and production

Soil analyses revealed an accumulation of NH_4^+ in the soils treated with ammonium fertilizer (Fig. 1a). As expected, NO_3^--based fertilization increased the NO_3^- content in soils (Fig. 1a). It should be pointed out that the use of the nitrification inhibitor DCD in the soil fertilization treatments containing NH_4^+ totally or partially blocked the formation of NO_3^-. Thus, NO_3^- contents were kept low in the soils treated with only NH_4^+, and slightly lower in the soils fertilized with both N forms than in those with the NO_3^--only treatment. Irrespective of N fertilization, inorganic SO_4^{2-} content in the soil increased with increasing S supply, ranging from 0.12 to 1.90 mol kg^{-1} (4.0 to 61.0 mg kg^{-1}) (Fig. 1c). Soil pH varied from 4.6 to 6.0 and varied with the N forms supplied, following the sequence: only NH_4^+ > NH_4^++ NO_3^- > without N > only NO_3^- (Fig. 1d). This expected variation in pH did not substantially change essential element concentrations in the plant tissue (data not shown), which were detected above the thresholds considered limiting for *B. brizantha* growth (CIAT 1981; De Bona and Monteiro 2010b).

Selected plant growth parameters (tillering, number of leaves, and chlorophyll content) and shoot production of *B. brizantha* were positively affected by N and S supply (Fig. 2). In N-supplied plants, a small addition of S (15 mg dm^{-3}) produced a huge increase in these variables. In general, the highest absolute values of the abovementioned variables were associated with the use of NO_3^- in N fertilization. In the case of shoot production, using only NO_3^- or both N forms intensified the S supply response (Fig. 2d). On average, shoot production increased with increasing S supply by 53, 145, and 196 % in plants grown with NH_4^+, NH_4^++ NO_3^-, or NO_3^- treatments, respectively.

Nitrogen concentrations in plant leaves increased up to 3-fold with the application of N fertilizers to the soil (Fig. 3a). The highest N concentrations in *B. brizantha* leaves were observed in plants treated with NH_4^++NO_3^-. Sulfur supply did not significantly affect the N content of newly expanded leaf tissue of the forage grass. On the other hand, N addition strongly enhanced plant responses to S supply as measured by S concentrations in grass leaves (Fig. 3b). Low S contents in soils associated with N fertilization led to

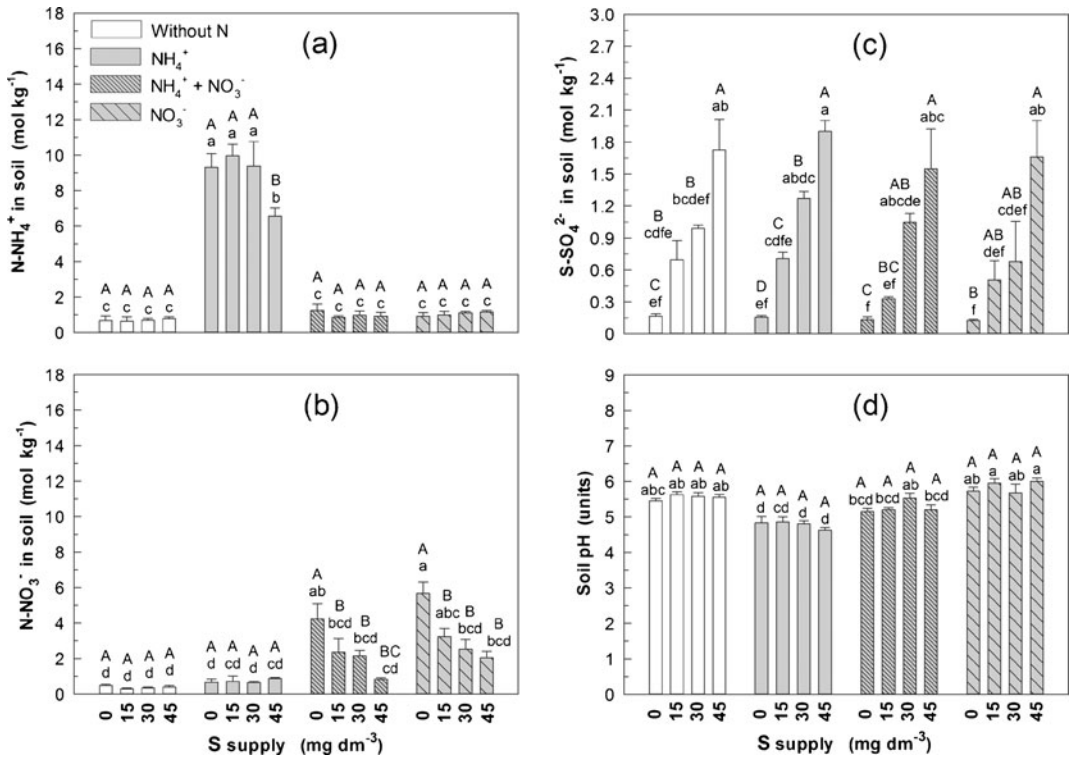

Fig. 1 Soil available N-ammonium (N-NH_4^+) (**a**), N-nitrate (N-NO_3^-) (**b**) and S-sulfate (S-SO_4^{2-}) content (**c**), and pH (**d**) changes as related to combinations of N forms [none (without N), NO_3^-, NH_4^+ or NH_4^++NO_3^- (30/70 %)] and S rates [none (0), 15, 30 or 45 mg dm^{-3}] applied to *Brachiaria brizantha* plants. *Bars* represent means ± SE. *Capital letters* denote significant differences among means in S treatments within each N treatment, while *lower case letters* denote significant differences among all treatments according to Tukey's test at $p \leq 0.05$; $n=4$

an unbalanced N:S ratio in grass leaves, raising this plant nutritional status index to exceed 40:1 (Fig. 3c).

In contrast to the N content data, free N-NH_4^+ and N-NO_3^- concentrations in *B. brizantha* leaves varied with different levels of soil S fertilization (Fig. 3d and e, respectively). Concentrations of N-NH_4^+ in grass leaves increased with increasing S rates and the highest values were observed in plants only treated with NH_4^+. No or low S caused an accumulation of free N-NO_3^- in the leaf tissues of plants cultivated without N fertilization or with NO_3^- (alone or as NH_4^++NO_3^-). As with S concentrations, free S-SO_4^{2-} accumulation in forage grass leaf tissue increased with increasing S rates (Fig. 3f).

NR and NiR activity, free amino acid and protein content

In spite of the difficulties and uncontrolled variation usually related to enzyme activity measurements in soil-grown plants, grass leaf NR and NiR activities (Fig. 4) showed a marked influence of S soil fertilization on these N assimilatory pathway enzymes. Soil S fertilization at the rate of 30 mg dm^{-3} enhanced leaf NR activity of plants fed with only NO_3^- as an N source by about 50 % (Fig. 4a). Combining NH_4^+ with NO_3^- in the N fertilization reduced the positive effect of S addition on *B. brizantha* leaf NR activity. It is interesting to observe that even under partial or exclusive NO_3^- nutrition, high S rates (>30 mg dm^{-3}) did not increase NR activity of the plants. Independent of N fertilization, grass leaf NiR activity was strongly depressed by the lack of S fertilization (Fig. 4b). On average, the addition of S enhanced leaf tissue NiR activity by approximately 130, 120, 40 and 70 % in plants without N fertilization, and with NH_4^+, NH_4^++NO_3^-, and NO_3^-, respectively.

Protein content of *B. brizantha* shoot tissues increased with increasing N inputs to the soil (Fig. 5a). However, the effect of N addition on protein synthesis was highly dependent on the interaction between N forms and S supply. Thus,

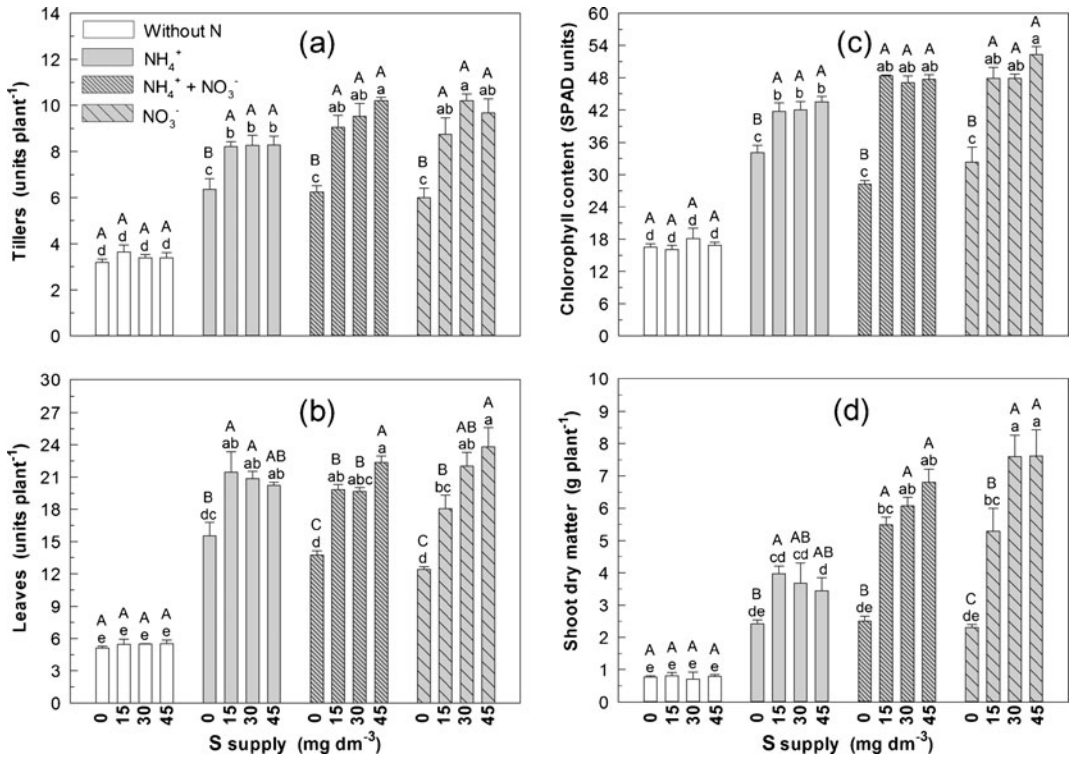

Fig. 2 Changes in total number of tillers (**a**) and leaves (**b**), leaf chlorophyll content (SPAD value) (**c**), and shoot dry matter yield (**d**) of *Brachiaria brizantha* plants as related to combinations of N forms [none (without N), NO_3^-, NH_4^+ or NH_4^++NO_3^- (30/70 %)] and S rates [none (0), 15, 30 or 45 mg dm^{-3}] applied to soils. *Bars* represent means ± SE. *Capital letters* denote significant differences among means in S treatments within each N treatment, while *lower case letters* denote significant differences among all treatments according to Tukey's test at $p \leq 0.05$; $n=4$

plants cultivated with an N source containing NO_3^- showed the lowest shoot protein content under S-limited conditions. Conversely, N-fertilized plants grown without an S supply accumulated high concentrations of free amino acids in the shoot tissue (Fig. 5b). According to detailed free amino acid screening analyses of plant shoots, asparagine (Fig. 5c) and proline (Fig. 5d) contents showed the strongest variation with varying N forms and S supply treatments. Free asparagine levels increased approximately 5-, 30-, and 40-fold in shoot tissues of S-deficient plants supplied with NH_4^+, NH_4^++NO_3^-, and NO_3^- as N sources, respectively. A significant accumulation of the free amino acid proline in shoot tissue was related to the use of NH_4^+ as the only N fertilizer source for *B. brizantha* plants, independent of S nutrition.

In general, N supply also increased the contents of some other free amino acids in grass shoot tissue, namely: serine, glutamine, alanine, aspartic acid, and glutamic acid (Fig. 6). Surprisingly, contents of the S-containing amino acids methionine and cysteine in plant shoot tissue were not substantially affected by soil S fertilization.

Nitrogen and S influxes in roots

Independently of N pre-treatment and/or the form of N supplied in the assay medium, short- and long-term S deprivation did not promote considerable changes in ^{15}N influx measured in *B. brizantha* roots (Table 1). In fact, marked alterations in ^{15}N influx were mostly related to the ^{15}N source. Thus, plants supplied with NH_4^+, NH_4^++ NO_3^-, and NO_3^- as ^{15}N sources showed an average ^{15}N influx rate of approximately 45, 92, and 75 μmol g^{-1} root DW h^{-1}, respectively.

In contrast to ^{15}N uptake, the absorption of ^{34}S was influenced by the N pre-treatment and the S nutrition of *B.*

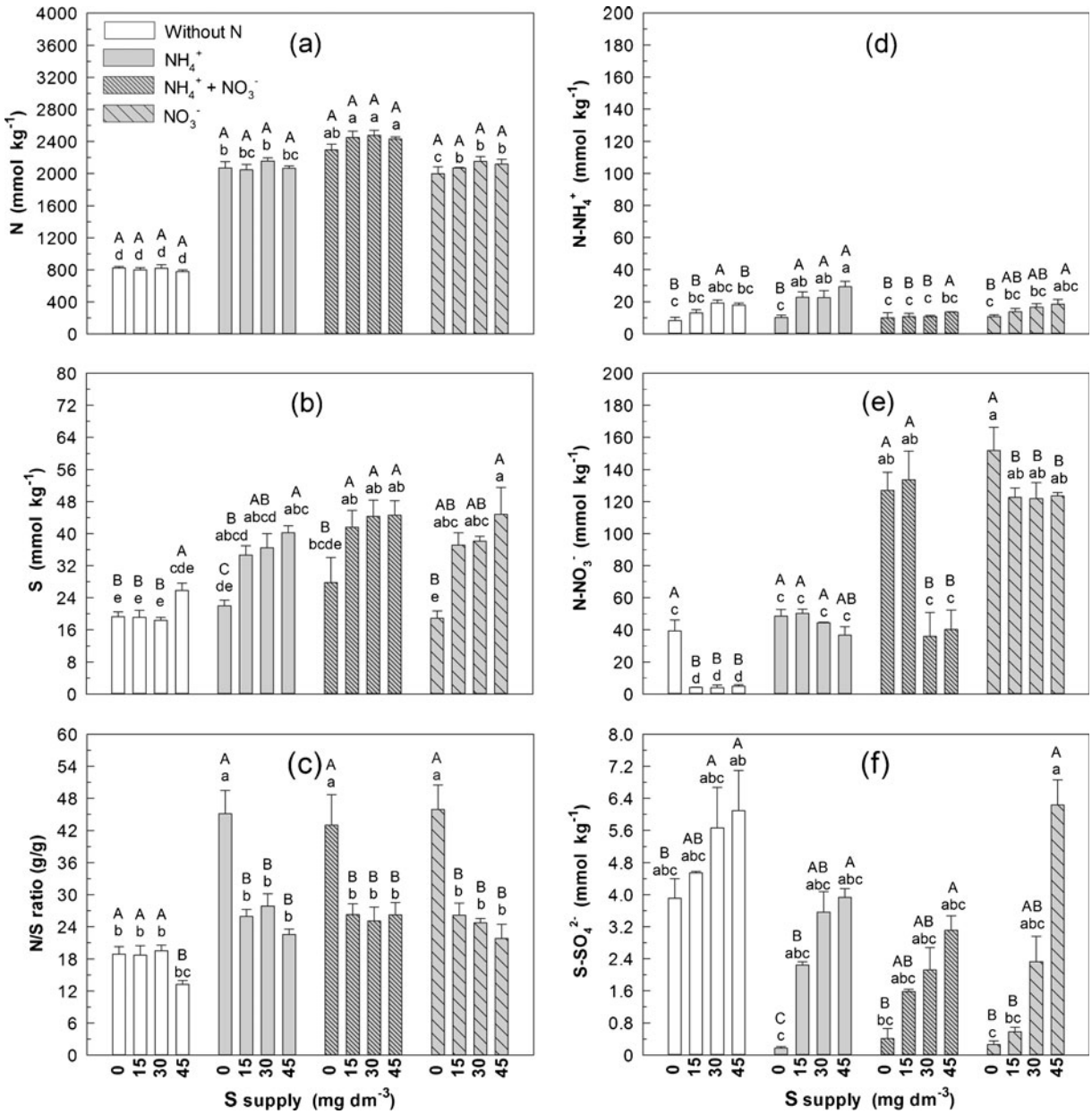

Fig. 3 Changes in total nitrogen (N) (**a**) and sulfur (S) (**b**), N/S ratio (**c**), N-ammonium (N-NH_4^+) (**d**), N-nitrate (N-NO_3^-) (**e**), and S-sulfate (S-SO_4^{2-}) (**f**) concentrations in *Brachiaria brizantha* leaves as related to combinations of N forms [none (without N), NO_3^-, NH_4^+ or NH_4^++NO_3^- (30/70 %)] and S rates [none (0), 15, 30 or 45 mg dm^{-3}] applied to soils. *Bars* represent means ± SE. *Capital letters* denote significant differences among means in S treatments within each N treatment, while *lower case letters* denote significant differences among all treatments according to Tukey's test at $p \leq 0.05$; $n=4$

brizantha plants (Table 2). Plants treated with an N source containing NH_4^+ (partially or exclusively) exhibited an increased root capacity response for S uptake under S deficiency or deprivation. On average, the ^{34}S absorption capacity of grass plants decreased with N treatments in the following order: NH_4^++NO_3^- (6.90 μmol g^{-1} root DW h^{-1}) > only NH_4^+ (4.93 μmol g^{-1} root DW h^{-1}) > only NO_3^- (3.77 μmol g^{-1} root DW h^{-1}) > low N (3.48 μmol g^{-1} root DW h^{-1}). Short-term S deprivation enhanced the ^{34}S influx rates of plant roots in all N treatments.

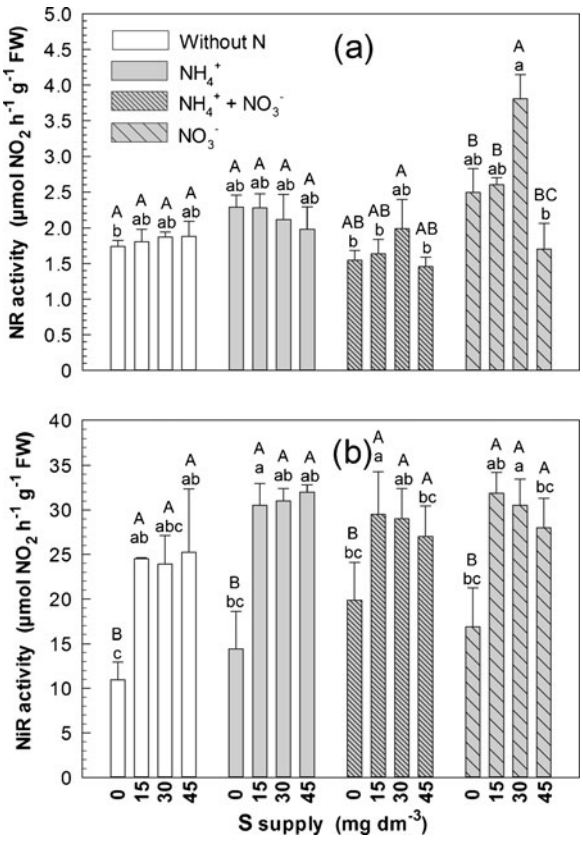

Fig. 4 Changes in nitrogen reductase (NR) (**a**) and nitrite reductase (NiR) (**b**) activity in *Brachiaria brizantha* leaves as related to combinations of N forms [none (without N), NO_3^-, NH_4^+ or NH_4^++NO_3^- (30/70 %)] and S rates [none (0), 15, 30 or 45 mg dm^{-3}] applied to soils. *FW* fresh weight. *Bars* represent means ± SE. *Capital letters* denote significant differences among means in S treatments within each N treatment, while *lower case letters* denote significant differences among all treatments according to Tukey's test at $p \leq 0.05$; $n=4$

Discussion

Agreeing with previous studies (Millard et al. 1985; Dubousset et al. 2009; Salvagiotti et al. 2009; De Bona and Monteiro 2010a; Varin et al. 2010), this work also confirmed that combined N and S soil fertilization is an efficient agricultural practice for improving plant growth performance and production. However, plant responses to S addition across various production, nutritional, and metabolic variables demonstrated a clear dependency on the form of N supplied. *B. brizantha* showed a moderate tolerance to high NH_4^+ content in soil solution (only incipient visual symptoms of phytotoxicity appeared and were characterized by leaf tip chlorosis and burn, and root growth suppression), but the strictly NH_4^+-fed plants were less responsive to S supply in terms of forage production and final dry matter yield than NH_4^++NO_3^-- or NO_3^--fed plants (Fig. 2). As N (Fig. 3a) and other nutrient concentrations in newly expanded leaf tissue (diagnostic leaves) were not limiting for full forage grass growth (CIAT 1981; De Bona and Monteiro 2010b), it is reasonable to suppose that the lower production in NH_4^+-fed plants can be caused by mild NH_4^+ toxicity stress effects. Accumulation of the free amino acid proline in shoot tissues of NH_4^+-fed plants reinforces this assumption (Fig. 5d). Proline is one of the major organic osmolytes. It accumulates in a variety of plant species in response to environmental stresses such as drought, salinity, extreme temperatures, UV radiation, heavy metals, and nutrient imbalances (including mineral toxicity) (Hare and Cress 1997; Ashraf and Foolad 2007). In addition to its role as an osmolyte for osmotic adjustment, proline helps alleviate stress (e.g., that caused by excess NH_4^+ in medium) by stabilizing sub-cellular structures (e.g., membranes and proteins), scavenging free radicals, and buffering cellular redox (Hare et al. 1999). Furthermore, the accumulation of proline under stressful conditions in many plant species has been correlated with stress tolerance, and proline concentrations have been shown to be generally higher in stress-tolerant than in stress-sensitive plants (Ashraf and Foolad 2007). Indeed, diminished plant growth performance due to excess NH_4^+ in medium is attributed, at least in part, to decreased cell size and number (Walch-Liu et al. 2000). Walch-Liu et al. (2000) investigating the effect of N forms on the growth of tobacco (*Nicotiana tabacum*) cultivated in nutrient solution, demonstrated by microscopic analysis of the epidermis of fully expanded leaves that cell number and cell size decreased 50 and 30 %, respectively, due to the application of NH_4^+.

The greatest production benefits with S use were observed in forage plants fertilized with NH_4^++NO_3^- and/or only NO_3^- as an N source (Fig. 2d). Although high energetic costs are required to reduce NO_3^- in the N assimilatory pathway (Bloom et al. 1992), this anionic N form can benefit plant growth because it is involved in promoting cytokinin synthesis and root-to-shoot translocation (Smiciklas and Below 1992; Wang and Below 1996; Walch-Liu et al. 2000) and, consequently, enhancing cell division and expansion, and axillary bud outgrowth (Ongaro and Leyser 2008).

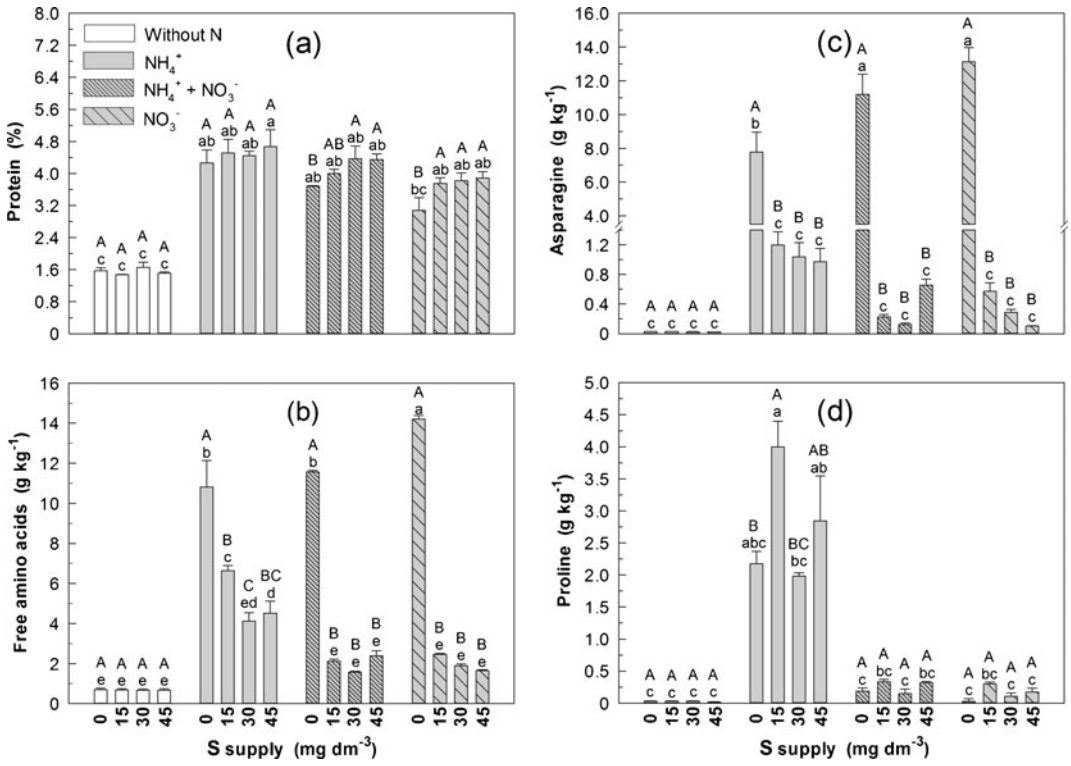

Fig. 5 Changes in protein (**a**), free amino acids (**b**), asparagine (**c**), and proline (**d**) content of *Brachiaria brizantha* shoot tissue as related to combinations of N forms [none (without N), NO_3^-, NH_4^+ or NH_4^++NO_3^- (30/70 %)] and S rates [none (0), 15, 30 or 45 mg dm^{-3}] applied to soils. *Bars* represent means ± SE. *Capital letters* denote significant differences among means in S treatments within each N treatment, while *lower case letters* denote significant differences among all treatments according to Tukey's test at $p \leq 0.05$; $n=4$

Reduced *B. brizantha* forage production due to S deficiency also reflected a general N:S imbalance, regardless of the N form supplied (Fig. 3c). However, the physiological and metabolic pathways linked to N and S cycles were altered in different ways by different forms of N. Thus, S-deficient plants supplied with NO_3^--containing fertilizers accumulated high amounts of N-NO_3^-, whereas exclusively NH_4^+-fed plants did not show the same pattern (Fig. 3e). The abovementioned accumulation of free N-NO_3^- in grass leaves was not accompanied by an increase in NR activity, although S supply did increase the activity rate of that enzyme (Fig. 4a). Given that N-NO_3^- availability was high in soils treated with NH_4^++NO_3^- or NO_3^- (Fig. 1b), these results do not support the assumption that NO_3^- is an important substrate for NR gene expression and activity (Reuveny et al. 1980; Galangau et al. 1988; Aslam and Huffaker 1989). According to Migge et al. (2000), the negative effects of S deprivation on leaf NR expression and activity may be a consequence of free amino acid accumulation. The results do in fact demonstrate a high accumulation of free amino acids, especially asparagine, in S-deficient plants (Fig. 5b and c). Asparagine is a transient soluble storage form of amino-N (Lea et al. 2007) and usually accumulates under S deficiency (Karmoker et al. 1991; Migge et al. 2000; Prosser et al. 2001; De Bona et al. 2011). The observed significant accumulation of transport amino compounds such as asparagine may have resulted from the stalling of protein synthesis for lack of S-amino acids in S-limited plants (Karmoker et al. 1991). De Bona et al. (2011) found greater asparagine accumulation in S-deficient barley plants fed with NO_3^- as an N source. The same association was observed in our study, in *B. brizantha* plants suffering from S deficiency, suggesting that asparagine accumulation in shoot tissue increases with the increasing NO_3^-/NH_4^+ ratio of the N treatment (Fig. 5c). It is very hard to isolate and quantify with precision the induction effect of NO_3^- availability on asparagine biosynthesis, since in soil conditions the control of N forms

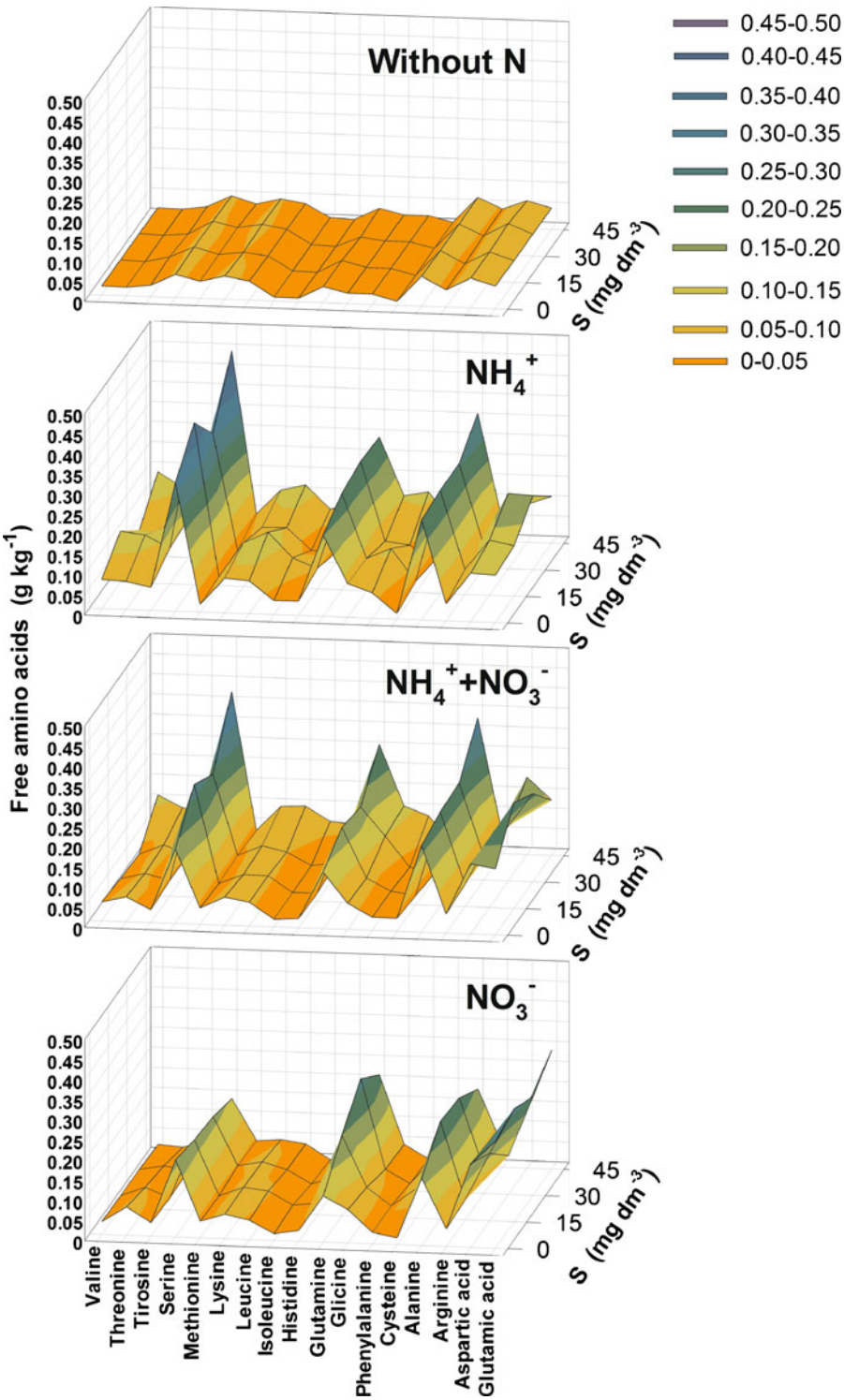

Fig. 6 Influence of combinations of N forms [none (without N), NO_3^-, NH_4^+ or NH_4^++NO_3^- (30/70 %)] and S rates [none (0), 15, 30 or 45 mg dm^{-3}] applied to soils on free amino acid concentration in the shoot tissue of *Brachiaria brizantha* plants. n=4

Table 1 Root ^{15}N influx in *Brachiaria brizantha* plants as related to combinations of N forms [Low N, NO_3^-, NH_4^+, or NH_4^++NO_3^- (30/70 %)], S supply [Low (−S) or high S (+S); long-term S limitation (S-deficient plants) or short-term S deprivation (S-deprived)], and ^{15}N sources [($^{15}NO_3^-$, $^{15}NH_4^+$, or $^{15}NH_4^+$+$^{15}NO_3^-$ (30/70 %)] added to the water-based influx assay medium. Values represent means ± SE; n=4

Treatments		^{15}N influx rate (μmol g^{-1} root dry weight h^{-1})					
		S-deficient plants			S-deprived plants (4 days)		
		$^{15}NH_4^+$	$^{15}NH_4^+$+$^{15}NO_3^-$	$^{15}NO_3^-$	$^{15}NH_4^+$	$^{15}NH_4^+$+$^{15}NO_3^-$	$^{15}NO_3^-$
Low N	−S	45±1.3	109±22	87±26	53±8.1	133±17	108±28
	+S	44±3.3	122±26	116±21	56±12	151±16	130±17
NH_4^+	−S	48±10	79±9.3	58±6.3	41±9.9	56±1.2	57±1.5
	+S	43±6.0	89±14	64±2.4	47±12	64±15	51±13
NH_4^++NO_3^-	−S	42±2.1	85±17	51±14	35±2.7	61±2.7	41±7.3
	+S	40±4.6	64±12	44±10	38±4.5	64±7.3	53±6.7
NO_3^-	−S	40±8.6	93±11	77±31	51±4.7	106±16	83±12
	+S	45±5.3	107±8.2	85±17	49±8.7	84±10	86±1.3

is not absolute. Thus, soil fertilized only with NH_4^+ showed low NO_3^- contents despite the use of nitrification inhibitors (Fig. 1b). In agreement with hypothesis posed by Migge et al. (2000) and De Bona et al. (2011), it can be concluded that asparagine accumulation probably inhibits NR activity in order to avoid an excess of soluble amino-N in shoot tissue cells of S-deficient plants growing in environments containing available NO_3^-.

The leaf enzyme NiR was mostly influenced by the S nutrition of *B. brizantha* plants (Fig. 4b). Thus, low S availability in soils (Fig. 1c) and concomitant low S concentrations in leaves (Fig. 3b and f) decreased NiR activity. Clearly, S requirements for the NiR prosthetic group Fe-S cluster biogenesis (Crawford et al. 2000) were the predominant factor in determining enzyme activity in forage grass leaves. As with NR, NiR activity did not show an induction by NO_3^- availability (Aslam and Huffaker 1989).

With regard to the nutritional value of the grass, these results demonstrate that although NH_4^++NO_3^-- or NO_3^--fed plants exhibited N concentrations in leaves tissues similar to or higher than those in NH_4^+-fed plants (Fig. 3a), protein content was more negatively affected by S deprivation when *B. brizantha* plants were grown in soil amended with NO_3^--containing fertilizers (Fig. 5a). These results were attributed mainly to the high accumulation of the free amino acid asparagine (Fig. 5c) and of unmetabolized N-NO_3^- (Fig. 3e) in shoot tissues of the grass plants.

Table 2 Root ^{34}S influx in *Brachiaria brizantha* plants as related to combinations of N forms [Low N, NO_3^-, NH_4^+, or NH_4^++NO_3^- (30/70 %)], and S supply [Low (−S) or high S (+S); long-term S limitation (S-deficient plants) or short-term S deprivation (S-deprived)] treatments. Values represent means ± SE; n=4

Treatments		^{34}S influx rate (μmol g^{-1} root dry weight h^{-1})	
		S-deficient plants	S-deprived plants (4 days)
Low N	−S	2.38±0.76	4.91±0.13
	+S	3.08±0.92	3.58±0.48
NH_4^+	−S	5.82±0.32	6.57±0.88
	+S	4.06±0.08	3.28±0.82
NH_4^++NO_3^-	−S	6.64±0.24	10.9±0.60
	+S	4.26±0.04	5.80±0.05
NO_3^-	−S	2.83±0.38	6.06±0.77
	+S	2.96±0.11	3.23±0.43

Surprisingly, the N influxes quantified using ^{15}N did not change due to plant S status or S shortages (Table 1). As a consequence, N concentrations in the leaves of *B. brizantha* remained unchanged despite S soil fertilization (Fig. 3a). By contrast, Clarkson et al. (1989) found that NH_4^+ and NO_3^- influxes of hydroponically-grown barley were strongly inhibited (approximately 50 %) after 5 day in S-deprived nutrient solution. According to presented results, *B. brizantha* apparently does not have this regulatory effect of S nutrition on N root influx, suggesting that N status and the ^{15}N form supplied are the major determinants of the N influx capacity of this forage grass. As expected, N-deficient plants showed a higher N root influx. However, this N limitation induction on the N root transporters (Xu et al. 2012) only occurred when NO_3^- was present in the ^{15}N assay solution. Studying high-affinity NH_4^+ and NO_3^- transport systems in plants through split-plot experiments, Gansel et al. (2001) observed that high-affinity NO_3^- transport is controlled by shoot-to-root signals of whole plant N demand, while NH_4^+ transport is predominantly dependent on the local N status of roots. In consequence, N deficiency mostly stimulates NO_3^- influx by a shoot-derived signal, while this putative signal is considered of minor importance relative to the effect of the local N status that dominates control over NH_4^+ influx (Loqué and von Wirén 2004). Besides nutritional and local N status induction effects, higher ^{15}N influx rates (more than 2-fold higher) in N-starved plants supplied with NO_3^- compared to NH_4^+ are a strong evidence that *B. brizantha* also has a preference for NO_3^- over NH_4^+ as N source (Kronzucker et al. 1997).

The supply of N as NH_4^+ in the assay solution strongly reduced N influx in *B. brizantha* roots, while the mixed N source promoted the highest influxes of ^{15}N (Table 1). This corroborates the results of this research that the highest N concentrations in leaf tissue were found in the soil-grown plants fertilized with NH_4^++NO_3^- as an N source (Fig. 3a). Studying N source synergism at the cellular level through the use of the technique of compartmental analysis by efflux and the radiotracer ^{13}N to measure cellular turnover kinetics in seedlings roots of rice (*Oryza sativa*), Kronzucker et al. (1999) concluded that frequently observed highest N influx with combined NH_4^+ and NO_3^- forms as N source can be attributed to an up-regulation of NH_4^+ uptake and metabolism by the presence of NO_3^-. Lewis and Chadvick (1983) also reported that barley plants reached their highest N influx rate with an N source based on a mixture of NH_4^+ and NO_3^-. In contrast to presented results, however, those authors noted that NH_4^+ stimulated N root influxes more than NO_3^-. Actually, it is expected that N influxes vary between plant species and the preferred N source (Kronzucker et al. 1997), and the natural predominance of NH_4^+ or NO_3^- ion transporters in their roots (von Wirén et al. 1997; Crawford and Glass 1998; Glass et al. 2001). It is important to emphasize that although the NH_4^+ ion markedly decreased N influx in *B. brizantha*, this effect did not directly impair the soil-grown NH_4^+-fed plants since the N concentration in leaves reflected a sufficient N status (De Bona and Monteiro 2010b). This supported the view that the growth and production inhibition observed in NH_4^+-fed soil-grown plants (Fig. 2) are more associated with NH_4^+ toxicity (Britto and Kronzucker 2002).

In contrast to N influx rates, the ^{34}S influx by S-deficient or S-deprived grass plants showed a dependency on N nutrition in terms of both plant N status and the N form supplied in the nutrient solution (Table 2). Thus, N deficiency decreased the S influx rate and, consequently, reduced the S supply response as measured by S concentrations in the shoot tissue of soil-grown plants (Fig. 3b). Clarkson et al. (1999) studying uptake and assimilation of S-SO_4^{2-} by S-deficient maize (*Zea mays*) cells, found that the response of S-starvation was strongly diminished in cells which had been deprived of an N source for 4 day. Those authors reported that high-N maize cells showed an S-SO_4^{2-} uptake rate twice that of low-N cells. Accelerated protein synthesis in N-sufficient plants may be responsible, at least in part, for the enhanced S influx rate in order to provide S-containing amino acids.

Supplying N nutrition only as NH_4^+ or as both N forms strongly increased the ^{34}S influx capacity of *B. brizantha* plants subjected to long- or short-term S limitation (Table 2). In a classic study, Van Beusichem et al. (1988) reported that the use of NH_4^+ instead of NO_3^- as an N source increased the S-SO_4^{2-} uptake of castor oil plants (*Ricinus communis*) by approximately 47 %. Likewise, Rehm and Caldwell (1970) tested the effect of NH_4^+ and NO_3^--fertilizers on S uptake in maize plants using ^{35}S, and found that supplying an NH_4^+ source greatly increased S-fertilizer absorption. Energy savings related to NH_4^+ assimilation (Crawford et al. 2000) and the maintenance of plant electroneutrality due to cation and anion uptake balance (Van Beusichem et al. 1988) should contribute to increased S influx rates in NH_4^+- and/or NH_4^++NO_3^--fed forage grass plants.

Independent of N status or pre-treatment combination, forage plants reacted to S shortages by increasing ^{34}S influx rates (Table 2). Clarkson et al. (1999) also reported that S-starved maize cells took up S-SO_4^{2-} at 8–10 times the rate of S-sufficient cells. Sulfur influx changes are linked to S-SO_4^{2-} transporters in plant roots (Buchner et al. 2004), which are down-regulated when external S supply is sufficient, but increase in plants during S deficiency (Hawkesford et al. 1993).

From a practical viewpoint, the use of N fertilizers containing both NH_4^+ and NO_3^- forms is strongly recommended for growing *B. brizantha* or similar species in low-S soils, since this practice increases N and S intake potential and thereby enhances plant growth, nutritional value, and dry matter yield. For instance, under drought conditions that can cause transient S limitation even in soils that are not S-limiting when water availability is sufficient, plants fed with a mixed N source are better able to uptake S from the soil and thus avoid, or at least reduce, the deleterious effects of S deprivation. It is important to emphasize that increasing the NO_3^- proportion in the N mixture requires a complementary increase in the S supply in order to avoid the accumulation of the free amino acid asparagine and impaired protein synthesis.

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