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Sulfur limitation increases nitrate and amino acid pools in tropical forages

Fabiana Schmidt[A], *Fabiano D. De Bona*[A,B], *and Francisco A. Monteiro*[A,C]

[A]Soil Science Department, University of São Paulo, Av. Pádua Dias 11, PO Box 9, Piracicaba, SP 13418-900, Brazil.
[B]National Wheat Research Center, Embrapa Trigo, Rodovia BR 285 294 km, PO Box 451, Passo Fundo, RS 99001-970, Brazil.
[C]Corresponding author. Email: famontei@usp.br

Abstract. Increasing the supply of sulfur (S) to forage plants can change their nitrogen (N) metabolism, causing changes in the N : S ratio that can potentially affect forage production and quality. The present study was focussed on revealing how supply (low, intermediate, high) of S affects amino acid composition and concentrations of total S, total N, sulfate-S, nitrate-N, and soluble protein in the leaves of tropical pasture species.

Greenhouse experiments were conducted in ground quartz (inert solid substrate) culture to examine the effect of S supply in two tropical species: *Panicum maximum* cv. Tanzania (Guinea grass) and *Stylosanthes guianensis* cv. Mineirão (stylo). Because legumes have greater S requirement than do grass species, application levels of S varied according to the species. Guinea grass was grown with 0.10, 0.55, 1.00, 1.45, and 1.90 mmol L^{-1} of S, and stylo with 0.10, 0.70, 1.30, 1.90 and 2.50 mmol L^{-1} of S. Plants of both species were harvested on two occasions.

Low S availability (0.10 mmol L^{-1}) caused a nutritional imbalance with N in Guinea grass and stylo plants, as shown by a high N : S ratio (>60 : 1), and high concentrations of nitrate-N and free amino acids in plant tissues. Increased S supply regulated the N : S ratio at values close to 20 : 1, which provided N and S concentrations more suitable for protein synthesis and optimum forage production for both forage species. Asparagine was the predominant amino acid present in S-limited Guinea grass, whereas arginine was more abundant in S-limited stylo. This result indicates that a limitation of S increases nitrate-N and free amino acids while decreasing plant growth rates and soluble protein concentrations in these forage species.

Additional keywords: amino acid composition, N : S ratio, nitrate concentration, sulfur fertilisation, tropical forage species.

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Introduction

Sulfur (S) is essential for plant growth and development. Because the metabolism of S is closely linked to that of nitrogen (N), changes in the N : S ratio can potentially affect the nutritional value of forage species. In plants supplied with adequate nutrient levels, the N : S ratio varies within a narrow range (20 : 1), reflecting the abundance of S in amino acids and proteins (Crawford *et al*. 2000). This close relationship between S and N clearly shows that S should be included in programs of balanced plant nutrition in order to optimise the growth and utilisation of forage resources.

In plants, S is absorbed by the roots as sulfate and transported via the xylem to the leaves, where it is reduced and incorporated into the amino acid cysteine. Cysteine is subsequently converted to methionine and/or proteins. Some plant proteins include S-containing amino acids, and for that reason, the combined availability of N and S plays an important role in protein synthesis and in plant growth and development. Coordination between the metabolic pathways of N and S must ensure sufficient fluxes of these elements in order to satisfy the minimum amino acid requirements for adequate protein synthesis.

Cysteine plays a crucial role in the synthesis of organic S compounds, and cysteine synthesis is the most important reaction that directly links plant N and S metabolism (Brunold *et al*. 2003). In addition to regulating methionine biosynthesis, cysteine is a precursor of glutathione (GSH), phytochelatins and co-factors (iron-S), essential vitamins such as biotin and thiamine, and S esters (coenzyme A) (Droux 2004; Saito 2004). Methionine is an important S-containing essential amino acid that is present in plants in small quantities. This amino acid receives its carbon (C) skeleton from aspartate, while the S portion is derived from cysteine.

Several studies have illustrated the complex regulatory links between N metabolism and S assimilation (Prosser *et al*. 1997; Migge *et al*. 2000; Hawkesford and De Kok 2006). Researchers studying plants subjected to prolonged S limitation have reported changes in the reserves of various metabolites followed by disruptions in the N metabolism, such as the inhibition of protein synthesis, the accumulation of free amino acids such as asparagine, arginine, and glutamine (Migge *et al*. 2000; De Bona *et al*. 2011), and low concentrations of cysteine and methionine in plant tissues (Nikiforova *et al*. 2006). In addition, high N : S

ratios can lead to an accumulation of N in a non-protein form, particularly as nitrate (NO_3^-) and soluble organic N (Haq and Carlson 1993; Prosser *et al*. 1997).

In the case of forage plants, understanding the metabolic and nutritional changes caused by S deficiency is important because suboptimum concentrations of essential amino acids and/or the accumulation of secondary products of the assimilatory pathways of N and S can reduce the nutritional value and dry mass (DM) production of forage. Since different plant species exhibit different pathways for metabolising N and S, the objective of this study was to examine the effect of S supply on N compounds in Guinea grass (*Panicum maximum* cv. Tanzania) and stylo (*Stylosanthes guianensis* cv. Mineirão).

Materials and methods

Growth conditions, treatments, and experimental design

Panicum maximum cv. Tanzania (Guinea grass) and *Stylosanthes guianensis* cv. Mineirão (stylo) plants were grown during summer under greenhouse conditions. The mean temperature was 30°C and photoperiod 16 h/8 h (day/night). Forage species were cultivated in 3.6-L plastic pots containing ground quartz as an inert substrate. Grass and stylo seeds were germinated in trays containing washed sand. Seedlings of both species were transplanted to the plastic pots 15 days after germination. Five grass or five stylo plants were grown in each pot. The experimental units for each species were distributed in complete randomised blocks with four replications.

Because legumes require more S than do grass species (Tallec *et al*. 2008), stylo treatments received greater application levels of S than the Guinea grass treatments. Sulfur was supplied via nutrient solution (Hoagland and Arnon 1950) to simulate a gradient of S supply (low to high) for the plants. The nutrient solution consisted of: (mmol L^{-1}) 1 KH_2PO_4, 5 KCl, 2 KNO_3, 5 $Ca(NO_3)_2$, 9 NH_4NO_3; and (μmol L^{-1}) 46 H_3BO_3, 9 $MnCl_2$, 0.73 $ZnCl_2$, 0.30 $CuCl_2$, 0.11 H_2MoO_4 [H_2O], 100 Fe(III)-EDTA. Five levels of S in the nutrient solution were tested for Guinea grass (0.10, 0.55, 1.00, 1.45, and 1.90 mmol L^{-1}) and for stylo (0.10, 0.70, 1.30, 1.90, and 2.50 mmol L^{-1}). The S in the nutrient solution was supplied as the salt $MgSO_4$. The concentration of magnesium (Mg^{2+}) was adjusted in all of the S rates, using an $MgCl_2$ solution, so that the final concentration of Mg^{2+} was 2 mmol L^{-1}. Ionic strength (1.30 atm) and pH (5.5) of the nutrient solution did not vary significantly across the different S treatments. Initially (5 days after transplanting), plants were grown in a diluted nutrient solution in which nutrient concentrations were 25% of those in the complete solution. After this period, both species were treated with the complete nutrient solution, which was renewed every 14 days. Pots were irrigated daily with deionised water.

Both forage species were harvested twice during the experiment. Guinea grass was harvested when plants started the senescence process in the mature leaves. Thus, the aboveground portion of the grass was harvested 35 days after transplanting (by cutting 5 cm above the root–aboveground transition) (Lavres *et al*. 2008) and subsequently after 30 days of regrowth (by cutting at ground level). In order to simulate natural grazing conditions, stylo was harvested when plants achieved 60 cm in height. Thus, the aboveground portion of the stylo was harvested 50 days after transplanting (by cutting 20 cm above the base of the plant) (Manfredini 2008) and 27 days later (by cutting at ground level). Stylo plants were allowed to grow and develop for 50 days before the first harvest in order to guarantee their regrowth and survival after defoliation.

Diagnostic leaves were the two most recent fully expanded leaf laminae for Guinea grass (Lavres *et al*. 2008) and the three fully expanded trifoliolate leaves closest to the plant apex for stylo (Manfredini 2008). Diagnostic leaves were sampled at both harvests and analysed for amino acid and soluble protein concentrations. Fresh plant material was immediately placed in liquid nitrogen and stored in a freezer at −80°C until analyses were performed. Plant material collected during the harvests was dried in a forced-ventilation oven at 65°C until it reached constant mass, and then was weighed. Dry diagnostic leaf samples were ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ) for subsequent determination of total S, total N, nitrate-N, and sulfate-S concentrations.

Total N, total S, nitrate-N, and sulfate-S concentrations in plant tissues

Total N and S concentrations in the diagnostic leaves were determined following methods described by Nelson and Sommers (1973) and Tabatabai (1982), respectively. The N : S ratio of diagnostic leaves was calculated as the ratio of total N and total S concentrations.

Quantification of sulfate-S concentrations in the diagnostic leaves of Guinea grass and stylo followed a modified protocol of Sinclair (1974). Briefly, 1 g of dry matter was placed in an Erlenmeyer flask and 25 mL of extractant solution containing glacial acetic acid, hydrochloric acid, and orthophosphoric acid was added. After shaking for 30 min, the contents of the flask were filtered and 2 mL of the extract was pipetted into a tube. The tube was placed in an S distiller, adapted from Johnson and Nishita (1952), and 4 mL of reducing solution based on hydroiodic, hypophosphorous, and formic acids was added. A tube was placed at the other end of the distiller with 40 mL of deionised water plus 10 mL of a receptor solution based on sodium acetate and zinc acetate [NaOAc–$Zn(OAc)_2$]. The extract was distilled for 1 h at 100°C in a heating block, where the sulfate-S it contained was converted to sulfide, which was collected in the receptor solution. The receptor solution containing the sulfide was then transferred to a 100-mL volumetric flask, with 10 mL of *p*-aminodimethylaniline + 2 mL of ferrous sulfate. The volume was completed with deionised water. After the mixture was left at rest for 20 min, sulfate-S was read in a spectrophotometer at a wavelength of 670 nm.

Nitrate-N concentrations were determined following a protocol described by Tedesco *et al*. (1995), using 0.1 g of dry material and 10 mL of extractant solution of KCl (1 mol L^{-1}). The extract was filtered and distilled in a Kjeldahl micro distiller that was modified as described by Tedesco and Gianello (1979).

Free amino acids in plant tissues

The extracts used to determine amino acids in the plants were derived from previously collected fresh plant material that had been stored in a freezer at −80°C. Material was macerated with a mortar and liquid N_2 with an added 10 mmol L^{-1} solution of CH_2O_2. Amino acids were determined via pre-column derivatisation with o-phthaldialdehyde and quantification by high-performance liquid chromatography (Jones *et al*. 1981).

Soluble proteins in plant tissues

The extracts used to determine soluble proteins were prepared with 1 g of fresh plant matter frozen at −80°C. Plant matter was ground with a ceramic mortar and pestle with liquid N_2 and insoluble PVPP (polyvinylpolypyrrolidone) added at 20% (w/w) of the sample. The extraction buffer used was potassium phosphate (100 mmol L^{-1}, pH 7.5) supplemented with EDTA disodium salt (1 mmol L^{-1}) and dithiothreitol (3 mmol L^{-1}). Extracts were centrifuged at 10 000*G* for 30 min at 4°C.

The concentration of soluble protein in the extracts was determined in triplicate with the protocol of Bradford (1976), using albumin from bovine serum (Sigma Chemical Co., St. Louis, MO) as a standard. Results were expressed as amounts of soluble protein accumulated in plants by multiplying the concentration of soluble protein (mg g^{-1} fresh weight) by the total fresh weight of the plant (g $plant^{-1}$).

Statistical analyses

Statistical analysis was carried out with SAS version 9.01 statistical software (SAS Institute 2004). The treatment factor in this study was S doses in the nutrient solution. Analysis of variance was performed for each forage species and harvest time separated. When *F*-tests revealed significant differences between S doses, linear, quadratic, and exponential regressions were carried out using the GLM (General Linear Model) command. Regression equations are reported with significance levels of the coefficients. The effect of S on the concentrations of free amino acids was assessed by comparing means via Tukey's test at a significance level of $P = 0.05$.

Results

Dry mass production and concentrations of total S, sulfate-S, total N, and nitrate-N in plant tissues

Aboveground DM production of Guinea grass increased to a maximum at an interpolated nutrient-solution S value of 1.26 mmol L^{-1} then declined at higher solution-S levels (Fig. 1*a*). At the first harvest, aboveground DM production of stylo increased linearly with increasing S availability (Fig. 1*b*), whereas at the second harvest, S levels >0.70 mmol L^{-1} were sufficient to yield maximum aboveground DM production.

Concentrations of total S (Fig. 2*a*, *b*) and sulfate-S (Fig. 3*a*, *b*) in the diagnostic leaves of both Guinea grass and stylo collected in the two harvests increased significantly with increasing S. At the first and second harvests, respectively, recently expanded leaves of Guinea grass supplied with S at 1.90 mmol L^{-1} had 58% and 68% higher concentrations of total S than plants supplied with 0.10 mmol L^{-1} (Fig. 2*a*). In general, total S concentrations in the diagnostic leaves of Guinea grass were highest at the end of the second growth period. Total S concentrations in the diagnostic leaves of stylo from the first harvest reached their maximum value at S levels of ~0.70 mmol L^{-1} in the nutrient solution (Fig. 2*b*). Higher levels of nutrient-solution S did not result in increased diagnostic leaf S concentrations in stylo collected at the first harvest. However, in diagnostic leaves collected at the second harvest, concentrations of total S showed a linear increase with increasing levels of nutrient-solution S.

In Guinea grass plants grown with S at 0.10 mmol L^{-1}, just 2% of total S accumulated as sulfate-S at both harvests. However, in plants grown with S at 1.90 mmol L^{-1}, sulfate-S accounted for 4% and 12% of total S in the first and second harvests, respectively. In stylo plants grown with S at 0.10 mmol L^{-1}, sulfate-S accounted for just 1% and 3% of total S in the first and second harvests, respectively. In stylo plants grown with S at 2.50 mmol L^{-1}, the percentage of sulfate-S in the first and second harvests was 27 and 23%.

Total N concentrations in the diagnostic leaves of Guinea grass was higher in plants grown with S at 0.10 mmol L^{-1} (i.e.

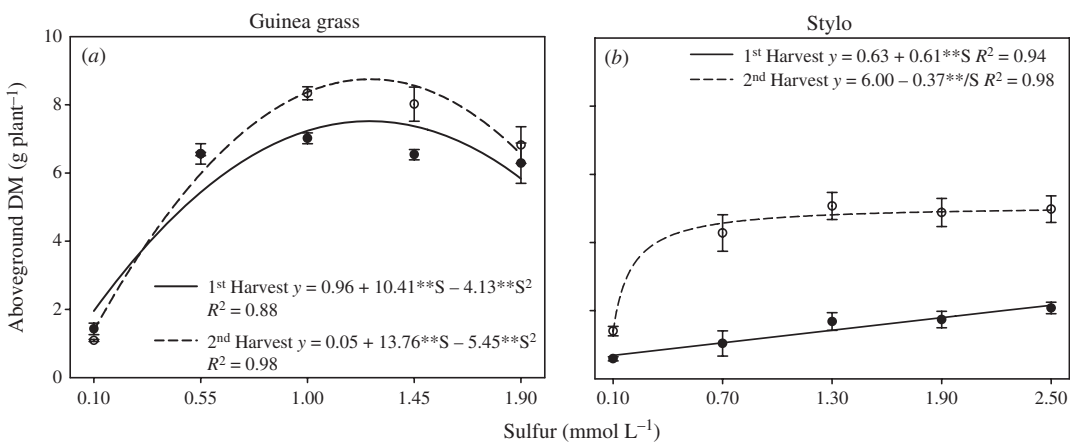

Fig. 1. Aboveground dry mass (DM) production in the first and second harvests of (*a*) Guinea grass and (*b*) stylo as related to sulfur supply in nutrient solution. Capped vertical lines are ± mean standard error ($n = 4$). Regression equations are reported with significance level of the coefficients; $^{**}P < 0.01$.

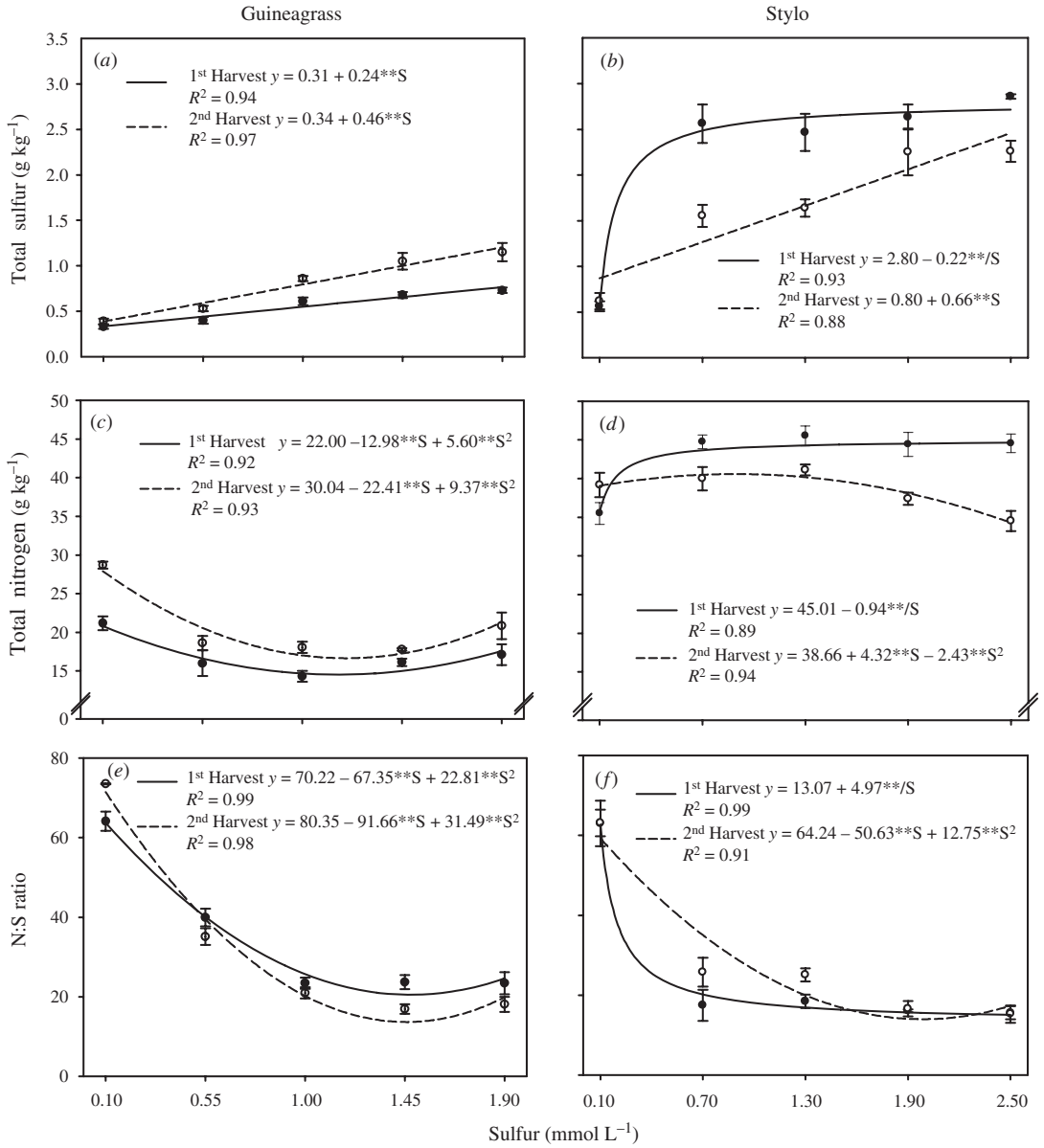

Fig. 2. Concentrations of total (*a*, *b*) sulfur and (*c*, *d*) nitrogen, and (*e*, *f*) N : S ratio in diagnostic leaves of Guinea grass and stylo sampled in the first and second harvests as related to sulfur supply. Capped vertical lines are ± mean standard error ($n = 4$). Regression equations are reported with significance level of the coefficients; $^{**}P < 0.01$.

S-deficient plants) than in plants grown at higher doses of S, for both harvests (Fig. 2*c*). The previously noted greater growth and DM production in Guinea grass with increasing S supply (up to 1.26 mmol L^{-1}; see Fig. 1*a*) led to a dilution of N, which was reflected in lower N concentrations in diagnostic leaves. This effect of N dilution due to the greater growth of plants supplied with higher rates of S was also observed in stylo at the second harvest (Fig. 2*d*). At the first harvest of stylo, total N concentration increased with nutrient-solution S level up to ~0.70 mmol L^{-1} and then remained unaltered with increasing S supply.

Nitrogen metabolism was affected by S supply in both forage species. Increased S supply resulted in significant reduction in nitrate-N concentrations (Fig. 3*c*, *d*). Indeed, nitrate-N concentrations in S-deficient Guinea grass plants were six and three times higher at the first and second harvests, respectively, than those in plants supplied with sufficient S (1.00 mmol L^{-1}). This suggests that reduction of nitrate-N may have been limited by low S availability to plants. Sulfur-deficient stylo accumulated approximately twice as much nitrate-N as adequately S-supplied plants (1.30 mmol L^{-1}) in both harvests.

Guinea grass grown at limiting doses of S (0.10 mmol L^{-1}) showed an N : S ratio of ~60 : 1 at the first harvest and ~70 : 1 at the second (Fig. 2*e*). Similarly, stylo grown under S limitation showed an N : S ratio of ~60 : 1 at both harvests (Fig. 2*f*). Regardless of the growth period, S supply of 1.00–1.90 mmol L^{-1} for Guinea grass and 1.30–2.50 mmol L^{-1}

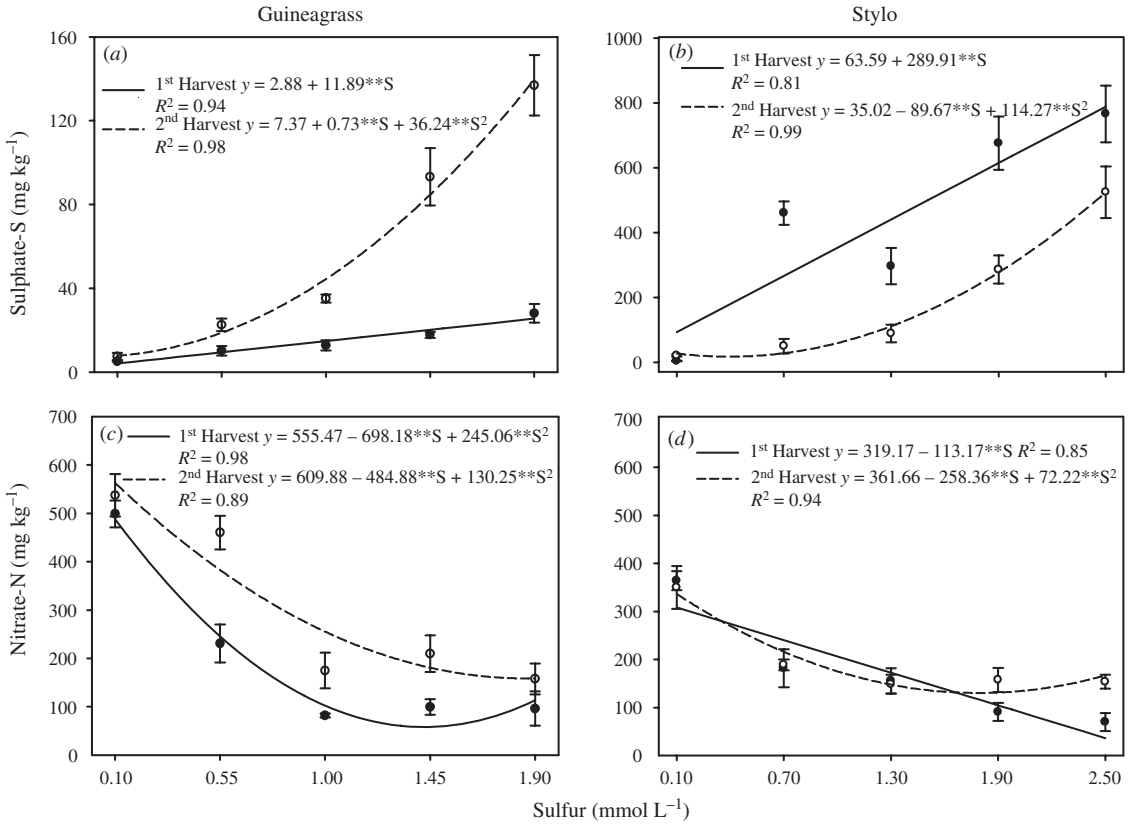

Fig. 3. Concentrations of (*a*, *b*) sulfate-S and (*c*, *d*) nitrate-N in diagnostic leaves of Guinea grass and stylo sampled in the first and second harvests as related to sulfur supply. Capped vertical lines are ± mean standard error ($n = 4$). Regression equations are reported with significance level of the coefficients; $^{**}P < 0.01$.

for stylo reduced the N : S ratio to ~20 : 1 in diagnostic leaves (Fig. 2*e*, *f*). Results also indicated that N : S ratios ~20 : 1 were associated with maximum production of aboveground DM in both Guinea grass and stylo plants (Fig. 4).

Concentrations and compositional distribution of free amino acids

Diagnostic leaves of Guinea grass and stylo plants grown under S limitation (0.10 mmol L^{-1}) contained greater concentrations of total free amino acids than those grown with adequate or excessive levels of S (Fig. 5*a*, *b*). Total free amino acid concentrations of Guinea grass plants grown with S at 0.10 mmol L^{-1} were ~80 and 90% higher at the first and second harvests, respectively, than those of plants supplied with higher S levels. Total soluble amino acid concentrations of stylo plants grown with S at 0.10 mmol L^{-1} were ~85% higher than those of plants grown at other S levels, for both harvests. Changes in free amino acid concentrations due to S limitation were accompanied by an increase in asparagine and arginine concentrations in the diagnostic leaves of Guinea grass and stylo, respectively (Table 1).

Detailed analysis of the primary free essential amino acids in the diagnostic leaf tissue of Guinea grass revealed that asparagine concentrations were ~90 times higher in plants grown with S at 0.10 mmol L^{-1} than in those grown with S at 1.90 mmol L^{-1}. Sulfur limitation also resulted in an increase in the concentrations of alanine, glutamine, glycine, proline, serine, threonine, and aspartic acid in diagnostic leaves of Guinea grass (Table 1). Increased S supply resulted in increased concentrations of methionine and cysteine, which contain the element. In the diagnostic leaves of S-deficient Guinea grass, the most abundant amino acids in decreasing order of concentration were asparagine > serine > aspartic acid > alanine > glycine > arginine > glutamic acid > glutamine. When S supply was increased to 1.90 mmol L^{-1}, concentrations of total amino acids decreased (Fig. 5*a*) and the most abundant amino acids in decreasing order of concentration were arginine > glutamic acid > methionine > tyrosine > valine > asparagine > leucine > phenylalanine (Table 1). Concentrations of S-containing amino acids (methionine and cysteine) in Guinea grass leaves increased dramatically with adequate (1.00 mmol L^{-1}) and excessive (1.90 mmol L^{-1}) S supply. Methionine concentrations were 88 and 95% higher in Guinea grass grown with adequate and excessive levels of S, respectively, than in S-deficient plants. Cysteine concentrations in Guinea grass tissue were 45 and 60% higher in plants with adequate and excessive levels of S, respectively, than in S-deficient plants.

Diagnostic leaves of S-deficient stylo plants (0.10 mmol L^{-1}) had arginine concentrations ~30 times higher than those of

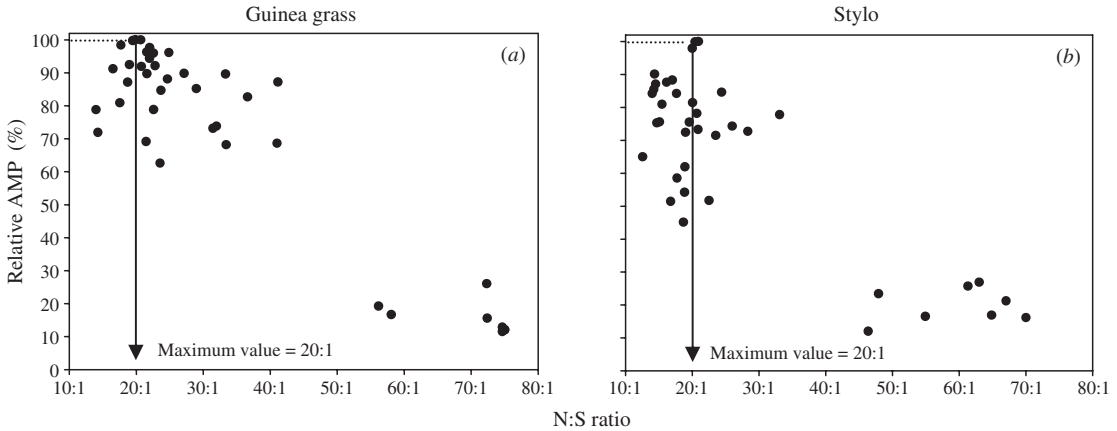

Fig. 4. Ratio of nitrogen to sulfur in the diagnostic leaves of (*a*) Guinea grass and (*b*) stylo associated with relative aboveground mass production (AMP). Results correspond to the values of the variables of all sulfur rates supplied in the two periods of plant growth ($n = 40$).

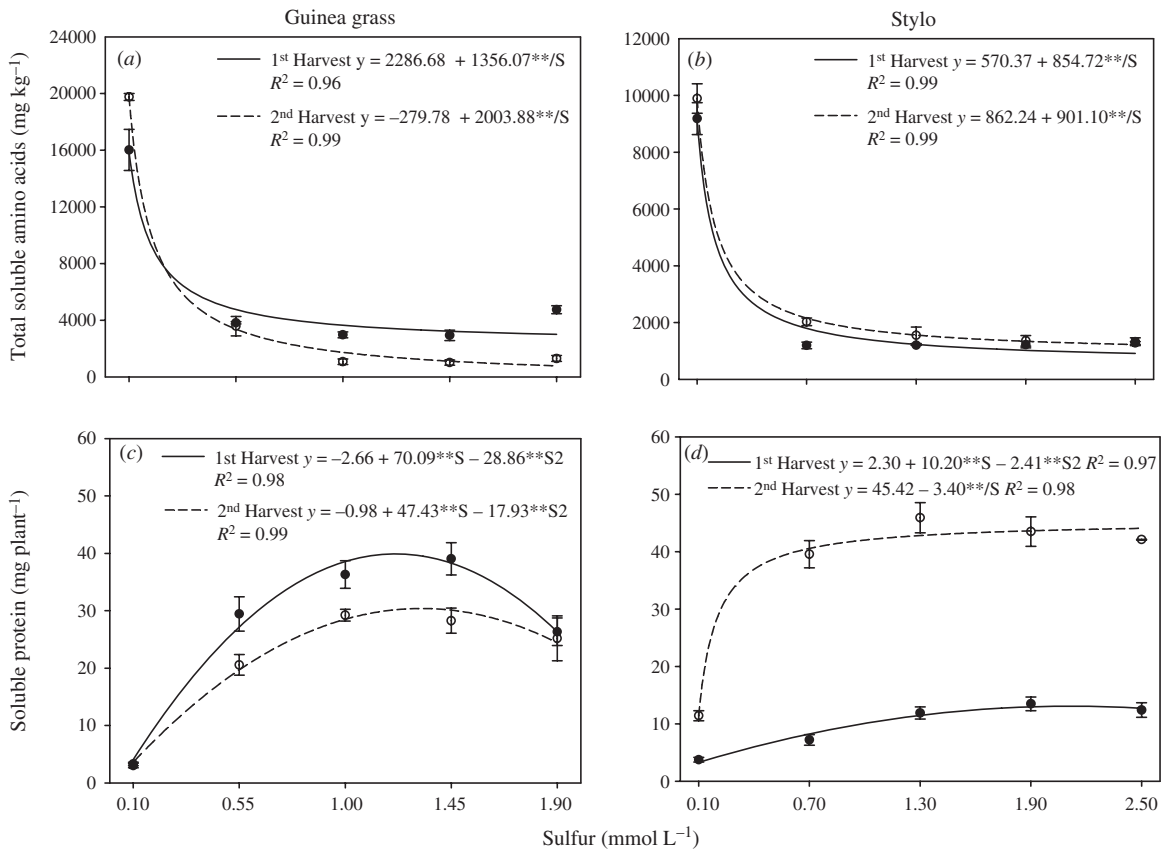

Fig. 5. (*a*, *b*) Total free amino acids and (*c*, *d*) soluble proteins in the diagnostic leaves of Guinea grass and stylo in the first and second harvests in response to sulfur rates. Capped vertical lines are ± mean standard error ($n = 4$). Regression equations are reported with significance level of the coefficients; $^{**}P < 0.01$.

plants grown with the highest S rate (2.50 mmol L^{-1}). Sulfur limitation also increased the concentrations of asparagine, histidine, isoleucine, lysine, threonine, and tyrosine in the diagnostic leaves of stylo (Table 1). In the diagnostic leaves of S-deficient stylo, the most abundant free amino acids in decreasing order of concentration were arginine > asparagine > glutamic acid > lysine > threonine > histidine. When S supply was increased to 2.50 mmol L^{-1}, concentrations of total free

Table 1. Concentrations (mg kg^{-1} fresh weight) of soluble amino acids in diagnostic leaves of Guinea grass and stylo grown under low (0.10 mmol L^{-1}), intermediate (1.00 and 1.30 mmol L^{-1}), and high (1.90 and 2.50 mmol L^{-1}) sulfur supply
Values represent the mean of two harvests since there was no significant difference between the harvests ($n = 8$). Within a row for each species, means followed by the same letter are not significantly different (Tukey's test at $P = 0.05$)

Amino acid	Guinea grass			Stylo		
	Low	Intermediate	High	Low	Intermediate	High
Aspartic acid	188.2a	103.1c	138.7b	92.6b	128.1a	126.5a
Glutamic acid	141.1c	243.9b	370.6a	206.0b	313.2a	331.8a
Alanine	185.3a	96.0b	97.4b	47.2b	81.8a	93.3a
Arginine	153.0c	404.1b	613.6a	8307.1a	383.7b	283.7b
Asparagine	16 209.0a	230.5b	178.3c	258.4a	97.4 b	77.1b
Cysteine	29.5c	53.3b	74.3a	19.8b	35.8a	10.8c
Glutamine	118.5a	54.8b	62.6b	30.7b	41.1a	36.5a
Glycine	161.2a	18.2c	34.0b	12.4a	9.3a	12.0a
Histidine	36.3b	26.1c	40.8a	100.6a	20.0b	25.0b
Isoleucine	16.9c	43.3b	75.5a	30.7a	9.9b	12.3b
Leucine	34.6c	87.1b	152.0a	16.8b	17.6b	24.5a
Lysine	30.7a	13.6b	24.4a	133.6a	16.1b	18.4b
Methionine	16.1c	131.2b	319.0a	14.6a	15.3a	7.1b
Phenylalanine	29.0c	66.3b	148.5a	12.7a	7.3b	11.8a
Proline	80.1a	42.1b	96.8a	35.3a	34.2a	45.0a
Serine	209.0a	89.8b	85.0b	64.3a	54.1a	57.2a
Threonine	76.3a	26.0b	34.6b	36.5a	14.6c	23.2b
Tyrosine	86.3c	169.6b	280.8a	102.3a	83.2b	86.6b
Valine	88.2c	119.2b	229.9a	11.8b	11.9b	17.3a
Total	17 889.3a	2018.2b	3056.8b	9533.4a	1374.6b	1300.1b

amino acids in the diagnostic leaves of the legume were lower (Fig. 5*b*) and their ranking altered. The three most abundant amino acids were glutamic acid > arginine > aspartic acid. Concentrations of the S-containing amino acids cysteine and methionine were low, even in plants grown with high doses of S (2.50 mmol L^{-1}), which shows a weak response of these compounds to S supply in this species.

Accumulated soluble protein

Soluble protein accumulation increased up to a certain level of S supply then decreased or remained constant in both species and at both harvests (Fig. 5*c*, *d*). The highest soluble protein concentrations of Guinea grass were reached at intermediate S levels of 1.21 and 1.32 mmol L^{-1} at the first and second harvests, respectively (Fig. 5*c*). Accumulated soluble protein in stylo at the first harvest reached its highest value at nutrient-solution S levels of 2.12 mmol L^{-1}. At the second harvest, the accumulation of soluble protein in the aboveground portion of stylo was approximately four times higher than at the first harvest and reached its highest constant value at nutrient-solution S levels of ~0.70 mmol L^{-1} (Fig. 5*d*).

Discussion

Although S is much less abundant than N in plant tissues, adequate supply of S is critical for the growth and development of higher plants, due to its role in regulating N metabolism (Dubousset *et al*. 2009; Kaur *et al*. 2011). This study provided evidence of the impact of S-limitation on the processes of N assimilation and metabolism, as well as incorporation of N into proteins, in Guinea grass and stylo plants. The S-deficient Guinea grass and stylo plants also showed a high N : S ratio in leaf tissue (60 : 1 and 70 : 1) and an accumulation of nitrate and free amino acids, reflecting a metabolic imbalance within the plants (Dijkshoorn and Van Wijk 1967). The increase in non-assimilated N (nitrate-N) and free amino acids is attributed to the partial interruption of the metabolic pathways of N assimilation and incorporation, which is illustrated by the low concentration of soluble protein and reduced production of DM of Guinea grass and stylo plants grown at suboptimal levels of S.

The large increase of accumulated nitrate-N in grass and stylo plants grown with low S supply corroborates results reported for other S-deficient plant species, such as maize (*Zea mays*, Friedrich and Schrader 1978), tobacco (*Nicotiana tabacum*, Migge *et al*. 2000), spinach (*Spinacea oleracea*, Prosser *et al*. 2001), and barley (*Hordeum vulgare*, De Bona *et al*. 2011). Low supply of S to plants compromises the activity of the nitrate reductase enzyme, causing nitrate-N to accumulate in plant tissues (Migge *et al*. 2000; De Bona *et al*. 2011). According to Migge *et al*. (2000), expression and activity of nitrate reductase in S-limited plants may be suppressed by the accumulation of amino acids in plant tissues. It is worth emphasising that high nitrate-N concentrations in forage plants can significantly reduce nutritional value, since an animal diet with high nitrate-N concentration (>0.7–1.0% nitrate-N) (Case 1957; Osweiler *et al*. 1985) can cause poisoning and a condition known as methemoglobinemia (Wright and Davison 1964).

Sulfur limitation increased the concentrations of soluble amino acids in Guinea grass and stylo tissues. In stylo much of this increase was accounted for by the amino acid arginine, while in Guinea grass it mostly involved asparagine. Asparagine and arginine are important N compounds that store and

transport soluble N in plants (Lea 1993). Increased asparagine and arginine concentrations in S-deficient plants may reflect a physiological and metabolic strategy to allocate excess reduced N (e.g. that derived from the reduction of nitrate-N, which cannot be used for protein biosynthesis) from the primary metabolism to leaves, in an attempt to maintain an adequate N : S ratio in plant tissues during periods of S deficiency.

The specific amino acid that accumulates most abundantly in S-deficient plants appears to vary with species. For example, studies of the temperate species perennial ryegrass (*Lolium perenne*) and barley have reported that S limitation led to increased concentrations of asparagine and aspartic acid (Cowling and Bristow 1979; Karmoker *et al*. 1991). By contrast, species such as tobacco, spinach, and beet (*Beta vulgaris*) grown in a substrate with low levels of available S showed striking increases in the concentration of arginine (Migge *et al*. 2000; Thomas *et al*. 2000; Prosser *et al*. 2001). Nikiforova *et al*. (2006), studying *Arabidopsis* plants in an S-deficient growth medium, reported increased concentrations of the amino acids serine and O-acetylserine.

It appears that the fluxes of N and carbon (C) that cannot be metabolised in the biosynthesis of S-containing amino acids due to S limitation are redirected through different physiological and biochemical pathways for compound synthesis, depending on the plant species. The higher concentration of asparagine in Guinea grass suggests that S deficiency in that species mostly affects the pathways of methionine and aspartate synthesis, which are linked by a complex regulatory network involving negative feedback relationships (Azevedo *et al*. 1997). Since S limitation in Guinea grass decreased concentrations of the amino acid cysteine, it seems reasonable to suppose that the conversion of cysteine and O-phosphohomoserine to cystathionine, in the biosynthesis of methionine, was also compromised. It is noteworthy that methionine concentration decreased in plants with low levels of available S. Migge *et al*. (2000) argued that increased levels of O-phosphohomoserine may have a negative effect in the initial stages of methionine synthesis, especially in the phosphorylation of aspartate. Aspartate that is not used in methionine biosynthesis may thus be converted to asparagine, which would explain the striking increase in the concentration of that amino acid in plant tissues.

In the case of the forage legume stylo, which contains a higher concentration of N than grasses, low levels of available S increased arginine synthesis through the urea cycle, in which the donor of C skeletons, analogous to oxaloacetate in the tricarboxylic acid cycle, is ornithine. It is possible that the higher concentration of arginine represents an intermediate step in the formation of the metabolite putrescine, which commonly accumulates in S-deficient higher plants (Nikiforova *et al*. 2005).

Increasing the supply of S to Guinea grass and stylo plants gradually corrected the imbalance in the N : S ratio in plant tissues, resulting in increased DM production and increased nutritional value of the forage, as shown by increased concentrations of sulfate-S, total S, S-containing amino acids, and soluble protein. Total S concentrations in diagnostic leaves responded strongly to added S in the legume, which indicates greater need for the element in the legume than in the grass. An N : S ratio of ~20 : 1 in the diagnostic leaves of Guinea grass and stylo collected at the first and second harvests was associated with maximum aboveground DM production (Fig. 4). Dijkshoorn and Van Wijk (1967) showed that, upon a plant reaching maturity, the N : S ratio tends to stabilise at 14 : 1 in grasses and at 17 : 1 in legumes. Monteiro *et al*. (1983) cultivated the forage legume siratro (*Macroptilium atropurpureum*) in an Entisol from the Cerrado biome treated with five rates of gypsum and observed the highest DM production, N concentrations, and nodular mass in plants with an S concentration of 1.7 g kg^{-1} in tissues and an N : S ratio of 20 : 1. However, plants grown in that soil without any added S exhibited an S concentration of 0.7 g kg^{-1} and an N : S ratio of 40 : 1. For the legume white clover (*Trifolium repens*), Monteiro (1986) obtained N : S ratios of 16 : 1 and 18 : 1 for plants cultivated in a Spodosol fertilised with S.

The nutritional balance of N and S in plants with adequate levels of S may be indicated by a decrease in free amino acids and an increase in S-containing amino acids (cysteine and methionine). The increased concentration of S-containing amino acids was accompanied by increased protein synthesis in the leaves of both forage species. In addition to a scarcity of the S-containing amino acids cysteine and methionine, the process of proteolysis strongly hinders protein formation and accumulation in S-deficient plants (Kaur *et al*. 2011). In general, stylo plants showed lower concentrations of cysteine and methionine than Guinea grass plants. The low concentrations of S-containing amino acids and their weak response to added S in stylo may be related to the rate of metabolism of these amino acids in protein synthesis (Crawford *et al*. 2000), which varies between species. Hirai *et al*. (2003), studying *Arabidopsis* plants, observed no changes in cysteine and methionine concentrations with varying levels of S.

Concentrations of cysteine and methionine in forage plants are extremely important and beneficial for animal diets (Amir and Hacham 2008). Unlike animals, plants can assimilate inorganic S as sulfate and reduce it to sulfite in the synthesis of S-containing amino acids (Leustek *et al*. 2000). As a result, forage plants represent one of the main sources of the S-containing amino acids that are an essential part of ruminant diets.

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