



N and S concentration and stoichiometry in soybean during vegetative growth: Dynamics of indices for diagnosing the S status

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ABSTRACT

Information regarding the pattern of sulphur (S) accumulation in shoots is scarce for field crops and few comprehensive analysis were performed on N:S stoichiometry. Particularly, the need to study the patterns of uptake, allocation and stoichiometry of S and N in soybean is two-fold. First, the main areas for soybean production in the world have been recently reported as S-deficient. Second, S concentration (%S) and N:S ratios are relevant for diagnosing S deficiency.

The aim of this work was to analyze, in a gradient of S availability, the dynamics of nitrogen concentration (%N), %S and N:S ratio in soybean lamina, stem and shoots during vegetative growth. Experiments were performed at Balcarce, Argentina during two growing seasons. Two soybean cultivars were evaluated: DM2200 (maturity group II) and DM4970 (maturity group IV), sown in optimum dates for the region (mid-November). DM2200 was also sown late (early January). We sampled crops between biomass $\approx 1 \text{ Mg ha}^{-1}$ and R5. Shoots were separated in lamina, stem and petiole, and pod.

The adjusted S dilution curve for S-sufficient treatments was attenuated in soybean ($S = 2.8 W^{-0.11}$), with no differences among cultivars of maturity groups II and IV, and sowing dates from November to January. For the same treatments, the N:S ratio was stable in shoots during vegetative growth (N:S = 12.2), supporting the use of a unique threshold for diagnosing the S status during this period. Sulphur concentration in stems was more responsive to the availability of S than %S in lamina. In addition, the concentration of N in stem tended to be constant with variation in %S while %N and %S correlated in lamina. This produced a greater response of N:S in stem than in lamina. Thus, the determination of %S and N:S in stem are good candidates as indicators of S status of soybean.

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1. Introduction

The pattern of nitrogen (N) accumulation and allocation in plants has been evaluated for several major crops. Despite some discrepancies, the concentration of N (%N) in shoots decreases during the growth cycle in dense canopies as the result of two processes: (i) the remobilization of N from shaded leaves at the bottom of the canopy to leaves at the top, assuming that N uptake rate is lower than crop growth rate and (ii) the increase in the proportion of plant structural tissues with low nitrogen concentration in comparison with metabolic tissues with higher %N (Lemaire and Gastal, 2009).

However, Divito et al. (2016) have shown that the N dilution curve during vegetative growth is much more attenuated in

soybean [*Glycine max* (L.) Merrill] than in other crops. In an environment where the phenological extremes span from maturity groups (MG) II to IV, the flat dilution curve resulted from the constant %N in lamina and stem and the early onset of pod formation.

Information regarding the pattern of sulphur (S) accumulation in shoots is scarce for field crops and few comprehensive analysis were performed on the N:S stoichiometry. Ciampitti et al. (2013) showed tight associations between N and S concentration in maize during the entire growing season, with a 1.14 faster dilution rate for N than for S. This coincides with the determinations of Vong et al. (2007) in oilseed rape and barley and Reussi Calvo et al. (2011) in wheat. Bender et al. (2015) recently described the patterns of nutrient accumulation in modern soybean cultivars, including N and S. However, this analysis focused on nutrient requirements for high yielding production and did not explicitly account for the stoichiometry of these nutrients.

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The main areas for soybean production in the world have been recently reported as S-deficient (Salvagiotti et al., 2012; Lucheta and Lambais, 2012) and S concentration (%S) and N:S ratio are relevant for diagnosing S deficiency (Salvagiotti et al., 2012; Kaiser and Kim, 2013; Divito et al., 2015). Consequently, it is necessary to study the patterns of uptake, allocation and stoichiometry of S and N in soybean.

We previously studied the dynamic of N dilution in soybean crops well supplied with sulphur (Divito et al., 2016). We also reported a good performance of %S and N:S for diagnosing the S status of soybean, based on information from 15 trials (Divito et al., 2015). However, data from these experiments precluded the analysis of the dynamics of %S and N:S during the vegetative growth. Thus, here we analyze data from two experiments previously presented in Divito et al. (2016) including S-deficient and S-sufficient crops. Our objective was to analyze, in a gradient of S availability, the dynamics of %N, %S and N:S ratio in soybean lamina, stem and shoots during vegetative growth.

2. Materials and methods

2.1. Crop husbandry, treatments and experimental design

The study was carried out at INTA Research Station, Balcarce, Argentina (37.5° S, 58.2° W, 130 m above sea level) during two growing seasons: 2010/11 (Se1) and 2012/13 (Se2). Rainfed crops were established under no-till on a fine, mixed, thermic Typic Argiudoll with a minimum effective depth of 1.5 m. Top-soil (0–0.2 m) pH was 6.5 (Se1) and 5.9 (Se2), soil organic matter was 56.0 (Se1) and 44.0 g kg⁻¹ (Se2) and P-Bray was 28.3 (Se1) and 26.8 mg kg⁻¹ (Se2). Soil N-NO₃⁻ in the 0–60 cm depth was 12.4 and 56.0 kg N ha⁻¹ for Se1 and Se2, respectively and soil S-SO₄⁻² in the same depth was 6.9 and 32.9 kg S ha⁻¹ for Se1 and Se2, respectively.

Experimental sources of variation were seasons, S rates, cultivars, and sowing dates. Gypsum (SO₄Ca₂H₂O, 16% S, 20% Ca) was broadcasted at crop emergence at rates of 0, 10 and 40 kg S ha⁻¹ (treatments S0, S10 and S40, respectively). Two indeterminate soybean cultivars were evaluated: DM2200 (MG II) and DM4970 (MG IV). These cultivars represent the phenological extremes for the region. Both cultivars were sown in optimum dates for the region (November 20th and 10th for Se1 and Se2, respectively). DM2200 was also sown on January 10th in both seasons. This combination allows the study of the effect of MG and sowing date on the patterns of biomass, N and S accumulation and partitioning. Seasons and combination of cultivars and sowing dates aimed at generating a wide range of growing conditions rather than assess the effects of individual sources of variation and their interactions. Hereafter, DM4970 and DM2200 sown in November are called G_{IV}-Nov and G_{II}-Nov, respectively and DM2200 sown in January is called G_{II}-Jan. Both are transgenic cultivars (glyphosate tolerant). The experimental design was a randomized complete blocks with three replicates. The experimental unit was 12 × 5 m. Further information about crop husbandry can be found in Divito et al. (2016).

2.2. Measurements

2.2.1. Phenology, growth, nitrogen and sulphur

Crop phenology was monitored every three days using the scale of Fehr and Caviness (1977).

The theory underlying the N dilution curve is restricted to the window between shoot biomass ($W \approx 1 \text{ Mg ha}^{-1}$) and the end of the vegetative period (Greenwood et al., 1990; Lemaire and Gastal, 1997). For $W < 1 \text{ Mg ha}^{-1}$ crops behave as isolated plants (Lemaire et al., 2007a,b) and during reproductive growth seeds conform an extra compartment in addition to metabolic and structural tissues

and translocation processes take place (Sadras and Lemaire, 2014). These concepts also apply for S dilution. We thus sampled crops between $W \approx 1 \text{ Mg ha}^{-1}$ and R5, when grain weight was negligible. Initial sampling coincided with the V6 stage for G_{IV}-Nov and R1 for G_{II}-Nov and G_{II}-Jan. Samples were also taken at R1, R3 and R5 for G_{IV}-Nov and at R3 and R5 for G_{II}-Nov and G_{II}-Jan.

At each sampling, shoots were collected from central rows in a 0.7 m² area and were separated into stem and petiole (hereafter called stem), lamina and pods. Samples were oven dried at 65 °C until a constant weight was reached, weighed, and ground (0.5 mm mesh). Nitrogen and S content in the samples were determined by dry combustion at 950 °C and 1350 °C, respectively and thermo-conductivity detection with a TruSpec CNS analyzer (LECO, St. Joseph, MI, USA).

2.2.2. Complementary measurements: soil water content

Soil water content (SWC) was estimated using the locally-calibrated model by Della Maggiora et al. (2002). Further information about the model can be found in Divito et al. (2016). Weather data were obtained from a weather station located 500 m from the experimental site.

2.3. Calculations

The decline in plant %N and %S was described using the negative power function proposed by Lemaire and Salette (1984) for N:

$$\%N \text{ or } \%S = aW^{-b} \quad (1)$$

The coefficient a represents the %N or %S at $W = 1 \text{ Mg ha}^{-1}$. Coefficient b is dimensionless and represents the ratio between the relative decline in %N or %S and the relative crop growth rate.

The S dilution curve of S-sufficient treatments was calculated following the procedure proposed by Justes et al. (1994). For each combination of cultivar and sowing date, this method considers the minimum %S observed at a given date among all S treatments that produced the maximum amount of W up to that date. It corresponds to a S at which W does not significantly increase with increasing S rate despite the increase in %S.

2.4. Statistical analysis

ANOVA was used to assess the effect of experimental sources of variation, i.e. S rate, variety, sowing date, and phenological stage on response variables using SAS PROC MIXED (SAS Institute, 1988). Least-squares mean tests were performed when treatment effects were significant (P value < 0.05).

Intercept and parallelism of the patterns of %N and N:S with %S were compared. All the equations were linearized by In-transformation for the study of N and S patterns (Niklas, 2006). Parameters of the equations were calculated from transformed data using model II of SAS PROC REG (SAS Institute, 1988) as proposed by Niklas (2006). Intercept and parallelism among variety-sowing date treatments were also compared from transformed data using ANOVA with SAS PROC MIXED (SAS Institute, 1988). All analyses considered a repeated measure model to incorporate the correlations for the errors arising from measurements on the same experimental unit through sampling dates.

3. Results

3.1. Growing conditions

Mean seasonal (November–April) solar radiation was 6.7% and 3.6% higher than the long-term median for Se1 and Se2, respectively (Fig. 1A). Photoperiod reaches a maximum in the summer solstice,

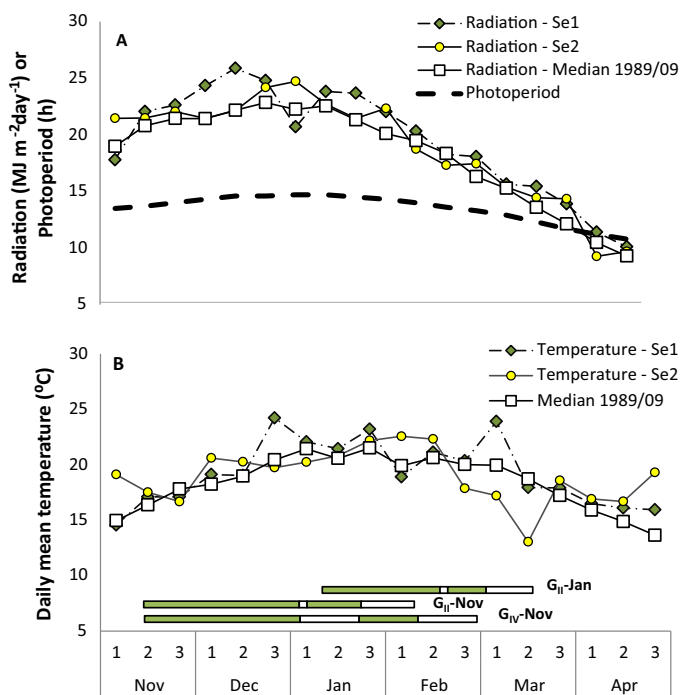


Fig. 1. Solar radiation and photoperiod (A) and daily mean temperature (B). Colored bars at the bottom of panel B indicate the periods between sowing-V6, V6-R1, R1-R3 and R3-R5 respectively (average of two seasons). Data from season 1 (Se1), season 2 (Se2) and median (1989/2009).

and falls 0.1, 0.6, 1.8 and 3.5 h in January, February, March and April, respectively (Fig. 1A). Mean seasonal temperatures were slightly higher than historical in both experimental seasons. During Se2, however, temperatures from end of February to mid-March were cooler than the long-term median (Fig. 1B).

During season 1, the soil water content of treatments G_{IV} -Nov and G_{II} -Nov was above the critical threshold of plant available water ($=0.6$) from crop emergence to V4, but it fluctuated between this threshold and 0.4 PAW during most of the period under study (Fig. 2A and B). During season 2, SWC was slightly below threshold from R3 for G_{IV} -Nov and from R5 for G_{II} -Nov. G_{II} -Jan did not suffer severe water deficit during the period under study, with exception of Se2, where SWC was slightly under the critical threshold during the vegetative period (Fig. 2C). Further information can be found in Divito et al. (2016).

3.2. Development, growth and dry matter allocation

The duration of the period emergence-R1 decreased following G_{IV} -Nov > G_{II} -Nov > G_{II} -Jan (Fig. 1B). The length of the R1-R5 period was similar between G_{IV} -Nov and G_{II} -Nov but it was shorter in G_{II} -Jan (Fig. 1B). On average for each cultivar-sowing date combination G_{IV} -Nov produced more shoot biomass than G_{II} -Nov and G_{II} -Jan when comparisons were done at the same phenological stage ($P < 0.01$). Differences between G_{II} -Nov and G_{II} -Jan were also significant ($P < 0.01$) but smaller.

Sulphur fertilization increased shoot biomass at R5 in 3 out of 6 combinations of treatments and season ($P < 0.05$). Responses ranged from 0.87 to 1.31 Mg ha⁻¹ (Table 1).

3.3. N and S concentration in plant parts

Nitrogen concentration in lamina and stem was constant among S rates at all crop stages for each cultivar-sowing date combination (Table 1). On average for each combination, the %N in lamina was constant as soybean developed in G_{II} -Nov in season 2 and G_{II} -Jan in

both seasons (Table 1). Contrarily, %N in lamina was lower ($P > 0.01$) at V6 than at R1, R3 and R5 in G_{IV} -Nov at both seasons and also at R1 than at R3 and R5 in G_{II} -Nov in season 1 (Table 1). %N in stem was constant with growing crops in five combinations and decreased in the first season for G_{II} -Nov (Table 1). %N in pods was higher at R5 than at R3 in all cultivar-sowing date combinations during season 1 and G_{IV} -Nov during season 2 whereas similar in G_{II} -Nov and G_{II} -Jan (Se2) with no differences among S rates (Table 1).

Sulphur concentration in lamina was constant with increasing S rates in most of the cultivar-sowing date combinations (Table 1). By contrast, stems %S increased with S fertilization in G_{IV} -Nov and G_{II} -Nov during both seasons (Table 1) and most phenological stages. Similar to %N, G_{IV} -Nov had a lower %S in lamina at V6 than at R1, R3 and R5 in both seasons (Table 1) and G_{II} -Nov had a lower %S in lamina at R1 than at R3 and R5 in season 1. Sulphur concentration in stems was constant with growing crops in most combinations and decreased in the second season for G_{IV} -Nov. Remarkably, the concentration of S in stems was more responsive to S rate than %S in lamina (Table 1). Sulphur concentration in pods was higher at R5 than at R3 in G_{IV} and G_{II} sown in November and similar between stages in G_{II} -Jan with no differences among S rates (Table 1).

The N:S ratio in lamina was similar among cultivar-sowing dates and S rates and slightly increased with increasing biomass ($P > 0.01$). The N:S was constant (14.1) ($P = 0.31$) for treatments with the minimum %S required to maximize shoot biomass (Fig. 3A). The N:S ratio in stem increased with increasing biomass in S0 treatments and decreased in S40. It was constant in S10. The critical threshold was 12.5 (Fig. 3B). N:S in stems was more responsive to S availability than in lamina.

The concentration of N in lamina increased with increasing %S ($P < 0.01$) (Fig. 4A). The pattern was similar among S rates ($P > 0.20$). Stem %N increased with %S in the non S-fertilized treatment but was less responsive than lamina %N ($P < 0.01$). Interestingly, stem %N was unresponsive to %S in S10 and S40 treatments (Fig. 4A). Thus, concomitant variation of %N and %S in lamina caused low variation of the N:S ratio with variable %S (Fig. 4B). In contrast, the decrease of N:S in stem was more pronounced as %S increased (Fig. 4B). These patterns were also evident in the low dispersion of N:S in lamina in comparison with N:S in stem (Fig. 3A and B). For each S rate, variations in %N, %S and N:S shown in Fig. 4 can be attributed to season, cultivar, sowing date and phenological stage at sampling (Table 1).

3.4. N and S dilution curves

Nitrogen dilution curves were attenuated and similar ($P > 0.47$) among S rates in all the combinations of cultivar and sowing date (Fig. 5A). Moreover, there were no differences among cultivar-sowing dates in the pattern of %N with increasing W ($P > 0.13$), so a unique function is proposed for treatments with the minimum %S required to maximize shoot biomass ($\%N = 35.3 W^{-0.08}$).

The concentration of S in shoot decreased with increasing biomass in the non S-fertilized treatments in all cultivar-sowing date combinations. The pattern of treatments S10 and S40 were similar to S0 in G_{II} -Nov ($P > 0.31$). Contrarily, %S in shoot was constant with increasing biomass in crops fertilized with 10 and 40 kg S ha⁻¹ for G_{IV} -Nov and G_{II} -Jan. Considering all cultivar-sowing date combinations the dilution curve of treatments S10 and S40 were similar ($P = 0.28$) and attenuated in comparison to the more pronounced dilution of S for treatment S0. The %S dilution curve of S-sufficient treatments was unique for all cultivar-sowing date combinations: $2.8 W^{-0.11}$ ($P < 0.01$) (Fig. 5B) and was parallel to the N dilution curve ($P = 0.28$). Parallelism between curves resulted in a constant N:S ratio (12.2) for S-sufficient treatments during the vegetative growth of soybean ($P = 0.12$) (Fig. 5C).

Table 1

Shoot mass (W), nitrogen (N) and sulphur (S) in lamina, stem and pod for cultivar DM4970 (MG IV) sown in November (GIV-Nov), DM2200 (MG II) sown in November (GII-Nov) and DM2200 sown in January (GII-Jan) in Season 1 and Season 2 under three rates of S fertilization. For each cultivar-sowing date (C-SD) and season combination different letters indicate differences between S rates (lower case letters) and phenological stages (PS) (capital letters) at P < 0.05 using Tukey.

C-SD	PS	S rate (kg ha ⁻¹)	W (Mg ha ⁻¹)		N (g kg ⁻¹)			S (g kg ⁻¹)								
					lamina	stem	pod	lamina	stem	pod						
Season 1																
GIV-Nov	V6	0	1.15	a C	41.0	b B	18.3	a A	3.01	a B	1.51	a A				
		10	1.23	a D	43.6	a B	18.0	a A	3.44	a B	1.60	a A				
		40	1.16	a D	42.6	a B	18.4	a A	3.36	a B	1.69	a B				
	R1	0	2.88	a B	52.0	b A	17.0	a A	3.82	a A	1.52	a A				
		10	2.87	a C	52.8	a A	18.4	a A	4.12	a AB	2.01	a A				
		40	3.06	a C	53.4	a A	17.5	a A	4.00	a AB	2.19	a AB				
	R3	0	4.73	a A	54.6	b A	15.9	a A	30.2	a B	3.73	a AB	1.56	b A	1.97	a B
		10	4.72	a B	54.8	a A	16.4	a A	30.0	a B	3.89	a AB	1.91	ab A	1.87	a B
		40	4.64	a B	56.4	a A	16.4	a A	30.4	a B	4.03	a AB	2.41	a A	1.97	a B
	R5	0	5.78	b A	56.3	b A	19.1	a A	38.1	a A	3.70	a AB	1.13	b B	2.50	a A
		10	6.72	a A	59.0	a A	19.6	a A	39.7	a A	4.32	a A	1.70	ab A	2.47	a A
		40	6.14	a A	58.6	a A	19.7	a A	38.6	a A	4.16	a A	1.97	a AB	2.53	a A
GII-Nov	R1	0	1.29	a B	47.1	a B	22.2	a A	3.49	a B	1.56	a A				
		10	1.26	a C	47.7	a B	24.1	a A	4.08	a B	1.60	a A				
		40	1.14	a C	45.3	a B	20.4	a A	3.50	a B	1.49	a B				
	R3	0	3.79	a A	58.3	a A	22.0	a A	38.0	a A	4.08	a BB	1.78	b A	2.65	a A
		10	3.86	a B	59.2	a A	21.9	a A	38.0	a B	4.59	a A	1.75	b A	2.33	a B
		40	3.74	a B	58.8	a A	21.5	a A	38.3	a B	4.15	a A	2.29	a A	2.65	a B
	R5	0	5.16	a A	55.7	a A	17.7	a B	42.8	a A	4.44	a A	1.58	ab A	2.99	a A
		10	5.31	a A	59.1	a A	18.1	a B	43.9	a A	4.67	a A	1.48	b A	2.70	a A
		40	5.45	a A	56.5	a A	16.5	a B	44.8	a A	4.24	a A	1.77	a AB	3.10	a A
	GII-Jan	R1	0	2.24	a B	56.8	a A	19.7	a A	4.52	a A	1.40	a A			
			10	2.26	a B	58.2	a A	20.1	a A	4.58	a A	1.81	a A			
			40	2.23	a B	58.1	a A	20.3	a A	4.49	a A	1.90	a A			
R3		0	2.61	a AB	57.4	a A	18.2	a A	34.7	a B	3.91	a A	1.49	a A	2.17	a A
		10	2.81	a AB	59.3	a A	21.4	a A	38.4	a B	4.71	a A	1.67	a A	2.09	a A
		40	2.84	a AB	59.6	a A	20.4	a A	38.9	a B	4.25	a A	1.96	a A	2.19	a A
R5		0	3.39	a A	55.5	a A	21.1	a A	41.6	a A	4.30	a A	1.26	a A	2.46	a A
		10	3.49	a A	55.6	a A	20.8	a A	42.9	a A	4.52	a A	1.27	a A	2.40	a A
		40	3.83	a A	55.0	a A	20.8	a A	41.6	a A	4.80	a A	1.73	a A	2.57	a A
Season 2																
GIV-Nov		V6	0	1.66	a D	38.6	b B	14.9	a A	2.72	a B	1.70	b A			
			10	1.61	a D	37.8	a B	15.9	a A	2.87	a B	2.08	ab A			
	40		1.59	a D	38.1	a B	15.7	a A	3.07	a A	2.20	a AB				
	R1	0	3.55	a C	48.8	b A	16.5	a A	3.06	a A	1.43	b AB				
		10	3.50	a C	51.0	a A	17.6	a A	3.53	a A	1.80	ab A				
		40	3.24	a C	50.5	a A	17.8	a A	3.53	a A	2.33	a A				
	R3	0	4.81	a B	45.6	b AB	14.7	a A	27.8	a B	3.08	a A	1.19	b B	1.58	a B
		10	4.97	a B	49.2	a A	16.5	a A	27.2	a B	3.33	a AB	1.67	a A	1.57	a B
		40	4.64	a B	48.6	a A	15.6	a A	29.6	a B	3.39	a A	1.79	a BC	1.77	a B
	R5	0	6.11	b A	44.0	b AB	15.3	a A	35.3	a A	2.77	b A	1.01	b B	2.00	a A
		10	7.42	a A	51.2	a A	14.9	a A	35.9	a A	3.55	a A	1.09	b B	2.07	a A
		40	7.14	a A	51.4	a A	15.4	a A	36.3	a A	3.56	a A	1.60	a C	2.27	a A
GII-Nov	R1	0	1.57	a B	40.4	a B	15.1	a A	2.74	b B	1.31	b A				
		10	1.56	a B	45.7	a A	16.6	a A	3.27	ab A	1.59	ab A				
		40	1.45	a C	46.5	a A	16.8	a A	3.43	a A	1.88	a A				
	R3	0	3.67	a AB	50.1	a A	17.7	a A	33.8	a A	3.04	a AB	1.23	b A	2.67	a A
		10	4.15	a AB	48.0	a A	17.5	a A	32.7	a A	2.98	a A	1.46	ab A	2.60	a B
		40	3.74	a B	47.9	a A	17.4	a A	31.2	a A	3.09	a A	1.69	a A	2.56	a B
	R5	0	3.97	b A	48.9	a A	15.4	a A	38.0	a A	3.35	a A	0.90	b A	3.10	a A
		10	4.43	ab A	49.0	a A	15.7	a A	37.6	a A	3.42	a A	1.26	ab A	3.19	a A
		40	4.84	a A	49.4	a A	16.0	a A	36.9	a A	3.60	a A	1.48	a A	3.14	a A
	GII-Jan	R1	0	1.81	a B	50.8	a A	19.8	a A	3.72	a A	1.77	a A			
			10	1.81	a B	48.7	a A	19.5	a A	3.50	a A	1.40	a A			
			40	1.90	a B	49.4	a A	19.2	a A	3.72	a A	1.65	a A			
R3		0	3.17	a A	50.1	a A	19.1	a A	42.0	a A	3.56	a A	1.38	a A	2.63	a A
		10	2.78	a B	49.2	a A	18.4	a A	41.8	a A	3.51	a A	1.30	a A	2.64	a A
		40	2.99	a A	47.6	a A	18.4	a A	39.3	a A	3.59	a A	1.51	a A	2.59	a B
R5		0	4.19	a A	48.3	a A	20.2	a A	47.1	a A	3.34	a A	1.38	a A	2.96	a A
		10	3.99	a A	47.5	a A	19.3	a A	48.1	a A	3.49	a A	1.40	a A	3.04	a A
		40	3.86	a A	46.6	a A	18.7	a A	47.9	a A	3.35	a A	1.71	a A	3.05	a A
ANOVA																
Season 1																
C-SD				<0.001	0.0347	0.0192	0.0018	<0.0001	0.0345	0.0021						
PS			<.001	<0.0001	0.0077	<0.0001	<0.0001	<0.0001	<0.0001							
S rate			0.212	0.0156	0.2779	0.9555	<0.0001	<0.0001	0.0042							
C-SD * PS			<0.001	<0.0001	<0.0001	0.0621	<0.0001	0.0231	0.0955							
C-SD * S rate			0.083	0.0315	0.8394	0.8939	0.0453	0.0008	0.2386							
PS * S rate			0.030	0.6746	0.666	0.7144	0.9014	0.0021	0.7278							
C-SD * PS * S rate			0.074	0.6623	0.7742	0.9982	0.1039	0.6652	0.9917							

Table 1 (Continued)

C-SD	PS	S rate (kg ha ⁻¹)	W (Mg ha ⁻¹)	N (g kg ⁻¹)			S (g kg ⁻¹)		
				lamina	stem	pod	lamina	stem	pod
Season 2									
C-SD			<0.001	0.4225	0.0392	0.0002	0.0051	0.355	<0.0001
PS			<0.001	<0.0001	0.111	<0.0001	<0.0001	<0.0001	<0.0001
S rate			0.011	0.6127	0.7007	0.4829	<0.0001	<0.0001	0.4877
C-SD * PS			<0.001	<0.0001	0.0014	0.1318	<0.0001	<0.0001	0.655
C-SD * S rate			0.010	0.0096	0.1597	0.1816	0.0002	<0.0001	0.1053
PS * S rate			0.004	0.1191	0.9686	0.707	0.0961	0.3324	0.5214
C-SD * PS * S rate			0.001	0.1264	0.6549	0.9942	0.0343	0.0215	0.9975

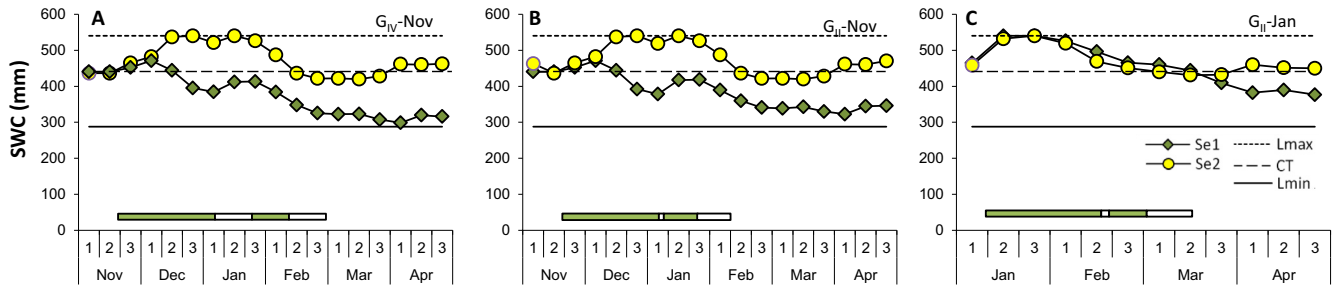


Fig. 2. Simulated soil water content (SWC) for cultivar DM4970 (MG IV) sown in November (G_{IV}-Nov) (A), DM2200 (MG II) sown in November (G_{II}-Nov) (B) and DM2200 sown in January (G_{II}-Jan) (C) in season 1 (Se1) and season 2 (Se2). Horizontal lines are upper (L_{max}) and lower limits (L_{min}) (Della Maggiora et al., 2000) and the limit for physiological water stress CT (Sadras and Milroy, 1996). Colored bars at the bottom of each panel indicate the periods between sowing-V6, V6-R1, R1-R3 and R3-R5, respectively (average of two seasons).

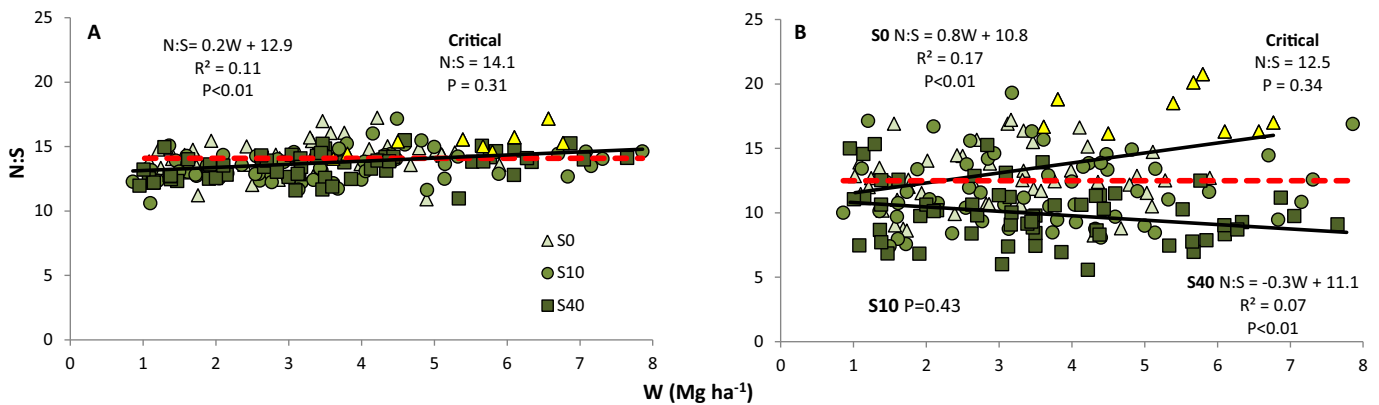


Fig. 3. N:S ratio in lamina (A) and stem (B) as a function of shoot biomass (W). Data from cultivar DM4970 (MG IV) sown in November (G_{IV}-Nov), DM2200 (MG II) sown in November (G_{II}-Nov) and DM2200 sown in January (G_{II}-Jan) in seasons 1 and 2 under three rates of S fertilization: 0 (S0), 10 (S10) and 40 (S40) kg S ha⁻¹. P indicates significance of regression. Yellow triangles indicate S deficient crops. Red dashed line represents the N:S function adjusted for treatment with the minimum shoot S concentration required to maximize shoot biomass. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

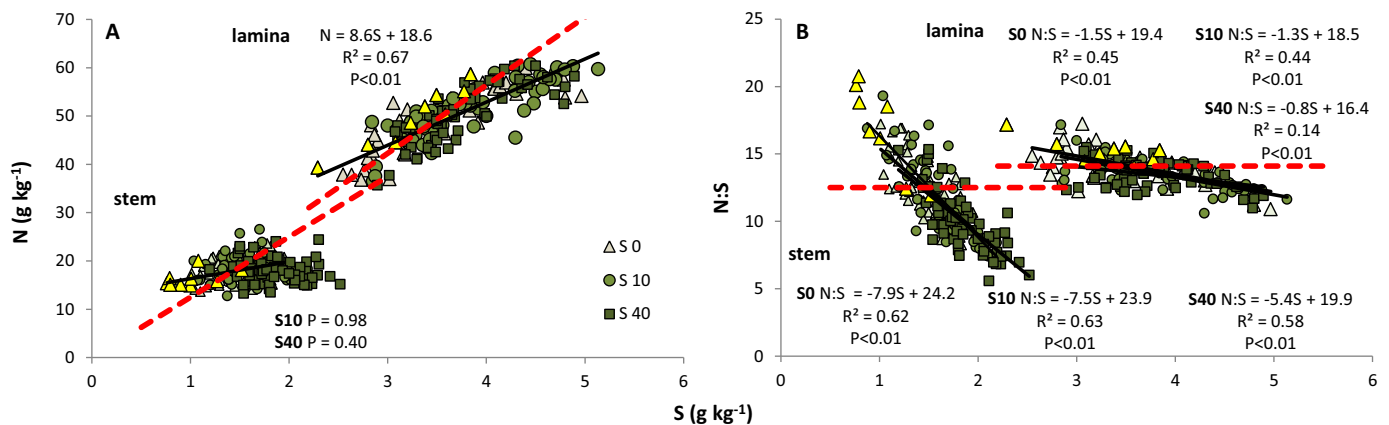


Fig. 4. N concentration (A) and N:S ratio (B) as a function of S concentration in lamina and stem. Data from cultivar DM4970 (MG IV) sown in November (G_{IV}-Nov), DM2200 (MG II) sown in November (G_{II}-Nov) and DM2200 sown in January (G_{II}-Jan) in seasons 1 and 2 under three rates of S fertilization: 0 (S0), 10 (S10) and 40 (S40) kg S ha⁻¹. P indicates significance of regression. Yellow triangles indicate S deficient crops. Red dashed line represents the N:S adjusted for treatment with the minimum shoot S concentration required to maximize shoot biomass. (14.1 for lamina and 12.5 for stem). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

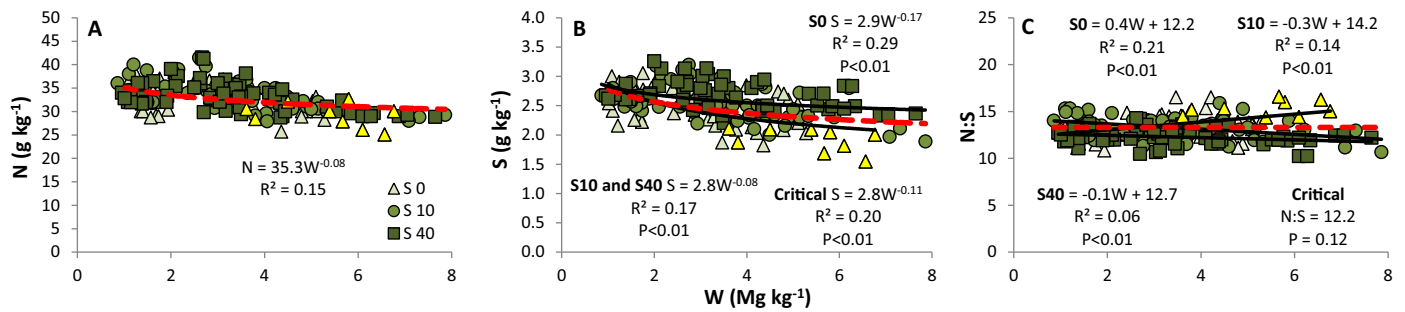


Fig. 5. Shoot nitrogen (N) concentration (A), sulphur (S) concentration (B) and N:S ratio (C) as a function of shoot biomass (W). Data from cultivar DM4970 (MG IV) sown in November (G_{IV} -Nov), DM2200 (MG II) sown in November (G_{II} -Nov) and DM2200 sown in January (G_{II} -Jan) in seasons 1 and 2 under three rates of S fertilization: 0 (S0), 10 (S10) and 40 (S40) kg S ha⁻¹. P indicates significance of regression. Yellow triangles indicate S deficient crops. Red dashed line represents the N, S and N:S functions adjusted for treatment with the minimum shoot S concentration required to maximize shoot biomass. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Growth and development

During season 1, G_{IV} -Nov and G_{II} -Nov suffered moderate water deficiencies (Fig. 2). These did not significantly affect crop growth as shoot biomass of each cultivar-sowing date was similar between seasons.

Greater shoot biomass of G_{IV} -Nov compared to G_{II} -Nov and G_{II} -Jan at the same phenological stage was consequence of the longer duration of the emergence-R1 period (Fig. 1B) (Divito et al., 2016). Differences in biomass accumulation between G_{II} -Nov and G_{II} -Jan were smaller. The longer emergence-R1 period in G_{IV} -Nov compared to G_{II} -Nov was related to the greater developmental rate of lower MG varieties when exposed to the same photoperiod (Setiyono et al., 2007). However, the shorter period of G_{II} -Jan was mainly associated with higher temperatures (Fig. 1B) (Ball et al., 2000). Divito et al. (2016) further discussed differences in shoot growth among cultivars and sowing dates.

Shoot biomass responses to S fertilization were low and evident in 3 out of 6 cultivar-sowing date-season combinations (Table 1). This is in accordance with previous investigations performed in the Southern Pampas, which indicate that the relative high organic matter content of soils (≈ 40 – 55 g kg⁻¹) attenuate S deficiencies (Reussi Calvo et al., 2011; Pagani and Echeverría, 2011). Responses to S fertilization are more frequent in this region when soybean is planted in a double crop following a winter cereal (Divito et al., 2015) or oilseed rape as a consequence of the removal of available SO₄⁻² from the soil by the preceding winter crop and the absence of a fallow period for S mineralization to restore soluble S in soil.

4.2. N and S concentration in plant parts

The lower %N and %S in lamina at V6 in comparison with R1 for G_{IV} -Nov was unexpected. In our previous work we proposed that the use of glyphosate for weed control at early soybean stages may have caused a temporary N deficiency (Divito et al., 2016). Lower nodulation and N fixation activity (King et al., 2001) and soybean chlorophyll content (Reddy et al., 2000; Reddy and Zablutowicz, 2003) were reported as a consequence of glyphosate applications at early stages. However, the similar pattern observed for %S and %N between V6 and R1 do not support this hypothesis and provide evidence that other mechanisms are involved. Interestingly, lower %S in lamina at V6 was also observed in S-fertilized treatments, so we dismissed that this pattern is caused by a temporary nutrient deficiency.

Constancy of %N in lamina with variations in S rate may be related to the mild S deficiency of crops in these experiments.

Accordingly, we previously determined that N concentration in leaves from the uppermost developed nodes were unresponsive to S in trials with low or no seed yield response to S fertilization (Divito et al., 2015). By contrast, we observed increases in leaf %N when S was applied to crops with pronounced deficiencies.

The low response of %S and N:S in lamina partially contradicts the good performance we reported for leaf %S and N:S for diagnosis of S deficiencies in soybean (Divito et al., 2015). However, in the former investigation leaf (with petiole) was sampled from the uppermost fully developed nodes which are more likely to be S-deficient given the low remobilization of S from bottom to top leaves (Anderson, 1996; Hitsuda et al., 2004).

Greater responsiveness to S addition of %S in stems than in lamina is in agreement with reports by Sexton et al. (1998) in pot and field grown soybean.

4.3. Stoichiometry between nitrogen and sulphur

N and S vary concomitantly because both nutrients are mainly related to protein content in plants (Schnug, 1997). Interestingly, concomitant variations were determined in all S rates in lamina, which indicates that both nutrients were in similar forms under the gradient of S supply. In stems, however, %N and %S were related only in S0, but not in S10 and S40, indicating that the excess of S in stems was allocated to alternative, non-protein structures.

Various physiological mechanisms support the observed patterns of %N with variations in %S in plant parts. When S becomes limiting for vegetative growth a slight decrease of S-containing amino acids in proteins is observed which generally leads to the accumulation of non-S-containing amino acids and O-acetyls erine (Prosser et al., 2001; Nikiforova et al., 2003; Saito, 2004). When S deficiency increases, total protein content is reduced because the amino acid composition is genetically determined (Schnug, 1997). This decrease in total protein involves diminutions in N content, which supports the concomitant variations of %N and %S in lamina (all S rates) and stem (treatment S0). Contrarily, the excess of S is accumulated as nonprotein amino acid S-methylcysteine (Randall and Wrigley, 1986), sulfolipids (Benning, 1998; Harwood and Okanenko, 2003; Frentzen, 2004) and sulfate (Randall and Wrigley, 1986). Particularly, Sexton et al. (1998) determined that excess of S in soybean was accumulated in stems as sulfate, which agree with the high responsiveness of %S to S rate (Table 1) and the independence of %N and %S in stems (treatment S10 and S40) (Fig. 4B).

Interestingly, concomitant variation of %N and %S in lamina caused low differences in the N:S ratio among S rates (Fig. 3A). By contrast, independence of the patterns of both nutrients in stem caused greater differences in the N:S ratio (Fig. 3B). This is in

agreement with Sexton et al. (1998), who documented that stems accumulated sulfate and showed wider variability in N:S ratio in response to S availability than other plant parts.

4.4. N and S dilution in shoot

The parameters of the N dilution curve adjusted for treatment with the minimum %S required to maximize shoot biomass ($\%N = 35.1 W^{-0.07}$) is similar to that we previously documented ($\%N = 37.0 W^{-0.08}$) (Divito et al., 2016). Interestingly, the N dilution curve is much more attenuated in soybean than in other crops (Lemaire and Gastal, 2007). We proposed that early pod onset and the constant %N in lamina and stem between R1 and R5 attenuated the N dilution curve (Divito et al., 2016). This is in line with Ney et al. (1997), who indicated that early flowering in legumes attenuated the N dilution.

As far as we know, there are no other published studies regarding the dilution of N in shoot under different S rates. The inverse comparison in maize i.e. S dilution curves for different N rates, showed steeper S dilutions in crops fertilized with increasing N rates (Ciampitti et al., 2013). A similar effect was expected for the N dilution curves of crops under increasing S supply based on the following two processes: i) S-sufficient crops reach each phenological stage with greater biomass accumulation than S-deficient crops, which implies younger plants with higher lamina:stem ratio at a given W in the former crops, and (ii) the enhancement of crop growth rate by S availability increases *de facto* the N uptake capacity of the crop (Sadras and Lemaire, 2014). For soybean, we demonstrated that the ratio lamina:stem has a low effect on the N dilution curve (Divito et al., 2016) so changes in the N dilution curve would occur if S fertilization increases W and N uptake rate increases proportionally. However, this was not observed in our experiments probably because of a low response of shoot growth to S fertilization.

Sulphur concentration in shoot also showed a low depletion with increasing biomass in the non-fertilized crops (Fig. 5B). A similar pattern was calculated for the S dilution curve of S-sufficient treatments. Of interest, the increase in shoot %S caused by S fertilization resulted in an absence of S dilution as the crop developed (Fig. 5B). This was observed in G_{IV} -Nov and G_{II} -Jan. The causes of the attenuated S dilution curve were similar to the previously exposed for N (early pod onset and constant %S in lamina and stem with growing crop). These results are in accordance with those published by Bender et al. (2015). From their data we calculated a low depletion of %N and %S from V7 to R5 (36.9–30.9 for %N and 2.6–2.1 g kg⁻¹ for %S).

Remarkably, when W was lower than $\approx 3 \text{ Mg ha}^{-1}$, the %S threshold calculated from the dilution curve was above the thresholds we define with data from 15 experiments and crops sampled between R1 and R3 (confidence interval 1.8–2.3 g S kg⁻¹) (Divito et al., 2015). This highlights that sampling date introduces variation to %S that must be considered for S diagnosis.

The parallelism between N and S dilution curves did not coincide with the faster dilution rate of %N reported for wheat, oilseed rape and maize by Reussi Calvo et al. (2011), Vong et al. (2007) and Ciampitti et al. (2013), respectively. However, our results were similar to those reported by Bender et al. (2015) in soybean. Calculations from their paper showed that N:S slightly increased from 14.1 to 15.0 from V7 to R5. This indicates that the slopes of the N and S dilution curves were similar. The threshold we propose for the period of vegetative growth (12.2) is between the confidence interval we reported previously from 15 experiments (10.6–13.6).

4.2. Implications for S diagnosis and concluding remarks

We previously defined a critical threshold for %S in shoots, for crops sampled between R1 and R3 without considering above-ground biomass at sampling (Divito et al., 2015). The results presented in the current paper demonstrate that, although the S dilution curve is attenuated in soybean, sampling date introduces variation to %S. Thus, is it imperative to consider W when using %S for S diagnosis.

In contrast, the stability of N:S ratio in shoots during vegetative growth (N:S = 12.2) supports the use of a unique threshold for diagnosing the S status during this period which is two-fold. First, it increases diagnosis accuracy. Second, it avoids the consideration of W, which is time consuming and is also an important source of variation.

Finally, the high responsiveness of %S and N:S in stem to S level makes these variables good candidates for diagnosing the S status of soybean. However, it must be considered that the supply of S in our experiments covered a range from moderate S deficiency to excess S. A better evaluation of the performance of %S and N:S in stems as indexes of the S status should involve more severe S deficiency.

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