Identifying sulfur deficient fields by using sulfur content; N:S ratio and nutrient stoichiometric relationships in soybean seeds

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ABSTRACT

Sulfur (S) fertilization has increasingly become an important issue in crop management. In Argentina S deficiencies have been reported, but there has been no success in finding soil tests for predicting soybean response to S fertilization. The objectives of this study were to: (i) evaluate seed S and N:S ratio as a tool for identifying S deficient soybean crops in field conditions and (ii) determine shifts in stoichiometric relationships among nitrogen (N), phosphorus (P) and S in soybean seeds which can be used for identifying S responsive sites under field conditions. Seed samples from 20 field experiments designed to explore response to S fertilization in soybean were collected in a large area in the Argentinean Pampas region. Seed yield (SY) and P (%P), S (%S) and N (%N) concentration in seeds were determined. Different data analyses were tested in order to find an S deficiency index. Moderate SY responses to S fertilization were observed in the present study (i.e. relative SY was never below 75%). seed S concentration increased in response to S fertilization in most responsive sites, and N:S ratio was governed by variations in seed S concentration. Stoichiometric relationships were more accurate for identifying S responsive sites than using single variables (e.g. seed S concentration or N:S). The isometric variations among N, P and S suggest that the accumulation of these nutrients is proportional in seed tissue. A significant increase in the intercept of the stoichiometric relationship between N and S in S deficient crops was observed. An S deficient index (SDI) was calculated based on this difference that can be used as a diagnostic tool for identifying S responsive sites in soybean. The present study shows a novel approach for using stoichiometric concepts in fertilizer management in soybean.

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1. Introduction

Sulfur (S), an essential nutrient for plants, is a constituent of key enzymes in plant metabolism and part of essential amino acids in seed storage proteins (Losak et al., 2010; Sexton et al., 1998c; Takahashi et al., 2011). Sulfur fertilization has increasingly become an important issue in crop management, especially after the reduction of atmospheric deposition due to control in S release from industry and the use of low S grade fertilizers (Scherer, 2001). Among plants used as crops, those from the *Brassicaceae* and *Leguminoseae* family appear as the most demanding of sulfur (Marschner, 1995). Soybean, the main crop in the Argentinean Pampas was historically cultivated without fertilizer addition; hence, crop nutrient demand relied only on the natural fertility of soils (Lavado and Taboada, 2009). Since nitrogen (N) is provided via biological fixation, reported seed yield reductions in soybean associated with nutrient constraints are mainly related with phosphorus (P) or S deficiency, which many times appeared simultaneously (Ferraris et al., 2002; Salvagiotti et al., 2004).

Best fertilizer practices involve the use of soil and/or plant tests capable of identifying sites that are more likely to respond to fertilizer addition. Soil tests developed for recognizing sites responsive

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to P fertilization has been successfully developed in the Pampas region, but there has been no success in finding soil tests for predicting soybean response to S fertilization. A preliminary analysis in the area (Vilche et al., 2002) showed that response to S fertilization was associated with a decrease in soil carbon levels and structural stability, and an increase in cropping history, erosion or previous fertilization. Lisle et al. (1994) and Zhao and McGrath (1994) suggested several chemical reagents which were positively related with soil S availability. However, field studies showed contradictory results, finding strong (Bailey, 1986; Blair et al., 1991) but also inconsistent relationships (Brennan and Bolland, 2006; Ferraris et al., 2004). Plant analysis has been proposed as a more reliable predictor of S responsive sites. Blake-Kalff et al. (2000) found that N:S ratio in leaf was a reliable indicator of S deficiency in wheat and in oilseed rape. Bailey (1986) found that herbage N:S ratio was a strong predictor of S deficiency in rape, barley and alfalfa in experiments performed in growth chambers. In field experiments, Grant et al. (2003) found that both N:S ratio and plant S concentration at flowering identified S deficient sites in canola. In soybean, Hitsuda et al. (2004) observed a strong relationship between seed yield and seed S concentration in controlled conditions, determining no increases in seed yield when seed S concentration was above 2.3 g S kg^{-1}, but no validation of this threshold was done under field conditions. In all cases, the strength of the relationship between nutrient content and crop response to S fertilization depended on which tissue is sampled and the sampling stage (Blake-Kalff et al., 2002).

In humid temperate regions, soil organic matter is the main source of S for a non-fertilized crop. Sulfur mineralization rates are not expected to vary greatly from year to year in sites with a prolonged and continuous cropping history unless organic sulfur is added to the soil (Eriksen, 2009; Knights et al., 2001). Therefore, S deficient sites would continue being deficient if they do not receive S fertilization (McGrath et al., 2002). In these situations, plant analysis at maturity (i.e. seed and/or vegetative tissues) may be used as a retrospective diagnosis for deciding S fertilization in the succeeding crop. In soybean, a great proportion of S is mobilized to reproductive organs during the seed filling period (Sexton et al., 1998c). Mobility of S in the plant is low, and thus, an early determination of S status may not relate with seed yield response. Mathot et al. (2009) found that, in swards dominated by grasses, total N and S content in aerial biomass were good predictors of S responsive sites. In wheat and rice, several studies (Randall et al., 1981, 2003; Reussi Calvo et al., 2011) separated S responsive and nonresponsive sites by concurrently analyzing N and S concentration in grains, characterizing S deficient sites by low S concentration and large N:S ratio in grain.

Many times deficiencies of different nutrients appear simultaneously in real conditions (Dobermann et al., 1998; Salvagiotti et al., 2004). Likewise, the addition of a nutrient at rates above the crop requirement may affect the relative contribution of other nutrients that are not involved in fertilization, affecting growth and nutrient internal balance. Nutrient ratios (as describe above for N and S) may be useful tools for managing multiple nutrient deficiencies, and thus, be a part of an integrated nutrient management strategy (i.e. balanced fertilization). Different approaches has been suggested for making multiple nutrient diagnoses, such as: (i) relating nutrient content in scatter plots in order to find the optimal nutrient combination that separate responsive sites (Randall et al., 1981), (ii) elaborating optimal nutrient indexes, e.g. DRIS methodology (Walworth and Sumner, 1987) and comparing them with actual values for identifying responsive crops or (iii) making algorithms which accounts for the interactions of total nutrient uptake and nutrient use efficiency (Janssen et al., 1990). Nutrient stoichiometry considers the relative proportions of elements (e.g. nutrients) in living organisms. This concept has been used for understanding changes at different levels of organization, from cell to ecosystem
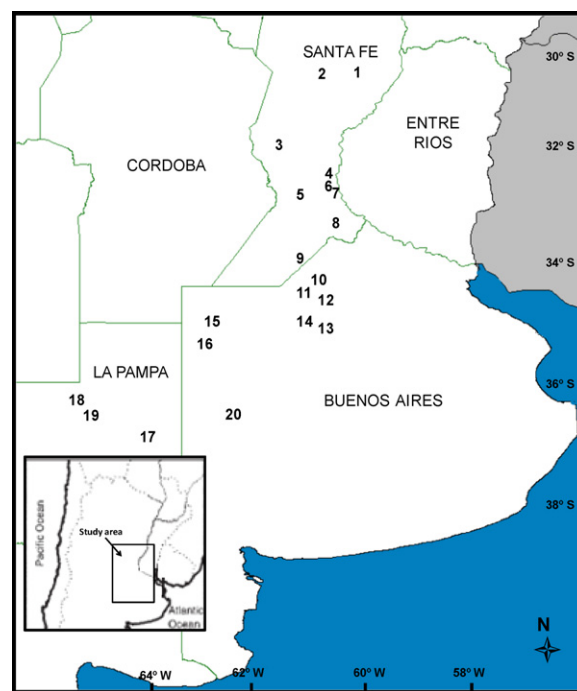

Fig. 1. Distribution of the experimental sites in the Argentinean Rolling Pampas. Characteristics of each site are described in Table 1.

(Elser et al., 1996), or for characterizing the allometry of nutrients in plants (Niklas and Cobb, 2006; Obeso, 2012; Sadras, 2006). Deviations from optimal allometry relationships among nutrients may be used for managing multiple nutrients in crops or for detecting nutrient imbalances, but practical application of this methodology for fertilizer management has not been reported. We expect that seed yield reductions in soybean in S-deficient fields is associated with a decrease in seed S content, and thereby a concomitant increase in the N:S ratio that may alter N–S stoichiometry in seeds. We also expect changes in the S:P stoichiometry in seeds in S deficient sites that deviate this relationship from the optimal.

The objectives of this study were to: (i) evaluate seed S and N:S ratio as a tool for identifying S deficient soybean crops in field conditions and (ii) determine shifts in stoichiometric relationships among N, P and S in soybean seeds which can be used for identifying S responsive sites under field conditions and used as a retrospective diagnosis tool for deciding S fertilization in the succeeding crop.

2. Materials and methods

2.1. Generalities and experimental design

Seed samples from 20 field experiments designed to explore response to S fertilization in soybean were collected. The experiments were performed between 2002 and 2004, in an ample area in the Argentinean Pampas region, between 30° and 37° lat. S from North to South, and from 65° to 61° long. W from West to East (Fig. 1). Soils included Entic and Typic Hapludolls in the West and Typic and Vertic Argiudolls in the most eastern part (Table 1). In each site the experiments had different treatment design, so, for the purposes of this study only non-S fertilized (control) and S fertilized treatment (at a rate of at least 12–24 kg S ha^{-1}) were analyzed. All experiments were arranged in a randomized complete block design with at least 3 replications. Table 1 summarizes the characteristics of the sites involved in this study. In all cases S fertilization was done at planting, using ammonium sulfate or calcium sulfate as S fertilizer source. In all cases P fertilization was performed in

Table 1
Site description, soil analysis at planting, S fertilizer and crop management in the experimental sites.

Site description				Soil analysis at planting							S fertilizer management			Crop management			
Site	Location	Season	Soil type	Bray1 P $(mg\,kg^{-1})$	$S\text{–}SO_4$ $(mg\,kg{-}1)$	pH	OC $(g\,kg^{-1})$	Clay	Silt	Sand	S rate $(kg\,ha^{-1})$	Source	Placement	YCC	Previous crop	Tillage	Cultivar
1	San Justo	2003–2004	TA	12	4	6.1	25.4	25	70	5	12	$CaSO_4$	Ba	>30	O	NT	A 6411 RG
2	La Pelada	2003–2004	TA	14	4	6.2	23.2	30	64	6	12	$CaSO_4$	Ba	>7	Mz	NT	A 6011 RG
3	Las Rosas	2000–2001	TA	36	10	5.7	16.9	27	71	3	15	$(NH_4)_2SO_4$	Br	>20	Sb	NT	DM 4800 RG
4	Maciel	2002–2003	TA	4	11	5.6	11.0	22	75	3	20	$CaSO_4$	Ba	80	Wh	NT	RA 505
5	Va Eloisa	2002–2003	TA	11	10	6	13.4	24	73	3	20	$CaSO_4$	Ba	15	Wh	NT	DM 4800 RG
6	Oliveros 1	2004–2005	TA	11	7	5.3	14.0	22	75	3	20	$CaSO_4$	Ba	>30	Sb	NT	TJ2049 RG
7	Oliveros 2	2004–2005	TA	9	10	5.3	14.2	22	75	3	20	$CaSO_4$	Ba	>30	Sb	NT	TJ2049 RG
8	Molina	2004–2005	VA	9	30	5.8	15.7	21	68	11	24	$(NH_4)_2SO_4$	Ba	>30	Wh	NT	DM 4800 RG
9	Wheelwhright	2003–2004	TA	11	3.6	5.4	14.0	25	62	13	15	$CaSO_4$	Br	>20	Sb	NT	A 3901 RG
10	La Trinidad 1	2004–2005	TA	19	4.3	6.1	15.7	23	49	28	15	$CaSO_4$	Br	>20	Mz	NT	DM 4800 RG
11	Arribeños	2004–2005	TA	12	n.a.	5.8	14.5	20	42	38	15	$CaSO_4$	Br	>10	B	NT	A 4303 RG
12	La Trinidad 2	2004–2005	TA	10	n.a.	5.9	16.9	23	49	28	15	$CaSO_4$	Br	>30	B	NT	DM 4800 RG
13	Junín	2002–2003	TH	12	14.8	5.5	15.1	14	26	60	15	$(NH_4)_2SO_4$	Br	>15	Mz	NT	A 3901 RG
14	Alem	2002–2003	TH	12	14.6	5.6	16.3	18	31	51	15	$CaSO_4$	Ba	5	Mz	CT	A 3901 RG
15	Gral. Villegas EEA	2004–2005	TH	24	13	5.8	11.6	20	31	49	20	$(NH_4)_2SO_4$	Br	6	Sb	NT	DM 4800 RG
16	Gral. Villegas	2004–2005	TH	16	15.2	5.9	13.4	12	29	59	20	$(NH_4)_2SO_4$	Br	3	Mz	NT	A 3550 RG
17	Alvear	2004–2005	EH	14	10.5	6	10.5	12	27	61	20	$CaSO_4$	Br	3	Mz	NT	DM 4800 RG
18	Luan Toro	2004–2005	EH	12	12.3	6.4	7.6	11	35	54	20	$CaSO_4$	Br	2	WF	NT	DM 4800 RG
19	Luan Toro	2004–2005	EH	16	6.4	6.5	6.4	8	33	59	20	$CaSO_4$	Br	2	WF	NT	DM 4800 RG
20	Mari Lauquen	2004–2005	EH	24	7.5	6.2	12.2	8	19	73	20	$CaSO_4$	Br	6	Mz	NT	DM 3200 RG

YCC, years under continuous cropping; n.a., not available.
Tillage: NT, no tillage; CT, conventional tillage.
Soil type: TA, Typic Argiudol; TH, Typic Hapludoll; EH, Entic Hapludoll; VA, Vertic Argiudoll.
Placement: Br, Broadcast; Ba, Banded.
Previous crop: Mz, maize; Sb, soybean; Wh, wheat; O, oat; WF, winter forage; B, barley.

Table 2

Mean values and analysis of variance for Seed yield (corrected to 0.13 kg H$_2$O kg grain^{-1}), seed N concentration (%N), seed S concentration (%S), seed P concentration (%P), seed N content (SeedN), seed S content (SeedS), Seed P content (Seed P) and N:S ratio in the different experimental sites for fertilized (F) and control (C) treatments.

Treatment (T)	Seed yield (kg ha^{-1})		%N (g kg^{-1})		%S (g kg^{-1})		%P (g kg^{-1})		N:S ratio		SeedN (kg ha^{-1})		SeedS (kg ha^{-1})		SeedP (kg ha^{-1})		N:P:S ratio	
Control (C)	3417		58.7		2.9		5.6		20.6		175		8.7		16.4		21:2.0:1	
Fertilized (F)	3718		59.2		3.3		5.6		18.4		192		10.5		18.2		18:1.7:1	
SE	47		0.2		0.03		0.05		0.24		2.5		0.2		0.3			
Site (S)	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F
1	2753	3538**	58.4	58.4	2.6	2.7	5.9	5.6	22.4	22.2	140	180**	6	8**	14	17**	22:2.2:1	22:2.2:1
2	3720	3557	60.7	61.5	2.7	3.0*	4.9	5.2	23.0	20.6*	196	190	9	9	16	16	22:1.8:1	22:1.8:1
3	3782	4224*	53.2	59.0**	2.0	3.1**	6.5	6.9*	26.3	18.9**	173	214**	7	11**	21	25**	26:3.2:1	26:3.2:1
4	2633	3142**	60.3	61.9+	2.5	3.1**	6.7	5.8**	24.5	19.8**	137	167**	6	8**	15	16	24:2.7:1	24:2.7:1
5	3491	3839+	57.2	59.3*	2.6	3.3**	6.3	6.4	22.8	17.9**	172	196*	8	11**	19	21+	22:2.5:1	22:2.5:1
6	3617	3510	59.7	61.0	2.8	3.5**	6.3	5.7*	21.1	17.5**	186	184	9	11*	19	17+	21:2.2:1	21:2.2:1
7	3728	3934	59.4	60.3	2.9	3.6**	6.2	5.8+	20.3	16.9**	190	204	9	12**	20	20	20:2.1:1	20:2.1:1
8	2185	2908**	58.7	61.3**	2.2	3.0**	5.1	4.9	27.2	20.5**	110	153**	4	7**	10	12*	27:2.4:1	27:2.4:1
9	4241	4661+	61.5	60.8	2.6	2.8	5.6	5.7	23.9	22.2	227	247+	9	11*	20	23*	24:2.2:1	24:2.2:1
10	4565	4710	60.7	57.7**	2.7	3.1**	5.0	5.4+	23.1	19.0**	241	236	11	13**	20	22+	23:1.9:1	23:1.9:1
11	2880	3131	60.3	60.1	3.2	2.9	4.6	5.4**	18.7	20.5	151	162	8	8	12	15*	19:1.4:1	19:1.4:1
12	3232	3628+	61.2	61.0	3.6	3.4	5.2	5.5	17.2	18.1	172	193+	10	11	14	17*	17:1.4:1	17:1.4:1
13	4490	4988*	60.2	60.1	3.1	3.4**	4.7	4.5	19.5	17.9+	235	261*	12	15**	18	20	19:1.5:1	19:1.5:1
14	3777	3711	60.3	61.4	2.9	3.5**	4.7	4.3	20.8	17.6**	198	198	10	11**	15	14	21:1.6:1	21:1.6:1
15	3634	3773	58.5	58.2	3.1	3.1	5.6	6.4**	19.0	19.1	185	191	10	10	18	21*	19:1.8:1	19:1.8:1
16	3630	3981+	57.8	57.4	3.3	3.5	5.1	5.2	17.4	16.5	182	199	10	12*	16	18	17:1.5:1	17:1.5:1
17	3609	4160*	58.1	57.0	3.3	3.4	5.1	5.1	17.5	16.6	182	206*	10	12*	16	18+	17:1.5:1	17:1.5:1
18	1546	1739	54.3	54.9	3.5	3.6	6.4	6.6	15.5	15.1	73	83	5	5	9	10	15:1.8:1	15:1.8:1
19	1994	2617**	55.5	56.1	3.5	3.6	6.3	6.4	15.9	15.6	96	128**	6	8**	11	14**	16:1.8:1	16:1.8:1
20	4834	4641	58.3	58.4	3.4	3.7	5.4	6.2**	17.1	16.0	245	236	14	15	23	25	17:1.6:1	17:1.6:1
F-test probability																		
S	<0.01		<0.01		<0.01		<0.01		<0.01		<0.01		<0.01		<0.01			
T	<0.01		<0.01		<0.01		0.15		<0.01		<0.01		<0.01		<0.01			
S × T	0.03		<0.01		<0.01		<0.01		<0.01		<0.01		<0.01		0.05			

+ Significant effect at the 0.10 probability level.
* Significant effect at the 0.05 probability level.
** Significant effect at the 0.01 probability level.
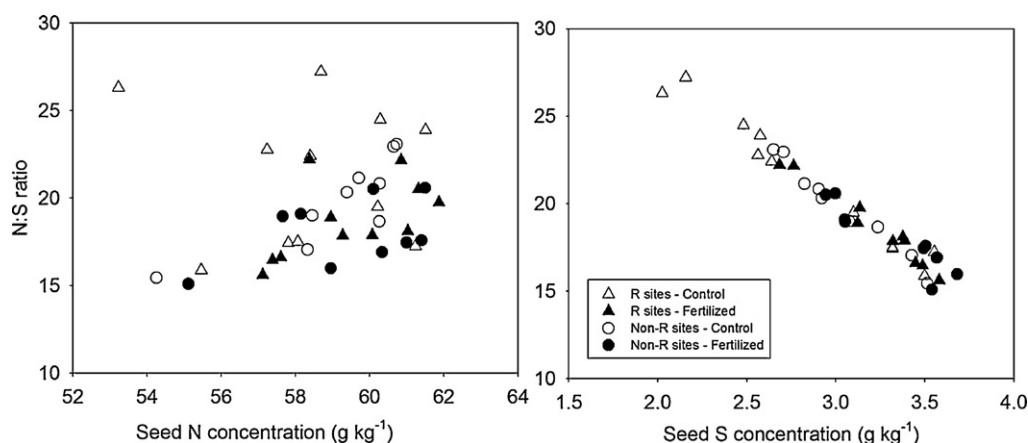
Fig. 2. Relationship between seed N concentration (left panel) or seed S concentration (right panel) and the N:S ratio. R sites and non-R sites are experiments that showed significant and non-significant response to S fertilization, respectively.

order to avoid P deficiency. Sulfate in soil was determined using ammonium acetate as extractant (Bardsley and Lancaster, 1965).

Seed yield (SY) was determined in each plot and adjusted to a standard moisture content of 0.13 kg H_2O kg grain^{-1}. Seed samples were ground to determine total N, P and S. Total N (%N) and S (%S) concentration was determined by the Dumas dry combustion method at 950 and 1350 °C, respectively, using a TruSpec CNS (LECO, St. Joseph, MI, USA) analyzer. Phosphorus concentration (%P) was determined colorimetrically after wet digestion. Seed N (seedN), S (seedS) and P (seedP) contents were determined by multiplying %N, %S and %P and seed yield on a dry matter basis. N:S ratio was obtained by dividing N and S concentration in seeds.

2.2. Data analysis

An overall ANOVA was performed using the PROC MIXED procedure in SAS for evaluating site and treatment (fertilized vs. non-fertilized) effects. Replication within site was used as an error term for evaluating site effect (Gomez and Gomez, 1984). In case of interaction, differences between treatments in each site were determined for SY, %N, seed S concentration and %P. Significant differences were determined at 1, 5 and 10% level using a LSD test. Sites that showed significant increases at 10% level for SY were considered as S responsive site. In order to test the usefulness of seed S for identifying S-responsive sites, three approaches were used:

(a) Plotting relative yield versus seed S concentration, N:S ratio and seedS. Relative yield was calculated as the quotient between SY in the unfertilized treatment and SY in the fertilized treatment in each site. Thresholds for the different variables were determined using the procedure described by Cate and Nelson (1965).
(b) Plotting %N or seedN versus seed S concentration or seedS as suggested by Randall et al. (1981).
(c) Establishing stoichiometric relationships among N, P and S in both fertilized and unfertilized plots in responsive and unresponsive sites. Relationships among N, S and P content in seeds were fitted using the logarithmic form of the model: $Y = a X^b$ (i.e.: $\log Y = \log a + b \log X$), where Y is S or P content in seeds, and X is N or P content in seeds (Niklas, 2006). Lines were fitted using the standardized major axis method. In this method, departure of each data point from the fitted line in the Y and X directions is considered, as the fitted line minimizes the sum of triangular areas between the line and each data point. Tests for detecting differences and/or shifts in the slope and intercept along

axis were performed (Warton et al., 2006). The software SMATR was use for fitting the lines, testing the significance of their parameters, and comparing slopes of different lines (Warton et al., 2006).

3. Results

3.1. Seed yield, N, S and P content

A wide range of environmental conditions were explored as showed by the ample range of maximum SY at each site, which varied from 1739 to 4988 kg ha^{-1} (Table 2). On average, S fertilization increased SY by ca. 301 kg ha^{-1} (ca. 9%), but a significant site × treatment interaction was observed (Table 2). In 11 out of 20 sites, S fertilization increased SY ($p < 0.10$; Table 2) ranging from 348 to 785 kg ha^{-1}. On average, S addition slightly increased (ca. 1%) %N and only in 4 out of 20 sites was a significant increase observed (Table 2), indicating that this variable was relatively stable across sites. Average seedN was 184 kg ha^{-1}, and as %N was not widely affected by S fertilization treatments, seedN showed significant responses in sites where SY increased significantly. On the other hand, seed S concentration increased ca. 14% in response to S fertilization. There was a significant site × treatment interaction, and positive response was significant in 10 out of 20 sites. In these sites, the mean increase in seed S concentration was 18%. Interestingly, only 5 sites that showed significant increases in SY also increased seed S concentration (Table 2). Therefore, seedS showed significant increases in 14 out of 20 sites, including not only 10 sites where SY increased significantly (average increase in seedS was 42% in these sites), but also 4 sites without a response in SY (average increase was 22% in these sites). Finally, %P increased in 5 and decreased in 3 sites (Table 2). SeedP increased ca. 11% in response to S fertilization, showing increases in 12 out of 20 sites. The N:S ratio varied from 15.1 to 27.2:1, and a significant effect of S fertilization was observed only when S% was affected by S fertilization (Table 2). Therefore, N:S ratio was closely associated with changes in seed S concentration rather than changes in %N (Fig. 2). On average, the N:P:S ratio in seeds was 21:2:1 for control treatments and changed to 18:1.7:1 when S was applied (Table 2).

3.2. Relationships between seed N and S and response to S fertilization

Sulfur concentration and N:S ratio in seeds of control treatment showed no consistent relationship with relative seed yield. Fig. 3
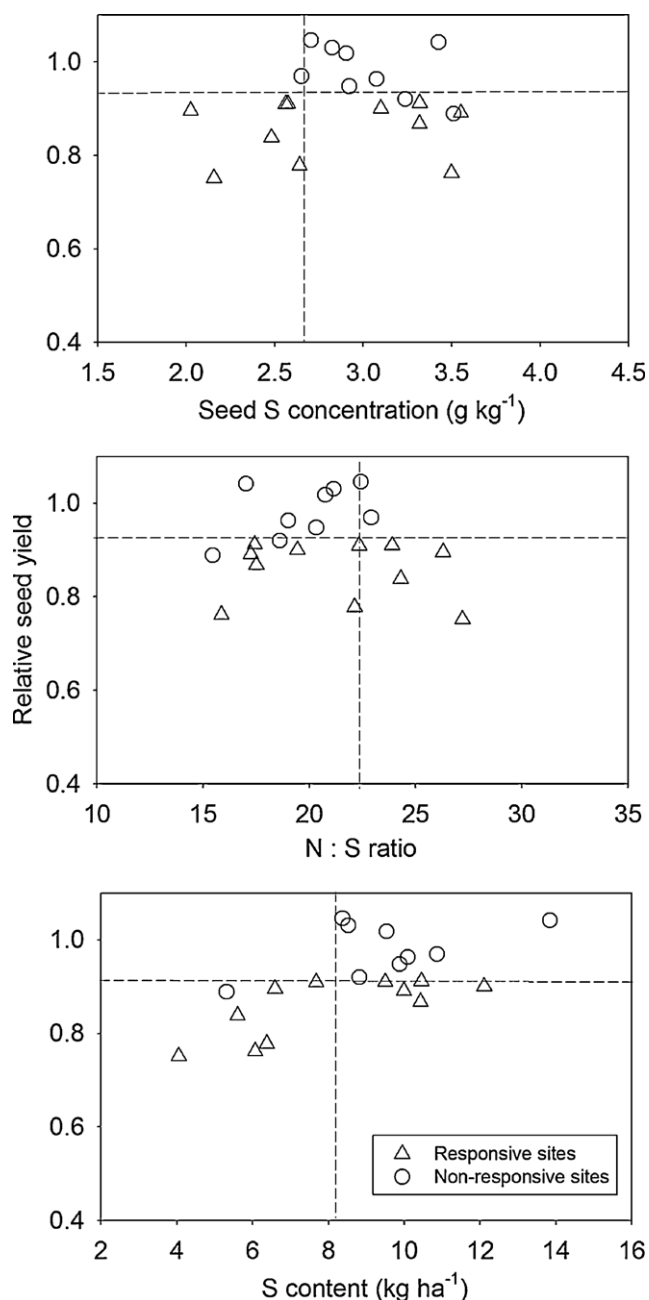
Fig. 3. Relationship between relative seed yield and seed S concentration (top panel), N:S ratio (middle panel) and seed S content (low panel). Responsive sites were those that significantly increased seed yield in response to S fertilization (see Fig. 4).

shows seed S concentration and N:S ratio values of 2.7 g S kg^{-1} and 22:1 as likely thresholds separating responsive and non-responsive sites using the Cate–Nelson procedure, but only 6 out of 11 responsive sites were identified as deficient in S (i.e. located in the left lower quadrant). Moreover, as N:S ratio was mainly governed by variations in seed S concentration (Fig. 2), sites identified as deficient below 2.7 g S kg^{-1} were the same sites that showed N:S ratio above 22:1. A more consistent relationship was observed when seedS was plotted against relative seed yield (Fig. 3), showing a threshold of 8 kg S ha^{-1} below which a site was identified as S deficient.

3.3. Identification of S deficient sites analyzing N and S content

Randall et al. (1981) suggested that sites responsive to S addition can be identified by relating N and S seed concentration as depicted in Fig. 4. Briefly, four quadrants are determined by intersecting lines that indicate an "optimal" seed S concentration or seedS and an "optimal" N:S ratio. Quadrant A should include the responsive sites (i.e. low seed S and no N limitations), while quadrants B and C should comprise the non-responsive sites (i.e. sites in which seed S is above the optimum). Optimal seed S concentration and N:S ratio were determined by maximizing the number of responsive sites in quadrant A. In the present study, optimal seed S concentration and N:S ratio were respectively 2.65 g S kg^{-1} and 22:1. All data associated with non-S deficiencies (i.e. control and fertilized treatments in non-responsive sites and fertilized treatments in responsive sites) were acceptably diagnosed (i.e. allocated in quadrant C), and 6 out 11 responsive sites were identified as deficient (i.e. allocated in quadrant A). No improvement in prediction occurs when relating seedN and seedS, using an "optimum" seedS of 8 kg S ha^{-1}.

3.4. N:P:S stoichiometry in seeds and response to S fertilization

Fig. 5 shows the stoichiometric relationships among N, P and S content in soybean seeds. The fitted function represents the relationship between N and S or P accumulation in seeds. Sulfur deficiency affected the N:S relationship, but no changes were detected in the P:S and N:P stoichiometry in seeds.

In absence of S deficiency (G2 = S fertilized treatment in responsive and unresponsive sites + control treatment in unresponsive sites) seedN scales almost isometrically with seedS and seedP, showing b values of 1.05 and 1.1 for S and P, respectively (Table 3). Likewise seedP and seedS are related by a factor of 0.96 (Table 3). Sulfur deficient crops (G1 = control treatments in responsive sites) had a different N:S relationship, showing a shift in intercept as detected by the SMA analysis ($p = 0.06$), but no changes in the slope were observed (Table 3).

Equating the exponential form of the relationship between seedN and seedS, using a common b (i.e. 1.05) but a different intercept (i.e. $a = 10^{\log a}$) for G1 (a_{G1}) and G2 (a_{G2}), for any amount of N, the seedS (G1): seedS (G2) ratio will be proportional to $(a_{G2}/a_{G1})^{1/b} = 0.88$. Therefore, for a given amount of N accumulated in seeds, sulfur deficient crops tended to accumulate 12% less S in seeds than crops with no S limitations (G2).

Sulfur deficiency did not modify P:S nor N:P stoichiometry, showing no shifts in either the intercept or the slope (Table 3), suggesting that changes in N:P stoichiometry were proportional independently of S deficiency.

4. Discussion

Moderate SY responses to S fertilization were observed in the present study, as reported in previous studies in the area (Gutierrez Boem et al., 2007; Salvagiotti et al., 2004). This response was considered moderate because relative SY was never below 75%, in contrast with reports in highly S deficient conditions, for instance pot experiments, where relative SY has been found to be as low as 35% of maximum SY (Hitsuda et al., 2004). Responsive sites in the present study were detected under different soil conditions which have been documented as likely causes of S deficiency, i.e. light soils, soils with low organic matter contents or that suffered erosion (Tisdale et al., 1986), and also in crops with diverse SY potential.

Classical methods for detecting nutrient thresholds below which a site is classified as deficient for a certain nutrient are often based in relationships among nutrient concentration and SY or
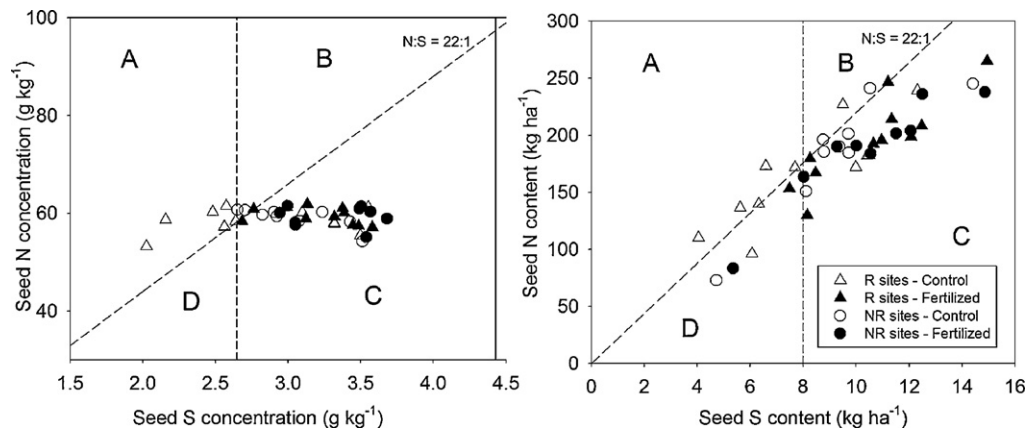
Fig. 4. Relationship between seed N and S concentration (left panel) and between seed N and S content (right panel). Critical values are from Fig. 3 – area A: S-responsive area; areas B and C: S-adequate area; D: S-unresponsive area (low N, high or low S).

Table 3
Parameters of the fitted log–log function showed in Fig. 5 using reduce major axis regression, for the relationships among seed N, S and P content for G1 (R sites – control, i.e. S deficient situations) vs. G2 (rest of the sites, i.e. non-S deficient situations). Wald test indicates shifts in log a or b between G1 and G2.

| Nutrient relationship	Data group	r^2	Slope comparison			Comparison of lines with common slope		
			b	$\log a$	p-Value[*]	b	$\log a$	p-Value[**]
N vs. S	G1	0.69	0.83	1.47			1.28	
	G2	0.83	1.10	1.18	0.18	1.05	1.22	0.06
P vs. S	G1	0.36	0.75	0.53			0.34	
	G2	0.72	1.00	0.25	0.33	0.96	0.28	0.32
N vs. P	G1	0.71	1.11	0.88			0.89	
	G2	0.76	1.10	0.91	0.95	1.10	0.90	0.89

[*] p-Value for comparison of b values.
[**] p-Value for comparison of intercepts ($\log a$) using a common slope.

relative SY (Evans, 1987). The assumption behind this analysis is that a decrease in the concentration of a nutrient reflects a deficiency that reduces SY. A rise in S concentration in response to S addition is a common feature in experiments in which S deficient conditions are severe, as those encountered in crops growing in pots or under hydroponics (Gaines and Phatak, 1982; Sexton et al., 1998b). Crops having seed S concentration greater than a threshold are not expected to increase SY when S is added (relative SY approaching 1), but they may increase seed S concentration as S rate rises, suggesting luxury consumption (Fernández et al., 2009; Goldman et al., 2002). These non-responsive sites are expected to occupy the upper-right quadrant in Fig. 3. However, in the present study, we observed a set of experiments (12, 16, 17 and 19, Table 2) with seed S concentration above an empirical threshold (i.e. greater than 3 g S kg^{-1}) that did not increase % S in response to S addition, but significantly boosted SY (i.e. low relative SY). This increase in SY at high seed S concentration may indicate an improvement in biomass accumulation (and thus SY) not directly related only with seed S concentration, suggesting an indirect effect of S addition that may have improved carbon (C) assimilation. Nitrogen is central in carbon metabolism, since it represents more than 70% of photosynthetic apparatus (Kumar et al., 2002). Sulfur is a constituent of key enzymes in N metabolism (Campbell, 1999; Friedrich and Schrader, 1978) and in the case of legumes is associated with enzymes responsible for biological N fixation (Benton and Peters, 2004). Salvagiotti et al. (2009) showed a rise in N uptake in response to S addition that increased biomass production via a larger radiation capture, finally reflected in a greater SY (Salvagiotti and Miralles, 2008). In addition, the increase in SY (i.e. a rise in C assimilation) due to S fertilization concomitantly diluted seed S concentration in seeds and did not show an increase in seed S concentration when S rate increased. This dilution effect was also

observed analyzing %N and SY in wheat, but in high N deficient conditions (Fischer et al., 1993). Therefore, seed S concentration alone is not a good predictor of SY response to S addition. In line with this reasoning, in the present study, we observed that S content in mass basis, a variable that integrates C assimilation, related better with relative seed yield. On the contrary, Hitsuda et al. (2004) proposed that seeds lots showing less than 2.3 g S kg^{-1} were more likely to respond to S fertilization. In the present study, we could not validate the proposed threshold. Two issues differed from the study of Hitsuda et al. (2004) that may explain the lack of fit of the relationship between SY and seed S concentration: (i) S deficiencies were moderate (relative SY above 0.75), as opposed to relative SY close to 0.35, and (ii) soybean positively respond to S fertilization in some sites where seed S concentration was above 3 g S kg^{-1}.

The results of this study suggest that N and S should be treated simultaneously for detecting S deficient sites. The N:S ratio was mainly driven by changes in seed S concentration as observed in other studies (Hitsuda et al., 2004), hence, this variable alone will have the same constraints for identifying sites responsive to S fertilization as those observed with seed S concentration. Relating seed S concentration (or seedS) and %N (or seedN) using the diagram proposed by Randall et al. (1981) successfully identified sites unresponsive to S fertilization (i.e. quadrant C, Fig. 4). The optimal N:S ratio threshold defined in this diagram was 22:1, larger than the 17:1 or 13:1 reported for wheat (Randall et al., 1981; Reussi Calvo et al., 2011), or 14:1 for rice (Randall et al., 2003). This is expected since N demand per unit of seed yield in soybean is ca. 2.5 larger than cereals (Sinclair and Wit, 1975). Dijkshoorn and van Wijk (1967) reported a theoretical N:S ratio of 40:1 for whole plants in different leguminous plants. Similar to the present study, Sexton et al. (1998a) showed N:S ratios in cotyledons of soybean seeds between 20 and 25:1 when S was not limiting. On the other
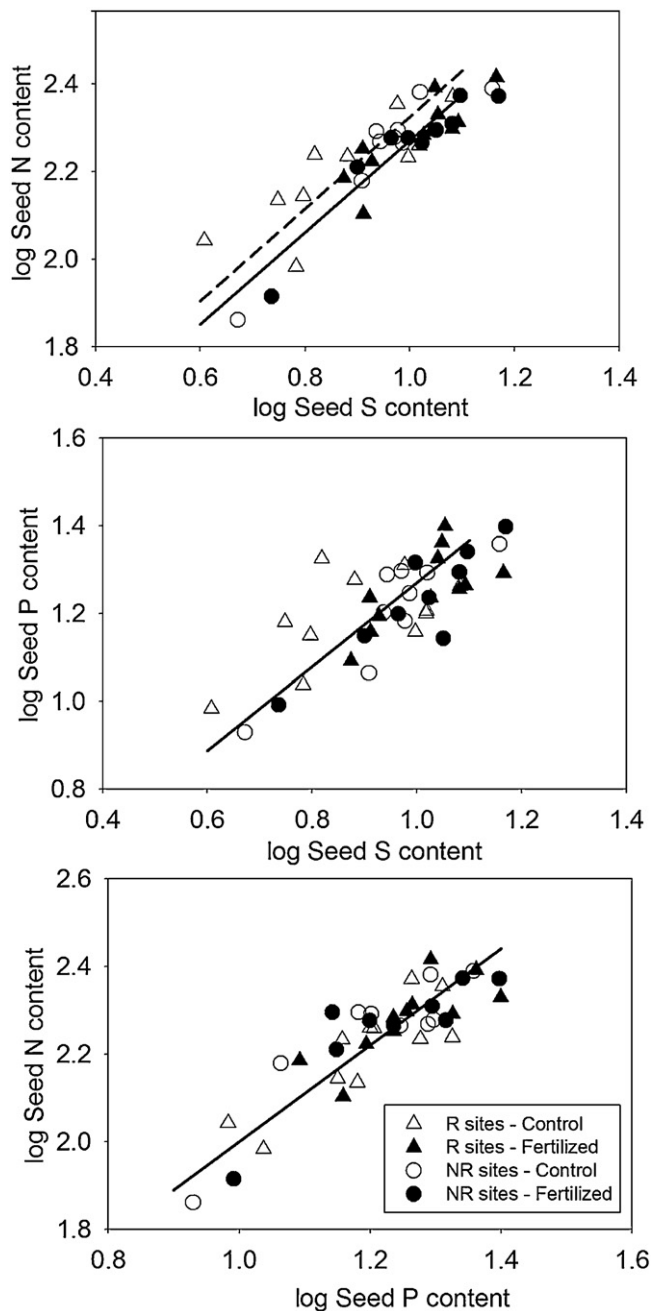
Fig. 5. Log–log bivariate plots showing the relationships between seed N, S and P content. Solid and dashed lines are reduced major axis regression curves for R sites – control and the rest of the sites, respectively. Parameters are shown in Table 3.

hand, this methodology was not accurate for separating responsive sites in the responsive quadrant (i.e. quadrant A in Fig. 4). Soybean (as other legumes) can regulate N uptake to match N demand (via biological N fixation or via soil N uptake). A narrow range of variation in %N has been reported in soybean (Salvagiotti et al., 2008) as observed in the present study. Therefore, great variations in %N are not expected unless biological N fixation is constrained (Leffel et al., 1992). In contrast, several studies successfully separated responsive sites in wheat and rice using this methodology (Randall et al., 1981, 2003). But, since cereals have large variations in %N in seeds (Van Duivenbooden et al., 1996), it will be easier to find situations where N cumulates disproportionally relative to S, and thus, this methodology may easily identify sites responsive to S fertilization.

In the present study, all relationship among nutrients (N:S, NP: and P:S) were isometric (i.e. scaling exponent, *b* equal 1). This type of relationship has been observed previously between N and C or P and C (Niklas, 2006; Obeso, 2012), but not for the N:P relationship. In leaves of various plant species, Niklas and Cobb (2006) proposed that N scaled with P by an exponent less than 1. Tissue composition may affect nutrient stoichiometry because nutrients accumulate in different proportions depending on specific physiological functions in the plant. At high relative growth rates, a reduction in the N:P ratio (i.e. a scaling component with values less than 1) is explained in terms of more investments in rRNA (major pool of P in cells) to produce proteins (principal pool of N) necessary for active growth (Agren, 2004). Therefore, stoichiometric relationships found in leaves which are in active growth (e.g. Niklas, 2006) may be different from seeds where the main physiological functions are related with storage and tissue maintenance. In the present study, the isometric relationship between N and P suggest that both nutrients were proportionally accumulated. Sadras (2006) suggested that phytate accumulation may play a role in altering N:P relationships in seed, especially at high P rates, but the present study showed no evidence for accepting changes in stoichiometric relationships, at least in soybean plants without suffering P deficiencies. Likewise, S deficiencies may lead to accumulation of non-S proteins (Sexton et al., 1998a) in seeds, which may change the proportionality of seed N and S content. In seeds of *Hedera helix*, Obeso (2012) showed an allometric relationship between S content and seed mass, but isometric variations between seed mass and N content, that suggest allometric relationships between N and S. However, the present study showed that S allocation in seeds varied isometrically with N content, even in responsive sites. In the present study, S deficient crops only modified the intercept of the stoichiometric relationship between N and S, the greater the value of the parameter, the larger the S deficiency. Therefore, this parameter may be used for diagnosing S deficiency in soybean. The N–S intercept for G2 group can be considered as an optimum N–S status (log a_{opt} = 1.22, then a_{opt} = 16.5). Then, in a determinate seed lot, seedN (N_{obs}) and seedS (S_{obs}) can be determined by multiplying seed yield from harvest and %N and seed S concentration determined in the laboratory. Then, these data will yield an observed N–S relationship equal to:

$$N_{obs} = a_{obs} \cdot S_{obs}^{b}$$

A unique *b* value was observed for responsive and unresponsive sites (i.e. 1.05), then the observed *a* (a_{obs}) will be:

$$a_{obs} = \frac{N_{obs}}{S_{obs}^{1.05}}$$

Then a S deficiency index (SDI) can be determined by comparing a_{obs} with a_{opt} (i.e. 16.5):

$$SDI = \frac{a_{obs}}{a_{opt}}$$

The greater the departure of SDI from 1, the larger the N–S imbalance.

In summary, stoichiometric relationships were more accurate for identifying S responsive sites than using single variables (e.g. seed S concentration, seedS or N:S) in soybean that showed moderate response to S fertilization. Relating %N and seed S concentration was useful to identify non-S deficient situations. The isometric variations among N, P and S suggest that the accumulation of these nutrients is proportional in seed tissue, which is characterized by a low metabolic activity. Changes in the intercepts of the stoichiometric relationship between N and S that reflected shifts in S content under constant N status in seed, may be use to define an S-deficiency index that can be used as a diagnostic tool for

identifying S responsive sites in soybean. The present study shows a novel approach for using stoichiometric concepts in fertilizer management in soybean. Further validation and follow-up research of this approach is necessary.

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