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Diagnosis of S deficiency in soybean crops: Performance of S and N:S determinations in leaf, shoot and seed

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ABSTRACT

The main areas for soybean production in the world have been recently reported as S-deficient. Chemical analyses of soil samples have not been successful for evaluating the S status of crops. It is generally accepted that plant tissue analyses are better than soil testing for predicting the necessity of S fertilization. Our aims were: (i) to analyze the patterns of S concentration (S_{conc}), N concentration (N_{conc}) and N:S ratios in soybean leaf, shoot and seed in response to S availability in soil, (ii) to determine the thresholds for S deficiency, and (iii) to evaluate the performance of the greenness index to assess S_{conc} and N:S in leaf.

Fifteen field experiments were performed during seasons 2012/13 and 2013/14. Sulphur fertilization increased seed yield in 9 out of 15 sites. The critical thresholds for S deficiency were 2.65, 2.06 and 3.93 g S kg^{-1}, for leaf, shoot and seed, respectively. For the same plant parts, the N:S critical thresholds were 13.90, 12.18 and 13.50, respectively. The use of N:S better diagnosed the S status than the analysis of S_{conc} in leaf and shoot. The relative seed yield was weakly associated with S_{conc} and N:S in seed. We propose the use of N:S in leaf for in-season assessment of S-status. We also found that the greenness index is sensitive to changes in the S status of the plant, which facilitates the diagnosis.

1. Introduction

In comparison to nitrogen (N) and phosphorus (P), sulphur (S) deficiencies in agricultural soils have received little attention. However, the intensification of crops rotations, the decrease of soil organic matter and the reduction of atmospheric inputs have depleted S pools in some soils (Scherer, 2001). As a consequence, crop deficiencies have been reported more frequently.

Soybean [*Glycine max* (L.) Merrill] is the most important grain legume crop, providing more than half of the world's vegetable oils and two-thirds of the world's protein meal. Interestingly, the main areas for soybean production in the world (i.e. Argentina, Brazil and United States) have been recently reported as S-deficient (Gutierrez Boem et al., 2007; Salvagiotti et al., 2012; Lucheta and Lambais, 2012; Kaiser and Kim, 2013).

Several soil tests have been developed to diagnose nutrient deficiencies. However, chemical analyses of soil samples have not been successful for evaluating the S status of crops (Scherer, 2009). It is generally accepted that plant tissue analyses are better than soil testing for predicting the necessity of S fertilization (Black Kalff et al., 2002).

Total sulphur concentration (S_{conc}) in shoots or in specific plant parts is widely used to assess the S status of crops (Schnug and Haneklaus, 1998; Black Kalff et al., 2002). However, S concentration varies with the sampled part of the plant and the crop development stage (Black Kalff et al., 2002). By contrast, when N supply to plants is close to the optimum (no deficiencies or excess) the N:S ratio is preferred to assess S deficiencies because it is less variable than S_{conc} (Dijkshoorn and van Wijk, 1967).

Irrespective of the index, some investigations have focused on the performance of the analysis of shoots or other plant parts. Thus, Kaiser and Kim (2013) determined a threshold (2.7 g S kg^{-1}) for yield response analyzing S_{conc} in soybean shoots sampled at V5 stage (scale proposed by Fehr and Caviness, 1977). Diagnosis based on leaf S_{conc} is easier because of the simplicity of sample collection. Thus, Hitsuda et al. (2004) and Kaiser and Kim (2013) identified thresholds for leaf S sufficiency for maximal seed yield in experiments performed in pots and under field conditions, respectively.

Seed has also been successfully utilized for diagnosing the S status of different crops (Randall et al., 1981, 2003; Reussi Calvo et al., 2011). Seed analysis does not allow the correction of deficiencies in

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the sampled crop, but can be used to characterize a soil and management practices retrospectively. In soybean grown in pots, Hitsuda et al. (2004) reported that the relative seed yield was better correlated with S_{conc} in seeds than in leaves. However, Salvagiotti et al. (2012) found that the S_{conc} or N:S in seeds did not correctly identify S responsive sites in field experiments.

The leaf greenness intensity is associated with the concentration of chlorophyll, which is also related to shoot S_{conc} (Hoefgen and Nikiforova, 2008; Pagani and Echeverría, 2012). Therefore, Hitsuda et al. (2004) reported that this index was associated with the seed yield of plants grown in pots under a gradient of S provision. As far as we know, leaf greenness has not been tested in field-grown soybean.

Then, some indexes of S sufficiency have been evaluated in soybean under diverse experimental conditions (i.e. pots or field experiments) while others remain to be evaluated. In recently published papers from field experiments Salvagiotti et al. (2012) and Kaiser and Kim (2013) evaluated some of them. However, the analysis of the patterns of N and S concentration and the definition of critical thresholds for S deficiency were limited in these experiments because only two S treatments were tested (no S addition and one S rate). Thus, it is relevant to make a comprehensive analysis from numerous field experiments with a wide range of S availability in order to identify reliable indicators of the S status of soybean crops. Our aims were: (i) to analyze the patterns of S_{conc}, N concentration (N_{conc}) and N:S ratios in soybean leaf, shoot and seed in response to S availability in soil, (ii) to determine the thresholds for S deficiency, and (iii) to evaluate the performance of the greenness index to assess S_{conc} and N:S in leaf.

2. Materials and methods

2.1. Experimental sites and design

Fifteen field experiments were performed during seasons 2012/13 and 2013/14 (Table 1). They were located in the northern, central and southern region of the Argentinean Pampas (Table 1) where soybean is the dominant crop. Trials were centered around Rafaela (31.3° S, 61.5° W) in the North, Teodelina (34.2° S, 61.5° W) in the Central region and Balcarce (37.5° S, 58.2° W) in the South. Some experiments were performed in timely sown sole-crop soybean and others in late planted soybean following winter cereals (Table 1). Geographical areas and sowing dates were selected with the aim of covering a wide range of growing conditions. Some trials were placed at INTA Research Stations (Balcarce and Rafaela) and others in farmers' fields (Table 1). Experiments in farmer's fields allow the evaluation of soybean response to S fertilization under common crop rotation and crop husbandry for each region. The experimental design was a randomized complete block with three replications. The dimension of the experimental units was 12 m × 5 m. Gypsum ($CaSO_4·2H_2O$, 16% S, 20% Ca) was broadcasted at crop emergence at rates of 0, 10, 20, 30 and 40 kg S ha^{-1} in 12 experiments and 0, 10 and 40 kg S ha^{-1} in 3 experiments.

Soybean seed was inoculated with *Bradyrhizobium japonicum* before sowing. Phosphorus was applied to all treatments as triple superphosphate (0–46–0) in order to avoid deficiencies (30 kg P ha^{-1}). All experiments were performed under no tillage. Weeds, diseases and insects were chemically controlled when required.

2.2. Soil sampling and laboratory procedures

At soybean emergence, composite soil samples (12 subsamples per block) were taken at depths of 0–20, 20–40, and 40–60 cm.

Table 1
Site description, soil analysis (0–20 cm depth for OM, pH and P-Bray and 0–60 cm to $N\text{-}NO_3^-$ and $S\text{-}SO_4^{-2}$) at sowing and crop husbandry in the experimental sites.

Experiment	Location	Season	Soil type[a]	OM (g kg-^{1})	pH	P-Bray (mg kg^{-1})	$N\text{-}NO_3^-$ (kg ha^{-1})	$S\text{-}SO_4^{-2}$ (kg ha^{-1})	Cultivar	Sowing date	Previous crop	Sampling date[b]	Site[c]
E1	South	2012/13	TA	44.2	5.9	26.8	58.3	32.9	DM 4970	08 Nov	Soybean	R2	ESt
E2	South	2012/13	TA	44.0	5.9	25.6	49.2	30.8	DM 2200	08 Nov	Soybean	R1	ESt
E3	South	2012/13	TA	45.5	6.5	15.2	38.8	25.2	DM 3810	15 Nov	Soybean	R1	FFi
E4	South	2013/14	TA	48.4	6.1	14.0	43.1	16.2	DM 3810	10 Nov	Maize	R2	FFi
E5	South	2013/14	TA	45.6	6.0	20.5	83.8	27.3	DM 3810	02 Nov	Soybean	R2	FFi
E6	Central	2013/14	TA	18.4	6.2	12.0	86.6	22.6	DM 4612	10 Nov	Maize	R3	FFi
E7	Central	2013/14	TA	20.8	6.1	14.9	53.9	19.4	DM 3810	22 Oct	Maize	R3	FFi
E8	Central	2013/14	TA	23.0	6.2	9.4	54.2	16.5	DM 4612	12 Oct	Maize	R3	FFi
E9	Central	2013/14	TA	19.4	6.1	25.5	46.3	14.2	DM 4612	12 Nov	Sorghum	R3	FFi
E10	North	2013/14	TA	23.6	6.3	46.9	20.3	22.9	DM 5900	05 Nov	Soybean	R3	Est
E11	South	2012/13	TA	43.4	6.2	13.1	27.1	24.7	DM 3810	12 Nov	Rye-grass (double crop)	R1	FFi
E12	South	2012/13	TA	45.1	5.7	23.2	52.6	33.2	DM 2200	10 Jan	Soybean	R3	Est
E13	South	2013/14	PP	36.6	6.3	17.1	29.0	13.1	DM 3500	12 Dec	Barley (double crop)	R3	FFi
E14	South	2013/14	TA	37.9	6.0	7.6	39.0	17.2	DM 3500	14 Dec	Barley (double crop)	R3	FFi
E15	South	2012/13	TA	44.6	6.3	15.8	22.5	25.1	DM 3500	8 Dec	Barley (double crop)	R1	FF

[a] TA and PP mean Typic Argiudoll and Petrocalcic Paleudoll, respectively.

[b] Sampling date of shoot, leaf and greenness index.

[c] ESt and FFi mean experimental station and farmer field, respectively.

Samples were dried at 30 °C and ground to pass a 2-mm sieve. Recognizable crop residues and roots retained on the 2-mm sieve were eliminated. Soluble and adsorbed S as sulfate ($S\text{-}SO_4^{-2}$) were extracted with $Ca(H_2PO_4)_2$ (Islam and Bhuiyan, 1998) and then determined by turbidimetry through barium chloride ($BaCl_2$) and Tween 80 as a stabilizer (Johnson, 1987). Organic matter (OM) was determined by the method of Walkley and Black (1934). Soil pH was determined at a 1:2.5 (w:w) soil-to-water ratio. Available P was determined following Bray and Kurtz (1945) and NO_3 by the method of Bremner and Keeney (1966).

2.3. Plant sampling and measurements

Crop phenology was monitored using the scale of Fehr and Caviness (1977). Shoot biomass was measured in 0.8 m^2 samples (2 sub samples of 0.4 m^2 per plot) between R1 and R3 (Table 1). At the same time, 20 uppermost fully developed trifoliate leaves (with petiole) were collected from central rows of the plots. After physiological maturity (R8), plants were harvested from a surface of 3.4 m^2 per plot. Soybean yield was adjusted to a standard moisture content of 0.13 kg H_2O kg $seed^{-1}$. Leaf, shoot, and seed samples were dried at 65 °C and ground to pass a 1-mm sieve. Total N and S in samples were determined by dry combustion and thermoconductivity detection with TruSpec CNS analyzer (LECO, St. Joseph, MI, USA).

The greenness index was determined at the same time of plant sampling using a Minolta SPAD-502 (Spectrum Technologies Inc., Plainfield, IL). Measurements were taken in the middle of the central leaflet from leaves of the uppermost fully developed nodes according to Fehr and Caviness (1977). The greenness index (GI) of each plot was the average of 20 readings. The sulphur sufficiency index (SSI) was calculated for each experiment as:

$$\mathrm{SSI} = \frac{\mathrm{GI}}{\mathrm{GI_c}}$$

where GI is the greenness index of the treatments and GI_c is the greenness index of the treatment with the minimum S rate required to maximize seed yield in the experiment (critical greenness index). Treatment with the minimum S rate required to maximize seed yield was calculated by an ANOVA and LSD test (0.05).

2.4. Weather data

There is a scarcity of meteorological stations, but topography, i.e. relatively uniform elevation in a flat region, and climatic characteristics allowed the use of weather data from sparse stations for general weather characterization. Lack of weather records in farmers' fields precluded finer analysis. Weather data of the Northern and Southern regions were obtained from INTA's weather stations at Rafaela and Balcarce. They were located between 0.5 and 12 km from the trials. Radiation and temperature data for the experiments in the Central region where obtained from Pergamino INTA weather stations, placed about 90 km from field experiments. Precipitation data of the Central region was obtained from stations placed between 1 and 15 km from the trials.

2.5. Statistical analyses

An overall ANOVA was performed using the PROC MIXED procedure in SAS for evaluating site and treatment (S rate) effects. Significant differences were determined at 0.05 level using a LSD test.

The relationship between variables was described with linear-plateau models:

$$Y = a + bX \quad \text{if } X \le c \qquad (1)$$

$$Y = a + bC \quad \text{if } X > c \qquad (2)$$

$$Y = a + bX \quad \text{if } X \ge c \qquad (3)$$

$$Y = a + bC \quad \text{if } X < c \qquad (4)$$

where a is the intercept and b is the rate of change in Y during the linear phase and c is the threshold X. Eqs. (1) and (2) were fitted for the relationship between relative seed yield and S_{conc} in leaf, shoot and seed, and for the relationship between the SSI and S_{conc} in leaf.

Eqs. (3) and (4) were fitted for the relationship between relative seed yield and N:S in leaf, shoot and seed and for relating the SSI and N:S in leaf. Linear-plateau models were developed using NLIN procedure of SAS computer software.

Relative seed yield was calculated for each site as the ratio between the yield of each treatment and the yield of the treatment that receives the maximum S rate (40 kg S ha^{-1}).

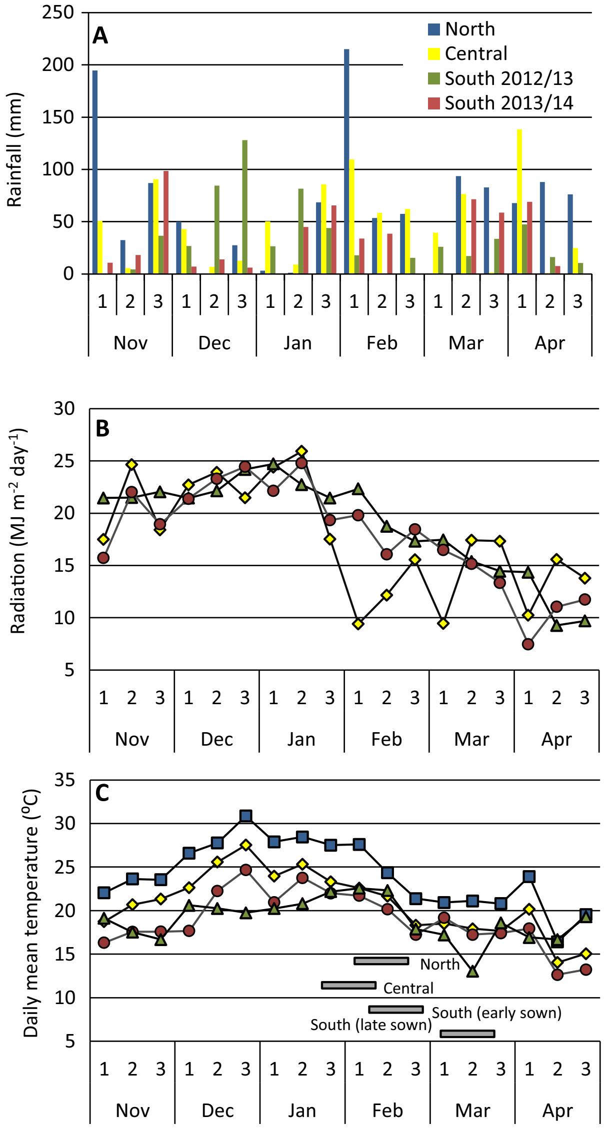

Fig. 1. Rainfall (A), solar radiation (B), and daily mean temperature (C). Colored bars at the bottom of the lower panel indicate the periods between R3 and R5 (critical period for seed yield determination). Data from Northern, Central and Southern regions (season 2013/14) and Southern region (season 2012/13).

3. Results

3.1. Growing conditions

Total precipitation for the period November to April was 1197, 865, 544 and 615 mm for the North, Central, South (2012/13) and South (2013/14), respectively (Fig. 1A). This is 54%, 35% and 13% above the historical median (1989/2009) for the North, Central and South (2012/13). Precipitation during 2013/14 was similar to the historical median for the period November to April. The amount and pattern of precipitation ensured sufficient water availability in the soil in the experiments of the Northern and Central regions during most of the soybean growing season. A period of water scarcity was determined from the second decade of February to the second decade of March during season 2012/13 and from the first decade of December to the second of January during season 2013/14 in the South. This coincided with the stages R5 to R6 (2012/13) and V4 to R1 (2013/14) in timely sown soybean and with crop emergence (2012/13) and R2 to R4 (2013/14) in late sown crops.

Radiation was similar between seasons in the Southern region from November to the first decade of April, when season 2013/14 had lower values (Fig. 1B). From November to late January and mid-March to late April, there were no larger differences in radiation between the Central and Southern regions. Central region had lower radiation during February and the first decade of March (Fig. 1B). Lack of data from the Northern region precluded the analysis of the pattern of radiation.

The average daily mean temperature for the period November to April was 24.1, 20.8, 18.9 and 19.0 °C for the North, Central, South (2012/13) and South (2013/14), respectively (Fig. 1C). This is 8.4%, 0.7%, 4.0% and 3.3% higher than the historical median (1989/2009) for each region or season. The Northern region had higher temperatures than the others regions/seasons during all the growing season. Differences between the Central and South were more marked during November to January and tended to diminish afterwards (Fig. 1C). Greater differences between seasons in the South were determined during the second decade of March, when season 2012/13 was markedly colder than 2013/14 (Fig. 1C).

3.2. Seed yield

On average for each site, soybean seed yield ranged from 1575 to 5338 kg ha^{-1} (Table 2) Maximum yield was found in the Central region whereas minimum yield was for double cropped soybean in the South-eastern region ($P < 0.01$) (data not shown). Sulphur fertilization increased seed yield in 9 out of 15 sites ($P < 0.05$) (Table 2). Experiment × S rate interaction was significant ($P < 0.05$) (Table 2), since the pattern of the response to S fertilization differed among experiments. Responses to S application were determined in experiments of the Central and Southern regions and ranged from 276 kg ha^{-1} (13.2%, compared with S0) to 742 kg ha^{-1} (40.2%, compared with S0). Remarkably, all the double cropped soybeans responded to S fertilization ($P < 0.01$) (23.7%, 40.0%, 14.9% and 13.2% compared with S0 for E11, E13, E14 and E15, respectively). In the experiments with response to S fertilization, seed yield was maximized with the application of 10 (4 experiments), 20 (4 experiments) or 30 (1 experiment) kg S ha^{-1}.

3.3. S and N concentration in leaf, shoot and seed

Sulphur concentration ranged from 1.6 to 3.6 g kg^{-1} in leaf, from 1.0 to 3.1 g kg^{-1} in shoot and from 2.5 to 5.3 g kg^{-1} in seed. This variable increased with the rate of S fertilization in 11, 14 and 11 experiments for leaf, shoot and seed, respectively ($P < 0.05$) (Table 2). In some experiments, the increases in S concentration in plant parts were related to increases in seed yield; whereas in others, the greater S_{conc} represented luxury uptake (Table 2). Compared with S0, maximum increases in S_{conc} were 40.0%, 68.8% and 110.0% for leaf, shoot and seed, respectively.

Nitrogen concentration in leaf, shoot and seed ranged from 29.1 to 48.2 g kg^{-1}, from 20.4 to 44.7 g kg^{-1} and from 53.8 to 65.0 g kg^{-1}, respectively. Sulphur fertilization affected N_{conc} in all plant parts (Table 2). Double cropped soybean showed the greatest increases in N concentration in response to S fertilization (maximum of 34.4%, 25.4% and 12.3% compared with S0 for leaf, shoot and seed, respectively) (Table 2). In some experiments, however, N_{conc} decreased when S was added. This effect was observed in shoot (E3, E4, E9 and E12; up to −12.4%, compared with S0) and in seed (E7; up to −2.3% compared with S0) (Table 2). N:S decreased in response to S fertilization and differences among treatments were observed in the same plant parts and experiments in which S_{conc} was also affected (data not shown).

The analysis of pooled data from all the experiments showed that N concentration increased ($P < 0.01$) with S_{conc} in leaf (Fig. 2A) and shoot (Fig. 2B), but these two variables were not associated in seed ($P = 0.14$) (Fig. 2C). N:S decreased with S_{conc} ($P < 0.01$) in leaf (Fig. 2D), shoot (Fig. 2E) and seed (Fig. 2F). The association between N:S and S_{conc} in seed was greater than in leaf and shoot.

3.4. Relation between relative seed yield and S status (S concentration and N:S ratio)

The critical thresholds for S concentration in leaf, shoot and seed were 2.65, 2.06 and 3.93 g S kg^{-1}, respectively, with no overlapping among their confidence intervals (Fig. 3A–C). Contrarily, the critical values for N:S in leaf, shoot and seed were similar, with values of 13.90 (Fig. 3D), 12.18 (Fig. 3E) and 13.5 (Fig. 3F), respectively.

Relative seed yield was strongly associated with N:S in leaf and shoot (Fig. 3D and E) and weakly related to S_{conc} in leaf, shoot or seed (Fig. 3A–C) and with N:S in seed (Fig. 3F).

3.5. Use of SSI to evaluate S concentration and N:S in leaf and relation with seed yield

Sulphur sufficiency index was related to leaf S_{conc} (Fig. 4A) and, more strongly, with leaf N:S (Fig. 4B) ($P < 0.01$). Regression analysis was conducted only for leaf because measurements of SSI were taken on this plant part. S concentration Moreover, relative seed yield was associated with SSI ($P < 0.01$) (Fig. 4C). Interestingly, the thresholds of the function that relates SSI with leaf S_{conc} and N:S (2.89 g S kg^{-1} and 15.1, for S_{conc} and N:S, respectively) were similar to the critical values we proposed for the relation between relative seed yield and S_{conc} or N:S in leaf (2.65 g S kg^{-1} and 13.90, for leaf S_{conc} and N:S, respectively). Similarity of the thresholds was supported by the overlapping of the confidence intervals (Figs. 3A and 4A for leaf S_{conc} and Figs. 3D and 4B for leaf N:S).

4. Discussion

4.1. Seed yield

Yield of timely sown soybean in the Central region was higher than in the Southern region. Greater rainfall and higher temperature during the critical period for yield determination (R3–R6 stages) in the former region explained these differences (Fig. 1). This coincides with Calviño and Monzón (2009) characterization of these environments. Low yield of double-cropped soybean in the South is a consequence of a short season with water deficit during most of the crop cycle and low temperatures during seed filling (Calviño et al., 2003). In our study, cold temperatures during the second decade of March (Fig. 1) likely reduced yield of late sown soybean in the Southern region in 2012/13.

Table 2

Seed yield, S and N concentration in leaf, shoot and seed of treatments fertilized with different S rates. Different letters indicate differences between S rates at $P < 0.05$ using Fisher protected LSD.

Experiment	S rate (kg ha^{-1})	Seed yield (kg ha^{-1})	S (g kg^{-1})			N (g kg^{-1})		
			Leaf	Shoot	Seed	Leaf	Shoot	Seed
E1	0	3602a	2.7c	2.1b	3.1	42.2	40.7a	55.5a
	10	3947a	3.3b	2.3b	3.4	45.6	44.7a	56.3a
	40	3647a	3.6a	2.8a	3.9	48.2	40.6a	56.4a
E2	0	2705b	2.5b	1.8a	3.3	40.7	29.2a	60.2a
	10	3375a	2.9ab	2.5a	3.4	44.8	31.0a	60.0a
	40	3408a	3.2a	2.4a	4.1	43.6	30.6a	61.5a
E3	0	3175c	2.8a	2.2a	3.3	44.7	32.2a	56.3a
	10	3374b	3.0a	2.4a	3.7	47.7	32.9a	56.4a
	20	3451ab	3.1a	2.3a	3.7	45.4	29.7ab	54.8a
	30	3496ab	3.2a	2.4a	3.7	43.2	31.7ab	54.8a
	40	3548a	3.2a	2.2a	4.0	45.9	27.4b	56.7a
E4	0	2867b	2.6d	2.0ab	2.6	39.6	23.3ab	56.8a
	10	3219a	3.0c	2.0b	3.9	40.8	22.3ab	56.5a
	20	2981ab	3.0c	2.4a	3.7	39.9	24.6a	56.6a
	30	3068ab	3.2b	2.3ab	4.0	39.4	21.2ab	55.9a
	40	2993ab	3.3a	2.3ab	4.0	40.5	20.4b	56.6a
E5	0	3294a	2.9b	2.0b	4.3	40.1	25.9a	58.6a
	10	3158a	2.9b	2.0b	5.0	38.5	26.2a	58.8a
	20	3216a	2.9b	2.0b	5.1	36.3	25.6a	58.5a
	30	3160a	3.1ab	2.2a	4.9	38.2	24.2a	58.5a
	40	3159a	3.2a	2.2a	5.0	40.5	26.3a	58.1a
E6	0	3271a	2.7b	2.1c	4.1	35.1	27.4a	58.3a
	10	3264a	2.8b	2.3bc	4.0	34.8	25.6a	58.2a
	20	3415a	3.0a	2.4ab	4.5	35.5	27.6a	58.3a
	30	3233a	3.1a	2.4ab	4.6	35.5	27.3a	58.0a
	40	3395a	3.0a	2.6a	5.0	35.9	26.4a	58.7a
E7	0	5106cd	2.6a	1.7b	4.1	41.5	25.9a	57.3ab
	10	5083d	2.7a	2.0a	4.5	41.3	25.5a	57.2ab
	20	5637a	2.8a	1.9ab	4.5	38.4	25.6a	56.9ab
	30	5359ab	2.6a	2.1a	4.7	37.8	25.5a	57.8a
	40	5222bc	3.0a	2.1a	4.5	40.0	26.2a	56.0b
E8	0	4068a	2.4c	2.0d	4.8	33.5	26.3a	54.6a
	10	4235a	2.5bc	1.9cd	5.0	34.2	23.4a	54.7a
	20	4084a	2.5bc	2.2bc	4.7	35.2	24.8a	54.5a
	30	4201a	2.6b	2.3ab	5.2	35.1	24.6a	55.4a
	40	4006a	3.1a	2.4a	5.3	36.7	24.2a	55.3a
E9	0	4268b	2.9b	1.8c	3.7	48.0	29.0a	57.3a
	10	4382b	3.2a	2.0c	4.5	46.0	27.4ab	58.4a
	20	4419b	3.3a	2.3b	4.5	47.5	27.0b	57.2a
	30	4515ab	3.3a	2.4ab	4.3	47.2	28.2ab	56.9a
	40	4779a	3.1ab	2.4a	4.8	39.6	27.4ab	58.1a
E10	0	3341a	2.7ab	2.4b	4.3	37.7	26.1ab	56.3a
	10	3352a	2.7ab	2.5ab	4.3	36.3	27.2a	56.7a
	20	3628a	2.6b	2.4b	4.6	37.3	25.0b	57.4a
	30	3522a	2.7ab	2.6a	4.6	37.6	25.2b	56.9a
	40	3549a	2.8a	2.4b	4.7	39.2	27.9a	56.8a
E11	0	2715b	2.9a	2.1d	2.5	45.1	33.7a	53.8a
	10	3167ab	3.0a	2.7c	3.1	45.1	35.1a	56.1a
	20	2993ab	3.1a	2.9b	3.5	44.5	35.4a	55.7a
	30	3359a	3.0a	2.8bc	3.6	43.0	34.9a	55.7a
	40	3148ab	3.0a	3.1a	3.8	44.3	35.9a	55.7a
E12	0	1601a	2.8a	2.9a	4.1	39.9	40.7ab	64.7a
	10	1449a	2.9a	2.8a	4.1	41.6	40.8a	65.0a
	40	1677a	2.9a	3.0a	4.4	42.4	38.5b	64.6a
E13	0	1853c	1.6b	1.5c	3.1	32.0	26.3c	55.4c
	10	2234b	2.2a	1.5c	3.8	39.4	28.0c	59.5b
	20	2289ab	2.4a	1.9b	4.4	40.7	28.4bc	60.9ab
	30	2309ab	2.6a	2.1ab	4.2	42.3	31.1ab	61.5ab
	40	2595a	2.7a	2.3a	4.5	43.0	32.3a	62.2a
E14	0	2104c	2.4b	2.0b	3.8	39.4	30.7a	62.2b
	10	2195bc	2.6ab	2.6b	4.1	43.1	33.0a	63.4ab
	20	2324ab	2.9a	2.5ab	4.3	43.7	32.4a	64.1a
	30	2207bc	2.8ab	2.3b	4.3	41.1	31.4a	64.5a
	40	2417a	2.8a	2.6b	4.4	41.6	32.8a	63.9a

Table 2 (*Continued*)

Experiment	S rate ($kg\,ha^{-1}$)	Seed yield ($kg\,ha^{-1}$)	S ($g\,kg^{-1}$)			N ($g\,kg^{-1}$)		
			Leaf	Shoot	Seed	Leaf	Shoot	Seed
E15	0	2091b	1.7c	1.0d	2.7	29.1	23.2b	54.5b
	10	2208a	2.0b	1.6c	3.5	32.3	27.8a	57.5a
	20	2207a	2.3a	1.8b	3.9	34.6	29.1a	57.6a
	30	2250a	2.3a	2.1a	3.9	33.9	29.0a	56.9a
	40	2367a	2.2a	2.0ab	4.1	31.9	29.0a	56.3a
Mean	0	3176	2.5	1.9	3.6c	3.9a	2.8	5.7
	10	3371	2.8	2.2	4.0b	4.1a	2.8	5.8
	20	3387	2.8	2.3	4.3a	4.0a	2.8	5.8
	30	3390	2.9	2.3	4.3a	4.0a	2.8	5.8
	40	3327	3.0	2.5	4.4a	4.1a	2.9	5.9
S rate		<0.01	<0.01	<0.01	<0.01	0.14	0.47	<0.01
Experiment		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
S rate × Experiment		0.02	<0.01	<0.01	0.48	0.30	<0.01	0.04

Responses to S application were determined in experiments of the Central and Southern region, according to previous research in these areas (Gutierrez Boem et al., 2007; Salvagiotti and Miralles, 2008; Reussi Calvo et al., 2011; Pagani and Echeverría, 2011). Responses were more frequent in double cropped soybean (Table 2), likely associated with the removal of available SO_4^{-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. In the experiments with response to S fertilization, maximum seed yield was observed with the application of 10–30 kg S ha^{-1}. The yield plateau at higher S rates is important for calibrating indexes of S status because confirms that experiments have S-sufficient treatments. This is not possible if only two S treatments were tested (no S addition and one S rate).

4.2. Patterns of S and N concentration in leaf, shoot and seed

Increases in S concentration in leaf, shoot and seed in response to S fertilization (Table 2) provide evidence that these variables could be used as indexes of S availability. Contrarily, it was not possible to determine a general pattern for N_{conc} in plant parts in response to S rate (Table 2). A review by Divito and Sadras (2014) provides robust evidence of the relationship between shoot S_{conc} and N_{conc} in legumes under a gradient of S availability. They reported that N_{conc} diminishes at a lesser extent than S_{conc} when S becomes limiting for plant growth. However, increases in shoot N_{conc} were reported in some cases under moderate S deficiency as a consequence of a larger reduction in shoot growth than in N uptake rate (Scherer and Lange, 1996). A similar pattern was also reported for moderated

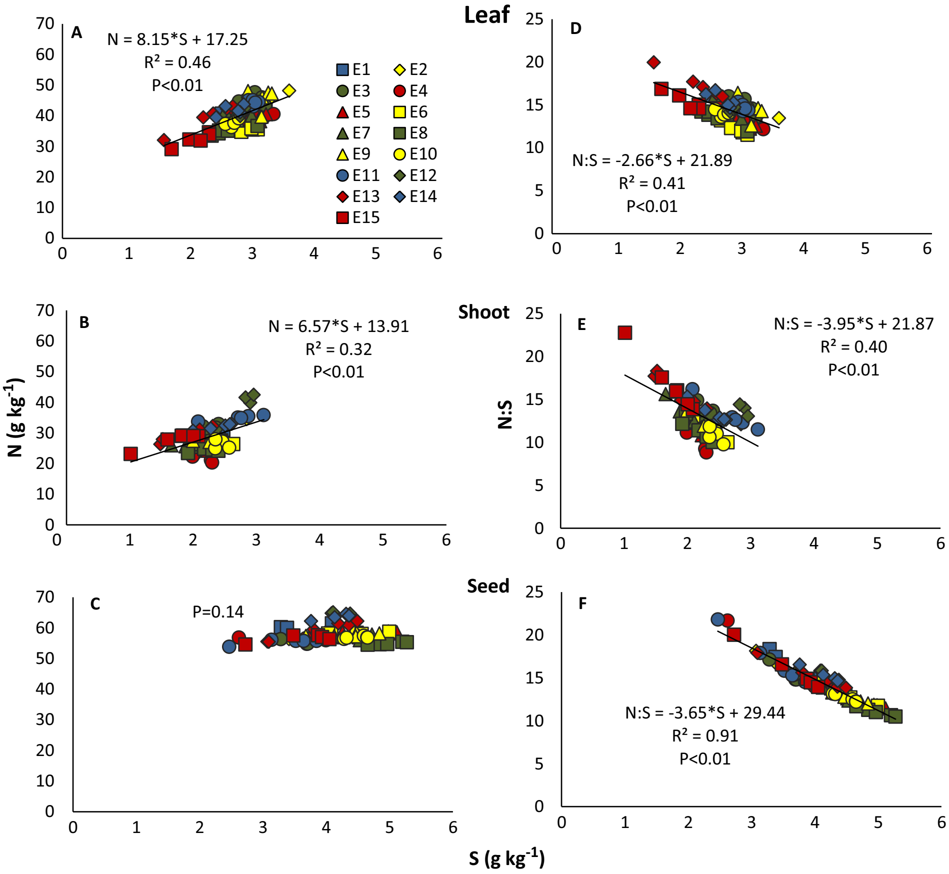

Fig. 2. Relationship between N concentration and S concentration in leaf (A), shoot (B) and seed (C) and between N:S ratio and S concentration in leaf (D), shoot (E) and seed (F). Data from 15 experiments each one with three to five S rates. *P* indicates significance of regression.

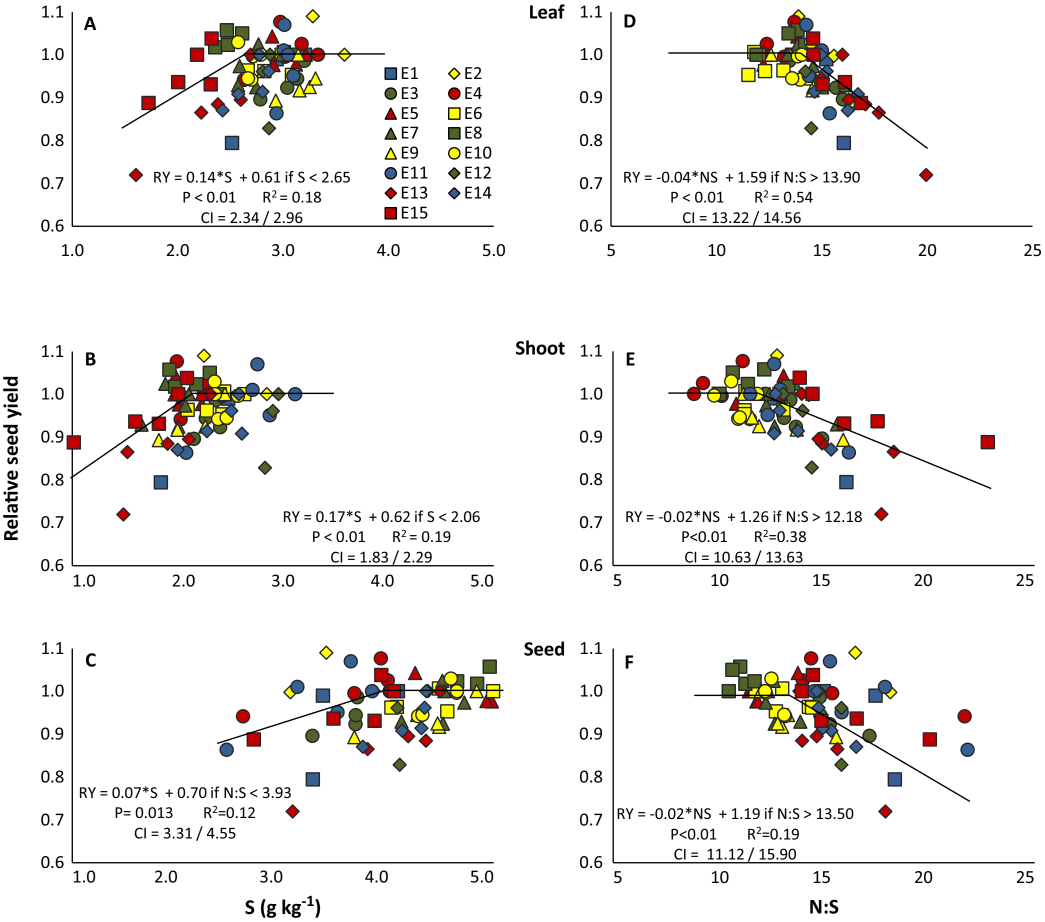

Fig. 3. Relationship between relative seed yield and S concentration in leaf (A), shoot (B) and seed (C) and between relative seed yield and N:S ratio in leaf (D), shoot (E) and seed (F). RY is the relative seed yield. CI is the confidence interval of the critical threshold (0.95). *P* indicates significance of regression. Data from 15 experiments each one with three to five S rates.

phosphorus deficiencies by Sulieman et al. (2013). In our experiments, variations in N:S ratio were mainly associated with changes in S_{conc} rather than with variations in N_{conc} in all plant parts. In seed, the N:S ratio was largely unresponsive to S supply.

The analysis of pooled data showed a pattern for N concentration in leaf and shoot in response to S concentration that could not be obtained when the experiment were analyzed individually (Table 2). So, in the pooled data, variations in S_{conc} and N_{conc} could be consequence of other causes besides the availability of S in soils. Soybean cultivar, environmental conditions or the phenological stage at sampling could affect S or N concentration in leaf or shoot. From a recent published paper by Bender et al. (2015) we calculated that modern soybean cultivars had a low depletion of N_{conc} and S_{conc} from R2 to R4 (34.4–32.7 and 2.4–2.2 g kg^{-1} for N_{conc} and S_{conc}, respectively). This indicates that sampling date within this range introduces low variation to the concentration of N and S in shoot. In contrast, Fontanive et al. (1996) and Sexton et al. (1998) reported reductions in S_{conc} with increasing shoot mass in the upper fully developed leaf and in aboveground biomass, respectively. However, the relevance of Fontanive et al. (1996) data is uncertain as they grew plants in pots, and the nutrient:biomass allometry of individual plants does not reflect that in field crops (Gastal et al., 2015). In our experiment, N_{conc} and S_{conc} in leaf and shoot were not associated with the phenological stage at sampling (data not shown). This could be consequence of a low depletion with increasing biomass or could be related to possible confounded effects among sampling date, environment and cultivars.

When sources of variation other than S availability affected S_{conc} in leaf and shoot, a concomitant change in N_{conc} was observed (Fig. 2A and B). These parallel change in S_{conc} and N_{conc} is the cause of the greater stability of the N:S ratio compared to S_{conc} (Dijkshoorn and van Wijk, 1967; Black Kalff et al., 2002). The low variation of N_{conc} in seed across a wide range of S_{conc} is in accordance with reports by Salvagiotti et al. (2012). In addition, this pattern agrees with results reported by Scherer (2001), who compiled information from various legumes and determined that total protein in seeds was slightly affected under S deficiency in comparison with the great decrease in the S-amino acids cysteine and methionine. Significant changes in amino acids composition in seeds but not in leaf or shoot is attributed to the fact that leaf proteins are mainly functional, while seed proteins are mainly for storage (Randall and Wrigley, 1986).

N:S requires two chemical determinations and compounds the two variances. Nonetheless, the N:S ratio in leaf or shoot reduced the variability of S_{conc} attributed to sampling date, cultivar or environment because of the concomitant variation of S_{conc} and N_{conc} (Fig. 2A and B). This is an advantage for diagnosing the S status. By contrast, in seeds, the stability of N_{conc} with variation in S_{conc} (Fig. 2C) indicates no benefits for using N:S instead of S_{conc}.

4.3. Critical thresholds for S sufficiency in leaf, shoot and seed

The threshold we propose for S concentration in leaf is lower than the value reported by Kaiser and Kim (2013) (3.1 g S kg^{-1}).

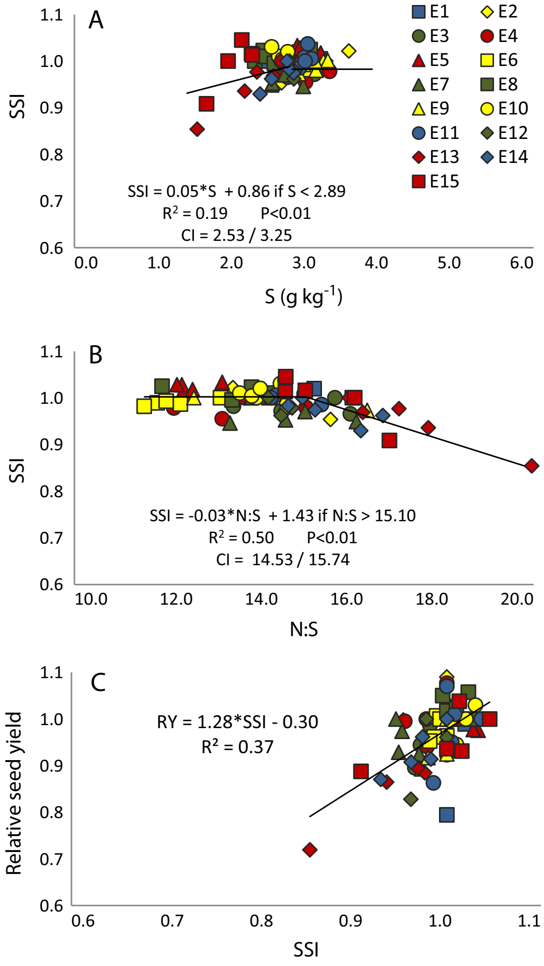

Fig. 4. Relationship between S sufficiency index (SSI) and S concentration in leaf (A), between SSI and N:S ratio in leaf (B), and between relative seed yield and SSI (C). RY is the relative seed yield. CI is the confidence interval of the critical threshold (0.95). *P* indicates significance of regression. Data from 15 experiments each one with three to five S rates.

Agrawal and Mishra (1994) determined lower critical values (ca. 2.0 g S kg^{-1}) for soybean crops sampled at flowering. This value, however, is in the range of S concentrations observed in the severe S-deficient treatments of our experiments.

The critical value we identified for S_{conc} in shoot (2.06 g S kg^{-1}) is lower than 2.7 g S kg^{-1}, the threshold proposed by Kaiser and Kim (2013). Most of our S-sufficient treatments had a S_{conc} in shoot lower than the threshold reported by these authors (Fig. 3B).

For seed S_{conc}, the critical value was higher than those proposed by Kaiser and Kim (2013) (3.3 g S kg kg^{-1}) and Salvagiotti et al. (2012) (2.7 g S kg^{-1}). However, Salvagiotti et al. (2012) indicated that this index did not have an acceptable performance given that several treatments with a relative yield below 90% had a seed S_{conc} greater than the critical value. These observations support the use of a higher threshold.

The threshold we determined for N:S in leaf is lower than that reported by Agrawal and Mishra (1994), who proposed a critical value of 16.0 for leaf sampled at flowering in field experiments. Hitsuda et al. (2004) also determined greater values for the N:S in leaf (from ≈16 to 24) but with no association with the relative seed yield of soybean plants growing in pots. Early reports of Dijkshoorn and van Wijk (1967) proposed to use a theoretical N:S ratio of ≈17 for assessing the S status of plants based on the protein-N:protein-S ratio observed in legumes shoots. As far as we know, no empirical N:S thresholds were reported for shoot of field-grown soybean.

The critical threshold we calculated for N:S in seed is lower than the value proposed by Salvagiotti et al. (2012) (22.0). This discrepancy is due to the lower S_{conc} and similar N_{conc} determined by those authors compared to our results. However, Salvagiotti et al. (2012) indicated that the N:S ratio was not suitable for S status diagnosis of soybean crops because ≈64% of the S deficient treatments had a N:S lower than 22.0. This indicates that a lower threshold for the N:S ratio in soybean seeds as the one indicated in our work is likely better at predicting the S status.

Other authors determined critical values for seed analysis of 13.0 and 17.0 for wheat (Randall et al., 1981; Reussi Calvo et al., 2011) and 14.0 for rice (Randall et al., 2003). Biological N fixation commonly ensures adequate N nutrition in legumes reducing variations in N_{conc} (Leffel et al., 1992). Contrarily, N_{conc} of cereals commonly depends on the provision of N from the soil and external sources (i.e. fertilizers, amendments) which can cause disproportionate accumulations of N in shoot. Thus, the performance of N:S as indicator of the S status would be better in legumes than in cereals because of the smaller variation in N_{conc}.

Relative seed yield was weakly related with S_{conc} in leaf ($R^2 = 0.18$), shoot ($R^2 = 0.19$) and seed ($R^2 = 0.12$). The association was noticeably improved by using N:S in leaf ($R^2 = 0.54$) and shoot ($R^2 = 0.38$) but not in seed ($R^2 = 0.19$). Remarkably, N:S in leaf showed the best performance, which was also reflected in the narrow range of the confidence interval for the critical threshold. An index integrating the concomitant variation of N_{conc} and S_{conc} in leaf and shoot over a large range of growing conditions reduced the variability attributed to sampling date, cultivar or environment. The stability of seed N_{conc} with variations in S_{conc} indicated no benefits for using N:S instead of S_{conc} in seed.

The N:S thresholds were similar for leaf, shoot and seed (Fig. 3D–F), indicating that N:S removed differences in S_{conc} among these plant parts (Fig. 3A–C). N and S vary concomitantly among plant parts because both nutrients are mainly related to protein content and variations in S-containing amino acids are small (Schnug, 1997).

4.4. *Use of SSI to evaluate S status of soybean crops*

The SSI allowed the characterization of the S status of soybean crops (Fig. 4A and B). S deficiencies reduce S-adenosyl methionine concentration, which is involved in chlorophyll synthesis (Hoefgen and Nikiforova, 2008). This supports the link between S status of plants and leaf greenness. Sulphur deficiency also increases leaf thickness, which is related to increases in the greenness index (Knops and Reinhart, 2000; Jifon et al., 2005). This effect was broadly reported for N (Peng et al., 1993). However, increases of leaf thickness are lower than decreases in chlorophyll content, which allows the use of the greenness index or the SSI to characterize deficiencies of these nutrients. Accordingly, Hitsuda et al. (2004) and Zhao et al. (2008) reported differences in the greenness index among soybean plants growing in pots under a gradient of S supply. As far as we know, we present the first assessment for using a greenness index under field conditions.

The thresholds of the function that relates the SSI with leaf S_{conc} and N:S were similar to the critical values we proposed for the relation between the relative seed yield and S_{conc} or N:S in leaf. Similarity between thresholds was more relevant for N:S because it has a narrower confidence intervals. This indicates that the greenness index is sensitive to changes in plant S status in the range of the proposed thresholds for leaf analysis. This relation should be validated with a large data set including more S responsive sites.

5. Concluding remarks

We determined critical thresholds for the diagnostic of the S status of soybean crops over a large range of growing conditions as driven by soil, weather and agronomy. These were 2.65, 2.06 and 3.93 g S kg^{-1}, for leaf, shoot and seed, respectively. For the same plant parts, the N:S critical thresholds were 13.90, 12.18 and 13.50, respectively. The performance of N:S was better than that of S_{conc} in leaf and shoot. Relative seed yield was weakly associated with S_{conc} and N:S in seed. Thus, we propose the use of N:S in leaf for in-season assessment of S-status.

N:S ratio in leaf and shoot removed variation attributed to sampling date, cultivar or environment. Contrarily, the stability of seed N_{conc} with variations in S_{conc} indicates no benefits for using N:S instead of S_{conc} in seeds. The SSI was sensitive to changes in the S status of the plant.

The thresholds we proposed should ideally be validated on an independent set of data. Further research should focus on the evaluation of the performance of leaf sampling and SSI at early developmental stages with the objective of avoiding S deficiencies throught early fertilization.

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