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N:P:S stoichiometry in grains and physiological attributes associated with grain yield in maize as affected by phosphorus and sulfur nutrition

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ABSTRACT

Balanced nutrition is necessary to reduce yield gaps in maize. Simultaneous phosphorus (P) and sulfur (S) deficiencies may be present in soils, so a P × S interaction is expected. In maize, yield is closely related to grain number (GN); thus, nutrient deficiencies impacting crop growth during GN formation can consequently impact yields. Grain nutrient concentration may reflect soil supplying capacity, and nutrient stoichiometry in grains can be used as an indirect indicator of nutrient deficiency for a retrospective diagnosis of sites responsive to P or S fertilization. The objectives of this study were to: i) determine maize response to increasing S fertilizer rates and to P addition; ii) analyze the effects of P, S, and their interaction on mechanisms involved in yield determination of maize; and iii) evaluate the effects of P and S shortage on stoichiometric relationships among N, P, and S content in maize grains. Two fertilization experiments were conducted on-farming conditions in 19 sites-year (SY) for analyzing grain yield response to increasing S fertilizer rates (E1), and studying the interaction between P and S fertilization on grain yield and physiological attributes associated with grain yield determination (E2), *i.e.* CGR_{CP}, $IPAR_{CP}$, RUE_{CP} and biomass at R6. Also N (%N), S (%S) and P (%P) concentration in grains were determined. Stoichiometric relationships among N, P and S in both P or S fertilized and unfertilized treatments in all SY were analyzed. Average grain yield response due to S and P addition was *ca.* 13 and 20%, respectively. Grain yield increased up to S fertilizer rates around 10 kg S ha^{-1}. P addition increased CGR_{CP} by 15–60% in 8 SY while S addition 12–16% in 2 SY. Both, RUE_{CP} and $IPAR_{CP}$ were positively associated with biomass production. P fertilization increased $IPAR_{CP}$ by 4%, but no S effect was observed. Before silking, P addition boosted cumulated radiation by 7%, but after silking no P or S effects were observed. A significant P × S interaction was observed for RUE_{CP}, since S fertilization increased RUE_{CP} by 14% only when P was not added. Independently of P or S shortage, grain N content scaled almost isometrically with grain S content, while N:P and P:S showed allometric relationships. Phosphorus deficiency did not modify N:S, N:P nor P:S stoichiometry. Likewise, S addition did not modify the N:P or N:S stoichiometry. A significant change in the intercept of the P:S relationship was observed in response to fertilization and may be used as a tool for identifying S responsive sites using grain nutrient analysis.

1. Introduction

Maize is the main cereal planted in the world, representing 26 and 37% of total cereal harvested area and production, respectively (FAO, 2013). Grain yield potential in maize may be close to 22–23 Mg ha^{-1} (Duvick and Cassman, 1999), however, variable yield gaps are observed in agricultural systems since several biotic and abiotic factors may reduce the maximum attainable yield in each particular field, *i.e.* the incidence of weeds, pests or diseases, water dynamics in the soil-plant-atmosphere system and/or nutrient availability (van Ittersum et al., 2013). Nutrient and water availability showed to be determinants for closing yield gaps, with different relative importance, depending on specific deficiencies

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characteristic of each agricultural system (Vitousek et al., 2009; Mueller et al., 2012; Ciampitti and Vyn, 2014).

An increase in yield gap due to nutrient shortage in a particular site depends on which nutrient is actually deficient, its mobility in the soil and crop demand, which is closely associated with grain yield. In maize, reports of nitrogen (N), potassium (K) and phosphorus (P) deficiency in agricultural systems have been widely reported (Bordoli and Mallarino, 1998; Dodd and Mallarino, 2005; Edmonds et al., 2013; Laboski et al., 2008; Salvagiotti et al., 2011). However, the effects of other nutrients on crop response to fertilization had less attention. Rego et al. (2007) showed response to the addition of micronutrients and sulfur (S) in low fertility soils. Response to S fertilization has been also reported in temperate areas. Under no tillage conditions and in loam soils in Minnesota, Rehm (2005) found increases up to *ca.* 11% in grain yield in maize. In the Pampas region of Argentina, in soils with many years of continuous cropping, partial loss of soil surface horizons through erosion, and reduced organic matter content, seed yield increased up to *ca.* 10% due to S addition in soybean and in doubled wheat-soybean crop (Gutierrez-Boem et al., 2007; Salvagiotti et al., 2004; Salvagiotti et al., 2012). Similar relative increases were determined in maize in the southern pampas (Pagani et al., 2012).

Under field conditions, many times two or more nutrients are simultaneously deficient, and thus, an interaction in fertilization response is expected. However, few studies have addressed nutrient interactions in the determination of grain yield. Salvagiotti et al. (2009) showed that response to S fertilization was strongly associated with N fertilization in wheat. On the contrary, Pagani et al. (2012) reported no interaction between N and S fertilization in maize.

Biomass production and its partition to reproductive sinks determine grain yield (van der Werf, 1996). Crop growth rate (CGR) depends on the ability of the canopy to intercept incoming photosynthetically active radiation (IPAR) and convert this radiation into new biomass, *i.e.* radiation use efficiency (RUE) (Sinclair and Muchow, 1999). In maize, grain number (GN) is closely associated with crop growth around silking stage (Cirilo and Andrade, 1994; Uhart and Andrade, 1995). Then, nutrient deficiencies affecting growth around this phenological stage may decrease GN and thus, grain yield. In wheat, Salvagiotti and Miralles (2008) showed that IPAR was more sensitive than RUE to varying S rates, as was also found by Plenet et al. (2000a,b) analyzing P deficiency in maize. These results suggest that under P or S deficiency, RUE is a conservative component of biomass production. Even when both nutrients may appear deficient, a comprehensive study of P × S interaction on grain yield in maize has not been made.

Grain nutrient concentration may reflect soil supplying capacity (Dobermann et al., 1996a,b), and thus it can be used as an indirect index of nutrient deficiency. Then, grain concentration can be used as a retrospective diagnosis for identifying responsive sites to fertilization. This is important especially for less mobile nutrients in soils, in which a building and maintenance fertilization strategy is planned. Different approaches may be used to analyze grain nutrient content for identifying responsive sites to fertilization, like nutrient stoichiometry. This methodology considers the relative proportions of elements (*e.g.* nutrients) in living organisms and have been used for characterizing allometry of nutrients in plants (Niklas and Cobb, 2006; Sadras, 2006). Deviations from optimal allometric relationships among nutrients may be used for detecting nutrient imbalances in crops. In soybean, Salvagiotti et al. (2012) identified S – responsive sites by analyzing seed S and N content using stoichiometric relationships. These authors found isometric relationships among N, P and S content in seeds, and a shorter accumulation of S as related with N in S-deficient sites. It is expected to find that P or S shortage would modify P:S stoichiometry in maize, and thus serve as a method for detecting P or S responsive sites.

The objectives of this study were to: i) determine maize response to increasing S fertilizer rates and to P addition; ii) analyze the effects of P, S, and their interaction on mechanisms involved in yield determination of maize; and iii) evaluate the effects of P and S shortage on stoichiometric relationships among N, P, and S content in maize grains.

2. Material and methods

2.1. Field experiments

Two fertilization experiments were conducted on-farm conditions in 19 sites in three growing seasons (*i.e.* 19 site-years, SY, Table 1), on Argiudoll and Hapludoll soils in the Argentinean Pampas region, between 32 and 35° Lat S from North to South, and from 62 to 60° Long W from West to East (Fig. 1).

Experiment 1 (E1–19 SY) was designed for analyzing grain yield response to increasing S fertilizer rates, and Experiment 2 (E2–18 SY) was planned for studying the interaction between P and S fertilization on grain yield, its numerical components and some physiological attributes associated with grain yield determination.

In E1, four S fertilizer rates (0, 7.5, 15 and 30 $kg\,S\,ha^{-1}$) were applied at planting using calcium sulphate as fertilizer (18% S). Triple superphosphate (20% P) at a rate of 150 $kg\,ha^{-1}$ was applied in order to avoid P deficiency. In E2, a P × S factorial experiment consisting of four treatments: P_0S_0, $P_{30}S_0$, P_0S_{30} and $P_{30}S_{30}$. In each treatment, subscript indicates nutrient rate, *i.e.* 0 or 30 kg of P or S ha^{-1} and fertilizers were the same as those used in E1. In both experiments, P fertilization was done at planting placing fertilizer in a band *ca.* 5 cm below and to the side of the seed. Sulfur fertilizer was applied broadcast just after planting

In both experiments, E1 and E2, treatments were arranged in a randomized complete block design with four replicates. All treatments received 150 $kg\,N\,ha^{-1}$ as urea at planting (46% N). In all cases, crop management followed current farmer technology.

2.2. Measurements

In each SY, a composite soil sample was taken to a depth of 20 cm before planting. Carbon content (Walkley and Black, 1934), Phosphorus P Bray I (Bray and Kurtz, 1945), S-sulphate soil content (Chesnin and Yien, 1950) and soil texture (*i.e.* clay, silt and sand proportion) by pipet method (Gee and Bauder, 1986) were determined. Carbon content was converted to organic matter (OM) using a conversion factor of 1.724.

At each SY, in each experimental unit from E2, above ground biomass was determined in a 0.5 m^2 area including 4 or 5 plants (depending on plant density at each SY), within the central five rows of each plot at R1 stage (Hanway and Ritchie, 1984), at around 20 days before ($R1_{-20}$) and after ($R1_{+20}$) R1 (*i.e.* critical period) and also at physiological maturity (R6 stage). Maize plants were clipped at the soil surface and samples were oven dried at 60 °C for 72 h and weighed. At R6, grains and vegetative structures were separated and harvest index (HI) was estimated as the relationship between grain and total aboveground biomass.

Crop growth rate (CGR_{CP}, biomass increase per unit area per day) for the period around R1 (*i.e.* critical period (CP)) was estimated as the slope of cumulated biomass and days after emergence using the three consecutive harvests ($R1_{-20}$, R1 and $R1_{+20}$).

In $R1_{-20}$, R1 and $R1_{+20}$, incident (I_0) and transmitted (I_t) radiation was measured using a line quantum sensor in eight SY (SYs 2, 5, 8, 9, 10, 11, 14, 15). Five determinations of I_t were obtained per experimental unit by placing the sensor at the soil surface and diagonally across center rows (Gallo and Daughtry, 1986), at around noon on totally sunny days. Incident radiation (I_0) was measured

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

Site description				Soil analysis at planting (0–20 cm)								Crop management				
Site–Year	Location	Season	Soil type	Bray1 P mg kg^{-1}	S-SO_4	pH	OC g kg-1	Clay %	Silt	Sand	Planting date	Plantdensity pl ha^{-1}	YCC	Previouscrop	Tillage	Hybrid
1	San Jerónimo	2003–04	VA	13.2	13.5	5.6	16.8	16.4	79.0	4.6	11-Sep	80,000	>50	Sb	NT	Cargill 280
2	Monje	2003–04	TA	11.8	8.2	5.7	12.8	14.4	81.8	5.9	11-Sep	80,000	>30	Sb	NT	NK 940
3	Oliveros	2003–04	TA	21.1	5.7	5.5	11.0	16.2	78.8	5.0	23-Sep	70,000	>10	Sb	NT	NK Siroco TDMax
4	Wheelwright	2003–04	TA	6.3	12.5	6.2	12.8	16.0	67.2	16.7	10-Oct	80,000	>20	Sb	NT	Dekalb 682 MGCL
5	Arroyo Dulce	2003–04	TA	7.3	5.3	5.9	9.3	19.0	58.0	23.1	29-Sep	80,000	>20	Sb	NT	Nidera Ax 840
6	El Dorado	2003–04	TH	11.4	12.0	5.6	18.6	8.0	44.3	47.7	14-Oct	72,000	1	Sb	CT	Pioneer 32G63
7	Junín	2003–04	TH	11.9	8.0	5.6	17.4	14.4	41.4	44.2	14-Oct	80,000	>20	Mz	NT	Pioneer 30R76
8	San Jerónimo	2004–05	VA	7.2	7.2	5.8	16.2	19.4	78.6	2.0	8-Sep	80,000	>30	Sb	NT	AW 190
9	Aldao	2004–05	TA	9.3	7.0	5.6	15.7	20.0	79.6	0.4	9-Sep	80,000	>20	Sb	NT	H 2750
10	Wheelwright	2004–05	TA	10.1	12.0	5.9	20.3	22.6	59.5	17.9	7-Sep	80,000	12	Wh/Sb	NT	Dekalb 696 MGMAV
11	Pergamino	2004–05	TA	5.2	7.6	5.8	16.2	21.8	63.9	14.3	14-Sep	80,000	>20	Sb	NT	Nidera Ax 882
12	Junín	2004–05	TH	6.0	8.9	5.4	13.9	13.0	29.8	57.2	22-Sep	76,900	>20	Wh/Sb	NT	Pioneer 30R76
13	O'Higgins	2004–05	AT	4.9	10.6	5.5	13.9	42.7	42.2	15.0	28-Sep	70,000	10	Ba/Sb	CT	Dekalb752 MGCLR5
14	Maciel	2005–06	AT	5.7	4.4	5.4	13.9	20.0	73.5	6.5	2-Sep	85,000	30	Sb	NT	NK900 TDMax
15	Cañada Rica	2005–06	VA	5.0	4.5	5.6	14.5	20.0	69.8	10.2	16- Sep	80,000	30	Sb	NT	AX882 MG
16	Colon	2005–06	TA	6.1	10.5	5.6	11.0	19.0	67.2	13.8	1-Oct	80,000	>20	Wh/Sb	NT	SPS 2721 MG
17	Pergamino	2005–06	TA	10.1	13.2	5.6	14.5	20.0	61.3	18.7	9-Sep	80,000	>20	Sb	NT	Dekalb 747
18	Junin	2005–06	TH	6.5	14.0	5.6	13.9	12.0	33.6	54.4	28-Sep	76,900	>20	Wh/Sb	NT	Pioneer 31Y04 MG
19	O'Higgins	2005–06	TA	4.0	16.0	5.7	15.7	14.0	41.1	44.9	19-Sep	72,000	10	Ba/Sb	NT	DM 2750

YCC = years under continuous cropping.
Tillage: NT = no tillage, CT = conventional tillage.
Soil type: TA = Typic Argiudol; TH = Typic Hapludoll; VA = Vertic Argiudoll.
Previous crop = Mz = maize; Sb = soybean; Wh/Sb = double cropped wheat/soybean; Ba/Sb = double cropped barley/soybean.

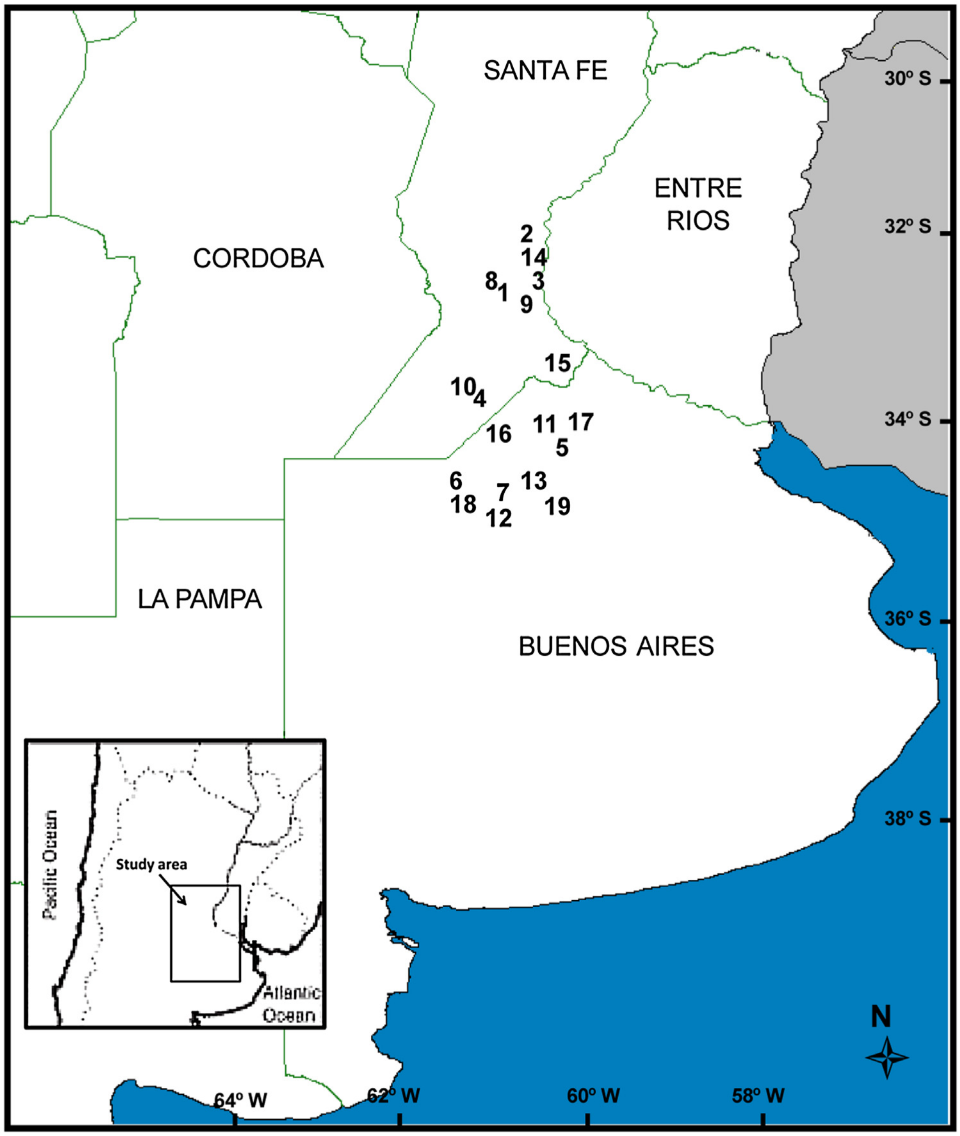

Fig. 1. Distribution of the experimental sites. Characteristics of each site are described in Table 1.

at the top of the canopy and the intercepted radiation (IPAR) was calculated as $[I_0 - I_t]/I_0$. A rate of change in daily canopy IPAR was estimated for each experimental unit dividing IPAR and days (d) between measurements as:

$$(\text{IPAR R1} - \text{IPAR R1}_{-20})/(\text{d R1} - \text{d R1}_{-20}) \text{ before R1}$$

$$(\text{IPAR R1}_{+20} - \text{IPAR R1})/(\text{d R1}_{+20} - \text{d R1}) \text{ for after R1}$$

Estimates of daily IPAR were then multiplied by incident solar radiation each day and summed to estimate cumulative IPAR between $R1_{-20}$ and $R1_{+20}$. Incident solar radiation each day was downloaded from NASA POWER database based on each siteís coordinates (Zhang et al., 2009).

At harvest, grain yield was determined in each plot and adjusted to a standard moisture content of 0.155 kg H_2O kg grain^{-1}. Individual grain weight (GW) was determined by counting and weighing a 500 grain sample and grain number per m^{-2} (GN) was calculated using grain yield and GW. A grain subsample from each plot was ground to determine total N, P and S. Total N (%N) and S (%S) concentration in tissue (expressed as g per kg) were determined by the Dumas dry combustion method at 950 and 1350 °C, respectively, using a TruSpec CNS (LECO, St. Joseph, MI, USA) analyzer (LECO, 2009a,b). Phosphorus concentration (%P) was determined colorimetrically after wet digestion. Grain N (GrainN), S (GrainS) and P (GrainP) content were determined by multiplying%N, %S and%P and grain yield on a dry matter basis. N:S, N:P and P:S ratio were obtained by dividing nutrient concentration in grains.

2.3. Data analysis

Analysis of variance was performed using the Proc Mixed procedure in SAS (Littell et al., 1996). In both experiments, replication nested within SY was used as an error term for evaluating SY effect (Gomez and Gomez, 1984). In case of interaction among factors the slice statement was used for detecting differences within each interaction. Quadratic, linear-plateau and quadratic-plateau regression was used to analyze the effects of increasing S rates on grain yield. Models were compared based on AIC and BIC coefficients, with the objective of determining which model best approximates the data. The AIC is calculated for every model and the “best” model is the one with the smallest AIC. The Bayesian information criterion (BIC) is another model selection criterion based on information theory but set within a Bayesian context.

The difference between the BIC and the AIC is the greater penalty imposed for the number of parameters by the former than the latter (Fabozzi et al., 2014). In case of similar goodness of fit we chose the simplest model. Parameters of the different equations were compared by a *t*-test. In responsive sites, agronomic efficiency (AE) of fertilizer S was estimated as the slope of the linear phase of the linear-plateau relationship between grain yield and S rate.

The proportion of variance in grain yield accounted for GN and GW was quantified by using r^2 values of linear regressions between grain yield and each numerical component as suggested by Sadras (2006). An inverse function (Andrade et al., 1999) was fitted to the relationship between grain number and CGR_{CP}: Grain number = a – b/CGR_{CP}, where parameter "a" indicates maximum grain number and parameter "b" represents the rate at which GN decrease with decreasing CGR_{CP}.

Stoichiometric relationships among N, P and S in both P or S fertilized and unfertilized treatments in all SY were analyzed. Relationships among N, S and P content in grains were fitted using the logarithmic form of the model: $Y = a X^b$ (*i.e.*: $\log Y = \log a + b \log X$), where Y was S or P content in grains, and X was N or P content in grains (Niklas, 2006). Two groups were created for analyzing the parameters of the stoichiometric relationships: (i) P or S deficient crops (G1) including control treatment in responsive sites (R sites) for each nutrient and (ii) no P or S deficiency (G2), which included P or S fertilized treatment in responsive and unresponsive sites (NR Sites), plus the control treatment in unresponsive sites. Lines were fitted using the standardized major axis method as described in Salvagiotti et al. (2012). This method considers the departure of each data point from the fitted line in the Y and X directions, and thus, the fitted line minimizes the sum of triangular areas between the line and each data point. The permutation of variables Y and X does not affect the line fitted by this method (*i.e.*, fitting Y *vs*. X or X *vs*. Y yield the same line). Tests for detecting differences and/or shifts in the slope and intercept along axis were performed (Warton et al., 2006). The software SMATR was used for fitting the lines, testing the significance of their parameters, and comparing slopes of different lines (Falster et al., 2006).

3. Results

3.1. Environmental conditions

Mean temperature in the period September-February (period in which maize cycle occurred) was 19.5, 19.6 and 20.2 °C for 2003–04, 2004–05 and 2005–06 seasons, respectively. Rainfall in the same period was respectively 429, 433 and 454 mm.

3.2. Grain yield response to increasing S fertilizer rate (E1)

The SY × S interaction showed a significant increase in grain yield in response to S addition in 8 SY (Table 2). On average, at these responsive sites grain yield increase was 1165 kg ha^{-1} (from 590 to 1881 kg ha^{-1}), representing in relative terms an increase from 8 to 20% (13% average).

Among the equations used to fit the relationship between grain yield and S fertilizer rate in the responsive sites, the linear-plateau model showed the lowest BIC and AIC values (Table 1 – Supplementary material), and therefore used for explaining grain yield response to S fertilization. Maximum grain yield was reached at S fertilizer rates between 6 a 17 kg S ha^{-1} (parameter c) depending on site (Table 2). The slope in the linear phase of the relationships (parameter b), that indicates the agronomic use efficiency ranged from 70 to 222 kg grain kg S^{-1}, with an average value of 149 kg grain kg S^{-1} (Table 2).

3.3. Grain yield and yield components response to P addition and the P × S interaction (E2)

Significant SY × S and SY × P interactions were observed for grain yield ($P < 0.01$), (Table 2 – Supplementary material), however the P × S interaction was not significant among sites ($P > 0.66$) (Table 3). P fertilization significantly increased grain yield in 10 out of 19 SY ($P < 0.05$). In these 10 P-responsive sites, average yield increase was 1678 kg ha^{-1} (20% over the non-P fertilized treatments). Likewise, S addition increased grain yield in 9 out of the 19 SY, with a mean response of 1302 kg ha^{-1} in these S-responsive sites, representing a relative 15% increment over the non-S fertilized treatments. In 4 SY significant increases in grain yield were observed for both P and S fertilization. Average agronomic use efficiency of P was 56 kg of grain per kg of P applied (ranging between 32 and 87). Likewise, mean agronomic use efficiency was 44 kg of grain per kg of applied S (between 32 and 61).

Grain number and Individual grain weight explained 63 and 20% of variation in grain yield. No SY × P or P × S interaction was observed for GW. On average, P addition increased GW by 3% (from 5 to 11% depending on SY) (Table 3). On the other hand, a significant SY × S interaction was observed, since only 2 out of 19 SY showed significant increases of GW in response to S addition.

3.4. Biomass production and CGR in response to P addition and the P × S interaction (E2)

Biomass at R6 was positively associated with grain yield ($r^2 = 0.52$). Response to P addition varied between sites from 9 to 25%, while increases in response to S fertilization ranged between 13 to 19%. As was observed with GY, no P × S interaction was detected. Harvest index was not affected by P or S fertilization treatments, and differences were only observed between SY. In average HI was 46%.

Crop growth rate in the critical period for GN determination was affected by either P or S addition, but this response depended on SY (Table 3 – Supplementary material). On average, CGR_{CP} was 26.3 g m^{-2} d^{-1}, and P fertilization increased this variable (from 15 to 60%) in 6 out of 19 SY ($P < 0.10$) while S addition increased 12–16%CGR_{CP} in 2 SY ($P < 0.10$). A P × S interaction was detected ($P < 0.05$). When P was not added, S fertilization increased CGR_{CP} by 10%, however when maize received P fertilization, no response to S fertilization was observed (Table 3). On the other hand, P fertilization increased CGR_{CP} independently of S addition.

Crop growth during silking was positively related with grain number (Fig. 2) with an inverse function. In general, parameter a in the function was greater in treatments that received P or S fertilization or both. Then, significant differences were observed when comparing P0S0 *vs* P0S30, P30S0 and P30S30.

3.5. Radiation interception and radiation use efficiency in response to P addition and the P × S interaction (E2)

On average, P fertilization increased cumulated IPAR during the critical period by 4% (Table 4). A significant P × SY interaction indicated that response to P fertilization occurred in 5 out of 8 SY. On the other hand, S addition did not increase $IPAR_{CP}$, and no P × S interaction was detected. Before silking in all SY but one (*i.e.* SY14) P addition boosted cumulated radiation by 7% (from 4 to 14% increase depending on SY). After silking no P or S effects were observed. At silking, intercepted radiation ranged between 78 and 91% depending on SY. A slight 2% average increase in this variable was observed in response to P addition. However, response to S fertilization depended on SY, a positive 7% increase was observed in SY 5, but a decrease in SY 10 and 15 (Table 4).

Table 2
Mean values, analysis of variance and probability values of the Site-year × S rate interaction separated by the slice statement in SAS for grain yield relative to four S fertilizer rates (S) evaluated in 19 site-year. In SY where significant increases in grain yield were observed, parameters of lineal-plateau regression equations between grain yield and S fertilizer rate is shown: a = grain yield when no S fertilizer is applied; b = grain yield increase at incrementing S rate; c = S rate at which maximum grain yield is reached. Significant F test probabilities (P < 0.10) are shown in italics. SE: standard error of the mean.

SY	S fertilizer rate (kg ha^{-1})				Slice Test	Parameters			r^2	Predicted grain yield at S rate = c
	0	7.5	15	30	*F-test probability*	a	b	c		
1	10909	10991	11118	11809	0.38	–	–	–	–	–
2	11659	11447	12710	12595	*0.03*	11413	70.0	16.8	0.71	12595
3	7924	7686	8115	8214	0.80	–	–	–	–	–
4	9596	11259	11394	11927	*0.01*	9596	221.7	9.3	0.93	11660
5	10548	10343	9793	10309	0.59	–	–	–	–	–
6	13153	13153	12694	12967	0.83	–	–	–	–	–
7	12003	13049	12753	12745	*<0.01*	12003	135.7	6.2	0.90	12489
8	14166	14407	14975	14381	0.53	–	–	–	–	–
9	11402	11662	11499	11848	0.87	–	–	–	–	–
10	7852	8840	8327	8952	*0.09*	7851	141.3	6.0	0.71	8707
11	13184	12244	13079	13430	0.18	–	–	–	–	–
12	9427	10483	10245	10434	*0.01*	9426	157.8	6.1	0.95	10387
13	8174	9351	8396	9241	*0.09*	8174	118.2	7.0	0.48	8996
14	9178	10974	10007	10876	*<0.01*	9178	198.8	7.1	0.73	10618
15	6546	6957	6932	7129	0.77	–				
16	9467	10539	11676	11829	*<0.01*	9456	147.2	16.1	0.99	11829
17	6698	7115	7717	7465	0.31	–	–	–	–	–
18	8631	9622	7670	7913	0.26	–	–	–	–	–
19	9455	9614	9109	9555	0.81	–	–	–	–	–
SE	570									
Variation	*F-test probability*									
SY	<0.01									
S	<0.01									
S × SY	0.02									

Table 3
Mean values, analysis of variance and probability values for grain yield, grain number per unit area, individual grain weight, crop growth rate in the critical period (CGR_{CP}), biomass at R6 and harvest index (HI) as affected by two sulfur (S) and two phosphorus (P) fertilizer rates in 19 site-year (SY) and SY × S and SY × P interaction. SE: standard error of the mean. Significant F test probabilities are shown in italics.

		Grain yield kg ha^{-1}	Grain number m^{-2}	Individual grainweight mg $grain^{-1}$	CGR_{CP} g $m^{-2}day^{-1}$		Biomass at R6 kg ha^{-1}	HI %
P rate	P0	9316	3345	278.5	24.6		18015	46.1
	P30	10360	3608	287.1	28.0		20221	45.6
	SE	106	36	1.7	0.4		252	0.7
					P0	P30		
S rate	S0	9468	3391	279.2	23.5	28.0	18435	45.6
	S30	10208	3565	286.3	25.7	28.0	19801	46.2
	SE	106	52	1.7	0.5		252	0.7
Variation		*F-test probability*						
SY		*<0.01*	*<0.01*	*<0.01*	*<0.01*		*<0.01*	<0.01
P		*<0.01*	*<0.01*	*<0.01*	*<0.01*		*<0.01*	0.44
S		*<0.01*	*<0.01*	*<0.01*	*0.02*		*<0.01*	0.41
P × S		0.86	0.72	0.77	0.02		0.08	0.19
SY × S		*<0.01*	*<0.01*	*<0.01*	0.75		*0.02*	0.07
SY × P		*<0.01*	*<0.01*	0.68	*<0.01*		*0.02*	0.23
SY × P × S		0.66	0.96	*0.02*	*0.02*		*0.05*	0.63

SY 3 and 5 was not included in the analysis for CGR_{CP}. SY 3, 18 and 19 were not included in the analysis for biomass at R6.

As opposed to $IPAR_{CP}$, a significant P × S interaction was observed for RUE_{CP}. When no P was added, S fertilization increased RUE_{CP} by 14%, however, when P was applied no significant differences were observed between S rates. Likewise, P fertilization increased RUE_{CP} by 16% under no S addition, but this difference was not significant when S was applied (Table 4). IPAR per kernel during the critical period (a variable that represents the source-sink ratio) significantly decreased by 15% when S was added, independently of SY. P fertilization also decreased IPAR per grain in 4 out of 8 SY, from 6 to 14%. Only one SY increases this variable in response to P addition.

Both, RUE_{CP} and $IPAR_{CP}$ were positively associated with biomass production in the critical period (Fig. 1 – Supplementary material), explaining 75 and 50% of biomass, respectively. Both relationships were not altered by either P or S fertilization.

3.6. N, S and P content in grains in response to P and S fertilization and the P × S interaction (E2)

In general, N, P or S content in grains (expressed as% or as kg of nutrient per unit area) responded positively to P and S addition, however, no P × S interaction was observed for all these variables (Table 5).

In average, S addition significantly increased%N in 2 out of 19 SY, but an average decrease of 6% was observed in 4 SY (Table 5). On the other hand, P supply showed an average increase of 3% in%N, showing no interaction with SY.

Sulfur fertilization increased%S from 5 to 41%, with no significant interaction with SY (Table 5). A significant P × SY interaction was observed, and%S increased from 21 to 70% in response to P fertilization in 5 out of 18 SY.

Table 4
Mean values, analysis of variance and probability values for $IPAR_{BefR1}$, $IPAR_{AftR1}$, $IPAR_{CP}$, RUE_{CP}, IPAR per grain and Intercepted radiation at R1, as affected by two sulfur (S) and two phosphorus (P) fertilizer rates in 7 site-year (SY) and SY × S and SY × P interaction. SE: standard error of the mean. Significant F test probabilities are shown in italics.

		$IPAR_{BefR1}$		$IPAR_{AftR1}$	$IPAR_{CP}$		RUE_{CP}		IPAR per grain		Int @ R1	
	MJ m^{-2}					g MJ^{-1}		MJ grain^{-1}		%		
P rate	P0	206.6		188.0	394.6		2.46		0.113		87.6	
	P30	221.2		188.6	409.9		2.61		0.109		89.1	
	SE	1.7		1.5	2.7		0.07		0.002		0.65	
							P0	P30				
S rate	S0	214.9		188.6	403.5		2.30	2.66	0.115		88.7	
	S30	212.9		188.0	400.9		2.63	2.56	0.107		88.1	
	SE	1.7		1.5	2.7		0.09		0.002		0.65	
		P0	P30		P0	P30	P0	P30	P0	P30	S0	S30
SY	2	174.1	193.6 **	204.6	379.0	397.9 **	2.91	3.14	0.091	0.097	85.7	87.3
	5	180.8	197.8 **	204.5	383.1	404.4 **	2.47	2.52	0.120	0.105 **	75.0	80.1 **
	8	190.5	202.8 **	241.0	435.3	439.8	2.80	2.82	0.102	0.092 *	95.3	93.9
	9	222.8	232.0 *	261.8	482.6	495.7 +	2.36	2.57	0.146	0.141	97.5	96.7
	10	217.8	248.6 **	142.1	357.2	393.2 **	2.37	3.20 **	0.106	0.106	85.8	82.9 +
	11	236.7	245.2 +	159.9	396.3	405.4	2.54	2.63	0.102	0.088 **	97.8	97.1
	14	227.9	231.7	123.9	397.6	399.6	2.05	1.87	0.150	0.141 +	91.1	90.0
	15	202.1	218.5 **	168.8	325.5	342.9 *	2.21	2.12	0.092	0.101 *	80.8	77.3 *
	SE	5.3		3.6	8.6		0.23		0.005		2.1	
Variation		*F-test probability*										
SY		*<0.01*		*<0.01*	*<0.01*		*<0.01*		*<0.01*		<0.01	
P		*<0.01*		0.69	*<0.01*		*0.03*		*<0.01*		0.02	
S		0.23		0.70	0.33		0.08		*<0.01*		0.42	
P × S		0.13		0.95	0.32		*<0.01*		0.57		0.85	
SY × S		0.17		0.24	0.09		0.68		0.12		*0.03*	
SY × P		*<0.01*		0.42	*0.05*		*0.01*		*<0.01*		0.13	
SY × P × S		0.98		0.71	0.99		0.15		0.98		0.23	

Within each SY, +, *, ** are significant effect of P or S rate at the 0.10, 0.05 and 0.01 probability level, as separated by the slice statement in SAS.

Table 5
Mean values, analysis of variance and probability values for grain N concentration (%N), grain S concentration (%S), grain P concentration (%P), grain N content (GrainN), grain S content (GrainS), grain P content (GrainP), N:P, N:S and P:S ration content in grains as affected by two sulfur (S) and two phosphorus (P) fertilizer rates in 18 site-year. SE: standard error of the mean. Significant F test probabilities are shown in italics.

		%N	%S	%P	GrainN	GrainS	GrainP	N:P ratio	N:S ratio	P:S ratio	N:P:S ratio
		g kg^{-1}			kg ha^{-1}						
P rate	P0	13.5	1.04	2.48	107	8.3	19.8	5.8	14.5	2.7:1	13:2.4:1
	P30	13.9	1.06	2.70	122	9.1	23.7	5.3	14.8	2.9:1	13.3:2.6:1
	SE	0.1	0.03	0.04	1.6	0.2	0.5	0.1	0.8	0.2	
S rate	S0	13.8	0.98	2.61	110	7.7	21.0	5.5	16.1	3.1:1	14.1:2.7:1
	S30	13.7	1.12	2.56	118	9.6	22.5	5.6	13.2	2.5:1	12.3:2.3:1
	SE	0.1	0.03	0.04	1.6	0.2	0.5	0.1	0.8	0.2	
Variation											
SY		*<0.01*	*<0.01*	*<0.01*	*<0.01*	*<0.01*	*<0.01*	*<0.01*	*<0.01*	*<0.01*	
P		*<0.01*	0.40	*<0.01*	*<0.01*	*<0.01*	*<0.01*	*<0.01*	0.67	0.43	
S		0.42	*<0.01*	0.18	*<0.01*	*<0.01*	*<0.01*	0.36	*<0.01*	*<0.01*	
P × S		0.10	0.37	0.65	0.93	0.38	0.42	0.33	0.20	0.41	
SY × S		*<0.01*	0.09	0.52	*<0.01*	*<0.01*	*<0.01*	0.54	0.35	0.35	
SY × P		0.25	*<0.01*	*<0.01*	*<0.01*	*<0.01*	*0.03*	*<0.01*	*<0.01*	*<0.01*	
SY × P × S		0.68	0.14	0.20	0.98	0.08	0.13	0.72	0.26	0.13	

SY 5 was not included in the analysis.

Finally, P fertilization increased %P from 9 to 43%, in 8 out of 18 SY (Table 4 – Supplementary material). Only 6 SY that showed significant increases in grain yield also increased%P. Sulfur fertilization showed no effects on %P across sites (Table 5).

The N:P ratio significantly decreased when P was added in 5 SY. In these sites, under P addition N:P ratio varied from 4.2 to 8.6:1. Sulfur fertilization did not affect N:P ratio (Table 5). Likewise, S fertilization also significantly decreased the N:S ratio from 16.1 to 13.2:1, across all SY. Likewise, in general, P addition did not modify the N:S ratio except for 2 SY (Table 5). Sulfur supply reduced the P:S ratio, from 3.1 to 2.5:1 averaging all SY. On the other hand, P addition did not affect this ratio, except for 3 SY (Table 5).

3.7. N:P:S stoichiometry in grains and response to P and S fertilization (E2)

Independently of P or S shortage, grain N content scaled almost isometrically with grain S content (*i.e.* the slope of the relationship between both nutrients are close to 1), suggesting that changes in N and S content in grains were proportional. On the contrary, when analyzing the effect of P fertilization, both N:P and P:S showed allometric relationships with average b values of 0.76 and 1.28, suggesting that P is accumulated in grains more than proportionally in relation with N or S. However, when analyzing the S fertilization effect, the N:P relationship was isometric (b = 0.95).

Phosphorus deficiency did not modify N:S, N:P nor P:S stoichiometry, showing no shifts in either the intercept or the slope

Table 6
Parameters of the fitted log–log function using reduce major axis regression, for the relationships among grain N, S and P content for maize crops that received 2 levels of P and S fertilization. Wald test indicates shifts in log a or b between G1 and G2. G1 = control treatments in responsive sites (R sites); G2 = no P or S deficiency included P or S fertilized treatment in responsive and unresponsive sites (NR Sites) + control treatments in NR sites.

Nutrient relationship	Treatment	r^2	Slope comparison			Comparison of lines with common slope		
			log a	b	p value*	b	log a	p value**
Response to P fertilization								
N *vs* S	G1	0.80	0.81	1.29			1.20	
	G2	0.57	1.03	1.10	0.26	0.92	1.20	0.95
N *vs* P	G1	0.47	0.83	0.96			1.04	
	G2	0.59	0.74	1.07	0.70	0.76	1.04	0.96
P *vs* S	G1	0.46	0.97	0.40			0.14	
	G2	0.63	1.38	0.04	0.25	1.28	0.13	0.97
Response to S fertilization								
N *vs* S	G1	0.86	1.07	1.07			1.18	
	G2	0.63	0.86	1.25	0.25	0.95	1.16	0.43
N *vs* P	G1	0.71	1.00	0.70			0.95	
	G2	0.56	0.73	1.09	0.22	0.95	0.99	0.18
P *vs* S	G1	0.66	1.07	0.38			0.32	
	G2	0.62	1.17	0.22	0.73	1.14	0.25	0.05

* *p* value for comparison of b values.
** *p* value for comparison of intercepts (log a) using a common slope.

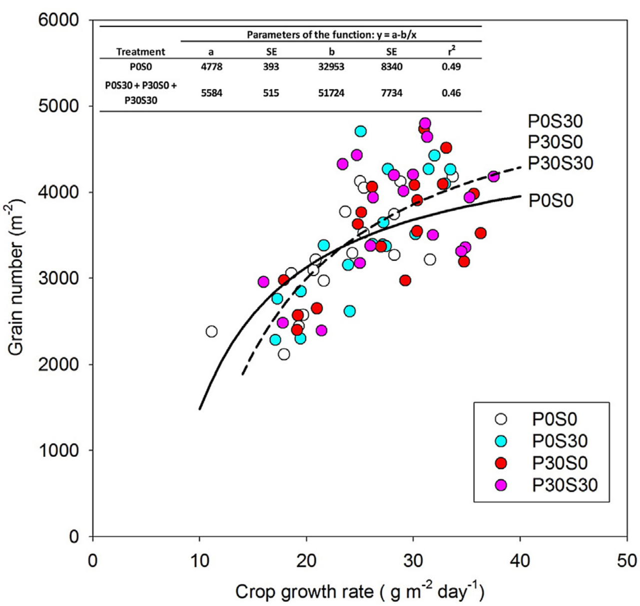

Fig. 2. Grain number as a function of CGR in the critical period (around silking) as affected by two sulfur (S) and two phosphorus (P) fertilizer rates in 18 site-year. Each point is the average of each treatment in each SY. Full and dashed lines are the fit for the P0S0 and the combined effect of P0S30 + P30S0 and P3S30 treatments, respectively, which showed significant differences ($P < 0.06$). The insert shows the parameters values and standard error (SE).

(Table 6). Likewise, S deficiency did not modify the N:P or N:S stoichiometry. However, even when the slope of the relationship between P and S content was not significantly modified, a significant change in the intercept (log a) was observed (Table 6). Then, when equating the exponential form of the relationship between Grain P and Grain S content using a common *b* factor (*i.e.* 1.14) but a different intercept (*i.e.* $a = 10^{\log a}$) for G1 (a_{G1}) and G2 (a_{G2}), for any amount of P, the grain S (G1): grain S (G2) ratio will be proportional to $(a_{G2}/a_{G1})^{1/b} = 0.86$. Therefore, for a given amount of P accumulated in the grains, S deficient crops (G1) tended to accumulate 14% less S in grains than crops with no S limitations (*i.e.* G2) (Fig. 3).

4. Discussion

Response to S fertilization depends on both, grain yield potential (associated with nutrient demand) and soil nutrient levels that are modified by soil organic matter content, soil texture or losses by erosion processes (Tisdale et al., 1986). In low-fertile soils of a tropical region, Weil and Mughogho (2000) showed high relative responses to S fertilization in maize (13–52%) in sites that averaged 4000 kg grain ha^{-1}, suggesting a great S deficiency in the soil. Likewise, in sandy soils of the USA, several reports showed grain yield increases up to 30% in response to S fertilization with S rates up to 80 kg S ha^{-1} (Bullock and Goodroad, 1989; Reneau, 1983). On the contrary, in loamy fine sand to silty clay loam soils, Rehm (2005) determined significant increases in grain yield up 8% at S fertilizer rates that ranged between 6.7 and 13.4 kg S ha^{-1}. These findings are similar to those in the present study, since S fertilization did not increase grain yield beyond *ca.* 10 kg S ha^{-1}, suggesting a moderate response to S fertilization in these medium to fine textured soils.

All sites with significant response to P addition showed soil P values below 15 ppm. Soil test calibration studies in the area under study suggest that the probability of response to P fertilization increase when soil P test is below 20 ppm (Barbagelata, 2011), therefore, all SY in the present study were under a moderate to slightly severe P shortage. Under these conditions, P and S effects on grain yield were additive, as no significant interaction was observed between both nutrients in grain yield and main yield components. This additive effect differs from the N × S interaction previously observed in wheat (Salvagiotti and Miralles, 2008) or maize (Rabuffetti and Kamprath, 1977), in which the effects of S addition were evident only when N was previously added.

The additive effect between P and S observed in the present study may be related to: (i) different mobility of both nutrients in the soil; (ii) different dynamics of nutrient availability during the crop cycle and/or (iii) the different relative demand of both nutrients during the cycle. In the first case, since P is less mobile than S, and acquired by the crop mainly by diffusion (Barber, 1962), then P availability will depend on root extension and P concentration in the soil (Baldwin et al., 1972). On the opposite, S moves in the soil mainly by mass flow, so water dynamics in soil is also important for S movement (Stevenson and Cole, 1999). Then, it is likely that P deficiency did not affect crop ability to acquire S, because S reaches crop roots by a different mechanism. Then, it is possible a

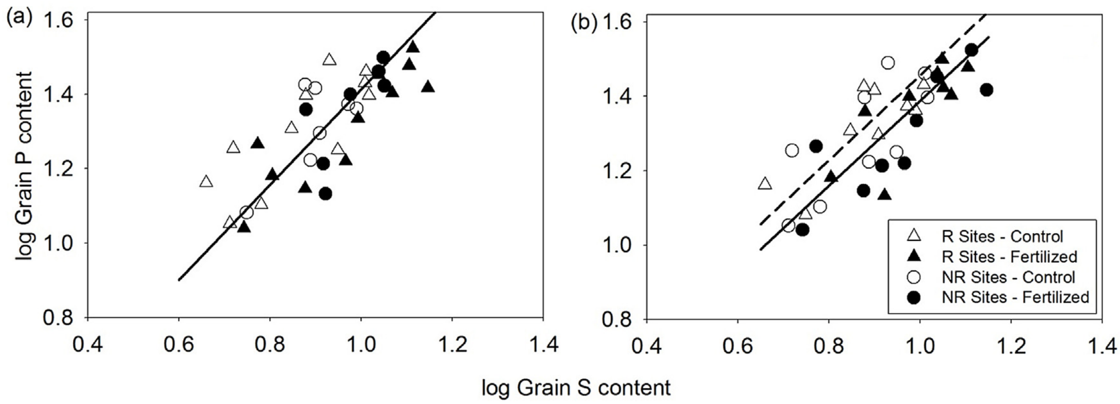

Fig. 3. Log-log bivariate plots showing the relationships between grain S and P content in response to phosphorus (a) and sulfur (b) deficiency. Dashed and solid lines are reduced major axis regression lines for R sites – Control (G1), and for R Sites – Fertilized and NR sites (G2), respectively. When no differences in parameters were detected a common line was fitted. Parameters are shown in Table 6.

positive response to S fertilization in P deficient soils, and thus no P × S interaction detected.

In the second case, many soils have low buffer power for S, so that replenishment of S in the soil solution is mainly by rainfall and/or organic matter mineralization. Then, S deficiency may become more severe later in the crop cycle when S demand increases and S level in the soil solution decreases. On the other hand, P in the soil solution is low but well buffered in many soils. Therefore, soil P availability does not change during crop cycle as P demand rises. Usually, P deficiency affects early stage of crop when root extension is limited.

Finally, in the third case, it may be thought that P demand is higher than S during early stages of the crop (related with tissues with high P concentration because of rapid growth). However, studies of nutrient uptake dynamics in maize (Ciampitti et al., 2013), showed that relative P and S uptake in V6 and R1 is similar for both nutrients, and thus affecting less the P × S interaction.

Whether these three situations occur, the dynamics of P and S in soil or in the plant do not overlap, and thus, it may be reflected in a non-interaction effect on grain yield. As was previously observed, grain yield was mainly associated with grain number per surface area (Andrade et al., 1999), and thus P and S response in GN was similar to that observed in grain yield. Also, most sites that showed significant response to P addition also showed increases in CGR_{CP}, a physiological variable associated with GN. Sulfur effect was less evident. Likewise, the relative response in this variable due to P addition was larger than in response to S fertilization, emphasizing that P shortage was more severe than S deficiency. When relating CGR_{CP} and GN, a positive non-linear relationship as previously reported was observed (Andrade et al., 1999; Maddonni et al., 2006). Both parameters of the equation were affected when each nutrient separately or together were applied; implying that crops under both P and S deficiency reached a lower maximum GN and a low decay rate as CGR_{CP} decrease.

In order to raise GN, crop management should maximize light interception and RUE during the critical period in which GN is defined (*i.e.* around silking) (Andrade, 1995). In the present study both variables were associated with biomass production in this period. Interestingly, P addition increased cumulative radiation in the period around silking, mainly by a significant effect in the period before silking. This result indicates that P shortage was evident early in the cropping season in accordance with reports that showed that the main effect of P fertilization is observed early in the season (Grant et al., 2001). In line with our results, Plenet et al. (2000a) observed that P deficient maize crops showed a delayed leaf area (and thus, light interception) early in the season, and these differences tend to disappear late in the season, since cumulated radiation after silking was not significantly affected by P shortage.

Phosphorus deficiency reduced RUE in the critical period, a variable associated with photosynthesis activity of the crop (Sinclair and Horie, 1989). Studies by Plenet et al. (2000b) in maize and by Sandaña and Pinochet (2011) in wheat, did not observe changes in RUE under P deficiency conditions, suggesting that changes in biomass production were more associated with light interception. However, P shortage in the present study was more severe than in these previous reports and reduced not only light interception, but also RUE. On the opposite, S shortage in the present study was not as severe as P deficiency, then, S addition did not affect this variable. In wheat, Salvagiotti and Miralles (2008) under moderate S deficient conditions, found changes in light interception but not in RUE.

As observed for most of grain yield components, no P × S interaction was observed in variables associated with N, P or S uptake. Phosphorus concentration in grain increased *ca.* 9% in response to P addition, but boosted P content (expressed in $kg\,P\,ha^{-1}$) by 20%. Likewise, S addition increased S concentration in grain by 14% and S content (expressed in $kg\,S\,ha^{-1}$) in 24%. These results showed the effects of both nutrients in grain growth, since not only increased P or S concentration but also C accumulation in grains. Similar results were observed in studies in soybean (Salvagiotti et al., 2012) indicating that nutrient uptake expressed in $kg\,ha^{-1}$ is a better indicator than nutrient concentration since it integrates both effects of nutrient uptake and of growth, avoiding the likely effect of nutrient dilution as grain yield increases.

Isometric relationships (*i.e.* b = 1) between N and S content in grains were observed as previously detected in soybean seeds (Salvagiotti et al., 2012), suggesting proportional changes in both nutrients, as was also observed for C:N and C:P relationships (Niklas, 2006) in different plant species. This isometric N:S relationship may be explained in terms of the proportional accumulation of both nutrients in grains since storage of sulfur in grains is controlled by accumulation of certain proteins in the endosperm (Wu et al., 2012), and both nutrients are needed for the formation of S-enriched proteins in maize grains (Singletary et al., 1990). On the other hand, the present study showed that N and S related allometrically with P, as was also observed by Niklas and Cobb (2006) analyzing C:P and N:P stoichiometric relationships during vegetative stages in different plant species. They explained these results in terms of a larger demand for P in young tissues that need more energy (*i.e.* ATP o rRNA) for organ differentiation and growth. In the present study, more accumulation of P in grains may be related to a luxury consumption of P under the P30 treatment, a P rate that was above P rates that usually optimizes grain yield (Salvagiotti et al., 2013). Some studies showed that crops accumulated P as phytate in grains when P fertilizer rates increased in special structures known as globoids (Modi and Asanzi, 2008), as opposed to S accumulation that depends on protein formation.

In the present study, S deficient crops only modified the intercept of the stoichiometric relationship between P and S. Therefore, this parameter may be used for diagnosing S deficiency in maize, as the greater the value of the parameter (log a), the larger the S deficiency. The P-S intercept for G2 group (crops well supplied with S) can be considered as an optimum S status (log a = 0.25, then a = 1.77). As an unique b value was observed for responsive and unresponsive sites (*i.e.* 1.14), the 'a' value of any grain lot may be estimated as: $a = grainP/grainS^{1.14}$, where grainP and grainS are the observed amount of P and S in the grains (*i.e.* in kg ha^{-1}). Sulfur deficient crops are expected to have 'a' values greater than 1.77. Further studies are needed to validate this preliminary optimum 'a' value.

5. Conclusions

Under the moderate S deficiency and a slightly more severe P shortage observed, maize response to P and S addition showed additive effects, suggesting that soil and plant processes that determine both P and S acquisition are independent.

Phosphorus deficiency reduced RUE and light capture mainly in the period before silking, decreasing biomass production in the critical period, and finally affecting grain number.

The stoichiometric N:P and P:S relationships were allometric and not modified by P deficiency. Under S deficiency only the P:S stoichiometric relationship was altered, and may be used as a "post-mortem" tool for identifying S responsive sites using grain nutrient analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fcr.2016.12.019.

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