



Does biological nitrogen fixation modify soybean nitrogen dilution curves?

Gabriel Santachiara^a, Fernando Salvagiotti^b, José A. Gerde^a, José L. Rotundo^{a,*}



^a Instituto de Investigaciones en Ciencias Agrarias de Rosario (IICAR, CONICET), Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Campo Experimental Villarino S/N, S2125ZAA, Zavalla, Santa Fe, Argentina

^b Departamento de Agronomía, EEA Oliveros INTA, Ruta 11 km 353, 2206, Santa Fe, Argentina

ARTICLE INFO

Keywords:

Fertilization
Mineral soil absorption
Rhizobium
Nutrition
Atmospheric nitrogen

ABSTRACT

Biological nitrogen fixation (BNF) in soybean [*Glycine max* (L.) Merr.] represents, on average, 60% of total nitrogen (N) uptake. Nitrogen dilution curves link aboveground crop N concentration (%N) to biomass accumulation (W). It has been reported that BNF is an energy-intensive process that might reduce biomass production per unit of captured N (physiological N use efficiency or NUE). This increased energy cost could lead to a more attenuated N (i.e. less efficient) dilution curve. However, there are no reports comparing N dilution curves for soybean crops differing in N source. Our objectives were to: (i) evaluate the impact of BNF on soybean N dilution curves and how it influences NUE, and (ii) establish independent N dilution curves for soil and atmospheric N. Our working hypothesis is that relying on BNF attenuates the N dilution curve and reduces NUE. The experiment consisted of a control and a fertilized treatment, 0 and 600 kg N ha⁻¹ respectively, applied to four soybean genotypes in order to establish two differential BNF situations. While the control and fertilized treatments had differential N accumulation from BNF, ~70% and ~16%, respectively, there were no differences observed in seed yield (~5000 kg ha⁻¹), NUE (~36 kg kg⁻¹) and only slight differences in total N uptake (~365 kg N ha⁻¹ in fertilized treatment compared to ~389 kg h⁻¹ in the control treatment). Results suggest that reliance on BNF for N does not influence substantially the attenuation of the N dilution curve and has no impact on NUE. The N dilution parameter (“b”) ranged from -0.128 to -0.218 among cultivars and fertilization treatments. The less negative values (more attenuated curve) corresponded to the fertilized plots likely associated with luxury N consumption. Interestingly, dilution curves from soil mineral N showed the typical dilution pattern, while N derived from the atmosphere followed a concentration pattern as the crop developed. This most likely reflects the continuous N flux from BNF to the plant as opposed to the decreasing soil mineral N supply. Recognizing these concentration/dilution curves for atmospheric and soil N has three immediate implications. First, the atmospheric N concentration curve might indicate an upper benchmark for evaluating symbiosis performance during crop development. Second, the concentration pattern observed for BNF could potentially help to reverse the observed decline in seed protein concentration in modern soybean cultivars. Third, the N concentration/dilution curves for the individual N sources could be incorporated into crop models for estimating BNF at different crop biomass levels during soybean development.

1. Introduction

Nitrogen accumulation is frequently the most limiting process to attain maximum yield in several crops (Plénet and Lemaire, 1999; Ziadi et al., 2010; Rotundo et al., 2014). Nitrogen plays a key role as a constituent of carbon assimilation enzymes like ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO; Rotundo and Cipriotti, 2017). It is also involved in leaf area generation and radiation use efficiency at the crop level (Sinclair and Horie, 1989). Soybean shows a strong positive correlation between seed yield and N uptake (Salvagiotti

et al., 2008; Rotundo et al., 2014), and has the highest N requirement compared to all other legumes and cereal crops (Sinclair and de Wit, 1975). To fulfill this high N requirement, soybean, like all legumes, utilizes two complementary sources for N uptake: soil mineral N absorption and atmospheric N via biological N fixation in association with rhizobium bacteria (Layzell, 1990).

On average, atmospheric N accounts for 60% of total N uptake (Salvagiotti et al., 2008). A regional survey in Argentina showed that the range of N derived from BNF varied from 46 to 71% in farmers' fields (Collino et al., 2015). However, recent evidence supports that

Abbreviations: BNF, biological nitrogen fixation; N, nitrogen; %N, nitrogen concentration; NUE, nitrogen use efficiency; W, above ground biomass accumulation

* Corresponding author.

E-mail address: jrotundo@unr.edu.ar (J.L. Rotundo).

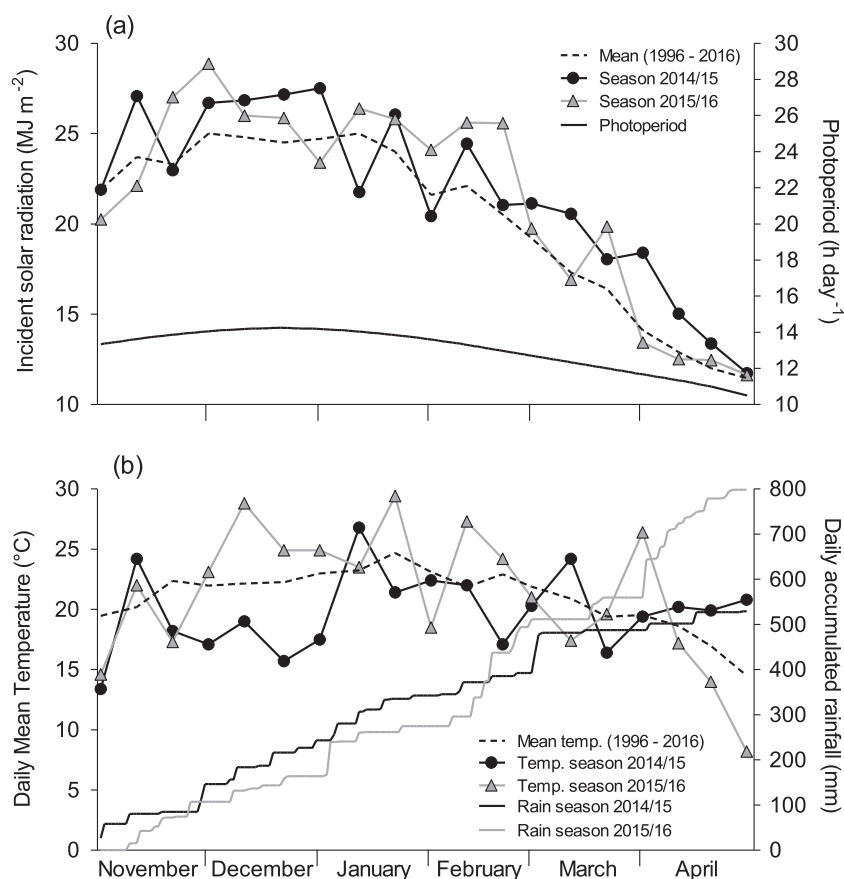


Fig. 1. Solar radiation 10-day average and daily photoperiod (a) and daily mean temperature 10-day average and daily accumulated rainfall (b) for the November–April period. Dashed lines show historic (1996–2016) solar radiation (a) and daily mean temperature (b).

high BNF is not required for maximizing yields when soil N supply is adequate for sustaining this potential yield, indicating a trade-off between both N sources (Santachiara et al., 2017). This view is also supported by the marginal soybean yield response to soil N fertilization with conventional fertilization practices (i.e. applying low N rates broadcast on the surface; Kaschuk et al., 2016). There may be, however, particular situations of high-yielding systems and/or unfavorable environmental conditions for nodulation where N fertilization can overcome N limitations (e.g. Salvagiotti et al., 2009; Cafaro La Menza et al., 2017). Regardless of the relative importance of each source, some evidence suggests that the BNF process entails an extra energy cost for the host as compared to soil nitrate absorption (Silsbury, 1977; Andrews et al., 2009). If this differential energy cost at the cellular level is expressed at a higher level of organization, then a reduction in physiological NUE may happen at the plant or crop scale. However, the impact of this extra energy cost to whole plant performance remains controversial (Salsac et al., 1984). For example, Kaschuk et al. (2009) suggested that rhizobial symbiosis stimulates photosynthesis due to increased carbon sink demand compensating any extra energy cost.

Physiological NUE may be defined as the production of biomass at physiological maturity per unit of accumulated N (Xu et al., 2012). High yielding cultivars from Argentina and the USA ranged from 28 to 35 kg of biomass produced per kg of N uptake (Rotundo et al., 2014). However, biomass production is dynamic, interacting with N uptake during the crop cycle, thus affecting NUE. Therefore, an approach relating plant N status to crop development is needed for analyzing differential energy cost on biomass production and NUE (Sadras and Lemaire, 2014). One such approximation is the concept of N dilution curve which relates aboveground N concentration to biomass accumulation, based on the equation proposed by Lemaire and Salette (1984):

$$\%N = aW^b \quad (1)$$

where W is total aboveground biomass (Mg ha⁻¹), parameter “a” is crop %N when W = 1 Mg ha⁻¹ and parameter “b” is a dimensionless coefficient, than when takes negative values, represents the rate of decline in %N as biomass accumulation progresses.

The *critical* N dilution is further defined as the N dilution curve for the minimum N concentration that maximizes biomass production (Gastal and Lemaire, 2002). This framework was used for comparing N capture dynamics and fertilization needs in the C4 crop maize (Plénet and Lemaire, 1999), and C3 crops wheat (Ziadi et al., 2010) and rice (Sheehy et al., 1998). The *critical* N dilution curve for non-legume species is determined by increasing levels of N fertilization to find the minimum N concentration that maximizes crop biomass. However, the construction of this critical curve is not possible for N-fixing species under normal conditions (Ney et al., 1997; Divito et al., 2016). Studies using non-fixing pea mutants and N fertilization show that N-fixing crops usually dilute N close to the critical dilution curve (Ney et al., 1997). Previous research in soybean showed no changes in the total N uptake dilution curve among genotypes and planting dates (Divito et al., 2016). However, no attempts were made to analyze if reliance more on BNF or soil mineral N absorption would modify N dilution curve parameters.

This study had two main objectives. The first objective was to evaluate whether BNF modifies the pattern of soybean N dilution during crop development and how this impacts NUE at maturity. Our working hypothesis was that BNF entails an extra-energy cost, and therefore it will lead to a more attenuated N dilution curve when compared to a full N fertilized treatment, expressing a reduced physiological NUE at maturity. The second objective was to establish, for the first time, individual N dilution curves for both soil and atmospheric

N. The hypothesis for this objective was that both N sources will dilute following a common pattern.

2. Materials and methods

2.1. Experimental conditions, treatments, and design

Two rainfed experiments were conducted during 2014/15 and 2015/16 growing seasons at Campo Experimental Villarino, located in Zavalla, Santa Fe, Argentina (33°1' S, 60°53' W; elevation 24.6 m). Soil was a silty clay loam Vertic Argiudoll, Roldán series and soybean was the previous crop in both years. Available soil water content at planting at 200-cm depth was 134 mm and 157 mm for the 2014/15 and 2015/16 growing seasons, respectively. Total rainfall for the November–April period was 529 mm and 781 mm for 2014/15 and 2015/16 growing seasons, respectively. A detailed description of mean daily temperature, accumulated rainfall, solar radiation, and photoperiod dynamics is included in Fig. 1. Soil N content (as nitrate) at 0–30 cm depth at planting and before N fertilizer application was 28.8 kg ha⁻¹ and 67.6 kg ha⁻¹ for 2014/15 and 2015/16 growing seasons, respectively.

Treatments consisted of a non-fertilized control (0 kg N ha⁻¹) and an N fertilized treatment which received 600 kg N ha⁻¹ applied at five growth stages at a rate of 125 kg N ha⁻¹: V2, V6, R1, R2, and R4 (Fehr and Caviness, 1977). Fertilizer was granulated urea (grade: 46-0-0) and was hand-broadcast between rows. Four cultivars (MG III: T2137, SPS3900 and MG V: DM5.1i, RA532) were evaluated in both seasons at 40 plants m⁻² plant population. Plots were over-seeded and hand-thinned to reach the target plant population. Each plot was twelve direct-drilled rows, 0.52 m apart, and 5.5 m long. Planting date was November 13th in 2014 and December 3rd in 2015. Weeds and pests were chemically controlled whenever necessary through the season. Seeds were inoculated at recommended rates with RizoLiq LLI[®] (Rizobacter Company, Argentina) containing both *Bradyrhizobium japonicum* (strain E109) and an osmo-protector to sustain the viability of the bacteria after seed pesticide applications. Compatible seed insecticide and fungicide Cruiser Advanced[®] (Syngenta Company, Argentina) were applied at a rate of 1 cm³ seed kg⁻¹ for both treatments.

Experimental design was a complete block design with N fertilization treatments and cultivars randomized within blocks. There were six blocks in 2014/15 and four blocks in 2015/16.

2.2. Biomass sampling and tissue N determination

Aboveground biomass was sampled weekly (starting at V6 and ending at R7) from a 1.04 m² area. This area corresponded to 1 linear meter from two adjacent rows. Since plots were hand-thinned, plant population was accurate and plots were homogenous. This relatively small sampled surface provided precise biomass estimation while allowed accommodating a large number of samples within each plot. In addition, border effects were prevented by a 0.25 m of non-sampled section per row. This approximation was successfully followed in Santachiara et al. (2017). Plants were hand-cut at the soil surface, bagged, and oven-dried at 60 °C before weighing. Seed yield was determined at physiological maturity in a 1.04 m² area of the two central rows. After biomass weighing, samples were milled, and passed through a 1-mm mesh. Seed and vegetative fractions at R7 were separated employing a thresher. Nitrogen concentration was determined in both vegetative and seed fractions using a Kjeldahl procedure (McKenzie and Wallace, 1954). Nitrogen use efficiency at R7 was defined as the ratio between total aboveground biomass and total N uptake (Xu et al., 2012). Nitrogen harvest index (NHI) was calculated as the ratio between seed N content and total N uptake.

2.3. Biological N fixation determination

Biological nitrogen fixation was determined using the xylem-sap

relative ureide abundance method (Peoples et al., 1989). The ureide method is based on the abundance of ureides (allantoin and allantoic acid) relative to amino acids (asparagine and glutamine) and nitrates in the xylem sap. Xylem sap was extracted from two to five additional plants every other biomass sampling date. Sap was extracted using a vacuum pump following Herridge et al. (1990). After extraction, sap samples were refrigerated in the field and conserved at -22 °C until laboratory determinations. Ureides, nitrate, and amino acid N concentrations were estimated colorimetrically following Young and Conway (1942), Cataldo et al. (1975), and Yemm and Cocking (1955), respectively. Relative ureide abundance was calculated as:

$$\text{Relative ureide-N(\%)} = \left(\frac{4U}{(4U + AA + N)} \right) \times 100 \quad (2)$$

where U, AA, and N are the molar concentration of ureides, amino acids, and nitrates, respectively (Herridge and Peoples, 1990). At each sampling date, Relative ureide-N (%) was multiplied by aboveground total N (kg N ha⁻¹) to estimate the amount of biologically-fixed N (kg ha⁻¹) following Herridge et al. (1990). Total BNF at physiological maturity (kg ha⁻¹) was calculated by adding the amount of biological N fixed at each sampling date. Percent BNF was calculated as the ratio between the amount of biologically fixed N (kg N ha⁻¹) and total N uptake at maturity. Finally, soil mineral N absorption was calculated as the difference between aboveground total N (kg N ha⁻¹) and the amount of biologically fixed N (kg N ha⁻¹). Soil mineral N included N derived from both organic matter mineralization and fertilizer applications, depending on the treatment.

2.4. Nitrogen dilution curve determination

Nitrogen concentration was estimated across cultivars, treatments, and sampling dates as the ratio between total N uptake (kg ha⁻¹) and aerial biomass (kg ha⁻¹). Tissue N concentration declines through the growth cycle following a negative power function (Lemaire and Salette, 1984; Eq. (1)). This function was fit for three plant N components: (i) total aboveground N including both atmospheric and soil mineral N, (ii) aboveground N derived only from soil mineral N absorption, and (iii) aboveground N derived only from the atmosphere via biological fixation. Parameter b takes negative values if there is a dilution pattern while values are positive if there is a concentration pattern. The model has two general assumptions (Lemaire and Salette, 1984). First, data points with biomass below 1 Mg ha⁻¹ need to be discarded to ensure full canopy cover. Second, the analysis cannot include data points after the seed-filling period (R5) since N remobilization and leaf senescence can alter the dilution patterns.

2.5. Data analysis

A mixed model ANOVA was conducted for seed yield (dry matter basis), N uptake at R7, NUE, NHI and percent BNF at R7. The model included year and blocks nested within year as random factors, while cultivar and fertilization treatment were considered fixed factors. Mixed model analyses were performed with the nlme package (Pinheiro et al., 2017) in R software (R-Core Team, 2013). Normality and residual homogeneity were tested using the Shapiro–Wilks test and the Brown and Forsythe test. The homogeneity of variances was confirmed with the folded F-test ($P < 0.05$). Fisher's protected LSD (least significant difference) was calculated for significant ($P < 0.05$) effects.

Power functions were fitted individually for each cultivar using GraphPad Prism 6.00 (GraphPad Software, La Jolla, California USA). For each cultivar, curves for the fertilization treatments were compared by an extra sum-of-squares F-test using GraphPad Prism 6.00 (GraphPad Software, La Jolla, California USA).

Table 1

Seed yield, total aboveground N uptake, biological N fixation (BNF), N use efficiency (NUE) and N harvest index (NHI) at physiological maturity (R7) for two contrasting N fertilization treatments (0 and 600 kg N ha⁻¹, respectively) and four soybean cultivars (DM5.1i, RA532, SPS3900 and T2137). Values are averages from two experiments (n = 6 in 2014/2015 and n = 4 in 2015/16). Different letters indicated significant differences at *P* < 0.05 within each source of variation.

Treatment	Cultivar	Seed yield (kg ha ⁻¹)	N uptake (kg ha ⁻¹)	BNF (%)	NUE (kg kg ⁻¹)	NHI (%)
0 kg N ha ⁻¹		4968	364.8 a	69.3 a	35.9	85.9 a
600 kg N ha ⁻¹		5081	388.6 b	15.7 b	36.2	81.3 b
	DM5.1i	4923	384.5	45.3	35.2	86.2
	RA532	4912	368.5	41.3	36.0	83.5
	SPS3900	4962	385.1	41.2	37.3	82.9
	T2137	5302	368.7	42.3	35.6	83.5
0 kg N ha ⁻¹	DM5.1i	4746	354.9	72.4	34.3	89.3
	RA532	5061	364.7	68.7	36.3	83.4
	SPS3900	4954	372.3	65.9	36.0	86.4
	T2137	5112	367.2	70.2	36.9	84.6
600 kg N ha ⁻¹	DM5.1i	5100	414.1	18.1	36.1	83.1
	RA532	4762	372.2	13.8	35.7	83.6
	SPS3900	4970	397.8	16.5	38.6	79.4
	T2137	5492	370.2	14.3	34.3	82.5
Source of variation		<i>P</i> value				
(Intercept)		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Treatment		0.5285	0.0458	0.0001	0.8467	0.0064
Cultivar		0.3604	0.5862	0.1375	0.7332	0.2941
Treatment x Cultivar		0.4924	0.3122	0.3362	0.5417	0.1821

3. Results

3.1. Seed yield, N uptake, biological n fixation, NUE, and NHI across cultivars and N fertilization

Average seed yield across years, cultivars, and N fertilization was ~5000 kg ha⁻¹ (Table 1). It was not affected by cultivar, fertilization, or the interaction between those factors (*P* > 0.05, Table 1). Total N uptake at physiological maturity was slightly increased by N fertilization (365 vs. 389 kg N ha⁻¹). Cumulative BNF at maturity was strongly inhibited by N fertilization. On average across cultivars, BNF was ~70% and ~16% for the control and fertilized treatments, respectively. No cultivar or interaction effect was detected. Nitrogen use efficiency was on average 36 kg biomass kg N⁻¹, and was not affected by any experimental factor. Nitrogen harvest index was ~5% significant reduced by N fertilization (Table 1).

3.2. Total aboveground N dilution across cultivars and N fertilization treatments

Nitrogen dilution curves were significant (*P* < 0.05) and explained 39–56% of variation across cultivars and N fertilization treatments (Table 2). The dilution curves differed between the non-fertilized control and the fertilized treatment for the cultivars evaluated (*P* < 0.05, Table 2). For all cultivars but T2137, N fertilization had a less negative dilution parameter “b”. Parameter “a” was only slightly modified by fertilization, without a clear pattern associated with the increase in N availability. For cultivar T2137, the main difference associated with N fertilization was the increase in parameter “a”. Overall, for all cultivars, the dilution curve was only slightly modified by N fertilization (Fig. 2). At any value of aboveground biomass, fertilization treatment increased total aboveground N concentration, although this effect was rather small.

3.3. Dilution pattern of aboveground soil and atmospheric derived N

Nitrogen derived from soil mineral absorption followed the typical dilution curve as expected from a negative power function (Fig. 3).

Table 2

Parameters of the total N dilution curve (%N = a W^b) corresponding to four cultivars (DM5.1i, RA532, SPS3900 and T2137) and two N fertilization treatments (0 and 600 kg N ha⁻¹). Parameter “a” indicates tissue N concentration at 1 Mg ha⁻¹ of total aboveground biomass. Parameter “b” indicates the degree of N dilution at increasing aboveground biomass. The regression coefficient (R²) indicates the fit of each function. *P* value compares regression curves between fertilization treatments within each cultivar.

N fertilization treatment	Parameter		R ²	<i>P</i> value treatment comparison
	a	b		
<u>Cultivar DM5.1i</u>				
0 kg N ha ⁻¹	4.049	-0.218	0.54 *	0.0082
600 kg N ha ⁻¹	3.771	-0.132	0.42 *	
<u>Cultivar RA532</u>				
0 kg N ha ⁻¹	3.687	-0.169	0.56 *	0.0065
600 kg N ha ⁻¹	3.694	-0.128	0.40 *	
<u>Cultivar SPS3900</u>				
0 kg N ha ⁻¹	3.919	-0.207	0.50 *	0.0015
600 kg N ha ⁻¹	4.049	-0.156	0.44 *	
<u>Cultivar T2137</u>				
0 kg N ha ⁻¹	3.75	-0.146	0.50 *	0.0067
600 kg N ha ⁻¹	4.029	-0.146	0.39 *	

* Indicates significant function fitting at *P* < 0.05.

Aboveground biomass explained 26–56% of variation in aboveground soil-derived N (Table 3). Consistently, N fertilization treatment modified soil-derived N dilution curves across cultivars. Nitrogen fertilization treatment increased parameters “a” and “b” (less negative value) (Table 3, *P* < 0.05). Both effects on curve parameters, as related to N fertilization, were visualized as a strongly attenuated dilution curve for soil-derived N (Fig. 3). On average across cultivars, soil-derived N dilution equations were %N = 3.467 W^{-0.648} and %N = 4.211 W^{-0.244} for the unfertilized and fertilized treatments, respectively.

The relationship between aboveground N concentration derived from the atmosphere and total aboveground biomass did not follow a dilution pattern (Fig. 4, Table 4). Contrary to N derived from the soil, N derived from the atmosphere increased as crop aboveground biomass

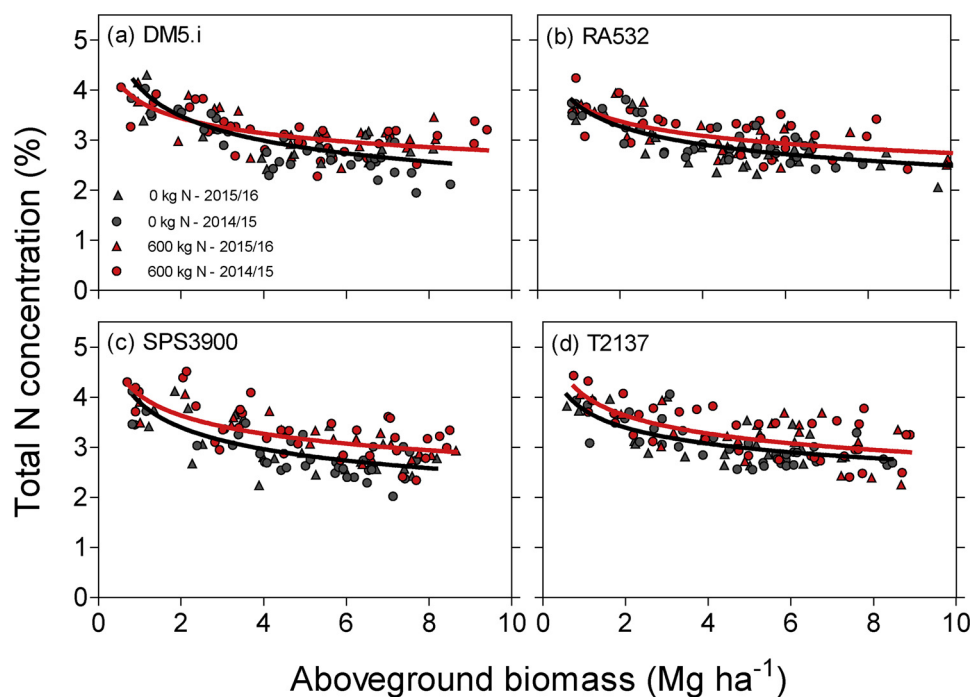


Fig. 2. Relationship between aboveground total N concentration and biomass for four cultivars evaluated in two experiments (2014/15 and 2015/16). Black dots and triangles correspond to a non-fertilized control and red dots and triangles correspond to a fertilized treatment. Each symbol is a single observation. Best-fit parameter values for these curves are in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increases (Fig. 4). This response was captured by a positive parameter “b”. Curves associated with this N fraction differed between N fertilization treatments ($P < 0.05$, Table 4). Although the relationship was not significant for the fertilized treatment in three cultivars, this treatment always reduced concentration of atmospheric-derived N, and parameter “b” was always positive denoting an increasing trend. Nitrogen derived from BNF showed the following equations: % N = $0.914 W^{0.341}$, and %N = $0.109 W^{0.686}$ for the unfertilized and fertilized treatments, respectively.

4. Discussion

Nitrogen fertilization reduced BNF ~80% relative to the control

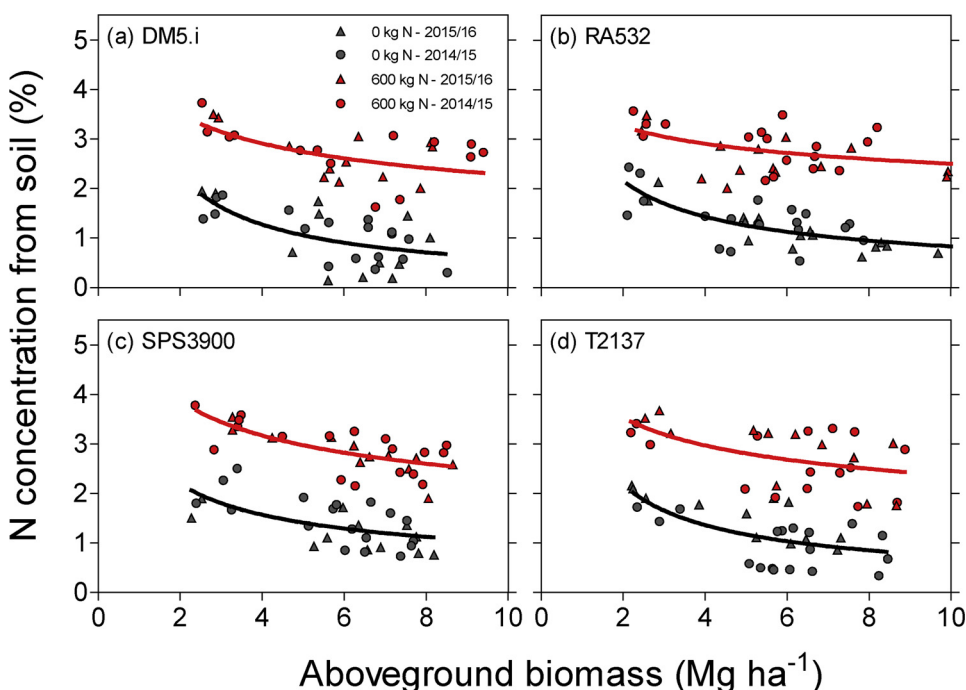


Fig. 3. Relationship between aboveground soil-derived N concentration and biomass for four cultivars evaluated in two experiments (2014/15 and 2015/16). Black dots and triangles correspond to a non-fertilized control and red dots and triangles correspond to a fertilized treatment. Each symbol is a single observation. Best-fit parameter values for these curves are in Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatment. However, the impact of N fertilization on other variables associated with crop growth was negligible (i.e. seed yield, N uptake, and NUE). However, some studies (Lamb et al., 1990; Scharf and Wiebold, 2003) have shown small increases in soybean yield when initial soil N was very low, constraining the development of a completely functional nodule system (van Kessel and Hartley, 2000). In the present study, early-season soil N availability in the control treatments was high enough to fulfill this requirement. These results are in agreement with previous observations where a lack of soybean response to N fertilization was observed (Semu and Hume, 1979; Herridge and Brockwell, 1988). Broadcast N fertilization usually promotes a N source replacement in detriment of BNF (Salvagiotti et al., 2009); our experiment modified N sources rather than total N uptake. Recent studies

Table 3

Parameters of the N dilution curve for soil-derived N ($%N = a W^b$) corresponding to four cultivars (DM5.1i, RA532, SPS3900 and T2137) and two N fertilization treatments (0 and 600 kg N ha⁻¹). Parameter “a” indicates tissue soil-derived N concentration at 1 Mg ha⁻¹ of total aboveground biomass. Parameter “b” indicates the degree of soil-derived N dilution across increasing amount of aboveground biomass. The regression coefficient (R²) indicates the fit of each function. P value compares regression curves between fertilization treatments within each cultivar.

N fertilization treatment	Parameter		R ²	P value treatment comparison
	a	b		
<i>Cultivar DM5.1i</i>				
0 kg N ha ⁻¹	4.087	-0.841	0.44 *	< 0.0001
600 kg N ha ⁻¹	4.227	-0.269	0.28 *	
<i>Cultivar RA532</i>				
0 kg N ha ⁻¹	3.252	-0.590	0.58 *	< 0.0001
600 kg N ha ⁻¹	3.650	-0.164	0.26 *	
<i>Cultivar SPS3900</i>				
0 kg N ha ⁻¹	3.056	-0.482	0.37 *	< 0.0001
600 kg N ha ⁻¹	4.718	-0.287	0.51 *	
<i>Cultivar T2137</i>				
0 kg N ha ⁻¹	3.474	-0.678	0.46 *	< 0.0001
600 kg N ha ⁻¹	4.250	-0.258	0.28 *	

* Indicates significant function fitting at P < 0.05.

(Cafaro La Menza et al., 2017) showed an increase in seed yield and N uptake in response to N fertilization, with seed yields above 6000 kg ha⁻¹. It is likely that the lack of response to N fertilization in our study is the result of a combination of a less than potential N demand and an adequate N supply from the combination of soil N + BNF (in the control treatment) or soil N + fertilizer N that replaced BNF in the fertilized treatment.

Models of N uptake in soybean assume that BNF acts as the primary N source only after soil mineral N is depleted (Sinclair et al., 2003). This observation is compatible with similar observed yields and N uptake in cultivars with contrasting contribution from BNF (Santachiara et al., 2017) and with a small effect of N fertilization on soybean yields (Salvagiotti et al., 2009). Soil mineral N absorption follows a pulse cycle depending on soil nitrate concentration, which is affected by N

Table 4

Parameters of the N dilution curves for atmosphere-derived N ($%N = a W^b$) corresponding to four cultivars (DM5.1i, RA532, SPS3900 and T2137) and two N fertilization treatments (0 and 600 kg N ha⁻¹). Parameter “a” indicates tissue soil-derived N concentration at 1 Mg ha⁻¹ of total aboveground biomass. Parameter “b” indicates the degree of soil-derived N dilution across increasing amount of aboveground biomass. The regression coefficient (R²) indicates the fit of each function. P value compares regression curves between fertilization treatments within each cultivar.

N fertilization treatment	Parameter		R ²	P value treatment comparison
	a	b		
<i>Cultivar DM5.1i</i>				
0 kg N ha ⁻¹	0.975	0.357	0.25 *	< 0.0001
600 kg N ha ⁻¹	0.152	0.591	0.20 ^{ns}	
<i>Cultivar RA532</i>				
0 kg N ha ⁻¹	1.007	0.242	0.23 *	< 0.0001
600 kg N ha ⁻¹	0.102	0.528	0.20 ^{ns}	
<i>Cultivar SPS3900</i>				
0 kg N ha ⁻¹	0.802	0.349	0.26 *	< 0.0001
600 kg N ha ⁻¹	0.100	0.815	0.14 ^{ns}	
<i>Cultivar T2137</i>				
0 kg N ha ⁻¹	0.871	0.416	0.39 *	< 0.0001
600 kg N ha ⁻¹	0.082	0.811	0.23 *	

* Indicates significant function fitting at P < 0.05.

fertilization, organic matter mineralization, and/or leaching processes (Johnsson et al., 1987). Soybean canopies may accumulate soil-derived N during periods of high soil nitrate supply. A dilution of N begins as a result of biomass accumulation until another pulse of nitrate supply occurs. However, Fig. 2 clearly shows that as the crop cycle advances to physiological maturity, crop growth rate was progressively higher than soil N supply, and thus, a dilution process occurred. In contrast, BNF represents a continuous N flux. The intensity of which varied depending on soil nitrate concentration (Arrese-Igor et al., 1997) and crop biomass production (Collino et al., 2015). Therefore, the larger crop production, the larger the BNF contribution, and thus, a concentration pattern is observed. This finding indicates that BNF is crucial for accumulating N in tissues even in situations where crop growth rate is high. As opposed, crop species that do not receive N via BNF, where soil or fertilizer are

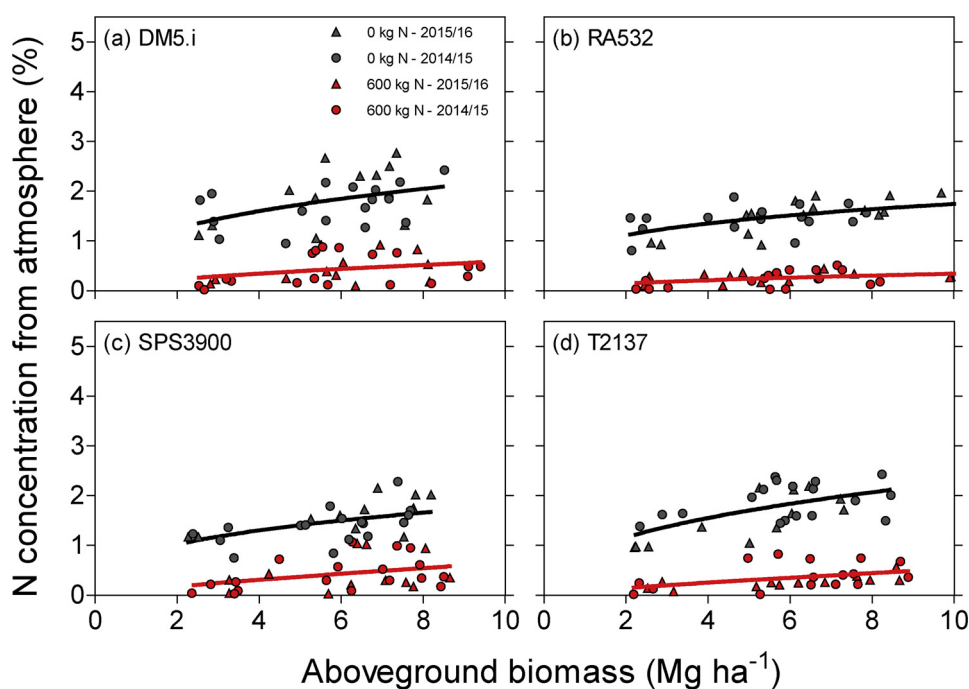


Fig. 4. Relationship between aboveground atmosphere-derived N concentration and biomass for four cultivars evaluated in two experiments (2014/15 and 2015/16). Black dots and triangles correspond to a non-fertilized control and red dots and triangles correspond to a fertilized treatment. Each symbol is a single observation. Best-fit parameter values for these curves are in Table 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5

Soybean N dilution curve parameters for total aboveground N from published literature. Maximum explored biomass is the biomass at the moment of last biomass sampling. Parameter “a” indicates tissue soil-derived N concentration at 1 Mg of total aboveground biomass. Parameter “b” indicates the degree of soil-derived N dilution across increasing amount of aboveground biomass.

Reference	Parameter a	Parameter b	Maximum explored biomass (Mg ha ⁻¹)
Ney et al. (1997)	3.024	-0.149	5.09
Bender et al. (2015) ^a	3.681	-0.105	6.38
Salvagiotti et al. (2009) ^a	4.363	-0.156	7.84
Divito et al. (2016)	3.700	-0.080	7.97
Gaspar et al. (2017) ^a	2.979	-0.066	6.10
This study	3.869	-0.163	7.64
Average	3.549	-0.111	6.84

^a Calculated from published raw data.

the only sources of N, show a sharper dilution curve (Lemaire, 1997).

The critical N dilution curve approximates the minimum N concentration that maximizes growth (Gastal and Lemaire, 2002). Nitrogen-fixing species like soybean usually dilute N close to this critical curve because N is usually not limiting growth since N is acquired via direct soil absorption or from atmospheric fixation (Ney et al., 1997). On average, across fertilization treatments and cultivars, total N concentration at 1 Mg ha⁻¹ biomass was 3.87 (parameter “a”). Parameter “b”, which is associated with the dilution pattern when taking negative values, was -0.163. These values are in agreement with other values found for soybean in the literature (Table 5). As previously described by Divito et al. (2016), soybean shows a more attenuated dilution curve compared to other legumes like pea (b = -0.33; Ney et al., 1997) or lucerne (b = -0.32; Lemaire et al., 1985), suggesting a lower NUE in the case of soybean. Divito et al. (2016) attributed this behavior to soybean early pod onset and constant leaf lamina and stem N concentration during the early reproductive period.

Relying more on BNF (i.e. comparing fertilized vs. control treatments) resulted in a slightly more pronounced N dilution pattern across the evaluated cultivars. Even though the dilution parameter “b” was more negative (i.e. more pronounced decline) for the control treatment, supporting a higher biomass production per unit of N, this has no major implications for seed yield, N uptake, or NUE. This implies that there is no apparent extra energy cost of relying on atmospheric N or that any extra cost is compensated by carbon assimilation stimulation (Kaschuk et al., 2009). Rhizobial symbiosis is a large carbon sink, consuming a significant proportion of recently assimilated photosynthates in soybean (Kaschuk et al., 2009). However, this demand may stimulate additional photosynthetic carbon assimilation via larger N fixation (Kaschuk et al., 2009). This extra photosynthesis could compensate for the theoretical extra energy cost of relying on atmospheric N, then, no major changes at the crop level would be observed regardless of the relative contribution of BNF vs. mineral soil N absorption. In fact, the more attenuated dilution observed in the N fertilization treatment also could be the result of luxury N uptake as was described by Salvagiotti et al. (2008), considering that identical seed yield can be attained by a wide range of N uptake. This agrees with the finding of reduced NHI in the N fertilized treatment. In any case, our working hypothesis was rejected because, despite a potential extra energy cost (Silsbury, 1977; Andrews et al., 2009), relying more on BNF did not substantially modify the N dilution curve, and had no influence on NUE, N uptake, or yield. Results suggest that under high soil N concentration there is substitution of BNF with soil N absorption (Arrese-Igor et al., 1997).

The second objective of our work was to establish individual dilution curves for soil mineral N and atmospheric N. Soil N fertilization strongly affected the dilution pattern of soil N and atmospheric N. Our work established for the first time the contrasting pattern of N concentration changes as related to biomass increase depending on the N

source. While soil mineral N concentration followed the expected dilution pattern, atmospheric N followed a concentrating curve as crop development progressed. The latter may have three immediate implications. First, this curve can indicate an upper benchmark for evaluating symbiosis performance during crop development (upper black curves in Fig. 4), and also a minimum benchmark indicating the minimum N derived from BNF because of inhibition from N fertilization (lower red curves, Fig. 4). This might be relevant, for example, for comparing the response to inoculants and/or fertilizer applications across biomass accumulation. Second, this concentration pattern sheds light on processes that may reverse the observed seed protein concentration decline in modern high-yielding soybean cultivars (de Felipe et al., 2016). It is expected that reducing N dilution in soybean canopies may result in increased seed N (and protein) concentration at maturity. The N concentration pattern of atmospheric N observed in the present study suggests that increasing BNF, and therefore N concentration at later reproductive stages, might result in less total N dilution and therefore increased seed N concentration. There is some empirical evidence suggesting that increasing BNF is related to increased seed protein concentration (Egli and Bruening, 2007). Third, currently BNF is modelled as the difference between crop N demand and soil N availability (Boote et al., 2008). Once soil mineral N is depleted, BNF is activated to match crop N demand. Then, N dilution/concentration curves for individual N sources, as determined in the present study, could potentially be incorporated in crop models to estimate BNF at different crop biomass during soybean development.

5. Conclusions

We found no evidence suggesting that relying more on BNF constitutes an extra energy cost affecting crop level performance or N dilution patterns in soybeans. Our results show, for the first time, the contrasting concentration changes of soil mineral N and atmospheric N sources along a biomass gradient. Soil mineral N followed a typical dilution pattern as biomass increased during development, as opposed to atmospheric N concentration which increased during crop development. This finding suggests that a continuous flux of atmospheric N from biological fixation is closely associated to N demand during crop development. Our results provide a benchmark to assess BNF performance, at any crop developmental stage, which is positively associated to biomass accumulation.

Acknowledgements

This research was funded by Agencia Nacional de Promoción Científica y Tecnológica, Argentina (PICT2011-1292 and PICT2014-3177). Santachiara, G., Gerde, J.A. and Rotundo, J.L. are members of CONICET, The Argentinean National Research Council. Authors want to thank Florencia Buldain for laboratory support and Sandra Huynh Truong for highly valuable comments on an earlier version of the manuscript.

References

- Andrews, M., Lea, P.J., Raven, J.A., Azevedo, R.A., 2009. Nitrogen use efficiency. 3. Nitrogen fixation: genes and costs. *Ann. Appl. Biol.* 155, 1–13.
- Arrese-Igor, C., Minchin, F.R., Gordon, A.J., Nath, A.K., 1997. Possible causes of the physiological decline in soybean nitrogen fixation in the presence of nitrate. *J. Exp. Bot.* 48, 905–913.
- Bender, R.R., Haegerle, J.W., Below, F.E., 2015. Nutrient uptake, partitioning, and remobilization in modern soybean varieties. *Agron. J.* 107 (2), 563–573.
- Boote, K.J., Hoogenboom, G., Jones, J.W., Ingram, K.T., 2008. Modeling nitrogen fixation and its relationship to nitrogen uptake in the CROPGRO model. In: Ma, L., Ahuja, L.R., Bruulsema, T.W. (Eds.), *Quantifying and Understanding Plant Nitrogen Uptake for Systems Modeling*. CRC Press, Boca Raton, FL, pp. 13–46.
- Cafaro La Menza, N., Monzon, J.P., Specht, J.E., Grassini, P., 2017. Is soybean yield limited by nitrogen supply? *Field Crop Res.* 213, 204–212.
- Cataldo, D.A., Haroon, M., Schrader, L.E., Youngs, V.L., 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci.*

- Plant Anal. 6, 70–80.
- Collino, D.J., Salvagiotti, F., Peticari, A., Piccinetti, C., Ovando, G., Urquiaga, S., Racca, R.W., 2015. Biological nitrogen fixation in soybean in Argentina: relationships with crop, soil, and meteorological factors. *Plant Soil* 392, 239–252.
- de Felipe, M., Gerde, J.A., Rotundo, J.L., 2016. Soybean genetic gain in maturity groups III to V in Argentina from 1980 to 2015. *Crop Sci.* 56, 3066–3077.
- Divito, G.A., Echeverría, H.E., Andrade, F.H., Sadras, V.O., 2016. Soybean shows an attenuated nitrogen dilution curve irrespective of maturity group and sowing date. *Field Crops Res.* 186, 1–9.
- Egli, D.B., Bruening, W.P., 2007. Nitrogen accumulation and redistribution in soybean genotypes with variation in seed protein concentration. *Plant Soil* 301, 165–172.
- Fehr, W.R., Caviness, C.E., 1977. Stages of Soybean Development. Iowa State University, Ames, IA.
- Gaspar, A.P., Laboski, C.A.M., Naeve, S.L., Conley, S.P., 2017. Dry matter and nitrogen uptake, partitioning, and removal across wide range of soybean seed yield levels. *Crop Sci.* 57, 2170–2182.
- Gastal, F., Lemaire, G., 2002. N uptake and distribution in crops: an agronomical and ecophysiological perspective. *J. Exp. Bot.* 53, 789–799.
- Herridge, D.F., Brockwell, J., 1988. Contributions of fixed nitrogen and soil nitrate to the nitrogen economy of irrigated soybean. *Soil Biol. Biochem.* 20, 711–717.
- Herridge, D.F., Peoples, M.B., 1990. Ureide assay for measuring nitrogen fixation by nodulated soybean calibrated by ¹⁵N methods. *Plant Physiol.* 93, 495–503.
- Herridge, D.F., Bergersen, F.J., Peoples, M.B., 1990. Measurement of nitrogen fixation by soybean in the field using the ureide and natural ¹⁵N abundance methods. *Plant Physiol.* 93, 708–716.
- Johnsson, H., Bergstrom, L., Jansson, P., Paustian, K., 1987. Simulated nitrogen dynamics and losses in a layered agricultural soil. *Agric. Ecosyst. Environ.* 18, 333–356.
- Kaschuk, G., Kuyper, T.W., Leffelaar, P.A., Hungria, M., Giller, K.E., 2009. Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? *Soil Biol. Biochem.* 41, 1233–1244.
- Kaschuk, G., Nogueira, M.A., de Luca, M.J., Hungria, M., 2016. Response of determinate and indeterminate soybean cultivars to basal and topdressing N fertilization compared to sole inoculation with *Bradyrhizobium*. *Field Crop Res.* 195, 21–27.
- Lamb, J.A., Rehm, G.W., Severson, R.K., Cymbaluk, T.E., 1990. Impact of inoculation and use of fertilizer nitrogen on soybean production where growing seasons are short. *J. Prod. Agric.* 3, 241–245.
- Layzell, D.B., 1990. N₂ fixation, NO₃⁻ reduction and NH₄⁺ assimilation. In: Turpin, D.T., Turpin, D.H. (Eds.), *Plant Physiology, Biochemistry and Molecular Biology*. Longman Scientific and Technical, Harlow, pp. 389–406.
- Lemaire, G., Salette, J., 1984. Relation entre dynamique de croissance et dynamique de prélèvement d'azote par un peuplement de graminées fourragères 1—Etude de l'effet du milieu. *Agronomie* 4, 423–430.
- Lemaire, G., Cruz, P., Gosse, G., Chartier, M., 1985. Etude des relations entre la dynamique de prélèvement d'azote et la dynamique de croissance en matière sèche d'un peuplement de luzerne (*Medicago sativa* L.). *Agronomie* 5, 685–692.
- Lemaire, G., 1997. *Diagnosis of the Nitrogen Status in Crops*. Springer, Berlin-Heidelberg pp. 237.
- McKenzie, H.A., Wallace, H.S., 1954. The Kjeldahl determination of nitrogen: a critical study of digestion conditions—temperature, catalyst, and oxidizing agent. *Aust. J. Chem.* 7, 55–70.
- Ney, B., Doré, T., Sagan, M., 1997. The N requirement of major agricultural crops: grain legumes. In: Lemaire, G. (Ed.), *Diagnosis of the Nitrogen Status in Crops*. Springer-Verlag Publishers, Heidelberg, pp. 107–118.
- Peoples, M.B., Faizah, A.W., Rerkasem, B., Herridge, D., 1989. Methods for Evaluating Nitrogen Fixation by Nodulated Legumes in the Field. Australian Centre for Int. Agric. Res., Canberra.
- Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., 2017. *nlme: Linear and Nonlinear Mixed Effects Models*. pp. 1–131. R package version 3. <https://CRAN.R-project.org/package=nlme>.
- Plénet, D., Lemaire, G., 1999. Relationships between dynamics of nitrogen uptake and dry matter accumulation in maize crops. Determination of critical N concentration. *Plant Soil* 216, 65–82.
- R-Core Team, 2013. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna. <http://www.R-project.org>.
- Rotundo, J.L., Cipriotti, P.A., 2017. Biological limits on nitrogen use for plant photosynthesis: a quantitative revision comparing cultivated and wild species. *New Phytol.* 214, 120–131.
- Rotundo, J.L., Borrás, L., de Bruin, J.D., Pedersen, P., 2014. Soybean nitrogen uptake and utilization in Argentina and united states cultivars. *Crop Sci.* 54, 1153–1165.
- Sadras, V.O., Lemaire, G., 2014. Quantifying crop nitrogen status for comparisons of agronomic practices and genotypes. *Field Crop Res.* 164, 54–64.
- Salsac, L., Drevon, J.J., Zengbe, M., Cleyet-Marel, J.C., Obaton, M., 1984. Energy requirement of symbiotic nitrogen fixation. *Physiologie Végétale* 22, 509–521.
- Salvagiotti, F., Cassman, K.G., Specht, J.E., Walters, D.T., Weiss, A., Dobermann, A., 2008. Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review. *Field Crop Res.* 108, 1–13.
- Salvagiotti, F., Specht, J.E., Cassman, K.G., Walters, D.T., Weiss, A., Dobermann, A., 2009. Growth and nitrogen fixation in high-yielding soybean: impact of nitrogen fertilization. *Agron. J.* 101, 958–970.
- Santachiara, G., Borrás, L., Salvagiotti, F., Gerde, J.A., Rotundo, J.L., 2017. Relative importance of biological nitrogen fixation and mineral uptake in high yielding soybean cultivars. *Plant Soil* 418, 191–203.
- Scharf, P.C., Wiebold, W.J., 2003. Soybean yield responds minimally to nitrogen applications in Missouri. *Crop Manage.* 2, 1–6.
- Semu, E., Hume, D.J., 1979. Effects of inoculation and fertilizer N levels on N₂ fixation and yields of soybeans in Ontario. *Can. J. Plant Sci.* 59, 1129–1137.
- Sheehy, J.E., Dionora, M.J.A., Mitchell, P.L., Peng, S., Cassman, K.G., Lemaire, G., Williams, R.L., 1998. Critical nitrogen concentrations: implications for high-yielding rice (*Oryza sativa* L.) cultivars in the tropics. *Field Crops Res.* 59, 31–41.
- Silisbury, J.H., 1977. Energy requirement for symbiotic nitrogen fixation. *Nature* 267, 149–150.
- Sinclair, T.R., Horie, T., 1989. Leaf nitrogen, photosynthesis and crop radiation use efficiency: a review. *Crop Sci.* 29, 90–98.
- Sinclair, T.R., de Wit, C.T.T., 1975. Photosynthate and nitrogen requirements for seed production by various crops. *Science* 189, 565–567.
- Sinclair, T.R., Farias, J.R., Neumaier, N., Nepomuceno, A.L., 2003. Modeling nitrogen accumulation and use by soybean. *Field Crops Res.* 81, 149–158.
- van Kessel, C., Hartley, C., 2000. Agricultural management of grain legumes: has it led to an increase in nitrogen fixation. *Field Crop Res.* 65, 165–181.
- Xu, G., Fan, X., Miller, A.J., 2012. Plant nitrogen assimilation and use efficiency. *Annu. Rev. Plant Biol.* 65, 1–30.
- Yemm, E.W., Cocking, E.C., 1955. The determination of amino-acids with ninhydrin. *Analyst* 80, 209–213.
- Young, E.G., Conway, C.F., 1942. On the estimation of allantoin by the Rimini-Schryver reaction. *J. Biol. Chem.* 142, 839–853.
- Ziadi, N., Bélanger, G., Clessens, A., Lefebvre, L., Cambouris, A.N., Tremblay, N., Nolin, M.C., Parent, L., 2010. Determination of a critical nitrogen dilution curve of spring wheat. *Agron. J.* 102, 241–250.