REGULAR ARTICLE

Contribution of different sources and origins of nitrogen in above- and below-ground structures to the partial nitrogen balance in soybean.

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Abstract

Aims Most studies that quantified the biological N fixation (BNF) and partial N balances of soybean have ignored the belowground structures. Our objectives were to evaluate the contribution of belowground structures to the partial N balance of soybean and to identify the origin (soil or BNF) and source (apparent remobilization or current gain during the seed-filling period) of seed N.

Methods Biomass, BNF, and N uptake coming from different N sources and origins, including belowground structures, were quantified in a two-year field study involving two soybean genotypes (MG IV and V) and two water availability conditions (rainfed and irrigated).

Results The inclusion of BNF-derived N present in belowground components (which averaged +12 kg N ha^{-1} at R$_7$) changed the results of the partial N balances from negative to positive. BNF was the main origin of seed N, accounting for 73 and 79% of seed N under water stressed and non-stressed conditions, respectively. Regarding the seed N source, apparent remobilization was the main contributor to seed N under water stress, whereas current N gain was the main contributor to seed N in unstressed plants.

Conclusions We conclude that i) the root system retains a relevant proportion of the atmospheric N fixed during the crop cycle and should be included in the partial N balance estimations; and ii) BNF is the main origin of seed N, even under contrasting growing conditions.

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Keywords Soybean · Biological nitrogen fixation · ^{15}N abundance method · Apparent N gain · Apparent N remobilization · Belowground N

Abbreviations

N	Nitrogen uptake
BNF	Biological nitrogen fixation
BNF-N	Nitrogen from biological nitrogen fixation
soil-N	Nitrogen intake from soil nitrogen
%Ndfa	Percentage of biological nitrogen fixation
BB	Belowground biomass

AB   Aboveground biomass
AB_{Veg} Vegetative aboveground biomass
Tot   Total biomass
PB   Partial balance

Introduction

Nitrogen (N) is an essential nutrient for plant growth that plays a key role in primary production in the biosphere (Gruber and Galloway 2008). It is directly involved in the photosynthesis process and the composition of structural and storage proteins, enzymes, amino acids, nucleic acids, and hormones (Kumar et al. 2002). One of the main challenges of agricultural N management is to provide enough N to sustain high yields, while improving soil fertility and minimizing N losses to the environment (Giller and Cadisch 1995; Ladha et al. 2020).

Meeting food security has led to a 35% increase in industrial N fertilizer consumption in the last 20 years (FAOSTAT Database 2021). The use of N fertilizers is heavily reliant on large amounts of energy (mainly fossil fuels) for industrial manufacturing, transportation, storage, and application to soils or plants (Erisman et al. 2008). An economically and ecologically attractive alternative for incorporating N to the agroecosystem is the inclusion of leguminous crops in the rotation. However, a leguminous crop has a positive N contribution to the agroecosystem only when N inputs through biological N fixation (BNF) are greater than N exports in the harvested product.

Soybean [*Glycine max* (L.) Merr.] is the most cultivated legume worldwide, occupying 123 million ha^{-1}, producing 340 million Mg yr^{-1} and accounting for about ca. 79% of global grain legume production, (FAOSTAT Database 2021). These figures highlight the relevance of the net contribution of BNF to the global N balance in soybean crops since they require c.a. 80 kg N per Mg of seed yield (Salvagiotti et al. 2008), from which 50–60% is derived from BNF (Ciampitti and Salvagiotti 2018). Assuming a global average N harvest index (NHI) (usually quantified without belowground biomass) of 73% (Salvagiotti et al. 2008), the net contribution of soybean BNF to agricultural soils could be either negative or slightly positive. In an extensive review, Ciampitti and Salvagiotti (2018) found that when the proportion of BNF in the plant N content

(%Ndfa) was below 72%, only 15% of cases had a positive partial N balance (difference between BNF derived N and N in seeds). However, these analyses were performed only with the aboveground BNF-derived N, without including belowground structures (Ciampitti and Salvagiotti 2018). Thus, the real contribution of BNF would be larger than that previously assumed by Ciampitti and Salvagiotti (2018). Studies that quantified BNF including belowground structures have been performed mostly under greenhouse conditions (Bacanamwo and Purcell 1999; Cassman et al. 1980; Hoogenboom et al. 1987; Kirda et al. 1989; Manavalan et al. 2010; Suematsu et al. 2017). However, this approach does not necessarily mimic plant and root growth under the complex soil-plant interactions occurring in the field. Rochester et al. (1998) estimated that 39% and 24% of total crop N at pod-filling and physiological maturity came from belowground components by quantifying the amount of ^{15}N label in the soil following foliar-feeding of ^{15}N-enriched urea solution to a field-grown soybean crop. On the other hand, Gelfand and Philip Robertson (2014) found at maturity only 3% of total soybean N in roots and nodules determined by physical recovery of nodulated roots from the soil, 91% from which came from BNF. These contrasting examples serve as an indication that the literature does not yet provide a solid basis for predicting N contribution from belowground biomass in soybean.

Besides BNF, the partial N balance depends on how much N is accumulated and finally exported in seeds. In soybean, as in most grain crops, seeds are the main sink for N, either acquired during the seed-filling period from soil and BNF (i.e. current N gain), or remobilized from vegetative structures. The amount and proportion of N remobilized to seeds depend on genotype (Kumudini et al. 2002; Masclaux-Daubresse et al. 2008; Zeiher et al. 1982) and environmental conditions (Gaspar et al. 2017; Muchow et al. 1993). In high-yielding soybean crops, Salvagiotti et al. (2009) determined that apparent N remobilization accounted for 35–42% of seed N, while Ortez et al. (2019) determined a larger proportion (ca. 59%). Santachiara et al. (2017) showed that seed N derived from BNF was greater than N derived from the soil. However, there are no antecedents of studies with field-grown soybean that simultaneously quantified the contribution of belowground structures

to N remobilization to seeds and the origin of this source (soil-N and BNF-N).

In the present study, we tested the following hypotheses i) the inclusion of the BNF-derived belowground N change the soybean N balance from negative to positive; ii) regardless of growing conditions, most seed N comes from BNF, rather than from soil N; and iii) the proportion of soil-N and BNF-N remobilized to seeds is similar. The objectives of this research were to: i) estimate the contribution of BNF from belowground structures to the soybean partial N balance, ii) quantify the relative proportion of soil-N and BNF in the seeds, and iii) estimate the relative proportion of soil-N and BNF-N that is remobilized from above and belowground structures to the seeds. The experimental approach consisted of a two-year field study (2016/2018) involving two soybean genotypes belonging to different maturity groups and two watering conditions (rainfed and irrigated) to test the hypotheses in a range of genotypes and environmental conditions.

Materials and methods

Experimental site and design

Two field experiments were conducted during the 2016/17 and 2017/18 growing seasons (Y) at INTA Oliveros Research Station, Santa Fe, Argentina (32°33′S, 60°51′W). The soil is a silt loam Typic Argiudol Maciel series, which is representative of the Northern Pampa Region (Rubio et al. 2019) and has 30, 750, and 220 g kg^{1}, of sand, silt, and clay, respectively, in the upper horizon (Mosconi et al. 1985). Topsoil (0–20 cm) organic carbon (Walkley & Black), extractable phosphorus (P Bray-1), and soil pH were 12.5 g C kg^{-1}, 12.2 mg P kg^{-1}, and 6.3. Treatments consisted of the combination of two genotypes belonging to maturity group (MG) IV (Sy4x1®) and V (DM53i53® IPRO) and two water supply conditions (WS): irrigated and rainfed. Seeds were inoculated with commercial *Bradyrhizobium japonicum* strains (E109, 2.5 ml kg^{-1} seed). Sowing dates were November 7th in 2016 and November 8th in 2017 and sowing density was 31 plants m^{-2} in both years. Previous crops were wheat (*Triticum aestivum* L.) as a cover crop in 2016 and maize (*Zea mays* L.) in 2017. Treatments were arranged in a randomized complete block design with four replications. Each plot was 10 rows wide (0.52 m row spacing) and 20 m long. Irrigated treatments were performed with a drip irrigation system. Weeds, diseases, and pests were chemically controlled whenever necessary during the season following local agronomic practices.

Plant sampling

Below- and above- ground biomass sampling was carried out at R_2, R_5, and R_7 development stages (Fehr and Caviness 1977). Physical recovery of roots and nodules biomass (hereafter belowground biomass) were obtained from undisturbed soil blocks 0.52 m wide (centered row), 0.35 m long, and 0.3 m deep taken in each experimental unit. Bationo et al. (2007) showed that the influence of management practices is more pronounced at 0.3 m depth than in the deeper soil layers, and suggest quantifying carbon and nutrient changes up to this depth. Several studies have shown that roots may be present up to 1.8–2 m (Dardanelli et al. 1997; Ordoñez et al. 2018), however few studies have quantified biomass up to these depths, which is relevant for estimating carbon and nutrient stocks. Fan et al. (2016) reported that 50% of soybean root biomass may be found in the upper 0.11 m and 68% in the upper 0.3 m of the soil. Then, we used these figures to extrapolate our 0–0.3 m in roots to the whole root system. We considered that nodule biomass was negligible below 0.3 m (Grubinger et al. 1982). Belowground structures were separated from soil by gentle hand-washing with a constant water flow. In each sampled soil block, all roots and nodules were obtained by straining water in excess in a 2 mm mesh to recover fine remains of detached roots. At all sampling times, belowground structures were separated into roots and nodules, and aboveground structures into leaves, stems, pods, podwalls, and seeds depending on the phenological stage. All structures were dried for 72 hs at 60 °C, and then weighed. At R_7, fallen leaves were collected and included for total biomass. At maturity, two central 15 m long rows were harvested. Seed yield was reported adjusted to a standard moisture content of 0.135 kg H$_2$O kg grain^{-1}. Harvest index was calculated as the ratio between seed and both above and total biomass (HI$_{AB}$ and HI$_{TB}$, respectively).

Nitrogen uptake and biological nitrogen fixation

The origin of N was i) N from biological fixation and ii) N coming from soil. For determining plant N and BNF, biomass samples from each plant structure were ground to a fine powder (0.5 mm mesh). Then, each sample was weighed into tin cups and total N concentration (%N) and ^{15}N natural abundance (δ^{15}N‰) (Supplementary Table S1) analysis was determined in a mass spectrometer (MBL - Stable Isotope Laboratory, Woods Hole, MA, USA). Nitrogen uptake in each structure was obtained by multiplying %N by its respective biomass. Belowground biomass N uptake (N-BB) was calculated as the sum of roots and nodules N. It is important to point out that this N-BB may be underestimated since the collected belowground biomass only represent a portion of the total belowground biomass. In addition, N released through rhizodeposition or senescent roots and nodules was not taken into account. Aboveground biomass vegetative N uptake (N-AB$_{Veg}$) was calculated as the sum of the N content in stems, leaves, pods, and podwalls depending on the phenological stage. At physiological maturity also seed N uptake (Seed N) was calculated. Total N uptake (N-Tot) was calculated as the sum of N-AB$_{Veg}$, Seed N (only at R$_7$ stage), and N-BB. The nitrogen harvest index was calculated as the ratio between seed N and both N in above and total biomass (NHI$_{AB}$ and NHI$_{TB}$, respectively).

Aboveground biological N fixation (%Ndfa) was determined using the natural ^{15}N abundance method (Shearer and Kohl 1986):

$$\%Ndfa = \frac{\delta^{15}N\ ref\ -\ \delta^{15}N\ soy}{\delta^{15}N\ ref\ -\ B} \times 100 \qquad (1)$$

Where B is δ^{15}N considering aboveground structures of soybean fully dependent upon N$_2$ fixation that was −1.032 ‰ (Collino et al. 2015), δ^{15}Nref is the ^{15}N natural abundance in a non-fixing crop relying only on soil N, and δ^{15}Nsoy is the ^{15}N natural abundance of soybean. An aggregate δ^{15}N for aboveground vegetative structures (i.e. sum of stems, leaves and podwalls) (Unkovich et al. 2008) and δ^{15}N for seed was used to calculate %Ndfa for aboveground vegetative and seed respectively.

Maize was the reference crop in each experimental unit (Supplementary Table S1).

Considering that the B value can be different between seed and vegetative structures, as demonstrated by Bergersen et al. (1992), a sensitivity analysis comparing the estimate of %Ndfa using the same B value of −1.032 for seeds and aboveground vegetative structures and the estimate of %Ndfa using −1.032 for aboveground vegetative structures and − 0.66 for seeds (Bergersen et al. 1992) to determine whether differences in the B value of different structures produce significant changes in the proportion of BNF and amount of BNF-N. No differences were observed between both approaches (Fig. S1, S2) with an RMSE 6.75%, and relative error 6%. Therefore, we use the local reference B value for aboveground structures to calculate %Ndfa of seed and aboveground vegetative.

Percentage of aboveground vegetative biological N fixation was used to calculate the amount of biological N fixation from the accumulated aboveground vegetative N uptake between phenological stages (E-R$_2$, R$_2$-R$_5$ and R$_5$-R$_7$), and the cumulative amount of biological N fixation was calculated as the sum of each phenological stage contributions (Herridge et al. 1990).

Assuming that the proportion of biological N fixation was similar when comparing aboveground and belowground structures (Carranca et al. 2015; Rymuza et al. 2020), then we used the proportion of BNF estimated in aboveground biomass to estimate belowground BNF-N (N$_{BNF}$BB).

Then total fixed N (N$_{BNF}$Tot) (kg N ha^{-1}) was determined as the sum of aboveground vegetative BNF-N (N$_{BNF}$AB$_{Veg}$), belowground BNF-N, and seed BNF-N depending on the phenological stage. Finally N uptake that did not came from biological N fixation was considered as coming from the soil-N.

A partial N balance was calculated as BNF-N minus N exported with seeds, but taking into account N$_{BNF}$AB for partial N balance aboveground (PB$_{AB}$), and N$_{BNF}$Tot for partial N balance total biomass (PB$_{TB}$), including physical recovery of belowground structures.

$$PB_{AB} = N_{BNF}AB - Seed\ N$$

$$PB_{TB} = N_{BNF}Tot - Seed\ N$$

Nitrogen sources for seed

Nitrogen sources for the seed were i) apparent N remobilized from vegetative structures and ii) apparent N gain during the seed-filling period.

Apparent N remobilization (N_{Remob}) to seeds from each vegetative structures (aboveground vegetative and belowground) was calculated, according to Ortez et al. (2019), as the difference in vegetative N between R_5 and R_7. The term "apparent" is used to indicate that remobilization estimates were derived by subtraction and not by direct measurement (Salvagiotti et al. 2009). These calculations do not take into account respiration and mobilization efficiencies (Dingkuhn and Le Gal 1996). Calculations (kg N ha^{-1}) were made for apparent remobilized N separately for each structure and origin, i.e. soil-N uptake (Soil-N $_{Remob}$) and BNF (BNF $_{Remob}$).

$$Apparent\ Soil - N_{Remob} = \left(R_{5\ Soil-N} - R_{7\ Soil-N} \right)$$

$$Apparent\ BNF_{Remob} = \left(R_{5\ BNF} - R_{7\ BNF} \right)$$

Apparent total N remobilization (Total Remob$_N$) was calculated as the sum of apparent Soil-N$_{Remob}$ and apparent BNF$_{Remob}$ from aboveground vegetative and belowground.

The proportion of apparent remobilized N to seeds (% Seed$_{Remob}$), was calculated as the ratio of apparent total N remobilized (kg N ha^{-1}) and total N in seeds (kg N ha^{-1}).

$$\%Seed_{Remob} = \frac{Total\ Remob_N}{Seed\ N} \times 100$$

The proportion of apparent N remobilized to seed from each N origin (i.e., BNF-N or soil-N), was calculated as the ratio of apparent total N remobilized from each origin to apparent total N remobilized (kg N ha^{-1}).

Finally, apparent N gain during the seed filling period (for both soil-N and BNF-N) was calculated as the difference between seeds N and apparent remobilized N and also calculated as a proportion of seed N.

Data analysis

Data were analyzed by analysis of variance using Infostat software (Di Rienzo et al. 2011) to evaluate the effects of year (Y), maturity group (MG), water supply (WS) and their interactions on all measured variables. In order to test Y effects, replication within year was used as error term (Gomez and Gomez 1984). Differences between PB$_{AB}$ and PB$_{TB}$ were compared as paired variables.

Results

Weather conditions

The distribution of rainfall and temperature during the crop season varied markedly between the two experimental periods (Fig. 1). Accumulated rainfall in the first year was 210 mm between E-R$_2$, 220 mm between R$_2$-R$_5$, and 192 mm between R$_5$-R$_7$, (total 630 mm). The irrigated treatment received only 50 mm before the pod-filling period. In contrast, in 2017/18 total rainfall was 44% lower compared to historical records, 140 mm, 67 mm and 38 mm for the E-R$_2$, R$_2$-R$_5$ and R$_5$-R$_7$ periods, respectively (total 245 mm) for the rainfed treatment. In this period, 270 mm were applied in the irrigated treatments.

The maximum average temperature during 2016/17 did not differ from the historical average, while in 2017/18 it was 9% and 13% warmer during R$_2$-R$_5$ and R$_5$-R$_7$ (Fig. 1).

Seed yield, biomass production and harvest index

Seed yield averaged 5110 and 2973 kg ha^{-1} in the 2016/17 and the 2017/18 seasons, respectively (Fig. 2) and showed a significant interaction Y x MG x WS ($p < 0.01$). Irrigation and genotype treatments showed no significant effects in the first season but greatly affected soybean performance in the second. In the latter season, seed yield averaged 3845 and 2100 kg ha^{-1} in the irrigated and rainfed treatments respectively (Fig. 2), and MG IV was 3336 kg ha^{-1}, 28% higher than MG V.

There was no significant Y x MG x WS interaction for above and belowground biomass at any phenological stage (Table 1). At R$_2$, aboveground

Fig. 1 Weather conditions during 2016/17 and 2017/18 crop seasons. Green line and shaded area represent the average, maximum and minimum air temperature range respectively. Staggered (blue) line and black bars represent accumulated rainfall and irrigation. Greenish and green arrows are phenological events for MG IV and V respectively. DAE; days after emergency
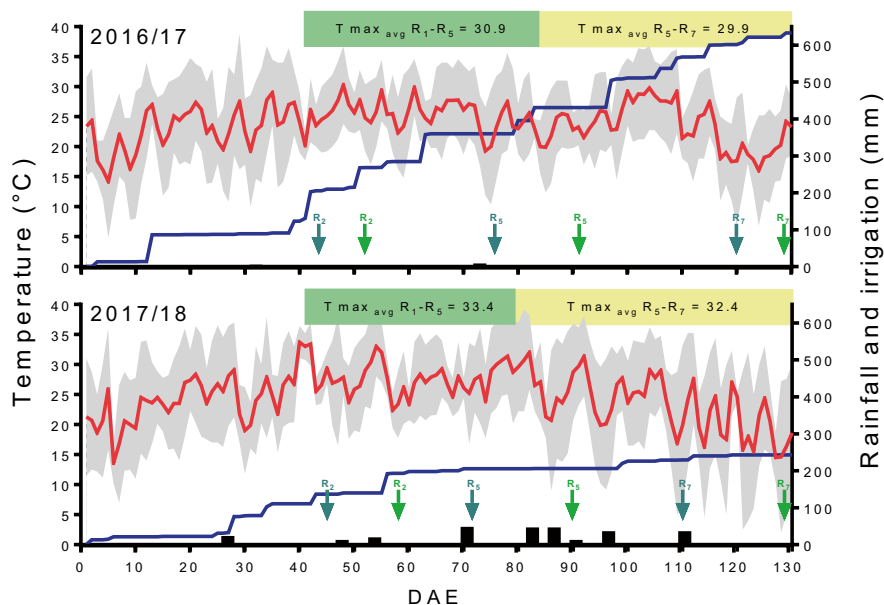
Fig. 2 Seed yield (kg ha^{-1}) adjusted to moisture content of 0.135 kg H$_2$O kg seed^{-1} in 2016/17 and 2017/18 growing seasons for two varieties belonging to MG IV and V under irrigated and rainfed conditions. Error bars represent the standard error of the mean
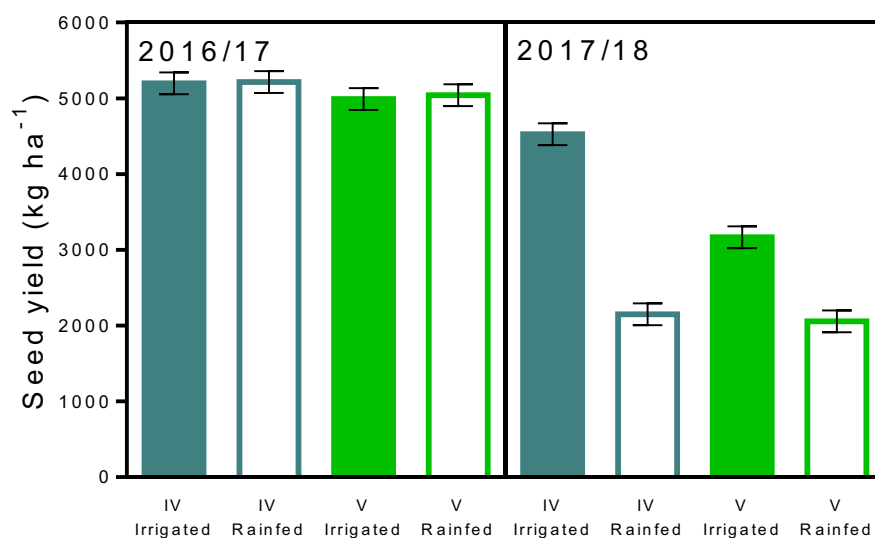
biomass was 2706 kg ha^{-1} for MG V, 84% higher than MG IV, however, this effect was lower for belowground biomass, where MG V averaged 624 kg ha^{-1}, 31% more than MG IV. Belowground biomass was 33% higher in 2017/18 (627 kg ha^{-1}) than in 2016/17 and 23% higher for rainfed (607 kg ha^{-1}) as compared with the irrigated treatment.

At R$_5$, the effects of the treatments on aboveground biomass were similar to what was observed at R$_2$. The Y x WS interaction was observed because in 2016/17 aboveground biomass averaged 6371 kg ha^{-1}, with no

differences between WS treatment, while in 2017/18 the irrigated treatment averaged 5993 kg ha^{-1}, 33% higher than the rainfed treatment (Table 1). Belowground biomass averaged 996 kg ha^{-1} at R$_5$, and a Y x MG interaction was observed, in which MG IV was 1116 kg ha^{-1}, 18% higher than MG V in 2016/17, but in 2017/18, MG IV was 874 kg ha^{-1}, 17% lower than MG V (Table 1).

At R$_7$, in 2016/17 there was no treatment effects on aboveground biomass that averaged 10,880 kg ha^{-1} but in 2017/18, the irrigated treatment was 11,304 kg ha^{-1},

Table 1 Belowground biomass at 0.3 m depth (BB), aboveground biomass (AB) and total biomass (TB = BB + AB) at the R_2, R_5, and R_7 stages in soybean as affected by two different maturity group (MG) and two different water supplies (WS) during two years (Y). Harvest index were calculated taking into account only aboveground biomass (HI_{AB}) or total biomass (HI_{TB}). Means, standard errors, ANOVA p-values for the Y, MG, and WS main effects, or their interactions are shown

MG	WS	R_2			R_5			R_7			HI_{AB}	HI_{TB}
		BB	AB	TB	BB	AB	TB	BB	AB	TB		
		kg ha^{-1}									%	
2016/17												
IV	IRRIGATED	354	1277	1631	1032	5513	6546	1194	10,789	11,983	0.42	0.38
	RAINFED	456	1547	2003	1200	6154	7354	1055	11,209	12,264	0.40	0.37
V	IRRIGATED	448	2438	2886	955	7194	8149	973	10,584	11,557	0.41	0.37
	RAINFED	631	2977	3608	934	6623	7557	863	10,940	11,803	0.40	0.37
2017/18												
IV	IRRIGATED	495	1486	1981	926	4715	5640	1284	11,355	12,639	0.34	0.31
	RAINFED	596	1587	2184	823	3978	4800	994	6368	7362	0.29	0.25
V	IRRIGATED	672	2809	3481	1099	7272	8371	1176	11,252	12,428	0.24	0.22
	RAINFED	745	2599	3344	996	5042	6038	1147	7092	8239	0.25	0.22
S. E.		41	189	214	55	332	338	101	415	433	0.01	0.0037
p value												
Y		<0.01	0.65	0.22	0.28	<0.05	<0.05	0.09	<0.01	<0.05	<0.0001	<0.0001
MG		<0.01	<0.01	<0.0001	0.98	<0.001	<0.0001	0.22	0.9	0.86	<0.0001	<0.0001
WS		<0.01	0.21	0.07	0.71	<0.01	<0.01	0.06	<0.01	<0.0001	<0.001	<0.0001
Y * MG		0.63	0.64	0.74	<0.001	0.13	<0.05	0.13	0.36	0.22	<0.0001	<0.0001
Y * WS		0.36	0.1	0.11	<0.05	<0.01	<0.01	0.81	<0.01	<0.0001	0.16	<0.001
MG * WS		0.67	0.94	0.99	0.24	<0.05	<0.01	0.33	0.52	0.4	<0.001	<0.001
Y * MG * WS		0.36	0.29	0.27	0.24	0.77	0.92	0.43	0.46	0.37	<0.01	<0.001

68% higher than in rainfed soybean. The average contribution of belowground biomass at this stage was 1086 kg ha^{-1}, without statistical differences between treatments (Table 1).

Averaging both seasons, belowground biomass recovered to a soil depth of 0.3 m accounted for 21, 15, and 10% of total biomass at R_2, R_5 and R_7 respectively (Table 1). In 2016/17 average HI_{AB} and HI_{TB} were 0.42, and 0.37, respectively, and no effects of the treatments were observed. Conversely, in 2017/18, HI_{AB} and HI_{TB} were 0.28 and 0.25, with a significant decrease in MG V of ca. 22%. This figures indicated that the inclusion of belowground structures will reduce the value of harvest index by 10%.

Total N uptake

The interaction Y x MG x WS on N uptake was significant only at R_7 (Tables 2 and 3). At R_2, total N uptake was 76 kg N ha^{-1} in 2017/18, and 64 kg N ha^{-1} in 2016/17 (Table 2), while MG V averaged 81 kg N ha^{-1}, 40% more than MG IV. Despite there were significant differences in belowground biomass in response to MG (Table 1), no differences were observed in belowground N (Table 2), which represented 13% and 18% of total plant N in 2016/17 and 2017/18 respectively.

At R_5, total N uptake averaged 164 kg N ha^{-1}, and a significant Y x WS interaction was observed (Table 2). In 2016/17, no differences among treatments were observed, averaging 173 kg N ha^{-1}, however, in 2017/18, irrigated treatment was 175 kg N ha^{-1}, 29% higher than in the rainfed treatment. Belowground N averaged 19 kg N ha^{-1} and represented 12% of total N uptake (Table 2).

At physiological maturity, total N uptake averaged 324 and 222 kg N ha^{-1} in 2016/17 and 2017/18, respectively, whereas in MG V was 254 kg N ha^{-1}, 13% less than MG IV (Table 3). There was no WS effects on total N uptake in 2016/17, but in 2017/18, the irrigated treatments had 70% more total N uptake than soybean under rainfed conditions. Seed N content in 2017/18 was 146 kg N ha^{-1}, 39% lower than 2016/17, mainly due the contrasting WS conditions in 2017/18 where irrigated plants had 80% more seed N than rainfed ones. Nitrogen in belowground biomass collected to 0.3 m accounted for ca. 4 and 8% of total plant N in 2016/17 and 2017/18, respectively (Table 3).

Seed N represented 74% and 66% of total N uptake in 2016/17 and 2017/18, respectively (Table 3) and as was observed with the biomass harvest index, the inclusion of belowground N implied an overall reduction of the N harvest index of ca. 6% (Table 3).

Biological N fixation

At R_2, soybean accumulated 49 kg N from BNF in above and belowground in 2016/17, representing 77% of total N uptake, dropping to 68% in 2017/18. Maturity groups IV and V showed an average %Ndfa of 67 and 77%, respectively (Table 2). Biologically N fixed in belowground structures was ca. 13% and 18% of total BNF at R_2 in 2016/17 and 2017/18, respectively (Table 2) and accounted for 11% of total N uptake.

At R_5, significant effects were detected in response to Y and MG for %Ndfa in total biomass, reaching values of 79% and 72% in 2016/17 and 2017/18, and 73 and 78% for genotypes MG IV and V, respectively (Table 2). In absolute terms, total BNF averaged 136 kg N ha^{-1} in 2016/17 and 113 kg N ha^{-1} in 2017/18. The differences observed due to water supply in 2017/18 explained the Y x WS interaction (P < 0.08), where irrigated soybeans fixed 1.32 times more N than the rainfed treatment. Biological N fixation in belowground structures contributed with ca. 11% and 13% of total biological N fixation at R_5 in 2016/17 and 2017/18, respectively, and accounted for 9% of total N uptake (Table 2).

At R_7, a significant WS effect was observed for %Ndfa, averaging 79% for the irrigated treatment and decreasing to 70% in the rainfed one (Table 3). In absolute terms, total BNF showed a significant Y x WS interaction mainly explained by 2017/18 WS differences. Total biological N fixation in 2017/18 averaged 112 kg N ha^{-1} in the rainfed treatments and enhanced to 218 kg N ha^{-1} in the irrigated treatment. In contrast, no effects of irrigation were observed in 2016/17, averaging 246 kg N ha^{-1}. Nitrogen derived from biological fixation in seeds averaged 175 kg N ha^{-1} in 2016/17. In 2017/18, this variable was 153 kg N ha^{-1} in the irrigated soybean, 1.9 times higher than the rainfed treatment. Biological N fixed in belowground was on average 12 kg N ha^{-1}, and represented 4% and 8% of total biological N fixation in 2016/17 and 2017/18, respectively, contributing with a respective 3% and 6% to total plant N uptake (Table 3).

Table 2 Nitrogen uptake (N) and fixed N_2 (N_{BNF}) in belowground biomass at 0.3 m depth (BB), aboveground vegetative biomass (AB_{Veg}) and total biomass (N-Tot) at the R_2, R_5 stages in soybean as affected by two different maturity group (MG) and two different water supplies (WS) during two years (Y). Means, standard errors, and ANOVA p-values for the Y, MG, and WS main effects, or their interactions are shown. Also the proportion of biological N fixation (Ndfa) is presented

MG	WS	R_2 - Total N uptake			R_2 - Fixed N2				R_5 - Total N uptake			R_5 - Fixed N2			
		$N\text{-}AB_{Veg}$	N-BB	N-Tot	$N_{BNF}AB_{Veg}$	$N_{BNF}BB$	$N_{BNF}Tot$	Ndfa	$N\text{-}AB_{Veg}$	N-BB	N-Tot	$N_{BNF}AB_{Veg}$	$N_{BNF}BB$	$N_{BNF}Tot$	Ndfa
		kg N ha⁻¹			kg N ha⁻¹			%	kg N ha⁻¹			kg N ha⁻¹			%
2016/17															
IV	IRRIGATED	36	6	42	26	4	30	68	147	22	169	116	17	133	79
	RAINFED	49	9	58	35	7	42	71	156	23	178	112	16	129	73
V	IRRIGATED	62	7	69	53	6	59	85	158	15	172	132	12	144	84
	RAINFED	75	9	84	59	7	66	78	159	16	174	125	12	137	79
2017/18															
IV	IRRIGATED	51	11	62	34	7	42	67	141	20	161	105	15	120	74
	RAINFED	54	14	69	33	9	41	61	114	19	134	77	13	90	67
V	IRRIGATED	77	13	91	49	8	57	63	168	21	188	123	15	138	73
	RAINFED	65	16	81	53	13	66	80	120	18	138	90	14	104	75
S. E.		6.0	1.2	6.3	5.62	0.93	5.95	5.03	10.4	1.5	10.5	9.41	1.3	9.77	3.12
p value															
Y		0.15	<0.001	<0.05	0.80	<0.001	0.50	<0.01	0.09	0.50	0.14	<0.01	0.79	<0.05	<0.05
MG		<0.001	0.18	<0.0001	<0.0001	<0.05	<0.0001	<0.05	0.13	<0.01	0.29	<0.05	<0.05	0.07	<0.05
WS		0.34	<0.01	0.13	0.27	<0.01	0.13	0.56	<0.05	0.77	<0.05	<0.05	0.30	<0.05	0.09
Y * MG		0.42	0.47	0.52	0.32	0.26	0.45	0.52	0.53	<0.01	0.29	0.93	<0.05	0.66	0.56
Y * WS		0.05	0.74	0.07	0.42	0.36	0.53	0.29	<0.05	0.30	<0.05	0.08	0.58	0.08	0.53
MG * WS		0.37	0.76	0.36	0.89	0.39	0.80	0.31	0.33	0.82	0.32	0.72	0.71	0.78	0.27
Y * MG * WS		0.39	0.86	0.42	0.59	0.10	0.42	<0.05	0.66	0.72	0.63	0.96	0.97	0.96	0.41

Table 3 Nitrogen uptake (N) and fixed N_2 (N_{BNF}) in belowground biomass at 0.3 m depth (BB), aboveground vegetative biomass (AB_{Veg}), seed (Seed), total biomass (N-Tot) at R_7 stage, and N derived from the atmosphere (Ndfa) in total plant (%) in soybean, as affected by two different maturity group (MG) and two different water supplies (WS) during two years (Y). Nitrogen harvest index were calculated taking into account only aboveground N (NHI_{AB}) or total N (NHI_{TB}). Means, standard errors, and ANOVA p-values for the Y, MG, and WS main effects, or their interactions are shown

MG	WS	R_7 - Total N uptake				R_7 - Fixed N_2					NHI_{AB}	NHI_{TB}
		N-AB_{Veg}	Seed N	N-BB	N-Tot	$N_{BNF}AB_{Veg}$	Seed N_{BNF}	N_{BNF}BB	N_{BNF}Tot	Ndfa		
					kg N ha^{-1}					%		
2016/17												
IV	IRRIGATED	77	256	17	351	64	197	14	275	78	0.77	0.73
	RAINFED	92	249	15	355	67	170	10	247	70	0.73	0.70
V	IRRIGATED	52	239	11	303	57	185	10	252	83	0.82	0.79
	RAINFED	65	212	10	287	55	149	7	212	74	0.77	0.74
2017/18												
IV	IRRIGATED	68	217	22	307	49	178	18	245	79	0.76	0.71
	RAINFED	41	100	16	157	14	78	10	102	65	0.71	0.64
V	IRRIGATED	75	159	18	253	50	127	13	191	75	0.68	0.63
	RAINFED	48	108	15	172	29	83	11	123	71	0.69	0.63
S. E.		7.4	8.6	2.22	13.5	5.93	13.6	2.22	16.5	4.55	0.02	0.02
p value												
Y		<0.05	<0.001	<0.01	<0.001	<0.001	<0.01	<0.05	<0.001	0.29	<0.01	<0.001
MG		0.09	<0.001	<0.05	<0.001	0.89	0.05	0.08	0.06	0.36	0.79	0.10
WS		0.21	<0.001	0.05	<0.0001	<0.01	<0.0001	<0.05	<0.0001	<0.05	<0.05	<0.05
Y * MG		<0.01	0.88	0.38	0.06	0.05	0.74	0.63	0.58	0.60	<0.01	<0.01
Y * WS		<0.01	<0.001	0.49	<0.0001	<0.01	<0.05	0.47	<0.01	0.94	0.35	0.87
MG * WS		0.88	0.08	0.37	0.22	0.62	0.23	0.26	0.19	0.42	0.40	0.40
Y * MG * WS		0.90	<0.01	0.64	<0.05	0.32	0.11	0.49	0.08	0.38	0.18	0.14

Apparent N gain and remobilization during the seed-filling period

Average apparent N remobilization and gain during the seed-filling period was 49 and 51%, respectively of total N content in seed (Fig. 3 a). However, a significant Y x WS interaction was observed for N gain, with no differences between WS treatments in 2016/17 and a significant reduction in apparent N gain in the rainfed treatment in 2017/18.

Apparent N gain during the seed filling period (R_5 - R_7) was 151 kg N ha^{-1} in 2016/17 (63% of total seed N). In 2017/18, apparent N gain in the irrigated

soybean was 105 kg N ha^{-1}, 3.8 times greater than the rainfed treatment, representing 53 and 26% of total seed N respectively (Fig. 3 a). On average, 29% of apparent N gain came from soil-N and 71% from BNF-N. In 2016/17, 27 and 73% of total apparent N gain came from soil-N and BNF-N, respectively, representing 17 and 50% of total seed N (Fig. 3 a). In contrast, in 2017/18, apparent N gain coming from BNF was ca. 84 and 52% of total apparent N gain for irrigated and rainfed treatments, respectively, representing 44% and 13% of total seed N (Fig. 3 a).

Average apparent N remobilized to seeds was 84 kg N ha^{-1} (Fig. 3 a), with the highest values in the

Fig. 3 a) Contribution (bar) and percentage (numbers) of apparent remobilized N (R_5 minus R_7 vegetative N) and apparent N gain (N acquired during seed filling period) (from BNF or soil-N) to seed N composition, and **b)** relative contribution of total apparent N remobilized to seed from aboveground structures (podwalls, leaves, and stems) and belowground (roots and nodule at 0.3 m depth) from different origins (BNF or soil-N), relative to two different water supply (Irrigated and Rainfed) during two successive years (2016/17 and 2017/18). Each column is the average of two varieties belonging to maturity IV and V . Error bars represent the standard error
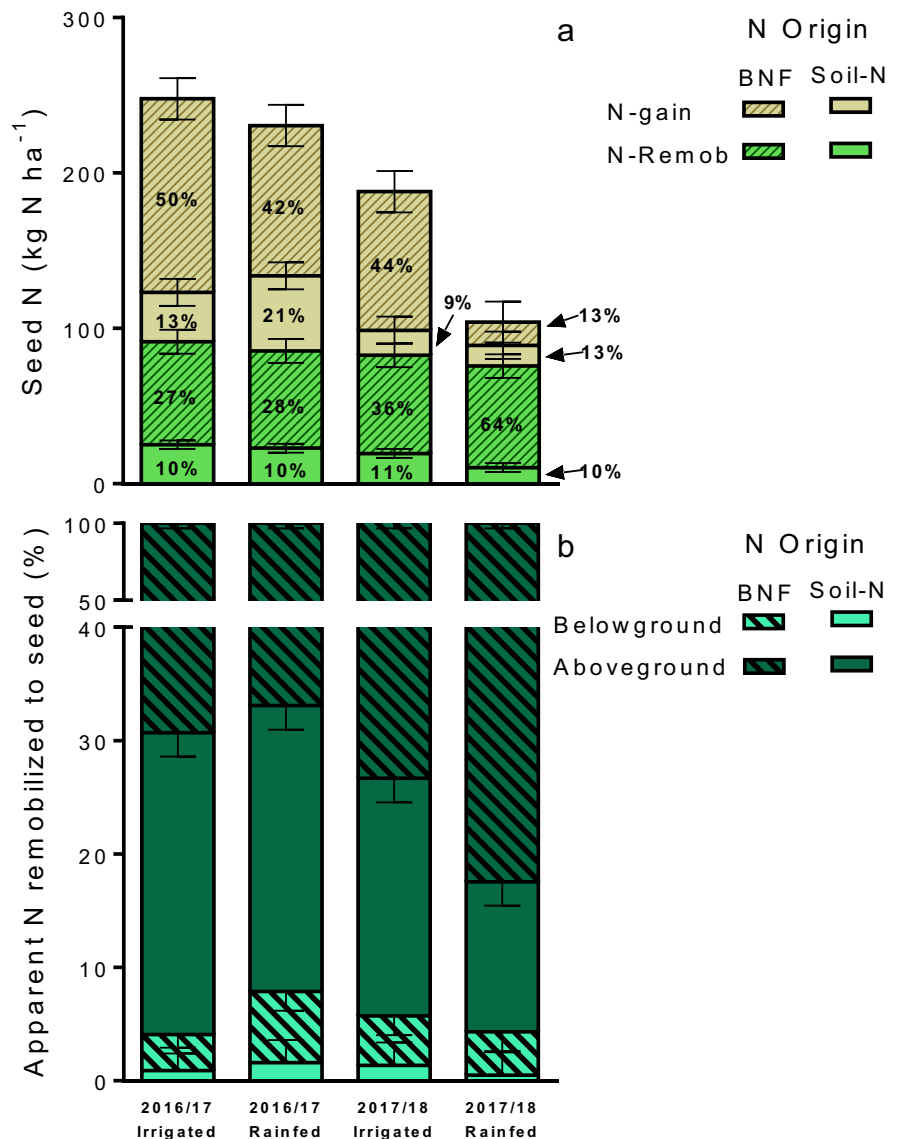
MG V that surpassed MG IV by 29% ($p < 0.05$) (94 and 73 kg N ha^{-1}, respectively). The proportion of seed N coming from apparent remobilization showed a significant Y x WS interaction, averaging 37% in 2016/17 with no effects of WS, but in 2017/18, apparent remobilized N in rainfed soybean plants represented ca. 74% of seed N, whereas 47% under irrigated conditions (Fig. 3 a).

In 2016/17, 27 and 73% of total apparent N remobilization came from soil-N and BNF, respectively, representing 10 and 27% of total seed N (Fig. 3 a). In contrast, in 2017/18, N coming from BNF apparent remobilization was ca. 76 and 86% of total apparent N remobilized to seeds for irrigated and rainfed treatments, respectively, representing 36 and 64% of total seed N (Fig. 3). Despite the origin (i.e. soil or BNF) of the apparent N remobilized to the seed, most came from aboveground vegetative structures (94.5%), while only 5.5% came from belowground apparent N remobilization (Fig. 3 b), with no significant differences between irrigation and genotype treatments. Average belowground apparent N remobilized to seeds was ca. 5 kg N ha^{-1}, of which 81 and 19% came from BNF and soil-N respectively (Fig. 3 b).

Partial N balance

The partial balance including either aboveground BNF or total BNF, showed no significant differences due to Y, WS or MG. When only aboveground BNF-N was included, the partial N balance was −3 and +6 kg N ha^{-1} in 2016/17 and 2017/18 respectively, but as expected, when belowground BNF-N was included, the values significantly increased ($p < 0.001$) to +7 and +19 kg N ha^{-1} (Fig. 4).

The slope of the regression between %Ndfa and the partial balance is a measure of the increase in the partial N balance per unit of %Ndfa. Solving the intercept for Y = 0 indicates the %Ndfa where a neutral balance (i.e. equal to 0) is reached. Figure 5 shows these regressions calculated on the basis of both total N and aboveground N partial balances. No differences were observed between the slopes of total N and aboveground N partial balances in both years (2016/17 $p = 0.76$, 2017/18 $p = 0.32$), indicating an increase in the partial balance of ca. 2.39 and 2.29 kg N ha^{-1} per unit of increase in %Ndfa in 2016/17 and 2017/18 respectively. The intercept was significantly different in each year (2016/17 $p < 0.06$,
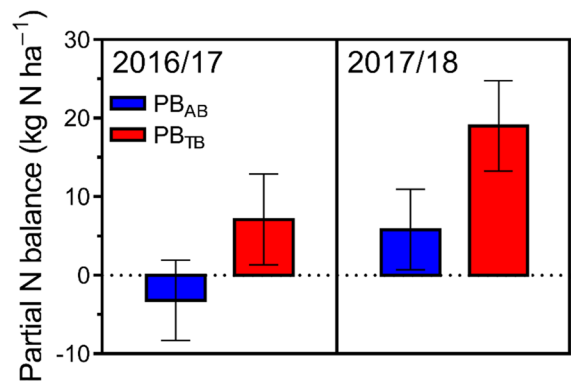

Fig. 4 Soybean partial N balance (kg N ha^{-1}) in the two growing seasons, calculated as; the biological N fixation minus N exported with seeds, taking into account biological N fixation located aboveground (PB$_{AB}$), or above and belowground structures recovered to a soil depth of 0.3 m (PB$_{TB}$). Each bar represents the average of two different maturity group (MG) under two water conditions (WS). Error bars represent the standard error

2017/18 $p < 0.01$). Solving both equations for Y = 0, if considering only BNF from aboveground biomass, positive balances were observed when %Ndfa was above 78 and 70%, for 2016/17 and 2017/18 respectively (Fig. 5). However, this value decreased to 74 and 65%, when including biological N fixation from belowground structures (Fig. 5).

Discussion

Biologically-fixed N, N uptake and N exports are closely related and define the partial N balance of legume crops and the net contribution of BNF to the agroecosystem. Recent reviews on field-grown soybean (Ciampitti and Salvagiotti 2018; Salvagiotti et al. 2008) estimated an average N requirement of 80 kg N per Mg of seed yield, and a contribution from BNF ranging from 0 to 372 kg N ha^{-1}, depending on the proportion of plant N derived from BNF. However, the authors indicated that these estimations should be interpreted with caution because most of published studies did not take into account the belowground structures and, thus N requirements would be greater than assumed. Likewise, there is a general consensus that quantifying belowground structures is crucial to understand the role of soybean in N cycling and approach the actual N balance
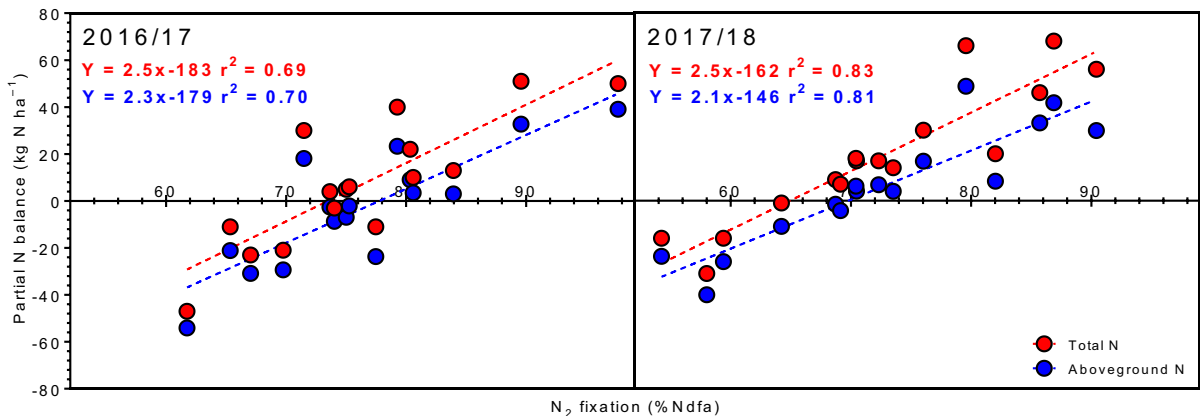
Fig. 5 Relationship between partial N balance (kg N ha^{-1}) and N$_2$ fixation (%Ndfa) for 2016/17 and 2017/18. Red and blue points represent total (including belowground N recovered to a soil depth of 0.3 m) and aboveground N, respectively

(Anglade et al. 2015; Ciampitti and Salvagiotti 2018; Unkovich et al. 2008). Uncertainty on the contribution of belowground components to the partial N balance is related to the intrinsically complex recovery of roots and nodules under field conditions and also to the role of root exudates. For this reason, ambiguity in estimating belowground N are mainly due to biomass rather than tissue N concentration quantification (McNeill et al. 1997). In our study, physical recovery of belowground structures were obtained with the monolith method in which root biomass and spatial heterogeneity are better quantified compared to the soil core method that may produce an overestimation of the root system (Nissen et al. 2008; Ping et al. 2010). In the present study, the belowground biomass physically recovered to a depth of 0.3 m accounted for 21, 15, and 10% of total biomass at R$_2$, R$_5$, and R$_7$, respectively. These proportions are in line with previous field studies performed under diverse environmental conditions (Cheng et al. 2003; Gelfand and Philip Robertson 2014; Mayaki et al. 1976; Roder et al. 1989). Fan et al. (2016) estimated that around 68% of the root system is concentrated in the top 0.3 m. Applying this proportion to our measurements, the belowground biomass of the whole rooting system would have reached 1517 kg ha^{-1} at maturity, representing 14% of the total biomass. Likewise, we found that the proportion of N contained in belowground structures averaged 16%, 12%, and 6% in R$_2$, R$_5$, and R$_7$, confirming the relatively low contribution of belowground structures, which are a relevant but not a primary sink for plant N (Salon et al. 2011).

Accurate estimations of belowground biomass are also important to estimate the carbon retained as organic matter. Previous reports showed that the proportion of carbon which is finally retained as stable organic matter can be 10-fold greater for belowground than for aboveground residues (Jackson et al. 2017; Kätterer et al. 2011; Mazzilli et al. 2015). This indicates that small changes in belowground residues could result in large differences in soil organic stable carbon formation. Compared to other crops that usually share the same rotation (e.g. maize and wheat in Argentina and USA), soybean has a low amount of residues returned to the soil, so a high proportion of this crop within a sequence affects soil organic C pools (Novelli et al. 2011; Varela et al. 2014). Since soil C and N dynamics are closely related, a similar decay in soil organic N is expected associated with the low amount of belowground residues. On the other hand, the significance of root exudates on crop N balances and the identification of the rhizodeposition origin (soil-N or BNF-N) is difficult to estimate under field conditions. Although interesting progress has been made to date on this topic (Fustec et al. 2010; Laberge et al. 2009; Wang et al. 2021), this is undoubtedly one of the areas where research efforts need to be intensified to get a closer approach to the real N balance.

In line with our first hypothesis, results from the 2016/17 season indicated that including belowground structures changed the partial N balance from negative to positive. In 2017/18, the partial N balance was positive even when not considering belowground

structures (Fig. 4). Our results agree with Ciampitti and Salvagiotti (2018), who indicated that positive partial balances should be expected when %Ndfa is greater than 73%. However, the partial N balance tended to be more positive in the second season, probably due to the lower yields and consequently lower N exports.

We found that positive balances can be achieved when total plant % Ndfa is above 65%, and that after including BNF-derived N in belowground recovered to a soil depth of 0.3 m, the partial N balance significantly increased ca.12 kg N ha^{-1}. Applying the correction mentioned above to estimate total root biomass based on measurements made in the upper 0.3 m (Fan et al. 2016), the amount of BNF-derived N present in the whole roots would rise to 16 kg N and the partial balances would turn out to 10 and 23 kg N ha^{-1} in 2016/17 and 2017/18, respectively. At the farm scale, although this additional 16 kg of N from BNF would not lead to generalized positive balances, it suggests that neutral balances are likely to be obtained. When scaled to large areas, e.g. at national or regional levels, estimated belowground N contributions reach a magnitude that clearly merit their inclusion into N budget estimations. For example, applying these estimations to the area planted with soybean in Argentina (19 million ha^{-1}; Dominguez and Rubio 2019), the belowground BNF-N contribution would reach ca. 300,000 Mg N per year.

It is important to highlight that, in the present study, we considered that the proportion of N derived from BNF in belowground structures was similar to that quantified in aboveground structures. This assumption was made based on previous studies that showed similar %Ndfa values for both aboveground and belowground structures (Carranca et al. 2015; Rymuza et al. 2020). A more precise estimation of the contribution of belowground structures, and thus the partial N balance, could be achieved if using a specific B value for roots and nodules, that usually have larger δ^{15}N values than aboveground structures (Shearer et al. 1980; Unkovich et al. 2008; Werner and Schmidt 2002), especially when high %Ndfa values are obtained. Okito et al. (2004) observed that including roots reduced the B value 24% as compared with the B value estimated only for aboveground biomass, and even reduced 45% when nodules were also included. Also, a refinement of the estimation of BNF may be done by using different B values for seed and

vegetative tissues. In the present study, the negative impact of the water deficit occurred in the 2017/18 season was stronger on total BNF-N (−47%) than on total soil N intake (−16%) and total dry matter accumulation (−42%), suggesting a higher sensitivity of BNF to the stress. (Purcell and King 1996; Purcell et al. 2004). These results would support that crops under water stress show decreased N uptake from both, BNF and soil-N, because the reduced phloem flow limits nodule activity (Serraj et al. 1999) and the reduced transpiration water flow limits soil N supply. In our experiments, the effects of water shortage on N uptake and BNF-N was larger than the effect of genotype, as depicted by the stronger effect of Y x WS than the Y x MG interaction. Bacigaluppo et al. (2011) demonstrated that different soybean maturity groups may explore different environmental conditions. However, the intensity of the water shortage in the second year of our experiment was large enough to avoid a "phenological escape" to this stress, with a large impact on seed yield, N uptake and BNF.

Seed N demand, which represents the output component of the partial N balance, can undermine overall N recycling if not supplied by BNF. Salvagiotti et al. (2021) observed that the relationship between seed yield and seed N is linear even in high-yielding soybean crops (around 6000 kg ha^{-1}), in which both soil-N and BNF usually may not support N demand (Cafaro La Menza et al. 2017; Ciampitti and Salvagiotti 2018). Bergensen et al. (1992) observed that the amount of BNF detected from the beginning to the end of the grain filling period was twice the amount detected at earlier stages. In the present study, adding "apparent current gain" (i.e. N acquired during the seed filling period) and apparent remobilized N, BNF accounted for 73 to 79% of total seed N, in stressed and non-stressed conditions respectively. This result fully supports our second hypothesis, which stated that regardless the environmental conditions, most of seed N come from BNF, rather than from soil N. In that sense, we could identify two contrasting situations: under non-water stress conditions (2016/17 and rainfed 2017/18), the seed BNF-N came mainly from the "apparent current N gain", as showed by Bergensen et al. (1992) while in water-stressed conditions came mainly from apparent remobilized BNF-N. In both cases, the partial N balance remained positive, due a high percentage (76%) of seed N from BNF.

Seed-N is supplied by N remobilized from vegetative structures, and from soil N intake and BNF during the seed filling period (Sinclair and de Wit 1975). From the perspective of leaving more N to the system (i.e. a net contribution of BNF), the larger the remobilization of N previously fixed from vegetative structures to the seeds, the lower the chances of having positive partial N balances. Likewise, a net contribution of BNF to the system may occur when current BNF gain increases, however, previous remobilization studies did not differentiate the origin of remobilized N to seed, a key aspect for N cycling in agroecosystems. Our research was designed to fill this gap by discriminating the ultimate origin (i.e. soil or BNF), of apparent N remobilized to seeds, and the final allocation of plant N to seed N. On average, the present study showed that belowground apparent remobilization accounted for 2.7% of seed N, and total vegetative apparent N remobilization represented 49% of seed N, in line with previous studies (Bender et al. 2015; Gaspar et al. 2017; Kumudini et al. 2002; Ortez et al. 2018). However, this proportion was highly dependent on the water status experienced by the crop, varying from 41% to 74% in well-watered and water-stressed plants, respectively. By contrast under water stressed conditions, the contribution of current apparent BNF gain to the seeds was more affected than without water stress. In such sense, collected evidence does not support our third hypothesis, since the BNF-N:soil-N ratio within vegetative apparentN remobilized to seeds was highly variable (range: 2.7 to 6.4). The variability was mainly regulated by the irrigation treatments, which increased the proportion of apparent remobilized N coming from BNF under water-stressed conditions. These figures indicate the relative importance of N source and origin. However, since the apparent remobilization and N gain are not direct measurements, specific measurements by ^{15}N enrichment will be needed to confirm these trends.

The contrasting water availability conditions explored allowed to achieve a wide range of yields (2000–5000 kg ha^{-1}), suggesting that the data obtained could be extrapolated to soybean crops growing under a wide range of conditions.

Conclusions

The results of this work filled a knowledge gap regarding the amount of BNF allocated belowground and its effects on the N balance of soybean crops under contrasting water availability conditions. On average, we found that belowground N accounted for 8% of total plant N and BNF allocated belowground represented 5 to 13% of total BNF, even when our data were adjusted to include potential additional N that may have been associated with nodulated roots deeper in the soil profile below 0.3 m. Although it should also be noted that such estimates are still likely to be conservative as the additional contributions of organic N released in the rhizosphere as exudates, or as the result of the turnover of root-hairs, fine roots and nodule senescence during soybean growth would not be detected by the physical recovery technique used in the current study. The soybean partial N balance was always positive (i.e. N inputs through BNF higher than N exported with the grains) when the N derived from BNF present in physical recovery of roots was included. If the belowground components are not taken into account and only the aboveground components are considered, the partial balance would be estimated as negative in our first experimental year. These results highlight the relevance of including belowground components when estimating N budgets of soybean crops at different scales.

Our study provides a novel perspective on the identification of the origin (soil-N or BNF) and source (apparent N remobilization or apparent N gain during the seed-filling period) of seed N and its relationship to the N balance. Biological N fixation was the main origin of seed N, accounting for 73 and 79% of seed N in water stressed and non-stressed conditions, respectively. In terms of the source of seed N, the relative contribution of apparent remobilization and apparent current gain depended on the growth conditions. Under water stress, apparent remobilization from vegetative structures was the main contributor to seed N, which was mainly explained by a higher relative apparent remobilization of BNF, rather than soil-N. In contrast, under well-watered conditions, apparent N gain during the seed filling period was the main contributor to seed N, mainly from BNF rather than from soil-N. Therefore, increasing N gain during the seed-filling period appears to be a key target for improving the partial N balance when designing crop management and breeding strategies for soybean crops.

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