Exploring Nitrogen Limitation for Historical and Modern Soybean Genotypes

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ABSTRACT

The United States (USA) and Argentina (ARG) account for over 50% of the global soybean [*Glycine max* (L.) Merr.] production. Soybean N demand is partially met (50–60%) by the biological nitrogen fixation (BNF) process; however, an unanswered scientific knowledge gap exists on the ability of the BNF process to fulfill soybean N demand at varying yield levels. The overall objective of this study is to explore the potential N limitation using different N strategies for historical and modern soybean genotypes. Four field experiments were conducted during 2016 and 2017 growing seasons in Kansas (USA) and Santa Fe (ARG). Twenty-one historical and modern soybean genotypes released from the 1980s to 2010s were tested under three N treatments: (i) control, without N application (Zero-N); (ii) 56 kg N ha^{-1} applied at R3-R4 growth stages (Late-N); and (iii) 670 kg ha^{-1} equally split at planting, R1, and R3–R4 growth stages (Full-N). Historical soybean yield gains, from the 1980s to 2010s, were 29% in the USA and 21% in ARG. Following the yield trend, seed N content increased for modern genotypes in parallel to the reduction on seed protein concentration. Regarding N treatments, Full-N produced 12% yield increase in the USA and 4% in ARG. Yield improvement was mainly related to increases in aboveground biomass, seed number (genotype effect), and to a lesser extent, to seed weight (N effect). This study suggests a potential N limitation for soybean, although there are still questions about the way in which N must be provided to the plant.

Core Ideas

- Yields (seed number) increased over time with modern soybean genotypes.
- Seed protein concentration decreased over time.
- Nitrogen fertilization impacted yield via changes on the seed weight.
- Nitrogen limited yields for high-yielding modern soybean genotypes.

SOYBEAN [*Glycine max* (L.) Merr.] is considered as the main source for vegetable oil and animal protein feed in the world (FAO, 2002). The United States (USA) and Argentina (ARG) account for more than 50% of the global soybean production (USDA-NASS, 2017). In the USA, more than 85% of the soybean area is in the Corn Belt region, where it is mainly planted in rotation with corn (*Zea mays* L.) (>60%). In ARG, soybean is primarily planted in the Rolling Pampas and Chaco regions, mainly after wheat (*Triticum aestivum* L.), and after corn to a lesser extent.

Soybean yield potential (Yp) is genetically determined and attained under ideal conditions (genotype × environment × management practices, G × E × M), assuming no limitations in resources (e.g., water and nutrient supply) and in absence of any biotic (e.g., insects, diseases) and abiotic (e.g., temperature, drought, salinity) yield-limiting factors (Evans, 1993). Yield gap between Yp and actual farmer yield (YA) is primarily defined by the interacting effect between genotypes (material selection), the environment (soil + weather), and management practices (e.g., planting date, nutrient and pest management).

A historical yield analysis for soybean showed that seed yield improved by 246% (1300 vs. 3200 kg ha^{-1}) from the 1930s to 2010s (Balboa et al., 2018). Annual seed yield increases of 31 kg ha^{-1} in the USA (Specht et al., 1999) and 28 kg ha^{-1} globally (Wilcox, 2004) were reported from the 1970s to 2000s. As yield increased, a negative effect on seed protein was recorded by Rowntree et al. (2013), with a 0.19 g kg^{-1} yr^{-1} decrease in seed protein for maturity group (MG) II and 0.24 g kg^{-1} yr^{-1} decrease for MG III, from the 1920s and 2000s. Changes in seed yield and seed protein concentration were a consequence of both genetic (Boerma, 1979; Specht and Williams, 1984; Voldeng et al., 1997; Wilson et al., 2014; de Felipe et al., 2016) and management practices (Frederick et al., 1991; Heatherly and Elmore, 2004; Bastidas et al., 2008; Bradley and Sweets, 2008).

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Abbreviations: ADM, aboveground dry biomass; ANOVA, analysis of variance; ARG, Argentina; BNF, biological nitrogen fixation; Full-N, 670 kg ha^{-1} equally split at planting, R1, and R3–R4 growth stages; HI, harvest index; Late-N, 56 kg N ha^{-1} applied at R3-R4 growth stages; MG, maturity group; UAN, urea ammonium nitrate; USA, United States; Zero-N, control, without N application.

Maximum soybean yields are affected by a balanced nutrition, with N as the nutrient with largest demand (Sinclair and de Wit, 1975; Egli, 1998; Roth et al., 2014; Bellaloui et al., 2015). Evidence shows that greater seed yield is associated with a large N requirement (Gaspar et al., 2017; Tamagno et al., 2017; Balboa et al., 2018). The main soybean N sources are provided by the biological N fixation (BNF) and soil N mineralization processes. The BNF process is the result of the conversion of atmospheric N_2 into ammonia (NH_3), and later on into N-containing organic components (Wright and Lenssen, 2013). However, only 50 to 60% of soybean N demand is usually met by the BNF (Salvagiotti et al., 2008). Nitrogen limitation for high-yielding soybean systems was recently proposed by Wilson et al. (2014), Cafaro La Menza et al. (2017), and Ciampitti and Salvagiotti (2018). Following this rationale, research on the interaction among plant N demand for soybean genotypes with high yield potential, N acquisition in soils with different supplying capacity (i.e., crop rotations, cover crops), and/or different capacities of Rhizobium to provide N via BNF (i.e., inoculation, soil pH corrections) is a gap in scientific knowledge that needs to be addressed.

The overall goal of this study was to explore N limitation for historical and modern soybean genotypes. Specific goals for this study were to (i) evaluate yield improvement and N limitation under historical and modern soybean genotypes, and (ii) study the contribution of contrasting N scenarios on seed yield and its components, N removal, and protein concentration.

MATERIALS AND METHODS
Experimental Sites

Four field experiments were conducted during 2016 and 2017 growing seasons in Rossville, KS (USA), and Oliveros, Santa Fe (ARG). All site-years were planted in corn–soybean rotations. Climate and soil characterization are presented in Table 1. Composite soil samples (10–15 cores) were collected before planting at 15- and 60-cm soil depth in the USA (39°07′ N; 95°55′ W). At the 15-cm soil depth, samples were analyzed for pH (Watson and Brown, 1998); organic matter (OM) (Combs and Nathan, 1998); P (Mehlich-P) (Beegle and Denning, 1998); cation exchange capacity (CEC); K, Ca, and Mg (Warncke and Brown, 1998); and for the soil samples at the 60-cm soil depth, only N-nitrate (N–NO_3) concentration was analyzed (Gelderman and Beegle, 1998). In ARG (32°33′ S; 60°52′ W), all soil samples were collected at the 20-cm soil depth, and soil pH, OM, N–NO_3, and Bray P-1 were analyzed using the same methodology conducted for the soil samples collected in the USA.

Treatments

A combination of 21 genotypes released in different decades and three N fertilizer treatments were evaluated. Release decades for soybean genotypes ranged from 1980 to 2010 and includes MG III and IV in both sites. For each site, the same soybean genotypes were evaluated for both years (Table 2). Nitrogen fertilization treatments consisted of three different strategies: (i) control with no N applied (Zero-N); (ii) late application of 56 kg N ha^{-1} at the reproductive stages, as described by Fehr and Caviness (1977), beginning of pod, R3, in the USA and full pod, R4, in ARG (Late-N); and (iii) all N provided by fertilizer at a rate of 670 kg N ha^{-1} equally split at planting, beginning of

flowering (R1), and R3 (USA)–R4 (ARG) stages (Full-N). The Full-N fertilized treatment was implemented to obtain a non-limiting N scenario, assuming a relative low dependence on the N-fixation process, and with the goal of supplying sufficient N to attain high-yielding soybean (6 Mg ha^{-1}). Nitrogen treatments were side dressed using liquid urea ammonium nitrate (UAN; N–P–K, 28–0–0), all applied via hand-held backpack sprayer. Prior to planting, all seeds were inoculated at commercial rate using liquid Vault NP (BASF, Ludwigshafen, Germany), active ingredient *Bradyrhizobium japonicum* at 3×10^9 colony forming units mL^{-1}, at 62 mL per 23 kg of seed.

All experiments were arranged as a split-plot design with four replications. At the USA site, the main-plot was the N treatment and the sub-plot was the genotype factor, whereas at the ARG site, the main plot was the genotype and N treatment was at the sub-plot factor. These differences on the experimental design were mainly due to the availability and how equipment operates in each research station; thus, experimental designs followed logistic convenience for each research station. The USA field plots consisted of four rows spaced at 76 cm with a plot size of 3.0 m wide by 10 m long. The ARG experimental plots had five rows spaced at 52 cm with a plot size of 2.6 m wide by 7.0 m long.

Crop Measurements

Aboveground biomass samples were collected from 1.5 m long in one of the two center rows in all plots before harvest. All collected biomass included not only the plant standing in the field (stem, pods, and grains), but the fallen leaves and petioles to determine total biomass. From each biomass sample, 10 plants were subsampled and fractioned into stems + petioles, leaves, and pod walls. All samples were dried at 65°C until a constant weight was achieved. Total aboveground dry biomass (ADM) was calculated as the sum of the dry weight of plant fractions (stem, leaves, pod walls, and seeds) at beginning of maturity (R7) in ARG and at full maturity (R8) in the USA. Biomass is expressed in kilograms of dry biomass per hectare (Eq. [1]).

$$\text{ADM at R7-R8 (kg ha}^{-1}) = \text{Dry biomass [stem + leaf + pod wall + seed](kg ha}^{-1}) \quad [1]$$

At harvest, the two center rows in each plot were harvested with a plot combine and one-kg of seed sample was collected in each plot. Individual seed weight was measured from a 1000 seed subsample. Then, seed number was estimated from the seed weight and seed yield information. Seed yield and seed weight were both adjusted to 0.130 kg H_2O kg^{-1}. Protein concentration (expressed in dry matter basis) was evaluated with the near infrared spectroscopy (NIR) using the samples collected at harvest with a Perten DA 7200 (Perten Instruments, Springfield, IL, USA). From the harvested seed samples, seeds of seven USA and four ARG genotypes representing all four release decades (1980s, 1990s, 2000s, and 2010s), were ground with a 1 mm mesh and N concentration analysis was conducted (AOAC, 1990). Seed N content, kg ha^{-1}, at harvest was calculated by multiplying the seed dry biomass (kg ha^{-1}) by N concentration (%) following (Eq. [2]).

$$\text{Seed N content (kg ha}^{-1}) = \text{Seed weight (kg ha}^{-1}) \times \text{N concentrations (\%)} \quad [2]$$

Table 1. Soil and climate characterization for 2016 and 2017 growing seasons in Rossville, United States (USA), and Oliveros, Argentina (ARG).†

Year	Location‡	Coordinates	Precipitation	T_{max}	T_{min}	Soil pH	CEC	OM	N-NO$_3$	Mehlich-P§	K	Ca	Mg
			mm	°C			meq 100 g^{-1}	%			mg kg^{-1}		
2016	Rossville, USA	39°07′ N; 95°55′ W	450	28	16	6.9	11.0	2.2	3.0	21	153	2074	202
	Oliveros, ARG	32°33′ S; 60°52′ W	742	31	17	5.5	–	2.1	6.3	12	–	–	–
2017	Rossville, USA	39°07′ N; 95°55′ W	523	29	16	7.3	5.8	1.3	2.7	13	90	951	95
	Oliveros, ARG	32°33′ S; 60°52′ W	688	28	19	–	–	2.5	23.5	9.5	–	–	–

† CEC, cation exchange capacity; OM, organic matter; T_{max}, maximum temperature; T_{min}, minimum temperature.
‡ At ARG, all soil samples were collected at 20 cm of depth; 2015–2016 and 2016–2017 growing seasons for this location.
§ At ARG, P test conducted was Bray P-1 instead of Mehlich-P.

Table 2. Description of field experiments conducted in Rossville, United States (USA), and Oliveros, Argentina (ARG) during the 2016 and 2017 growing seasons.

Year	Location†	Genotype‡	Year released	Maturity group	Planting date
2016	Rossville, USA	P3981, Williams82, 9391, 9392, P93B82, 93B67, 93M90, 93Y92, 94Y23, P35T58R, P39T67R, P31T11R, and P34T43R2	1980, 1981, 1987, 1991, 1997, 2001, 2003, 2009, 2013, 2013, 2013, 2014, and 2014	3, 3, 4, 3.8, 3.8, 3.9, 3.9, 3.9, 4, 3, 4, 3.1, and 3.4	12 May
	Oliveros, ARG	Williams, A4422, DM49, A3910, DM4800, DM3700, NS4955, and SRM3988	1984, 1988, 1990, 1994, 2000, 2003, 2014, and 2015	3, 4, 4, 3, 4, 3, 4, and 3	9 Nov.
2017	Rossville, USA	P3981, Williams82, 9391, 9392, P93B82, 93B67, 93M90, 93Y92, 94Y23, P35T58R, P39T67R, P31T11R, and P34T43R2	1980, 1981, 1987, 1991, 1997, 2001, 2003, 2009, 2013, 2013, 2013, 2014, and 2014	3, 3, 4, 3.8, 3.8, 3.9, 3.9, 3.9, 4, 3, 4, 3.1, and 3.4	18 May
	Oliveros, ARG	Williams, A4422, DM49, A3910, DM4800, DM3700, NS4955, and SRM3988	1984, 1988, 1990, 1994, 2000, 2003, 2014, and 2015	3, 4, 4, 3, 4, 3, 4, and 3	14 Nov.

† 2015–2016 and 2016–2017 growing seasons for ARG location.
‡ Each position for genotype is related to its respective position on released year and maturity group.

Lastly, pods samples (collected prior to harvest) were dried, mechanically separated into pod walls and seeds, and then weighed for the calculation of the harvest index (HI) parameter, obtained as the ratio of seed biomass related to total ADM at harvest, both expressed in dry basis (Eq. [3]).

$$HI = \frac{\text{Seed biomass (kg ha}^{-1})}{\text{Total ADM (kg ha}^{-1})} \qquad [3]$$

Statistical Analyses

The effect of genotype, N, and their interaction was tested with a mixed model, fitting the main plant traits evaluated in this study: seed yield, seed number, seed weight, harvest index, dry biomass at R7-R8, seed N content, and seed protein concentration. Genotype and N were considered fixed effects, while blocks and years were considered random. Each site (USA and ARG) was analyzed independently considering their experimental design. All statistical analyses were performed with the R software (R Software, 2017). As a first step, the Levene's test was conducted using the car package in R program (Fox and Weisberg, 2011) for testing the homogeneity of variance across years for all evaluated traits. When variances were not homogenous, a model comparison was performed by first, adding the weight = varIdent and correlation = corAR1 functions using the nlme package in R (Pinheiro et al., 2017). Then models were compared using the Akaike information criterion (AIC), Bayesian information criterion (BIC), and the P value. Analysis of variance (ANOVA) was conducted for each response variable and the results were considered significant when the P value was smaller than 0.05. Regression lines were plotted for yield vs. seed number, seed weight, and seed protein traits. Then, the residuals of those relationships were plotted against the year of release of the respective genotypes to explore real effects of those
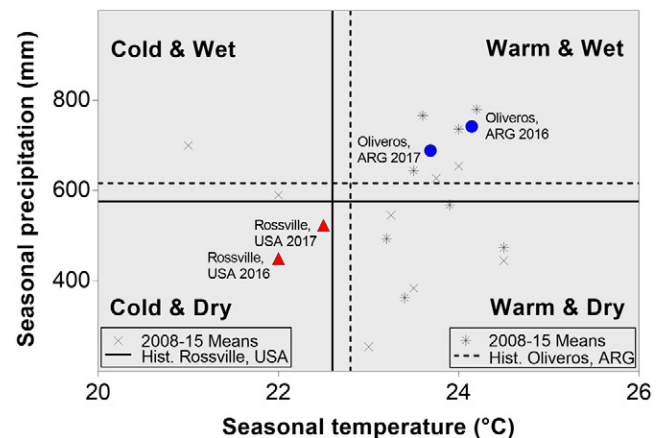

Fig. 1. Thirty-year historical mean, 2008 to 2015 seasonal trends, and 2016–2017 growing season means for temperature and precipitation characterization at Rossville, United States (USA), and Oliveros, Argentina (ARG). Red triangles, USA 2016 and 2017; Blue circles, ARG 2016 and 2017; (×) 2008 to 2015 USA trend; (*) 2008 to 2015 ARG trend; solid lines, 30-yr historical mean for the USA; and dashed lines, 30-yr historical mean for ARG.

plant traits over time (adjusted by yield). Regression analyses (Motulsky and Christopoulos, 2003) and figures were executed using Graph Pad Prism 7 Software.

RESULTS AND DISCUSSION
Environmental Conditions

Seasonal precipitation, maximum (max), and minimum (min) temperatures were recorded throughout both 2016 and 2017 growing seasons at all sites (Table 1). Environmental conditions were compared to the 30-yr historical mean, and with the last 8-yr (2008–2015) seasonal trend. The 2016 and 2017 seasonal mean temperatures were close to the 30-yr historical line with approximately only 1°C of deviation (Fig. 1). As for

Table 3. Overall means and analysis of variance (ANOVA) for N and genotypes on seed yield, seed number, seed weight, harvest index (HI), aboveground dry biomass (ADM) at R8, N exported in seed, and seed protein at harvest for Rossville, United States (USA), field experiments as summary of the 2016 and 2017 growing seasons.

Genotype, year released	N dose	Seed yield	Seed no.†	Seed weight	HI	ADM R8‡	Seed N content	Seed protein
		kg ha^{-1}	seeds m^{-2}	mg seed^{-1}	%	———— kg ha^{-1} ————		g 100 g^{-1}
P34T43R2, 2014		3685	2537	145	0.41	8255	–	–
P31T11R, 2014		4019	2901	140	0.44	8509	–	–
P39T67R, 2013		4750	3366	137	0.42	9935	–	–
P35T58R, 2013		4216	2768	143	0.38	10250	205	38.6
94Y23, 2013		3894	2872	142	0.38	9638	–	–
93Y92, 2009		4204	3073	140	0.37	10092	–	–
93M90, 2003		3486	2354	143	0.36	9185	196	40.3
93B67, 2001		3016	2260	143	0.42	7397	184	40.5
P93B82, 1997		3598	2384	146	0.35	8904	200	40.5
9392, 1991		2882	2134	137	0.36	7926	172	40.0
9391, 1987		3152	2249	143	0.37	8395	174	39.1
Williams82, 1981		2702	1967	144	0.37	7325	–	–
P3981, 1980		2753	1926	145	0.30	8533	161	41.0
	(i) Zero-N	3385	2465	140	0.38	8176	176	39.9
	(ii) Late-N	3476	2469	141	0.39	8237	–	–
	(iii) Full-N	3837	2634	147	0.37	9975	193	40.1
N dose		<0.05*	ns	<0.01**	ns	<0.01**	ns	ns
Genotype		<0.001***	<0.001***	ns	<0.05*	<0.001***	<0.001***	<0.001***
N dose × genotype		<0.01**	ns	ns	ns	ns	ns	ns

* Significant at the 0.05 level.
** Significant at the 0.01 level.
*** Significant at the 0.001 level.
† ns, nonsignificant at the 0.05 probability level.
‡ Fallen leaves were collected from the ground for biomass estimation at the R8 stage.

precipitation, all site-years were within the 150 mm range out of the historical line. The 2017 season was the closest to the 30-yr historical at both sites. The growing seasons evaluated in this study were comparable to the ones experienced during the last 8-yr period (2008–2015).

Yield Improvement and Nitrogen Limitation for Historical and Modern Genotypes

Nitrogen fertilizer ($P < 0.05$ in the USA and $P < 0.001$ in ARG), genotype ($P < 0.001$ for both sites), and their interactions ($P < 0.01$ for both) resulted in a significant effect on soybean yields (Tables 3 and 4). These results suggested that the magnitude of yield response to N differed among genotypes, with yield responses to N occurring more frequently with high-yielding level (modern varieties) and less likely at the low-yielding level.

There is large evidence that soybean seed yield has increased over time (Wilcox et al., 1979; Specht and Williams, 1984; Rowntree et al., 2013; Wilson et al., 2014; Balboa et al., 2018). In the USA, seed yield ranged from 1998 to 6115 kg ha^{-1}, whereas in ARG yield ranged from 2210 to 6470 kg ha^{-1}. At both sites, modern genotypes (released in the 2010s) presented higher yields compared with older materials (1980s, 1990s, and 2000s) (Fig. 2A and 2B). When comparing average yield across all three N fertilization strategies for the modern (2010s) vs. old (1980s) genotypes, yield increased from 2909 to 4073 kg ha^{-1} in the USA (29% increase), and from 3911 to 4964 kg ha^{-1} in ARG (21% increase). A similar study contrasting old (low yield potential, released in the 1930s) vs. modern (high yield potential, released in the 1990s) genotypes observed a 30% seed yield increase linked to longer duration of leaf area, and greater

biomass (Kumudini et al., 2001). Globally, seed yield increased from 1100 to 2600 kg ha^{-1} from 1961 to 2014 (FAO, 2017).

Primary yield drivers resulted from the combination of efforts in plant breeding and fine-tuning on management practices. About half of soybean yield improvement is attributed to genetic changes and the other half to improved agronomic practices and their interaction (Rowntree et al., 2013). Among relevant management practices to be highlighted are earlier planting dates (Conley and Santini, 2007; Bastidas et al., 2008), use of conservation tillage, narrow row spacing, reduction of harvest losses (Heatherly and Elmore, 2004), and improvement in weed control (Bradley and Sweets, 2008). On the other hand, reported genetic improvements in plant traits are related to disease resistance (Foulkes et al., 2009), longer seed filling (Gay et al., 1980), shorter vegetative period favoring longer reproductive periods (Shen and Liu, 2015), and a reduction in lodging (Specht and Williams, 1984). Balboa et al. (2018) summarized that soybean yield improvements (from 1922 to 2015), primarily impacting biomass production, resulted from both fine-tuning management practices and advances in genetics.

In general, as related to yield response to N, when averaged across all genotypes, the Full-N treatment presented a positive impact on seed yield at both the USA and ARG sites (Tables 3 and 4). When overall N supply is not enough, the plant will start remobilizing N from vegetative fractions to the seed, causing a reduction in the photosynthetic capacity, and consequently limiting seed yields (Sinclair and de Wit, 1975). Hence, the addition of N fertilizer becomes an alternative for meeting plant N demand and, ultimately, increasing soybean seed yield. In the USA, the Full-N treatment increased seed yield by 12% relative to Zero-N

Table 4. Overall means and analysis of variance (ANOVA) for N and genotypes on seed yield, seed number, seed weight, harvest index (HI), aboveground dry biomass (ADM) at R7, N exported in seed, and seed protein at harvest for Oliveros, Argentina (ARG) field experiments as summary of the 2016 and 2017 growing seasons.

Genotype, year released	N dose	Seed yield	Seed no.†	Seed weight	HI	ADM R7‡	Seed N content	Seed protein
		kg ha⁻¹	seeds m⁻²	mg seed⁻¹	%	——————— kg ha⁻¹ ———————		g 100 g⁻¹
SRM3988, 2015		4816	2745	175	0.40	10,334	–	–
NS4955, 2014		5200	3149	164	0.42	10,445	259	35.3
DM3700, 2003		4353	2589	168	0.44	8,694	–	–
DM4800, 2000		4389	2446	179	0.41	9,169	210	35.1
A3910, 1994		3912	2484	157	0.38	8,947	–	–
DM49, 1990		4120	2596	158	0.44	8,187	206	37.4
A4422, 1988		4144	2507	165	0.38	9,281	212	37.3
Williams, 1984		3705	1983	188	0.39	8,152	–	–
	(i) Zero-N	4290	2568	168	0.41	9,051	221	36.4
	(ii) Late-N	4256	2512	169	0.41	8,983	215	36.2
	(iii) Full-N	4443	2607	170	0.41	9,420	230	36.3
N dose		<0.001***	<0.01**	<0.001***	ns	<0.01**	<0.01**	ns
Genotype		<0.001***	<0.001***	<0.001***	ns	<0.001***	<0.001***	<0.001***
N dose × genotype		<0.01**	ns	<0.01**	ns	ns	<0.001***	ns

** Significant at the 0.01 level.

*** Significant at the 0.001 level.

† ns, nonsignificant at the 0.05 probability level.

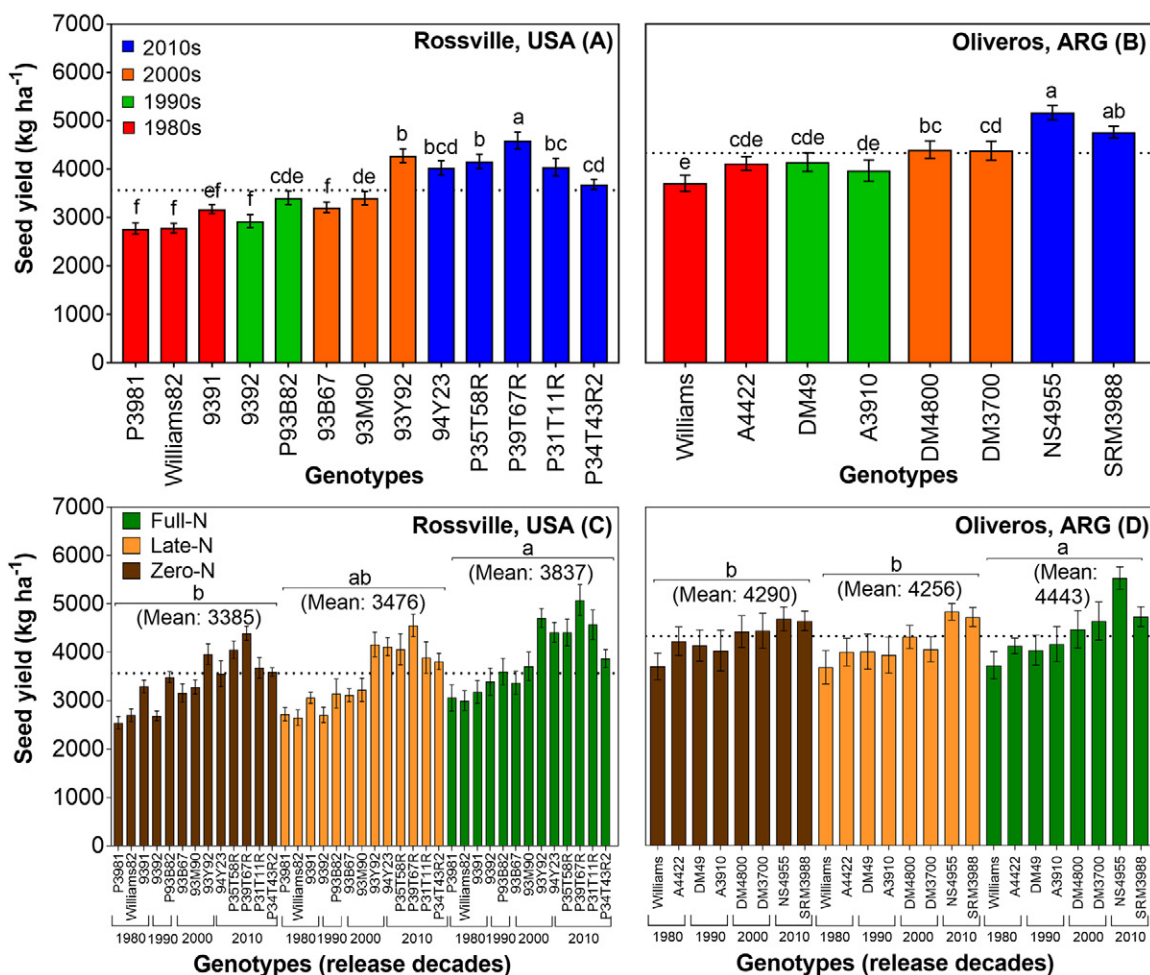‡ Only leaves present in main stem at the R7 stage were included.

Fig. 2. Seed yield (kg ha⁻¹) for 21 historical and modern soybean genotypes as general mean of three N fertilization doses (A and B), and seed yield for three N doses and 21 genotypes (C and D) during the 2016 and 2017 growing seasons at Rossville, United States (USA), and Oliveros, Argentina (ARG). Upper panel: colors represent genotypes of four released decades: 1980s (red), 1990s (green), 2000s (orange), and 2010s (blue). Bottom panel: colors represent three N doses: Zero-N (brown), no N applied; Late-N (yellow), 56 kg N ha⁻¹ at R3–R4 stages; and Full-N (green), 670 kg ha⁻¹ split at planting, R1, and R3–R4 stages. Each bar shows the mean and standard errors of the mean. Dashed lines show the overall mean for each site. Different letters indicate significant differences at P ≤ 0.05.

(Fig. 2C), while Late-N did not statistically differ from the other N treatments. In ARG, the positive response to Full-N was documented with a 4% increase in seed yields relative to the other N treatments (Fig. 2D). For this site, Zero-N and Late-N did not show significant yield differences. In overall, yield response to the Full-N fertilization was observed in both locations, indicating a different yield threshold to express the potential N limitation to satisfy plant nutrient demand (3562 kg ha^{-1} overall in the USA and 4330 kg ha^{-1} overall in ARG) and emphasizing the complexity of different soil N supply and ability of N fixation at these sites. In the current study, yield response to N was observed in environments below the defined yield threshold for high-yielding soybean (4500–5000 kg ha^{-1}) presented by Salvagiotti et al. (2008). The latter indicates that the yield response to N application is not strictly dependent on the yield environment, but other factors affecting the soil N × BNF × plant interaction, playing a significant role. It has been widely assumed that in high-yielding environments the likelihood of N limitation increased due to the intrinsic positive relationship of N requirement and seed yield (Gaspar et al., 2017; Tamagno et al., 2017). A recent study conducted in Nebraska (USA) and Balcarce (ARG) fields with yield levels ranging from 2500 to 6500 kg ha^{-1} concluded that an overall 11% yield response can occur above 2500 kg ha^{-1} yielding environments (Cafaro La Menza et al., 2017). For the current study, N response was observed only in genotypes yielding above 3240 kg ha^{-1} in the USA and 3702 in ARG (Fig. 2C and 2D) in the control conditions (Zero-N). Similar findings were presented in Wilson et al. (2014), reporting yield response to N fertilizer with modern genotypes (high-yielding) rather than older (low-yielding) counterparts. These results suggest that on these sites, BNF and soil N were not capable to fully meet plant N demand, and thus, yield increases were observed when adding N in a non-limiting approach (Full-N treatment).

As for the Full-N scenario, it should be properly acknowledged that this high N fertilizer rate was implemented with the only objective of answering the research question if N presented a limitation for soybean yield formation and seed quality composition (i.e., protein). Although we did not attempt to do any economic and environmental N footprint examinations, it is clear that this method will be far-off any profitable and/or sustainable threshold. Application of N at early reproductive stages could have a positive impact on seed yield supplementing N to the plant when N demand is at a high rate, but some studies have reported no effect from this practice (Gutiérrez-Boem et al., 2004; Barker and Sawyer, 2005). In agreement with these studies, our results showed no significant yield response to Late-N applications (Fig. 2C and 2D), suggesting that a late-season low fertilizer N rate (56 kg ha^{-1}) was not able to improve attainable yields in these environments, because of the trade-off between soil N (or fertilizer) and BNF (Salvagiotti et al., 2009).

Biomass, Seed Number, and Individual Seed Weight

Seed yield increase was related to increases in ADM by harvest and in response to both historical to modern genotypes and to the addition of N as a full rate (Full-N) (Fig. 3). Previous scientific literature (Kumudini et al., 2001; Balboa et al., 2018) has reported increases on ADM as the main factor driving soybean yield improvements over the last decades. In the present study,

greatest accumulation of ADM was observed with modern genotypes (2010s). Overall, 8600 kg ha^{-1} of ADM was accumulated by harvest in the USA and 9100 kg in ARG (Fig. 3A and 3B). The differential ADM accumulation at the two sites can be explained by the interaction of factors affecting crop growth such as management practices, genetic traits, weather [precipitation (Muchow, 1985), temperature (Hadley et al., 1984)], among others. In agreement with the literature, ADM accumulation expressed the yield potential of each site, with ARG recording greater ADM (Fig. 3) and yields (Fig. 2).

In the current study, the application of the full fertilizer N rate (Full-N) resulted in greater biomass (18% more in the USA and 4% more in ARG) for both sites relative to the control treatment (Fig. 3C and 3D). A recent study (Bender et al., 2015) reported increases in biomass in response to fertility (N, P, K, S, and Zn) applications, but primarily attributed to an increased rate of biomass gain during the seed-filling period (from R5 to R7 growth stages).

Moreover, response to genotype and N factors was also observed for seed number and seed weight. Cafaro La Menza et al. (2017) documented seed yield increases linked to superior biomass, seed number, and seed weight. Biomass improvement for genotypes were mainly related to increases on seed number (Fig. 3A.1 and 3B.1) rather than seed weight (Fig. 3A.2 and 3B.2), whereas fertilizer N primarily impacted seed weight (Fig. 3C.2 and 3D.2) rather than seed number (Fig. 3C.1 and 3D.1).

Seed number averaged 2532 seeds m^{-2} in the USA and 2563 seeds m^{-2} in ARG. Seed number was mainly impacted by the genotype factor at both sites, when comparing three old vs. three modern genotypes in the USA and two old vs. two modern in ARG, 1980s vs. 2010s (Fig. 3A.1 and 3B.1). Observations above the 1:1 ($y = x$) line showed greater seed number for the 2010s genotypes, with the downside observed for the 1980s soybean materials.

In general, greater individual seed weight was observed in ARG with an overall average across all treatments of 169 mg seed^{-1} relative to 143 mg seed^{-1} observed in the USA. This yield component was mainly impacted by the N application at both sites (Fig. 3C.2 and 3D.2), with Full-N presenting greater seed weight relative to Zero-N conditions. Observations above the 1:1 ($y = x$) line showed greater individual seed weight for Full-N condition with the downside for the counterpart (Zero-N). At ARG, both factors (N application and genotype) influenced the seed weight. The combined effect of genotype and N resulted in a significant interaction (Table 4), with greatest seed weight observed for the oldest genotype (Williams) with N fertilizer applications.

Lastly and in agreement with previous findings (Cafaro La Menza et al., 2017), HI was not affected by N treatments. Greater HI was observed in the USA site with modern genotypes (Table 3); the same behavior was reported in the synthesis analysis presented by Balboa et al. (2018), finding HI increases over time. In the current study, the lowest HI value was 0.30 (P3981, released in 1980) while the maximum was 0.44 (P39T67R, released in 2014).

Nitrogen Exported and Seed Protein Concentration

Historical changes in seed yield presented also implications for changes in N uptake. To achieve high yields, soybean
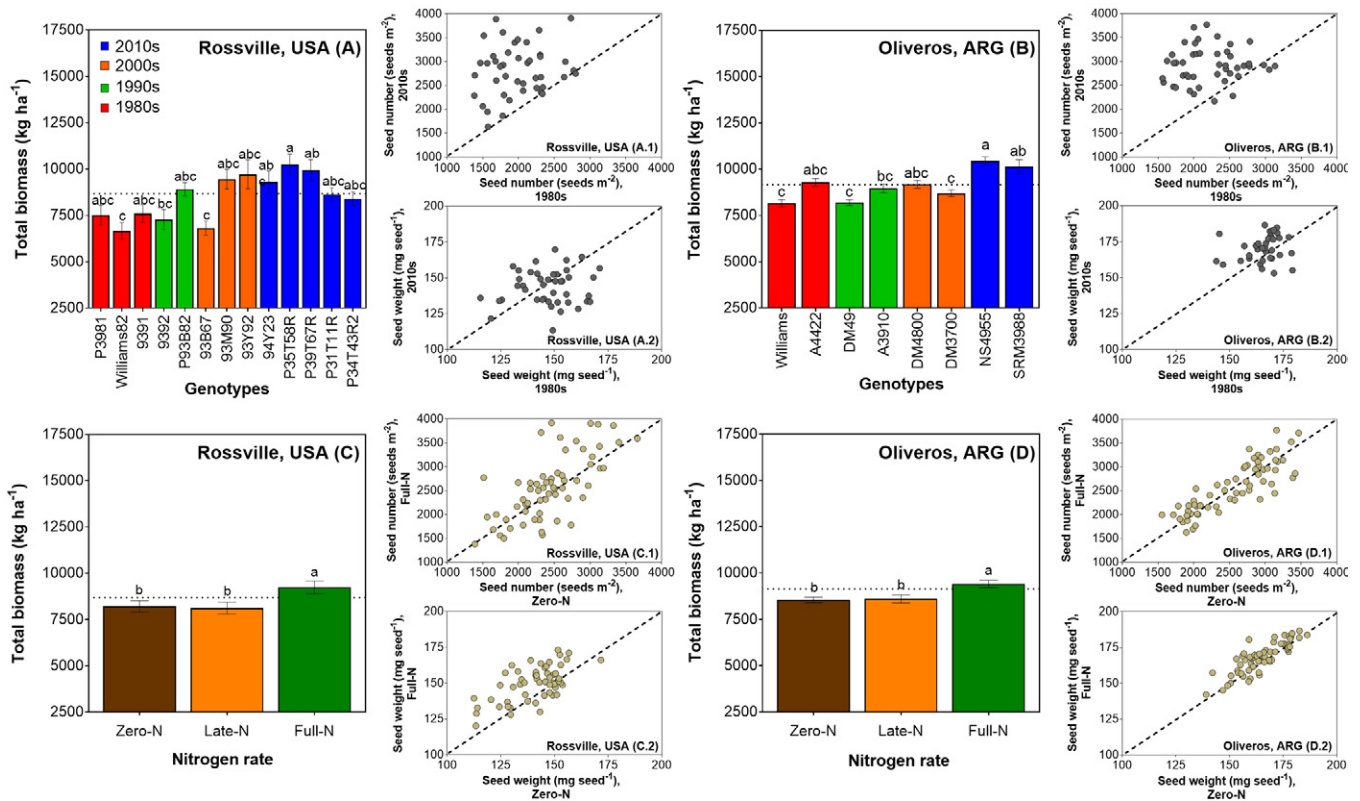
Fig. 3. Total aboveground biomass (kg ha^{-1}) for 21 historical and modern soybean genotypes (A and B), and three N doses (C and D) at Rossville, United States (USA), and Oliveros, Argentina (ARG) during the 2016 and 2017 growing seasons. Upper panel: colors represent genotypes of four released decades: 1980s (red), 1990s (green), 2000s (orange), and 2010s (blue). Bottom panel: colors represent three nitrogen rates: Zero-N (brown), no N applied; Late-N (yellow), 56 kg N ha^{-1} at R3-R4 stages; and Full-N (green), 670 kg N ha^{-1} split at planting, R1, and R3-R4 stages. Each bar shows the mean and associated standard errors of the mean. The dashed lines show the overall mean at each site. Different letters indicate significant differences at $P \leq 0.05$. Scatter plot insets on (A and B) compare seed number (seeds m^{-2}) and individual seed weight (mg seed^{-1}) for contrasting genotypes (1980s vs. 2010s), and insets on (C and D) compare the same traits for contrasting N rates (Zero-N vs. Full-N). Dashed lines in all scatter plot insets show the 1:1 ($y = x$) lines.

plants must attain high photosynthesis rates and accumulate large amounts of N in seeds (Salvagiotti et al., 2008). Nitrogen exported in seeds ranged from 115 to 272 kg N ha^{-1} in the USA and 84 to 386 kg N ha^{-1} in ARG. As for seed yield, modern genotypes (released after 2010) presented the largest amount of N removed (Fig. 4A and 4B). Increases of 19% in the USA and 23% in ARG were observed when comparing overall N removal for modern (2010s) relative to older (1980s) genotypes, averaging all N fertilization conditions.

Although N is the main factor determining protein concentration, increases in seed yield are usually linked to decreases of seed protein concentration due to a dilution effect. The inverse relationship between seed protein concentration and seed yield have been, historically, reported in the scientific literature (Hartwig and Hinson, 1972; Sebern and Lambert, 1984; Wehrmann et al., 1987). Wilcox et al. (1979) reported lower seed protein concentration for modern relative to older genotypes of MG II released from 1927 to 1974. Moreover, a comprehensive and recent study (Rowntree et al., 2013) explored seed protein concentration changes over 115 different genotypes of MGs II and III released from 1923 to 2008, and reported decreases of seed protein concentration with a linear fashion but with larger reduction (ca. 21%) for MG III.

Protein concentration in seed varied between 34 and 45% in the USA and between 28 and 44% in ARG. Protein concentration decreased as yield increased over the decades, registering

the lowest seed protein concentration with the modern, 2000s and 2010s, genotypes (Fig. 4C and 4D). In the USA, a 3.3% decrease (absolute terms) in seed protein concentration was registered, which in turn represented a ~8% in relative terms when comparing 2010s vs. 1980s soybean materials. In ARG, seed protein concentration was reduced by 1.1% in absolute terms and by 3% in relative terms when comparing 2000s and 2010s to 1980s and 1990s genotypes. Comparable decreases in seed protein concentration (from 41 to 38%) were observed in a recent study (Cafaro La Menza et al., 2017) when seed yield increased from 2500 to 6000 kg ha^{-1} in response to addition of N fertilizer. The latter results are in agreement with the seed protein concentration decreases over time for MGs II and III released between 1923 and 2008 (Wilson et al., 2014).

Effect of N applications on the total seed N exported was not observed in the USA (Table 3). Nonetheless, an interaction effect of genotype × N was observed in ARG. The largest amount of seed N exported was a function of the Full-N condition and the modern (NS4955) genotype (data not shown). The Zero-N exported 221 kg ha^{-1} of seed N, whereas the Full-N exported 230 kg ha^{-1} (Table 4) representing a ~4% additional N removal. This additional 4% in seed N content is the N needed for obtaining yield response to N addition as suggested by Salvagiotti et al. (2009). The same authors reported an additional 18 kg ha^{-1} of seed N when comparing control vs. the N treatment (264 vs.
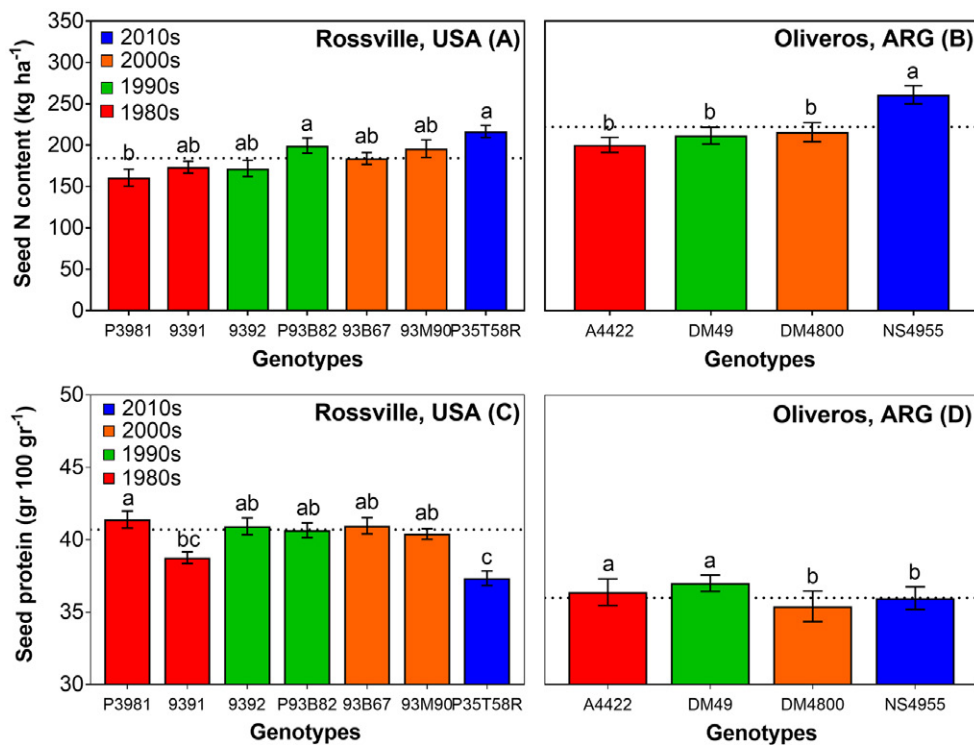
Fig. 4. Seed N content (kg ha⁻¹) and seed protein concentration (g 100 g⁻¹ seed), both in dry basis, for 21 historical and modern soybean genotypes of different release decades as general mean of three N fertilization doses for Rossville, United States (USA), and Oliveros, Argentina (ARG) during the 2016 and 2017 growing seasons. Each color groups genotypes in four release decades: 1980s (red), 1990s (green), 2000s (orange), and 2010s (blue). Each bar shows the mean and associated standard errors of the mean. The dashed lines show the overall mean for each location. Different letters indicate significant differences at $P \leq 0.05$.

282 kg N), suggesting that a ~6% of additional N directed to the seed for increasing yield without inhibiting the BNF process.

As for the seed protein concentration, N treatments effects were not observed at any locations (Tables 3 and 4), suggesting that the differential N was not high enough for impacting seed protein concentration. Cafaro La Menza et al. (2017), with overall yields of 4500 kg ha⁻¹ found minor but measurable protein concentration increases (relative ~4%, which in turn represented 1.5% in absolute terms) in soybean in response to the application of N at high rate (330–640 kg N ha⁻¹) for both the USA and ARG. However, the yield levels explored in our study (overall 3946 kg ha⁻¹) were not as high as those reported by Cafaro La Menza et al. (2017).

Historical Trends in Plant Traits: Seed Number, Weight, and Protein Concentration

The analyses of regressions lines (yield vs. seed number, yield vs. individual seed weight, yield vs. seed protein concentration) and analysis of residuals of those relationships were performed for each location (Fig. 5).

Strong and positive relationship ($R^2 = 0.81$, $P < 0.0001$, and $n = 214$ in the USA; $R^2 = 0.87$, $P < 0.0001$, and $n = 192$ in ARG) was observed between yield and seed number (Fig. 5A). In agreement with previous literature (Rotundo et al., 2012), seed number is one of the main yield components increasing with yield. However, different seed yield to seed number slopes were documented for the two sites, showing that ARG attained similar yields with lower seed number (and thus greater seed weight) as compared with the USA. Residuals of this relationship were plotted against the year of release for all tested

genotypes. Lines with deviations from zero are those lines where the P value of the deviation test is smaller than 0.05. Residuals of the seed yield and seed number relationship did not show statistical deviation from zero (Fig. 5B) in either site, suggesting that seed number changes were mainly governed by the productivity level, regardless of the year of release of each genotype.

When looking to the yield and individual seed weight relationship, only ARG showed a positive and significant trend with higher yields related to higher individual seed weight (Fig. 5C). In the USA, the slope of this regression was not different than zero, suggesting no differences in seed weight among yield levels and supporting the idea that higher yields were primarily related to increases in seed numbers. Residuals of the seed yield and seed weight relationship did not show any departure from zero for the USA, but it was slightly different than zero for ARG (Fig. 5D), although slopes were not statistically different between locations. Similarly to the seed number result, it was concluded that differences on the individual seed weight were mainly driven by the different yield conditions in both locations rather than an effect of time of release of genotypes. Summarizing, current results are in agreement with findings reported by Rotundo et al. (2012), where seed number was the main component driving increases in seed yield in the USA and ARG.

Greater seed protein concentration (~3%, absolute terms) was obtained in the USA relative to ARG (Fig. 5E). The latter result is expected due to the yield levels attained in each location (yields, ARG > USA) and explained with the documented trade-off between yield and seed protein concentration (Hartwig and Hinson, 1972; Wilcox et al., 1979; Sebern and Lambert, 1984; Wehrmann et al., 1987; Rowntree et al., 2013). A significant
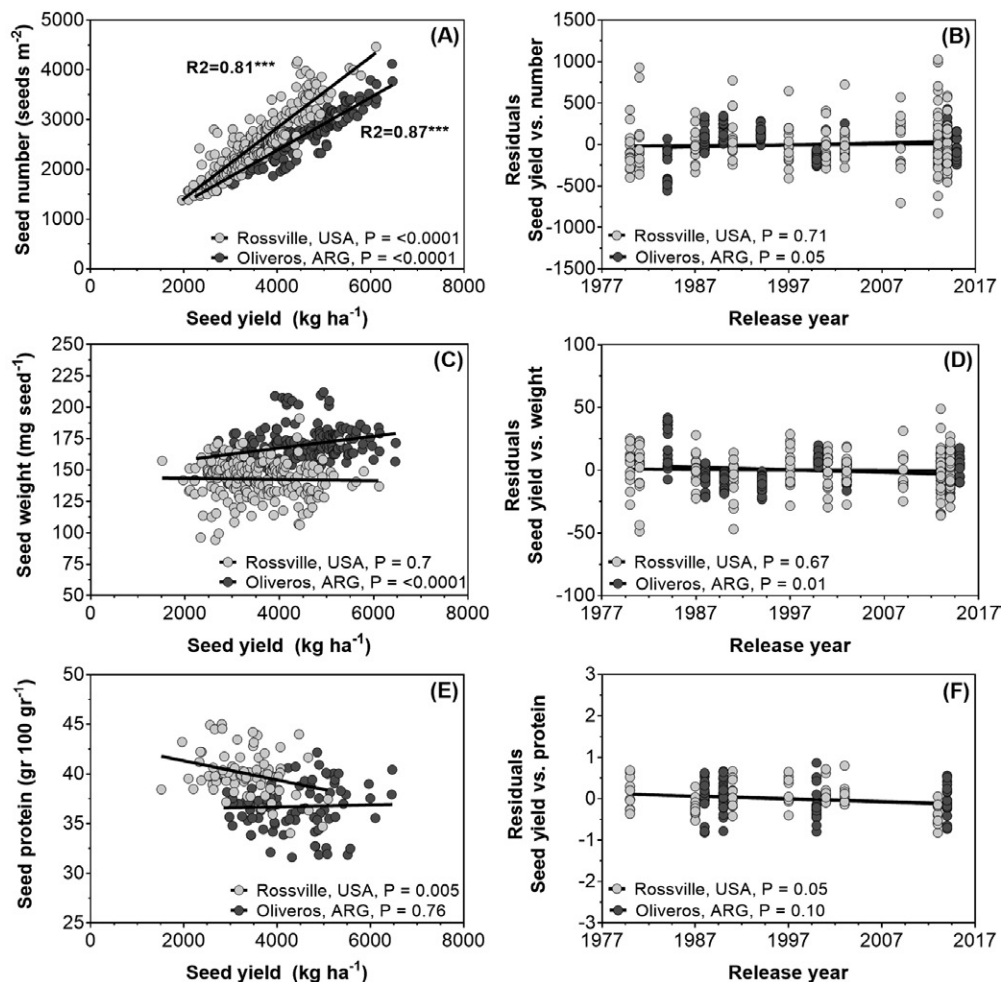
Fig. 5. Seed number (seeds m^{-2}; A and B), seed weight (mg seed^{-1}; C and D), and seed protein concentration (g 100 g seed^{-1}; E and F) traits for Rossville, United States (USA), and Oliveros, Argentina (ARG) during the 2016 and 2017 growing seasons. First column (left): seed yield (kg ha^{-1}) and trait regressions; and second column (right): residuals of the seed yield and trait relationships plotted against year of release of genotypes.

and negative slope was observed only in the USA, suggesting a greater potential limitation of N, reflected with greater yield increases in the USA for Full-N vs. Zero-N. Furthermore, this effect was translated into less N allocated to seeds when moving from low to high yields. As for the residuals plot of the seed yield and protein concentration relationship, no statistical departures from zero were observed in either location (Fig. 5F). These findings reinforced that yield and seed protein concentration were predominantly driven by the productivity level rather than solely year of release for the soybean materials tested.

Results of seed number, individual seed weight, and seed protein concentration suggested: (i) yield differences implied differences (either increases or decreases) on the seed number, seed weight, and seed protein concentration traits in most cases where each environment portrayed different strategies for achieving yields (Fig. 5A, 5C, and 5E); and (ii) changes on seed number, seed weight, and seed protein concentration were mainly governed by the productivity level rather than by the year of release of the soybean materials (Fig. 5B, 5D, and 5F).

CONCLUSIONS

Increases in seed yield were documented when comparing the progress from historical (1980s) to modern (2010s) soybean genotypes in the USA (+29%) and in ARG (+21%). Historical

changes in seed yield were also reflected in the seed N removal, which increased by +19% in the USA and by +23% in ARG. Seed protein concentration was decreased as productivity increased, 3.3 and 1.1% decreases (in absolute terms) for the USA and ARG, respectively, comparing modern with historical genotypes.

Nitrogen limitation (comparing seed yield between the Full-N [without limitation of N] vs. Zero-N [control]) varied between 12% in the USA to 4% in ARG. In the USA, additional 17 kg N ha^{-1} of seed N content was required to increase yields, whereas in ARG, this shortage of seed N content was 9 kg N ha^{-1}. The additional N requirement in seed ([Full-N – Zero-N], seed N content) was not overcome by the late-season N application of 56 kg N ha^{-1} at R3-R4, potentially reflecting the trade-off between the supply of N from the BNF and fertilizer N.

The genotype effect was reflected as yield improvement over time, primarily obtained with changes in biomass and seed number. Nitrogen limitation predominantly impacted seed weight, pinpointing that N limitation might occur during the seed filling period when conditions are favorable for maximum yield.

Future research should improve the knowledge related to exploring the management factors that permitted not only to improve seed yield but also to sustain or improve seed quality (i.e., seed protein concentration) and specially how to manage

the interaction of N in crop × soil N × BNF for satisfying plant N demand in high-yielding soybean systems.

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