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Mineral nitrogen impairs the biological nitrogen fixation in soybean of determinate and indeterminate growth types

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ABSTRACT

Soybean (*Glycine max* Merrill) crop production in Brazil relies mainly on biological nitrogen fixation (BNF) for nitrogen (N) supply. Recent adoption of indeterminate growth-type genotypes has raised doubts on the need for supplemental mineral N that might negatively affect the BNF. We assessed the effects of mineral N on BNF attributes of soybean genotypes grown in central and southern Brazil. Genotypes were inoculated with *Bradyrhizobium* sp. and/or received mineral N in three sets of experiments. In the first set, two genotypes received increasing rates of mineral N in nutrient solution, which consistently reduced the BNF. In the second set, mineral N applied at sowing and/or topdressing reduced nodulation and ureides-N in determinate and indeterminate growth-type genotypes. In the third set, mineral N applied at R5.3 stage, foliar or as topdressing, did not increase grain yield in four field experiments. Mineral N impaired BNF irrespective of the growth type and had no effect on grain yield.

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Glycine max; ureides; nitrogen fertilization; mineral N; nodulation

Introduction

Soybean (*Glycine max* Merrill) is one of the most important food crops worldwide (Rodrigues-Navarro et al. 2011). In Brazil, about 33.8 million ha were cropped in the 2016/17 cropping season, mainly in the central-west and southern regions (CONAB 2017).

Nitrogen (N) is essential for plant growth, participating in several metabolic pathways and in the synthesis of molecules such as proteins, nucleic acids, hormones, and chlorophyll (Epstein 1999). Due to the high protein content in grains, about 40%, soybean requires large amounts of N (Rodrigues-Navarro et al. 2011; Kinugasa et al. 2012), about 80 kg per metric ton of grains. The sources of N to plants are soil, via mineralization of organic matter, non-biological N_2 fixation (e.g. lightning, combustion, volcanism), biological N_2 fixation (BNF) (Hungria et al. 2006a), and even N fertilizers (Ray, Heatherly, and Fritschi 2006; Salvagiotti et al. 2008). Considering that the N reservoirs in soil organic matter are limited and are depleted quickly after a few cropping cycles, and that N fertilizers have usually an efficiency below 50% (Hungria et al. 2006a), BNF is currently the most sustainable way—economically and environmentally—to provide N for soybean.

BNF results from a highly specific symbiosis with *Bradyrhizobium* strains (Hungria et al. 2005), which supplies 50–60% of soybean demands (Salvagiotti et al. 2008) but may reach up to 94%, even for high yields (Hungria et al. 2006a). The high efficiency of Brazilian soybean genotypes on taking more

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N biologically is probably the result of breeding programs that are generally prioritized relying on the BNF process instead of mineral N fertilization. Hence, BNF greatly contributes to the high economic competitiveness of the Brazilian soybean in the world market (Hungria and Mendes 2015).

Nevertheless, changes in the soybean cultivation scenario, including more productive, early, and indeterminate growth-type genotypes, have led some farmers to use supplementary fertilization with mineral N, even without scientific support for the effectiveness of this practice. However, mineral N supply may impair the N_2 fixation capacity of soybean (Gan et al. 2003; Ray, Heatherly, and Fritschi 2006; Kinugasa et al. 2012). Several studies on the use of mineral N in soybean show conflicting results (Salvagiotti et al. 2008), which are attributed to several biological and non-biological causes (Ray, Heatherly, and Fritschi 2006; Heitholt et al. 2007). Use of mineral N may increase soybean grain yield under very specific situations, but this practice is rarely economically viable (Ray, Heatherly, and Fritschi 2006; Salvagiotti et al. 2008).

The aim of this study was to assess the effects of mineral N on BNF traits in soybean genotypes of determinate and indeterminate growth types under greenhouse and yield under field conditions.

Materials and methods

Set I—greenhouse conditions with sterile substrate

The indeterminate growth-type genotypes BRS 360 RR and BMX Potência RR were grown in a nutrient solution in a greenhouse. Five surface-sterilized seeds (1 min 70% ethanol; 4 min 0.5% sodium (Na) hypochlorite; ten rinses in autoclaved distilled water (H_2O)) were sown in Leonard jars containing autoclave-sterilized substrate (sand + vermiculite, 1:1 v/v), and inoculated with *Bradyrhizobium japonicum* (SEMIA 5079) and *Bradyrhizobium elkanii* (SEMIA 587) (commercial peat-based inoculant applied to deliver 1.2×10^6 cells per seed). Just after emergence, plantlets were thinned to two per jar and started to receive a modified Hoagland's nutrient solution containing 0, 5, 10, 15, 25, 50, 75, 100, or 125 mg L^{-1} of N as ammonium nitrate for 4 weeks. Non-inoculated controls with 0 or 80 mg L^{-1} of N were included as negative and positive controls for N, respectively, but were not considered in the statistical analysis. The experimental design was completely randomized, with six replications.

Thirty-five days after emergence (DAE), plants were collected and assessed for nodulation, flow of ureides-N in the xylem sap, and N concentration in shoots.

Set II—greenhouse conditions with non-sterile soil

Fourteen soybean genotypes, seven of indeterminate and seven of determinate growth types, were assessed (Table 1). The experiment was conducted in a greenhouse using Typic Acrudox (USDA 1999) soil taken at a depth of 0–20 cm from a soybean production area. The results of soil chemical analyses before liming and fertilization and particle size are presented in Table 2. Dolomitic lime (2.2 g kg^{-1}) was applied to raise the soil pH calcium chloride ($CaCl_2$) to 6.5 after 30 days of incubation. The pots filled with 4 kg of this soil received magnesium sulfate (1.15 g pot^{-1}) and potassium hydrogen phosphate (0.293 g pot^{-1}). A solution containing the following micronutrients such as cobalt (Co) (Cobalt (II) Sulfate Heptahydrate ($CoSO_4 \cdot 7H_2O$)—0.028 mg pot^{-1}), molybdenum (Mo) (Sodium Molybdate Dihydrate ($Na_2MoO_4 \cdot 2H_2O$)—0.1 mg pot^{-1}), and boron (B) (boric acid (H_3BO_3)—10 mg pot^{-1}) was also applied.

Immediately before sowing, seeds were inoculated with peat inoculant as described in set I. Five seeds were sown per pot and thinned to two plantlets seven DAE. Each genotype received the following: no mineral N (0 N); 100 mg pot^{-1} of mineral N (urea) at sowing (100 N at sowing); 100 mg pot^{-1} of mineral N (ammonium nitrate) as topdressing at 32 DAE (100 N at 32 DAE); or 200 mg pot^{-1} of mineral N, 50% at sowing (urea) and 50% as topdressing at 32 DAE (ammonium nitrate) (100 N at sowing + 100 N at 32 DAE). Plants were watered daily to maintain 70% of soil water holding capacity.

Table 1. Characteristics of soybean genotypes used in set II: maturation group, maturation time, cycle, height, flower color, protein content, oil content, and sowing period.

Genotype	Maturation group	Maturation (days) [†]	Cycle (days)	Height (cm)	Flower color	Protein content (%) [‡]	Oil content [‡]	Sowing period (preferential)
				Indeterminate growth-type genotypes				
BRS 283	6.5	Early	120–126	85–105	Purple	36.6	21.6	05 Oct–05 Dec
BRS 284	6.3	Early	120–126	80–100	Purple	38.7	20.4	10 Oct–05 Dec
BMX Potência RR	6.6	Mid-early	125–140	95–112	White	39.4	19.7	15 Oct–15 Nov
BMX Turbo RR	5.8	Very-early	110–122	80–105	Purple	38.7	20.0	15 Oct–15 Nov
BMX Força RR	6.2	Early	118–128	81–107	White	40.4	20.1	15 Oct–15 Nov
Nidera 5909 RR	5.9	Mid-early	110–125	72–92	Purple	37.2	18.2	20 Oct–10 Dec
V Max RR	6.1	Very-early	110–115	76–103	White	39.2	23.2	20 Oct–20 Nov
				Determinate growth-type genotypes				
BRS 184	6.7	Mid-early	122–128	68–95	Purple	39.0	24.2	15 Nov–05 Dec
BRS 232	6.9	Mid-early	126–132	67–93	Purple	40.9	19.5	25 Nov–05 Dec
BRS 317	6.6	Mid-early	122–128	80–110	White	37.1	22.1	20 Oct–05 Dec
BRS 295 RR	6.5	Mid-early	118–126	70–95	White	39.1	19.9	20 Oct–05 Dec
Embrapa 48	6.8	Mid-early	122–128	60–95	White	39.1	21.4	25 Nov–05 Dec
BRS 255 RR	6.7	Mid-early	122–128	70–94	White	39.9	23.3	20 Oct–05 Dec
BRS 294 RR	6.5	Early	120–126	64–92	White	38.5	19.0	25 Oct–30 Nov

[†]Maturation groups: Very-early (100–112 days); early (113–122 days), and mid-early (123–130 days). Maturation for the state of Paraná, Southern Brazil; values may vary according to the season.
[‡]Contents of protein and oil in seeds.

The experimental design was a randomized complete block, with a 2 (growth types) × 4 (N treatments) factorial arrangement and five replications of each genotype.

Plants were collected at 52 DAE and assessed for shoot dry weight (DW) and root DW, number of nodules, nodule DW, ureides-N in dry leaves and petioles, and amide-N, ammonium-N, and nitrate-N in leaves. Twenty fresh nodules were collected randomly and stored at −20°C for analysis of soluble carbohydrates. The nodule DW/number of nodules ratio resulted in the specific mass of nodules.

Table 2. Chemical characteristics and particle size at 0–20 cm depth of soils used in the sets of experiments.

		mg dm^{-3}	cmolc dm^{-3}						%		g kg^{-1}		
Set	pH	P	H + Al	Ca	Mg	K	SB	CEC	V	C	sand	silt	clay
II greenhouse [†]	4.7	2.14	5.6	4.02	0.64	0.31	4.97	10.5	47	1.93	732	30	238
III field, BRS 379 Topdressing	4.8	15.08	7.05	3.86	1.33	0.32	5.51	12.6	44	2.95	324	146	530
III field, BRS 379, Foliar	4.8	9.94	6.85	3.55	1.21	0.39	5.15	12.0	43	2.75	300	163	537
III field, BMX Ativa, Topdressing	5.1	6.15	6.22	3.86	2.23	0.32	6.41	12.6	51	2.88	294	118	585
III field, BMX Ativa, Foliar	5.0	4.52	6.03	3.79	2.00	0.22	6.01	12.0	50	2.80	267	135	598

[†]Before liming and fertilization. pH in 0.01 mol L^{-1} $CaCl_2$; P extracted in Mehlich I solution; H+Al determined in SMP buffer; Al, Ca, Mg, K extracted in 0.5 mol L^{-1} KCl; SB = sum of basis (Ca + Mg + K); V = % of basis occupying the CEC.

Set III—field experiments

Four independent field experiments were conducted in the 2012/2013 crop season in Typic Acrudox soil (USDA 1999) in Ponta Grossa PR, southern Brazil, with application of mineral N as urea. Two genotypes (BRS 379 RR—indeterminate or BMX Ativa RR—determinate growth type) received 0, 50, 100, or 200 kg ha^{-1} of urea (45% N) as topdressing or 0, 5, 10, or 15 kg ha^{-1} of urea as foliar application using a carbon dioxide (CO_2)-pressurized sprayer, at R5.3 stage (Fehr and Caviness 1977). The experimental design was a randomized complete block with four replications. Field plots consisted of six soybean rows of 7 m, separated two rows apart. Data on soil physical and chemical properties before the application of mineral N are shown in Table 2.

The genotype BRS 379 RR was sown on 19 November 2012, receiving 124 kg ha^{-1} of 0–20–20 fertilizer in-furrow, resulting in eight plants m^{-1} spaced 0.45 m between rows. The preceding wheat (*Triticum aestivum* L.) crop received 413 kg ha^{-1} of 14–33–00 fertilizer. The genotype BMX Ativa RR was sown on 11 November 2012, receiving 200 kg ha^{-1} of simple superphosphate in-furrow and 150 kg ha^{-1} of potassium chloride in the surface, resulting in 12 plants m^{-1} spaced 0.5 m between rows. In all crops, seeds were inoculated with commercial peat-based inoculants with *Bradyrhizobium* sp. to supply 1.2×10^6 cells per seed. At physiological maturity, plants were assessed for grain yield in the four central rows of the field plots, adjusted to 13% moisture.

Plant analysis

After drying, shoots of plants from sets I and II were ground (<18 mesh), and 100 mg was digested in sulfuric acid in duplicate to determine N shoot concentration. N was quantified by spectrophotometry with the green salicylate method (Searle 1984).

Plants from set I were assessed for the rate of ureides-N transportation (Vogels and van der Drift 1970) in xylem sap collected according to Hungria (1994). Dried leaves and petioles from set II were ground separately in a micromill (0.1 mm mesh), and ureides-N content was determined in extracts from 300 mg of dry tissue as above. Leaf extracts were assessed for amide-N (Boddey et al. 1987), ammonium-N (Mitchell 1972), and nitrate-N (Cataldo et al. 1975), with minor changes for the determination of ammonium-N in which sodium hypochlorite was replaced by sodium dichloroisocyanurate (Hungria 1994). Soluble carbohydrates in fresh nodules were based on the methods of Haissig and Dickson (1979) and Ou-Lee and Setter (1985).

Statistical analysis

First, pre-requisites for normality and independence of errors, the non-additivity of the model, and homogeneity of variances (Shapiro and Wilk 1965; Tukey 1949; Anderson and McLean 1974) were checked using SAS (SAS Institute 2009). The significance of the analysis of variance—ANOVA—was checked with SAS (SAS Institute 2009). Once verified that statistical significance ($p \leq 0.05$) for treatment occurred, Tukey's test was applied with SAS (SAS Institute 2009) for single effects and SANEST for interactions (Zonta, Machado, and Silveira-Júnior 1982), obtaining the Tukey Honest Significant Difference (HSD) at $p = 0.05$ to compare qualitative treatments, or regression analysis for quantitative treatments using the software SISVAR (Ferreira 2008).

Results

Set I

Under controlled conditions and sterile substrate, the number of nodules for the genotype BMX Potência RR fitted a quadratic model, describing an initial increase, followed by a decrease as the doses of N increased, whereas BRS 379 fitted a linear decreasing model (Figure 1A). For nodule DW, both genotypes fitted linear decreasing models (Figure 1B). The rate of ureides-N transported in sap also decreased sharply, fitting a quadratic model for the genotype BRS 360 RR, but with no effect for the

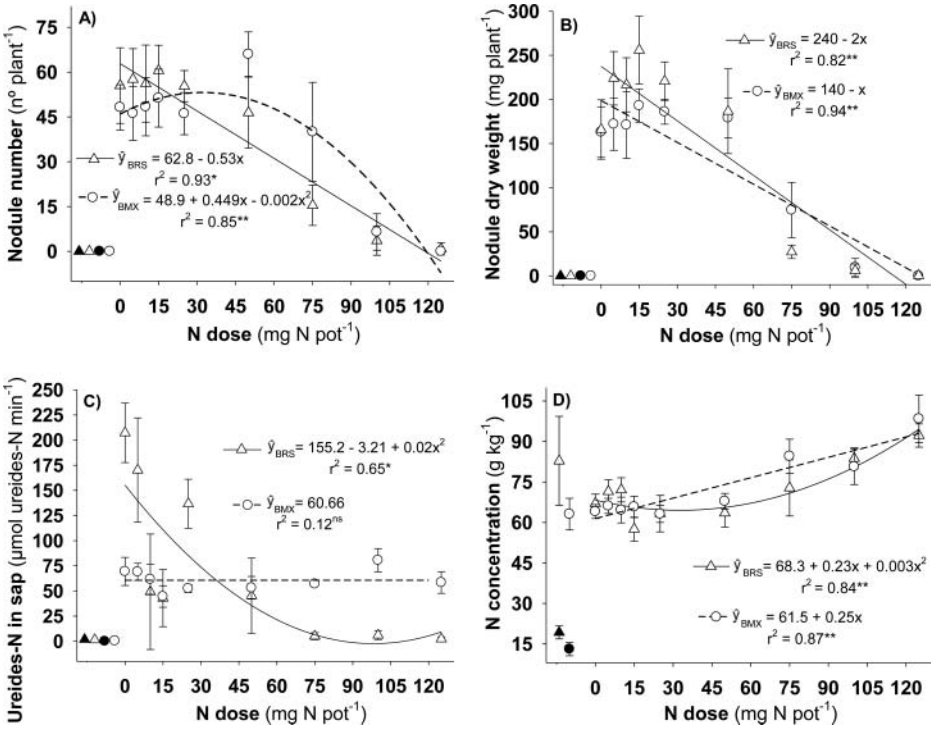

Figure 1. Number (A) and dry weight of nodules (B), ureides-N transportation rate in sap (C), and N concentration in shoots (D) at 35 days after emergence in two indeterminate growth-type genotypes (BRS 360 RR and BMX Potência RR) inoculated with *Bradyrhizobium* sp. and receiving increasing doses of mineral N in nutrient solution once a week, for 5 weeks (set I). Symbols:▲ BRS 360 non-inoculated, without N (Ni–N);△ BRS 360 Ni, with 80 mg of N pot^{-1} (80 N);△ BRS 360 + *Bradyrhizobium*;● BMX Potência Ni–N;◯ BMX Potência Ni + 80 N;◯ BMX Potência + *Bradyrhizobium*. Vertical bars represent the standard deviation. * $p \leq 0.05$; ** $p \leq 0.01$; ns = non-significant.

genotype BMX Potência RR (Figure 1C). At doses ≤25 mg pot^{-1} of N, the BRS 360 RR genotype showed higher rates of ureides-N transportation than BMX Potência RR, but values were under the BMX Potência genotypes at doses >50 mg pot^{-1} of N. Both genotypes had an increase in N concentration in shoots with the doses of N in the solution, linear for BMX Potência and quadratic for BRS 379 (Figure 1D).

Set II

Indeterminate growth-type genotypes had higher root DW, shoot DW, and number of nodules than the determinate type (Table 3). Effect of N on root DW was non-significant, but the treatment 100 N at sowing + 100 N at 32 DAE increased shoot DW, whereas decreased the number of nodules compared with the treatment 0 N and 100 N 32 DAE. Nodule DW had significant interaction between growth types and N treatments; higher nodulation occurred with only BNF (0 N), and decreased with the addition of mineral N. Considering the growth type, indeterminate had more nodule DW than determinate ones when mineral N was applied at sowing. Plants grown exclusively with BNF (0 N) had higher specific nodule weight.

Ureides-N in leaves decreased with the addition of mineral N in both genotypes (Table 4). Plants grown with only BNF had higher values for both growth types than plants that received mineral N, either at sowing or as topdressing. Considering the genotype within each level of N treatments, the indeterminate ones had higher contents. Ureides-N in petioles had a slight variation between

Table 3. Effect of supplemental N on plant growth (shoot DW; root DW) and nodulation (number of nodules; nodule DW; and specific mass of nodules) of determinate and indeterminate growth-type soybean genotypes inoculated with *Bradyrhizobium* sp. (set II).

	N treatment				
Growth type	0 N	100 N sowing	100 N 32 DAE	100 N sowing + 100 N 32 DAE	Average
			Root DW (g pl^{-1})		
Determinate	2.1	2.2	2.3	2.2	2.2 b
Indeterminate	2.6	2.4	2.4	2.5	2.5 a
Average	2.3 A	2.3 A	2.4 A	2.3 A	
ANOVA	Treat. N (N) = ns	Growth type (G) = *	N × G = ns		
Tukey HSD		HSD = 0.16			
			Shoot DW (g pl^{-1})		
Determinate	8.6	8.1	8.8	9.0	8.6 b
Indeterminate	9.0	9.1	9.2	9.7	9.2 a
Average	8.8 B	8.6 B	9.0 AB	9.4 A	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N × G = ns		
Tukey HSD	HSD = 0.43	HSD = 0.23			
			No. nodules (no. pl^{-1})		
Determinate	67.0	55.2	63.1	45.6	57.7 b
Indeterminate	73.5	64.2	69.0	56.5	65.8 a
Average	70.2 A	59.7 AB	66.0 A	51.1 B	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N × G = ns		
Tukey HSD	HSD = 11.2	HSD = 5.9			
			Nodules DW (mg pl^{-1})		
Determinate	202 a A	133 b B	128 a B	75 b C	134
Indeterminate	206 a A	155 a B	143 a B	130 a B	158
Average	204	144	135	102	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N×G = *		
Tukey HSD			HSD_G = 21.2		
			HSD_N = 28.3		
			Specific mass of nodules (mg nod^{-1})		
Determinate	3.1	2.5	2.1	1.7	2.4 a
Indeterminate	3.1	2.5	2.1	2.4	2.5 a
Average	3.1 A	2.5 B	2.1 B	2.0 B	
ANOVA	Treat. N (N) = *	Growth type (G) = ns	N × G = ns		
Tukey HSD	HSD = 0.63				

Means followed by the same letter, lower case in columns to compare growth types, and upper case in lines to compare N treatments, do not differ significantly by Tukey's HSD test ($p = 0.05$). For ANOVA *, significant at $p \leq 0.05$; ns, non-significant. ($n = 35$, seven varieties of each growth type). No mineral N (0 N); 100 mg pot^{-1} of mineral N (urea) at sowing (100 N at sowing); 100 mg pot^{-1} of mineral N (ammonium nitrate) as topdressing at 32 DAE (100 N at 32 DAE); and 200 mg pot^{-1} of mineral N, 50% at sowing (urea) and 50% as topdressing at 32 DAE (ammonium nitrate) (100 N at sowing + 100 N at 32 DAE).

genotypes, depending on the N treatment; stronger effects occurred for N treatments in which mineral N decreased the concentration of ureides-N, especially when applied at sowing (Table 4).

Indeterminate genotypes had higher concentrations of nitrate-N in all N treatments. In the treatments that received N at sowing or as topdressing at 32 DAE, the determinate growth-type genotypes showed the lowest and the highest concentrations of nitrate-N, respectively. The indeterminate genotypes, on the other hand, were less affected by N treatments (Table 4). Ammonium-N had a slight variation between growth types within each N treatment. However, the application mineral N as topdressing, with or without mineral N at sowing, increased the ammonium-N in leaves in the determinate genotypes in comparison with plants with only BNF. Amide-N varied according to the growth type in response to N treatments. The determinate genotypes had higher concentrations in plants with only BNF, but lower when supplemented with N. Regarding the effects of N treatments, amide-N decreased with supplemental N at sowing in comparison with plants with only BNF, and increased in both treatments that received mineral N as topdressing, in both growth-type genotypes.

The N concentrations in shoots were not as high in N-supplemented plants compared with plants with only BNF, and this effect was more evident in the determinate genotypes (Table 4). There was less N accumulation in determinate genotypes supplemented with N, whereas indeterminate genotypes

Table 4. Effect of supplemental N on the concentrations of ureides-N in leaves and petioles, nitrate-N, ammonium-N and amide-N in leaves, concentration and total N accumulated in shoots, and concentration of soluble carbohydrates in fresh nodules from determinate and indeterminate growth-type soybean genotypes inoculated with *Bradyrhizobium* sp. (set II).

	N treatment				
Growth type	0 N	100 N sowing	100 N 32 DAE	100 N sowing + 100 N 32 DAE	Average
			Ureides-N in leaves (μmol g^{-1})		
Determinate	2.7 b A	1.4 b D	2.2 b B	1.9 b C	2.1
Indeterminate	3.0 a A	1.6 a C	2.5 a B	2.3 a C	2.3
Average	2.8	1.5	2.3	2.1	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N × G = *		
Tukey HSD			$HSD_G = 0.05$		
			$HSD_N = 0.06$		
			Ureides-N in petioles (μmol g^{-1})		
Determinate	18.6 a A	10.8 b D	16.1 a B	15.3 a C	15.2
Indeterminate	18.2 b A	11.1 a D	15.9 b B	15.4 a C	15.2
Average	18.4	11.0	16.0	15.3	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N × G = *		
Tukey HSD			$HSD_G = 0.17$		
			$HSD_N = 0.23$		
			Nitrate-N in leaves (μmol g^{-1})		
Determinate	0.7 b C	0.6 b D	0.9 b A	0.8 b B	0.8
Indeterminate	1.0 a B	1.0 a B	1.1 a AB	1.2 a A	1.1
Average	0.9	0.8	1.0	1.0	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N × G = *		
Tukey HSD			$HSD_G = 0.05$		
			$HSD_N = 0.07$		
			Ammonium-N in leaves (μmol g^{-1})		
Determinate	2.4 b B	2.4 b B	2.7 a A	2.7 a A	2.6
Indeterminate	2.5 a A	2.6 a A	2.6 b A	2.6 a A	2.6
Average	2.4	2.5	2.7	2.6	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N × G = *		
Tukey HSD			$HSD_G = 0.08$		
			$HSD_N = 0.11$		
			Amide-N in leaves (μmol g^{-1})		
Determinate	2.3 a C	1.2 b D	2.6 b A	2.4 b B	2.2
Indeterminate	2.1 b C	1.5 a D	3.0 a A	2.6 a B	2.3
Average	2.2	1.4	2.8	2.5	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N × G = *		
Tukey HSD			$HSD_G = 0.04$		
			$HSD_N = 0.05$		
			N in shoots (g kg^{-1})		
Determinate	27.5 a A	18.0 b D	25.4 a B	24.5 b C	23.9
Indeterminate	27.0 b A	19.0 a C	25.6 a B	25.7 a B	24.3
Average	27.2	18.5	25.5	25.1	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N × G = *		
Tukey HSD			$HSD_G = 0.45$		
			$HSD_N = 0.61$		
			N accumulated in shoots (mg pl^{-1})		
Determinate	236 a A	146 b C	223 b B	222 b B	207
Indeterminate	243 a A	173 a B	236 a A	248 a A	225
Average	240	159	229	235	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N × G = *		
Tukey HSD			$HSD_G = 9.39$		
			$HSD_N = 12.53$		
			Soluble carbohydrates in fresh nodules (mg g^{-1})		
Determinate	49.8 a A	34.3 b C	39.6 b B	35.2 b C	39.7
Indeterminate	48.4 b A	38.7 a C	45.9 a B	38.8 a C	42.9
Average	49.1	36.5	42.7	37.0	
ANOVA	Treat. N (N) = *	Growth type (G) = *	N x G = *		
Tukey HSD			$HSD_G = 1.33$		
			$HSD_N = 1.77$		

Means followed by the same letter, lower case in columns to compare growth types, and upper case in lines to compare N treatments, do not differ significantly by Tukey's HSD test ($p = 0.05$). For ANOVA *, significant at $p \leq 0.05$; ns, non-significant. ($n = 35$, seven varieties of each growth type). No mineral N (0 N); 100 mg pot^{-1} of mineral N (urea) at sowing (100 N at sowing); 100 mg pot^{-1} of mineral N (ammonium nitrate) as topdressing at 32 DAE (100 N at 32 DAE); and 200 mg pot^{-1} of mineral N, 50% at sowing (urea) and 50% as topdressing at 32 DAE (ammonium nitrate) (100 N at sowing + 100 N at 32 DAE).

had reduction when received N only at sowing. Both growth-type genotypes had the same N accumulation when N was supplied only via BNF (Table 4).

The concentration of carbohydrates in nodules also decreased with supplemental N in both growth types (Table 4). In the plants with only BNF, indeterminate genotypes had less soluble carbohydrates, but there was an inversion when plants were supplemented with mineral N.

Set III

Yields did not respond to the application of mineral N at R5.3 stage, either as topdressing, or as foliar application, irrespective of the growth type (Figure 2). Yields ranging from 4500 to 4800 kg ha^{-1} were obtained.

Discussion

The average soybean yield in Brazil at the 2016/17 growing season was 3338 kg ha^{-1} (CONAB 2017), but there are reports of yields over 7000 kg ha^{-1}. Recently, questions have arisen about the need of

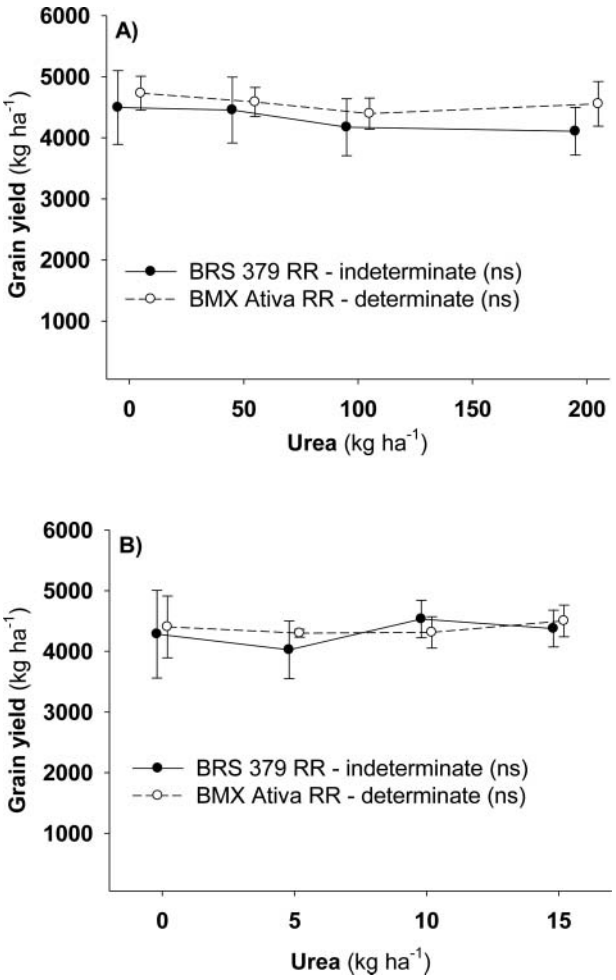

Figure 2. Grain yield as a function of urea application as topdressing (A) or leaf application (B) at R5.3 stage on grain yield of two growth-type soybean genotypes (BRS 379 RR and BMX Ativa RR), under field conditions in the season 2012/13 (set III). Vertical bars represent the standard deviation. ns = non-significant ($p > 0.05$).

supplementary mineral N in indeterminate growth-type soybean varieties, especially in highly productive environments (Salvagiotti et al. 2008). In the expectation to reach higher yields, farmers have been induced to use mineral N without scientific criteria, despite previous reports stating that fertilization with mineral N can negatively affect the BNF in soybean of determinate growth type (Gan et al. 2003; Hungria et al. 2006b).

Plants supplemented with mineral N in nutrient solution (set I) either at sowing or as topdressing under greenhouse conditions (set II) showed significant decreases in traits related to BNF like nodulation, specific mass of nodules, and ureides-N, emphasizing on the negative effect of supplemental mineral N. The reduction in nodulation was more pronounced in the treatment that received mineral N at sowing and as topdressing, demonstrating that the higher the amount of mineral N supplied, the more intense the negative effect on nodulation, and consequently the BNF-related parameters. In a trial under field conditions, the application of 50 or 100 kg ha^{-1} of N at sowing reduced the number and nodule DW, and limited the amount of N fixed biologically and the grain yield (Hungria et al. 2006b). Since the 1960s, it has been reported that supplemental mineral N negatively affects the BNF, reducing nodulation in legumes (Allos and Bartholomew 1959; Weber 1966). Despite reduction in number, DW and specific mass of nodules in N-supplemented treatments, plants presented between 46 and 74 nodules and between 70 and 200 mg of nodules per plant in set II. Plants having between 15 and 30 nodules and mass of nodules between 100 and 200 mg under field conditions can fulfill the amount of N required by soybean (Hungria, Campo, and Mendes 2007). The determinate growth genotypes, however, presented less than 100 mg of nodules when supplemented with mineral N, which resulted in lower N concentration and less N accumulation in the shoots. As a result, plants relying only on BNF presented N concentration and accumulation, respectively, 20% and 10% higher than plants supplemented with mineral N (set II). Differences in nodulation traits between genotypes were also observed in set I in nutrient solution, showing that N may differentially affect BNF in different genotypes (Gan et al. 2003). Soybean genotypes selected under rhizobial inoculation during the breeding process like in Brazil are more responsive to BNF (Hungria et al. 2006a; Hungria and Mendes 2015), and probably are more sensitive to supplemental N fertilizers like BRS 360 RR, whereas genotypes selected under application of N fertilizers would have limited BNF capacity (Chang, Lee, and Hungria 2015).

In some legumes as soybean, N is transported from nodules to shoots mainly as ureides-N (allantoin and allantoic acid), which can be used to estimate the BNF (King and Purcell 2005). Herridge and Peoples (1990) confirmed by ^{15}N isotope dilution technique the theory that the higher and more efficient the BNF, the higher the ureides-N contents in plant tissues. In N-supplemented treatments, in nutrient solution or in soil, at sowing or as topdressing, mineral N resulted in decrease of ureides-N contents in sap, leaves, and petioles, demonstrating reduction in the synthesis of ureides-N as a consequence of the BNF impairment (Hungria et al. 2006b; Ray, Heatherly, and Fritschi 2006).

Root DW and Soybean shoot DW responses to mineral N vary and even when positive, usually do not result in a grain yield increase (Heitholt et al. 2007; Hungria et al. 2001; 2005). Mineral N supply could have some positive effect, if any, only under environmental limitations like drought stress, excessive acidity, high temperatures, inadequate inoculation, or incompatibility with chemicals used in seed treatment, sub-dosing and uneven inoculation, or availability of mineral N in soil enough to hinder the BNF process (Hungria et al. 2005; Heitholt et al. 2007; Kinugasa et al. 2012). Despite some increase in shoot DW, mineral N leads to restriction in nodulation, and consequent decreases in ureides-N contents, N concentration and accumulation (Deaker, Roughley, and Kennedy 2004; Ray, Heatherly, and Fritschi 2006), and nitrogenase activity (Kinugasa et al. 2012). In contrast, higher concentrations of N, ureides-N, and accumulated N in plants relying only on BNF are indicative of the BNF efficiency (Herridge and Peoples 1990; Deaker, Roughley, and Kennedy 2004). Independent of the growth type, plants that received mineral N as topdressing (set II) were less affected than plants that received mineral N at sowing because they developed initially without interference of mineral N and relied on BNF until the topdressing fertilization at 32 DAE. This effect is probably the reason why soybean is more prone to respond negatively to mineral N at early growth stages than at late growth stages (Gan et al. 2003; Ray, Heatherly, and Fritschi 2006), as the early inhibition of the BNF process makes plants more dependent

on external N inputs. However, applications as topdressing also had negative effects and application at both sowing and as topdressing was the most negative condition.

The proportion between different types of N compounds transported to shoots depended on the source of N provided, biological or mineral. In this study, the ureides-N decreased due to supplemental N, especially when applied at sowing, whereas nitrate-N, ammonium-N, and amide-N increased compared with plants with only BNF. Decrease of ureides-N and increase of nitrate-N in N-fertilized soybean have been observed, since mineral N is absorbed from the soil mostly as nitrate (Riedell et al. 2011).

Decrease in the concentration of soluble carbohydrates in nodules of plants supplemented with mineral N, compared with plants without supplemental N, also emphasizes the negative effect of mineral N on the translocation of energy sources to the nodules. A reduction of carbohydrate contents in nodules may lead to flaws in the metabolism of C, compromising the provision of malate, which is the main energy source for bacteroids to use in the BNF process (Kaschuk et al. 2010). Limitations of energy sources caused by mineral N at any developmental period impair with the C metabolism in soybean nodules and consequently the BNF process.

In the four field experiments (set III), late N application at R5.3 stage did not affect plant yield, in contrast to the statement that soybean is likely to respond to mineral N for yields above 4500 kg ha^{-1} (Salvagiotti et al. 2008). This argument is based on the fact that the C sink at late reproductive stages goes predominantly to reproductive structures and starves the nodules and, consequently, the biological supply of N for high yields would be hindered. However, newly formed nodules in secondary roots and N biologically fixed at earlier stages and accumulated in the shoots have shown to be sufficient to support high yields without the need of supplemental N (Hungria et al. 2006a; 2006b). In fact, N concentrations in recently mature leaves of soybean by the time of application of N fertilizer were 53–54 g kg^{-1} for BRS 379 RR and 46–50 g kg^{-1} for BMX Ativa RR. Even for lower concentrations of N in leaves (26–42 g kg^{-1}) observed at R1 stage, there was no response to mineral N at 200 kg ha^{-1} (de Luca, Nogueira, and Hungria 2014). Yields of soybean relying on BNF, even in highly productive environments, can be similar or higher than plants receiving N fertilizers (Hungria et al. 2005; 2006b; Hungria, Campo, and Mendes 2007; Rodrigues-Navarro et al. 2011; de Luca, Nogueira, and Hungria 2014), but with less economic and environmental costs.

Results shown here emphasize the negative effects on BNF if mineral N is applied at early stages of plant development, and the absence of effects on grain yields when applied at late stage. Thus, the use of mineral N in soybean crop must be avoided. Inoculation with effective *Bradyrhizobium* sp. strains under good inoculation practices will assure the bacterial survival and biologically provide N required even for high grain yield potentials and avoid increases in production costs and risks to environment and human health.

With the intensification of agricultural systems, breeding of new, highly productive soybean genotypes should take into account their potential for biological N_2 fixation. BNF must be considered environmentally and economically as the most viable tool for providing N to soybean, independent of the growth type, contributing to the sustainability of the crop production systems.

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