

Can Additional Inoculations Increase Soybean Nodulation and Grain Yield?

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ABSTRACT

Highly productive soybean [*Glycine max* (L.) Merr.] genotypes require that large amounts of N be supplied throughout the growth cycle to attain high yields. The nutrient can be obtained through biological N₂ fixation by elite bradyrhizobial strains if a set of fully functional nodules is available. This study was conducted during two crop seasons in the central-west region of Brazil to investigate if additional inoculations via spray applications of bradyrhizobia at different stages of the growth cycle can result in increased nodulation and grain yield. Even though the soils had high populations of soybean bradyrhizobia, seed inoculation at sowing increased grain yield by 28 and 27% in the first and second seasons, respectively, compared to the non-inoculated control. Additional inoculations performed until the R6 stage significantly increased soybean nodulation, which might indicate that plants were able to overcome the limitations of autoregulation of nodulation, allowing the development of new functional nodules to sustain the increased demand for N at latter stages of the growth cycle, especially during pod filling. In response to the increased nodulation, grain yield gains of 2 and 7% were obtained in the first and second experiments, respectively, relative to inoculation at sowing only. Our results indicate that soybean roots can form new nodules in response to additional inoculations, promoting increased N₂ fixation and higher grain yield.

Core Ideas

- A full set of N₂-fixing nodules can supply soybean N demands.
- Seed inoculation increased nodulation and grain yield of field-grown soybean.
- Additional spray inoculations (V1, V3, V6, R1, or R3) resulted in further increases.
- New nodules and grain yield increases can be achieved by additional inoculations.

SOYBEAN IS one of the most important commodities in the portfolio of Brazilian export products, accounting for approximately 13% of all the country's exportations (CONAB, 2016; Embrapa Soja, 2016). The crop is highly demanding on N, requiring around 80 kg of N to produce 1000 kg of grains (Hungria and Mendes, 2015). The utilization of chemical N fertilizers would cost around US\$15 billion every year, making soybean cultivation unattractive under Brazilian conditions (Hungria and Mendes, 2015). The supply of N for soybean in Brazil relies exclusively on the inoculation with elite strains of N₂-fixing bradyrhizobia (Hungria et al., 2006; Hungria and Mendes, 2015). Soybean growth based on biological nitrogen fixation (BNF) is also a reality in Brazilian neighboring countries, such as Argentina, Paraguay, and Bolivia (Hungria et al., 2006; Hungria and Mendes, 2015). In addition to economic advantages, BNF with soybean in South America contributes to reduce the release, into the atmosphere, of millions of tonnes of CO₂ resulting from the production of chemical fertilizers (Sá et al., 2017)

Most of the nodules present on the roots of legumes are formed by the inoculant applied to the seed before sowing (Bhuvaneshwari et al., 1981; Calvert et al., 1984). Previous studies (Kosslak and Bohlool, 1984; Reid et al., 2011; Tanabata and Ohshima, 2014; Wang et al., 2014) have demonstrated that the host exerts autoregulation of nodulation (AON), controlling the number of nodules and causing the majority of them to be formed near the root crown. In soybean, AON has been shown to be activated as early as 4 d after inoculation, suppressing further nodulation (Kosslak and Bohlool, 1984). Initial nodule formation is critical, and only a set of fully functional nodules can supply the adequate amount of N to the crop. However, symbiosis aging (Van de Velde et al., 2006), and environmental stresses (Matamoros et al., 1999; Puppo et al., 2005) may cause nodule senescence and cessation of BNF, generally coinciding with the stage of pod filling (Lawn and Brun, 1974; Bethlenfalvai and Phillips, 1977; Neves and Hungria, 1987; Barradas et al., 1989). Since AON has been mainly studied at early stages of plant growth, little is known if further boosts

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Abbreviations: AON, autoregulation of nodulation; BNF, biological nitrogen fixation.

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of nodulation can occur later on during the growth cycle, and neither is it known if latter nodules can contribute to the crop's N nutrition and promote yield increases.

Recent studies have shown that when initial nodulation fails, remedial spray inoculation at early stages of plant growth can promote nodulation and the crop's recovery, resulting in satisfactory grain yield (Zilli et al., 2008; Hungria et al., 2015). However, there is no information about the effects of additional inoculations when initial nodulation is successful, or if plants can overcome AON. The presence of N₂-fixing nodules throughout the growth cycle might represent a key strategy to supply the nutrient for highly productive plant genotypes.

In this study, we have investigated if additional spray inoculations performed at different stages of the growth cycle could promote further nodulation and grain yield increases in field-grown soybean.

MATERIALS AND METHODS

This study consisted of two field experiments, conducted in the 2014/2015 and 2015/2016 cropping seasons, at the experimental farm of the Universidade Estadual Paulista (UNESP), Ilha Solteira Campus, in Selvíria, central-west region of Brazil [51°22' W, 20°22' S, 335 m altitude, and Köppen-Geiger's Aw climate (Alvares et al., 2013)], MS, Brazil.

Both experiments were planted in an area where inoculated soybean was grown before. Irrigation by central pivot sprinklers was available. Composite samples of the top 20-cm layer from the soil of the experimental area were collected 30 d before sowing, and subjected to chemical and granulometry analyses, as described before (Hungria et al., 2015). Dolomitic lime (28% CaO, 20% MgO) was applied to the soil to raise base saturation to 60%. In addition, the population of autochthonous bacteria capable of nodulating soybean was estimated by the most probable number (MPN), according to O'Hara et al. (2016). Soil chemical and microbiological properties, as well as granulometry are shown in Table 1.

Field plots measured 35 m² each, and had seven rows, spaced by 0.45 m. Plots were separated by 0.5 m-wide rows and 1.5 m-wide terraces to avoid cross contamination from surface flushes containing bacteria or fertilizers that may occur in consequence of heavy rainfall. In both seasons, all plots were fertilized with 300 kg ha⁻¹ of the 00-20-20, N-P-K formulation. N-fertilizer control plots (see treatment descriptions below) received 200 kg N ha⁻¹ as urea, split 50% at sowing and 50% at R1 soybean growth stage (Fehr and Caviness, 1977). For both experiments, cultivar BMX Potência RR (Brasmax, Grupo Genética do Brasil) was employed. Seeds were treated with fungicides (Vitavax + Thiram, 250 mL 100 kg⁻¹ seeds) prior to inoculation and sowing.

In 2014/2015 the experiment was set up under conventional soil tillage, whereas for the 2015/2016 experiment a no-tillage system was implanted. To produce straw for soil coverage for the no-tillage system of the 2015/2016 cropping season, cover crops were grown during fallow in the experimental area. Three different plant species were sown: maize (*Zea mays* L.), for a maize/soybean cropping system, brachiaria [*Urochloa* (= *Brachiaria*) *ruziziensis*], for a pasture/soybean cropping system, and *Crotalaria spectabilis*, for a green manure/soybean cropping system. No fertilization was performed for the fallow

Table 1. Chemical and microbiological properties and granulometry of the soil from the experimental area.

Property	Value
Chemical properties	
pH (CaCl ₂)	4.8
P (resin)	20 mg dm ⁻³
Organic matter	22 g dm ⁻³
K	3.4 mmol _c dm ⁻³
Ca	17 mmol _c dm ⁻³
Mg	11 mmol _c dm ⁻³
H + Al	47 mmol _c dm ⁻³
Al	2 mmol _c dm ⁻³
V (base saturation)	40%
Granulometry	
Clay	54%
Sand	33%
Silt	13%
Microbiological properties	
Bradyrhizobial (soybean) population	2.1 × 10 ⁷ cells kg ⁻¹

crops, except for maize, which received 100 kg N (urea) ha⁻¹ and 50 kg K (KCl) ha⁻¹, at the V5 stage (five visible leaves). All other cultural conditions followed the technical guidelines for each species. All crops were desiccated with herbicides at the beginning of the cropping season, and the straw was handled with a rolling knife tool.

In both seasons, eight inoculation treatments were evaluated: T1–non-inoculated control; T2–non-inoculated, N-fertilized control (200 kg N ha⁻¹); T3–standard seed inoculation (peat) (SI); T4–SI + spray inoculation at V1; T5–SI + spray inoculation at V3; T6–SI + spray inoculation at V6; T7–SI + spray inoculation at R1; and T8–SI + spray inoculation at R3. V1, V3, V6, R1, and R3 are different vegetative (V) and reproductive (R) stages of plant development, as described by Fehr and Caviness (1977). In the 2014/2015 crop season, a randomized block design was employed, whereas in 2015/2016 a 3 × 8 factorial design (three fallow crops and eight inoculation treatments) was adopted. In both seasons, the experiments had four replicates.

Standard seed inoculation was performed right before sowing, with peat-based inoculant containing strains SEMIA 5079 (*Bradyrhizobium japonicum*) and SEMIA 5080 (*B. diazoefficiens*). Inoculum dose was of 6 g kg⁻¹ seeds (to obtain 1.2 × 10⁶ cells seed⁻¹). The inoculant was mixed with a 10% sucrose solution at 250 mL 50 kg⁻¹ seeds to improve inoculant adherence to seeds, as described before (Hungria et al., 2006). For spray inoculations, liquid [7 × 10⁹ colony forming units (CFU) mL⁻¹] inoculant containing the same strains as above was diluted in water for a final volume suitable to deliver 150 L ha⁻¹. Spray inoculation was accomplished by means of a coastal sprayer, directing the sprays toward the soil, always at the end of the afternoon (5:00 p.m. h, Brazilian daylight-saving time). Spray inoculation treatments were implanted on the same plots in both seasons. Right after spraying, all plots received sprinkler irrigation to deliver a 10 mm water table.

Samplings for determination of nodulation parameters (nodule number and dry matter), as well as plant biomass occurred at the V4, R2, R4, and R6 stages. Five plants in a row were collected from each plot at each sampling time. Roots and shoots were separated in the laboratory, carefully washed and allowed

to dry in a 65°C oven for approximately 72 h. Nodules were then removed from the roots and allowed to dry for another 72 h and were counted and weighed. Dry shoots were also weighed.

Plant nutritional status was evaluated at the R2 stage by collecting the third fully developed leaf and its petiole from 30 plants of each plot, according to van Raij et al. (1996). The material was employed for the determination of N, P, K, Ca, Mg, S, Cu, Fe, Zn, and Mn contents, according to the methodology of Malavolta et al. (1997).

At physiological maturity, 15 m² from each plot were collected to estimate final plant stand, plant height, position of insertion of the first pod, number of branches per plant, number of pods per plant, grain yield (on a 13% moisture base), and the weight of 100 grains (on a 13% moisture base). Grain protein content was determined from a sample of grains, according to Malavolta et al. (1997).

All results were subjected to the Shapiro–Wilk normality test and to an ANOVA at $p < 0.05$. Treatment means were compared by the Scott and Knott test, also at $p < 0.05$.

RESULTS

All inoculation treatments significantly increased the number of nodules per plant in the 2014/2015 experiment, when compared to the non-inoculated controls without and with N-fertilizer (Table 2). Significant effects of additional inoculations were observed at the R6 growth stage, when nodulation promoted by additional inoculations performed at V1, V3, V6, and R1 was significantly superior to that of plants from both controls and those that had received seed inoculation at sowing only (Table 2). In early evaluations, inoculation resulted in nodules mainly in the root crown, and later on the primary and mainly on the secondary roots (data not shown). In the no-tillage experiment of 2015/2016, the fallow crop significantly affected the number of nodules produced on soybean plants at all samplings, with positive effects for *U. ruziziensis* and *C. spectabilis* (Table 2). Once again, all inoculation treatments promoted significantly better nodulation than the controls at all samplings, and additional inoculations performed at V3, V6, and R1 significantly increased the number of nodules observed at the R6 sampling, when compared to the other treatments (Table 2).

Although without statistical difference, inoculation resulted in a 2.3- to 3.8-fold increase in nodule dry weight (Table 3). Nodule biomass responded significantly to the fallow crops in the 2015/2016 no-tillage experiment, with positive effects for *U. ruziziensis* and *C. spectabilis* (Table 3).

Plant biomass estimated at all samplings was not affected by any treatment in the two crop seasons (Table 4) but, similar to the results of nodule number and biomass, the fallow crop significantly affected plant biomass measured at the V4 growth stage in the 2015/2016 no-tillage experiment, with positive effects for *U. ruziziensis* and *C. spectabilis* (Table 4).

Shoot N content was significantly increased by all inoculation treatments relative to the non-inoculated controls in the 2014/2015 experiment, and by the addition of N fertilizer and all inoculation treatments in the 2015/2016 experiment (Table 5). No effects of the fallow crop were observed in the no-tillage experiment (Table 5). Phosphorus, K, Ca, Mg, S, Cu, Fe, Zn, and Mn contents in the shoots were not affected by any treatment in either experiment (Table 5).

Table 2. Nodule number (no. plant⁻¹) of soybean in response to standard seed inoculation with peat at sowing (SI), and additional inoculation by spray at the different growth stages. Data represent the means of four replicates and non-inoculated (NI) controls without or with N (200 kg of N ha⁻¹) were also included.

2014/2015 Cropping season				
Treatments	Samplings			
	V4	R2	R4	R6
Inoculation (I)				
NI	6b†	11b	15b	12c
NI + N	7b	12b	15b	13c
SI	19a	28a	38a	31b
SI + V1	24a	31a	39a	38a
SI + V3	18a	29a	42a	39a
SI + V6	–	30a	42a	40a
SI + R1	–	30a	45a	38a
SI + R3	–	–	36a	34b
<i>F</i> test				
<i>p</i> value	0.0032	0.0475	0.0268	0.0479
CV %	28.3	24.3	22.5	28.4
2015/2016 Cropping season				
Treatments	Samplings			
	V4	R2	R4	R6
Fallow crop (FC)‡				
<i>Z. mays</i>	14b	20b	18b	18b
<i>U. ruziziensis</i>	20a	35a	33a	30a
<i>C. spectabilis</i>	19a	30a	30a	28a
Inoculation (I)				
NI	10b	18b	16b	14c
NI + N	10b	20b	18b	14c
SI	20a	33a	30a	25b
SI + V1	23a	35a	30a	27b
SI + V3	22a	37a	36a	36a
SI + V6	–	34a	35a	35a
SI + R1	–	32a	32a	33a
SI + R3	–	–	30a	28b
<i>F</i> test				
<i>p</i> value (FC)	0.0034	0.0426	0.0375	0.0023
<i>p</i> value (I)	0.0076	0.0001	0.0247	0.0026
<i>p</i> value (FC × I)	0.1369	0.7489	0.0946	0.0795
CV %	29.3	20.4	21.1	24.3

† ns, statistically not significant; values followed by the same letter were statistically different ($p < 0.05$).

‡ *Zea mays*, *Urochloa* (= *Brachiaria*) *ruziziensis*, *Crotalaria spectabilis*.

Final plant stand, plant height, position of insertion of the first pod, number of branches per plant, and number of pods per plant were not affected by any treatment in any experiment (data not shown). However, significant differences in the weight of 100 grains were observed, with significant positive effects of N fertilizer and all inoculation treatments in the 2014/2015 experiment, and of all the inoculation treatments in the no-tillage experiment of 2015/2016 (Table 6). No significant differences due to the fallow crop were observed. No significant differences were observed in the protein contents of the grains in any of the experiments (Table 6).

Grain yield was significantly improved in response to all inoculation treatments in the first experiment, in comparison with both non-inoculated controls, with and without N fertilizer (Table 6). Significant differences due to the fallow crop were

Table 3. Nodule biomass (mg plant⁻¹) of soybean in response to standard seed inoculation with peat at sowing (SI), and additional inoculation by spray at the different growth stages. Data represent the means of four replicates and non-inoculated (NI) controls without or with N (200 kg of N ha⁻¹) were also included.

2014/2015 Cropping season				
Treatments	Samplings			
	V4	R2	R4	R6
Inoculation (I)				
NI	14ns†	58ns	90ns	76ns
NI + N	15	65	90	82
SI	49	151	228	202
SI + V1	55	167	250	251
SI + V3	45	160	256	257
SI + V6	–	162	265	264
SI + R1	–	162	274	270
SI + R3	–	–	238	238
F test				
p value	0.1233	0.7651	0.4671	0.8513
CV %	16.9	28.8	22.0	22.4
2015/2016 Cropping season				
Treatments	Samplings			
	V4	R2	R4	R6
Fallow crop (FC)‡				
<i>Z. mays</i>	41ns	100ns	110b	124b
<i>U. ruziziensis</i>	64	189	228a	225a
<i>C. spectabilis</i>	57	162	204a	204a
Inoculation (I)				
NI	28 ns	88ns	94 ns	97ns
NI + N	30	96	106	98
SI	64	172	180	185
SI + V1	69	189	204	202
SI + V3	64	204	245	270
SI + V6	–	180	224	273
SI + R1	–	160	211	244
SI + R3	–	–	192	210
F test				
p value (FC)	0.0962	0.0768	0.0476	0.0357
p value (I)	0.5487	0.6283	0.2632	0.0897
p value (FC × I)	0.9862	0.8632	0.1314	0.1293
CV %	23.4	29.5	20.3	26.3

† ns, statistically not significant; values followed by the same letter were statistically different ($p < 0.05$).

‡ *Zea mays*, *Urochloa* (= *Brachiaria*) *ruziziensis*, *Crotalaria spectabilis*.

observed in the second experiment, favored by *U. ruziziensis* and *C. spectabilis*. In 2015/2016, soybean yield also presented impressive significant responses to all additional inoculations when compared to seed inoculation alone or to N fertilization (Table 6).

DISCUSSION

Nitrogen fixation by the association between legumes and rhizobia has long been exploited in agriculture as an environment-friendly means to deliver the N that plants need to grow and produce grains. In Brazil, as well as in other countries of South America, soybean is grown successfully at the expense of the inoculation of seeds at sowing with selected elite strains of bradyrhizobia, with no need of supplementary N fertilizer (Hungria et al., 2006; Hungria and Mendes, 2015; Kaschuk et al., 2016; Saturno et al., 2017). However, one question remains:

Table 4. Plant biomass (g plant⁻¹) of soybean in response to standard seed inoculation with peat at sowing (SI), and additional inoculation by spray at the different growth stages. Data represent the means of four replicates and non-inoculated (NI) controls without or with N (200 kg of N ha⁻¹) were also included.

2014/2015 Cropping season				
Treatments	Samplings			
	V4	R2	R4	R6
Inoculation (I)				
NI	2.3ns†	16.4ns	18.0ns	18.8ns
NI + N	2.3	17.9	18.9	19.3
SI	2.9	17.8	19.9	19.3
SI + V1	2.6	19.1	20.7	19.8
SI + V3	2.5	18.5	19.6	20.4
SI + V6	2.4	17.9	19.6	19.8
SI + R1	2.4	17.9	19.6	20.6
SI + R3	2.7	18.3	19.9	19.9
F test				
p Value	0.9820	0.0972	0.1982	0.6870
CV %	13.5	11.8	9.7	9.0
2015/2016 Cropping season				
Treatments	Samplings			
	V4	R2	R4	R6
Fallow crop (FC)‡				
<i>Z. mays</i>	2.2b	16.1ns	16.9ns	17.2ns
<i>U. ruziziensis</i>	2.4a	16.4	17.0	17.5
<i>C. spectabilis</i>	2.3a	16.0	16.6	17.4
Inoculation (I)				
NI	2.2ns	15.6 ns	16.6 ns	17.6ns
NI + N	2.3	15.4	16.5	17.4
SI	2.3	16.1	17.0	17.3
SI + V1	2.3	15.6	16.6	17.0
SI + V3	2.3	15.8	16.6	17.1
SI + V6	2.2	15.4	16.5	17.2
SI + R1	2.3	15.2	16.8	17.2
SI + R3	2.4	15.8	16.8	17.8
F test				
p value (FC)	0.0470	0.9902	0.4072	0.0721
p value (I)	0.3192	0.2972	0.1002	0.7289
p value (FC × I)	0.7091	0.6870	0.1097	0.5791
CV %	9.4	16.2	13.5	18.3

† ns, statistically not significant ($p < 0.05$).

‡ *Zea mays*, *Urochloa* (= *Brachiaria*) *ruziziensis*, *Crotalaria spectabilis*.

how high can yields really go? In other words, is there room for more nodules to be formed on the roots during plant growth, resulting in more fixed N₂ available for the plants and, consequently, sustain higher yields?

It has been widely documented that the nodulation of legume roots is a process that occurs under a very stringent control by the plant, known as AON (Kinkema et al., 2006; Reid et al., 2011; Tanabata and Ohyama, 2014). As demonstrated by Kosslak and Bohlool (1984), AON in soybean can be triggered as early as 4 d after inoculation. Several mechanisms, at both the physiological (e.g., Li et al., 2009; Ikeda et al., 2011), and molecular (e.g., Reid et al., 2011; Kassaw et al., 2015; Wang et al., 2014) levels have been implicated in the control AON by plants. In any event, root nodulation seems to be restricted to a limited number of nodules allowed by the plant to be formed.

Table 5. Nutrient contents in the leaves of soybean plants from both experiments.

Treatments	2014/2015 Cropping season									
	N	P	K	Ca	Mg	S	Cu	Fe	Zn	Mn
	g kg ⁻¹						mg kg ⁻¹			
Inoculation (I)										
NI†	40.3b‡	3.9	18.4	9.8	3.5	3.0	22	260	35	77
NI + N	41.4b	3.9	18.9	9.9	3.4	3.1	23	278	36	79
SI	45.3a	3.7	18.2	10.0	3.6	3.2	21	274	34	80
SI + V1	46.3a	3.4	17.8	10.1	4.0	2.9	23	267	36	85
SI + V3	46.3a	3.7	18.6	10.5	4.1	2.8	21	259	35	84
SI + V6	45.3a	3.5	18.4	10.3	3.6	2.9	21	257	36	84
SI + R1	45.4a	3.6	18.3	9.9	3.9	3.0	20	267	36	85
SI + R3	45.3a	3.8	18.0	10.0	4.0	3.1	22	262	35	81
F test										
p value (I)	0.0466	0.2703	0.9862	0.1932	0.2345	0.0928	0.1853	0.9832	0.1542	0.9720
CV %	9.3	5.4	12.3	15.2	5.5	7.3	20.3	28.3	18.7	29.8
Treatments	2015/2016 Cropping season									
	N	P	K	Ca	Mg	S	Cu	Fe	Zn	Mn
	g kg ⁻¹						mg kg ⁻¹			
Fallow crop (FC)§										
<i>Z. mays</i>	48.7	3.7	19.5	10.3	3.5	3.0	22	268	34	89
<i>U. ruziziensis</i>	46.6	3.6	18.6	9.9	3.9	3.1	20	250	36	83
<i>C. spectabilis</i>	49.1	3.6	19.5	10.4	4.0	2.9	22	279	38	80
Inoculation (I)										
NI	41.1b	3.9	18.5	10.4	3.6	3.2	20	284	36	79
NI + N	46.3a	3.7	18.3	9.3	3.8	3.1	22	250	38	83
SI	49.3a	3.6	19.6	10.3	4.1	3.0	22	267	34	85
SI + V1	48.3a	3.9	19.0	10.8	3.9	2.9	24	269	36	79
SI + V3	47.3a	3.3	18.4	9.1	3.7	2.7	20	278	35	83
SI + V6	48.5a	3.6	19.1	10.5	4.2	3.3	23	256	36	82
SI + R1	47.5a	3.5	18.3	9.6	3.7	2.8	20	276	35	76
SI + R3	48.9a	3.7	18.9	10.0	4.0	3.0	21	266	37	80
F test										
p value (FC)	0.0396	0.7612	0.1172	0.1987	0.1079	0.2932	0.2087	0.1093	0.5889	0.1097
p value (I)	0.1769	0.1876	0.8751	0.1092	0.7321	0.6538	0.5373	0.8752	0.1073	0.1860
p value (FC × I)	0.8591	0.1802	0.1203	0.4861	0.1272	0.9769	0.3476	0.6482	0.8579	0.7652
CV %	12.2	7.4	14.3	17.2	17.3	12.1	21.1	17.9	28.5	20.5

† Treatments: Non-inoculated (NI) controls without or with N (200 kg of N ha⁻¹), seed inoculation with peat at sowing (SI), and supplementary inoculation by spray at the different plant growth stages.

‡ For each nutrient, values followed by different letters were statistically different ($p < 0.05$), and in the columns without letters were not statistically different.

§ *Zea mays*, *Urochloa* (= *Brachiaria*) *ruziziensis*, *Crotalaria spectabilis*.

Autoregulation of nodulation has been mainly described in early nodulation events; however, it is possible that the process occurs dynamically throughout plant's growth cycle. Interestingly, Caetano-Anollés et al. (1991) performed sophisticated experiments in which they removed all the mature nodules from soybean plants, leading to the development of new fully functional nodules. Their results demonstrated that rather than initiating new infections, soybean plants somehow "woke up" dormant pre-existing infections, since the new nodules appeared in the same regions of the roots where the initial nodules had been formed. As it has been already demonstrated (Bhuvanewari et al., 1981), roots are only transiently susceptible to infection by rhizobia, arguing against the idea of nodules derived from new infections. In contrast, in our experiments, the newly formed nodules seem to have originated from new infections, since plants that originated from conventional seed inoculation presented significantly fewer nodules than those that received additional spray inoculations performed at the V1,

V3, V6, and R1 growth stages. If the newly formed nodules of our plants were to be originated from pre-existing infections, no significant differences were to be expected, since all plants were subjected to the same growth conditions in both experiments.

Our results suggest that soybean plants responded to additional inoculations. One possibility is that the plants might have been able to overcome AON. This response may be related to the continuous demand of the plants for N, as their growth cycle progresses. In the inoculated treatment, we applied no N fertilizer for the soybean plants, forcing them to grow "on the edge", perhaps encouraging new nodulation boosts to supply the N demanded by the plants as they grew. In a similar situation, N-limited *Medicago truncatula* plants stimulated the growth of pre-existing, as well as the generation of new nodules, as demonstrated by Jeudy et al. (2010). Therefore, our results give high support to the source-sink theory, of increasing BNF rates under high plant growth demands (Neves and Hungria, 1987; Kaschuk et al., 2012), and also highlight the

Table 6. Weight of 100 grains, grain protein content, and grain yield of soybean in response to standard seed inoculation with peat at sowing (SI), and additional inoculation by spray at the different growth stages. Data represent the means of four replicates and non-inoculated (NI) controls without or with N (200 kg of N ha⁻¹) were also included.

2014/2015 Cropping season			
Treatments	Weight of 100 grains g	Grain protein content %	Grain yield kg ha ⁻¹
Inoculation (I)			
Control	15.1b†	63.0 ns	3266c
N control	15.9a	62.3	3872b
SI	16.2a	65.7	4166a
SI + V1	16.1a	64.3	4323a
SI + V3	16.0a	64.9	4373a
SI + V6	16.4a	65.5	4314a
SI + R1	16.3a	66.3	4158a
SI + R3	16.2a	67.4	4116a
F test			
p value	0.0311	0.2693	0.0462
CV %	4.1	11.2	19.9
2015/2016 Cropping season			
Treatments			
Fallow crop (FC)‡			
<i>Z. mays</i>	16.5 ns	66.4 ns	4045b
<i>U. ruziziensis</i>	16.4	65.3	4611a
<i>C. spectabilis</i>	16.3	65.0	4788a
Inoculation (I)			
Control	15.7b	63.4	3104c
N control	15.9b	65.0	3252c
SI	17.0a	66.3	3937b
SI + V1	17.0a	65.4	4298a
SI + V3	17.2a	66.4	4314a
SI + V6	16.9a	64.3	4221a
SI + R1	16.8a	65.1	4290a
SI + R3	16.8a	66.0	3950b
F test			
p value (FC)	0.5862	0.1896	0.0472
p value (I)	0.0027	0.2683	0.0001
p value (FC × I)	0.5913	0.2875	0.1386
CV %	5.5	29.9	13.9

† ns, statistically not significant; values followed by the same letter were statistically different ($p < 0.05$).

‡ *Zea mays*, *Urochloa* (= *Brachiaria*) *ruziziensis*, *Crotalaria spectabilis*.

high plasticity of soybean, being capable of increasing nodulation and BNF capacity when demanded (de Luca et al., 2014). However, other possibilities, as hormonal effects, should also be investigated, as they might have implications in the formation of new nodules.

The contribution of BNF may be seriously compromised by nodule senescence, which can be induced by symbiosis aging (Neves and Hungria, 1987; Van de Velde et al., 2006), or by environmental stresses (Matamoros et al., 1999; Puppo et al., 2005). Natural nodule senescence generally coincides with the stage of pod filling, when plants require large amounts of N (Lawn and Brun, 1974; Bethlenfalvai and Phillips, 1977; Neves and Hungria, 1987). In this regard, Kassaw et al. (2015) have postulated the existence of a second nodule suppression system related to plant nutrient status, especially N; the authors have

observed that when the nodules on the roots of *M. truncatula* plants were no longer able to supply the plants with N, AON was suppressed. The plants in our experiments may have exhibited similar mechanisms, especially if we take into account the elevated demand for N associated with the high yields that soybean achieves under our growth conditions, and that the impressive responses to additional inoculations occurred at the reproductive stages of the soybean growth cycle.

Taking grain yields into account, even though the soil presented a high population of rhizobia capable of nodulating soybean, estimated at 2.1×10^7 cells kg⁻¹, seed inoculation increased yield by 28 and 27%, in the first and second seasons, respectively, confirming the reported benefits of reinoculation commonly observed in Brazil (Hungria et al., 2006; Hungria and Mendes, 2015). However, noteworthy were the results demonstrating that additional inoculations promoted, in the first season, on average, increases of the magnitudes of 2, 10, and 30% relative to seed inoculation only, and to the non-inoculated controls with or without N fertilizer, respectively; in the second season, increases of even higher magnitude (7, 30, and 36%, respectively) were observed. Increases in yield due to supplementary inoculation strongly suggest that N limitation may have occurred under our experimental conditions, forcing new boosts of nodulation to supply the crop with the necessary amount of N to support high yields.

Therefore, we may conclude that there is, indeed, room for new nodules to be formed on soybean roots in response to additional inoculations, culminating in increased N₂ fixation and grain yield.

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