Inoculum Rate Effects on the Soybean Symbiosis in New or Old Fields under Tropical Conditions

Mariangela Hungria,* Ricardo Silva Araujo, Elson Barbosa Silva Júnior, and Jerri Édson Zilli

ABSTRACT

Soybean [*Glycine max* (L.) Merr.] can highly benefit from inoculation with elite strains of *Bradyrhizobium*, selected for high capacity of N_2 fixation. However, to achieve the benefits, the strains must be capable of effectively nodulate the host, and inoculum rate may be critical, especially under stressing tropical environmental conditions. We performed 10 field experiments, in four crop seasons and four Brazilian states, including soils without and with established populations of soybean bradyrhizobia, to investigate the effects of inoculum rates, consisting of zero, 0.6×10^6, 1.2×10^6, and 2.4×10^6 colony forming units (CFU) per seed. Nodule number and dry weight were evaluated at early flowering and grain yield and N content in grains at the physiological maturity. Satisfactory nodulation and grain yields were obtained with 1.2×10^6 CFU seed^{-1}. However, there were sites with and without established population of bradyrhizobia where maximum nodulation and/or yields were achieved with inoculum rate of 2.4×10^6 CFU seed^{-1}, and that could require even higher rates. Therefore, we suggest the adoption of the minimum inoculum rate of 1.2×10^6 cells of *Bradyrhizobium* seed^{-1} for soybean planting in the tropics, to achieve maximum contribution of biological N_2 fixation.

Core Ideas

- Inoculum rate with elite strains is critical to achieve the benefits of biological N_2 fixation.
- The minimum rate of 1.2×10^6 cells of *Bradyrhizobium* seed^{-1} was determined to benefit biological N_2 fixation.
- Soybean can highly benefit from biological N_2 fixation.
- Some sites required 2.4×10^6 cells seed^{-1} and could respond to higher rates.

SOYBEAN PLANTS can establish a symbiotic relationship with N_2–fixing bacteria of the genus *Bradyrhizobium*, from which they can obtain nearly all the N necessary to fulfill growth requirements and achieve high yields. In Brazil, soybean is a major crop, and yields have been considerably improved by the inoculation of seeds with selected elite strains of *Bradyrhizobium* (Hungria et al. (2006a, 2006b); Hungria and Mendes, 2015). However, such benefits are only attainable if the inoculated bacteria actually form effective nodules on the host plant's roots.

Seed inoculation is an efficient strategy to introduce effective bradyrhizobia to soybean rhizosphere (Deaker et al., 2004; Hungria et al., 2005), being the results directly affected by the number of inoculated bacteria. The influence of the inoculum rate applied to the seed on nodule formation in legumes has long been subject of studies (e.g., Brockwell et al., 1988; Papakosta, 1992; Patrick and Lowther, 1995; Sparrow and Ham, 1983; Thornton, 1929). However, no conclusive data about the adequate inoculum rate to maximize the benefits from nodulation and N_2 fixation are available, and no one yet is sure whether more is better or worse.

The influence of inoculum rate on the outcome of the expected traits is not fully understood, not only in the case of nodule bacteria and legumes, but also regarding other types of plant–microorganisms interactions. For example, Zhang et al. (2001) did not observe positive effects of increasing the inoculum rate of bacilli plant growth promoting rhizobacteria (PGPR) on induced systemic resistance to leaf blight (*Cercosporidium personatum*) in peanut (*Arachis hypogeae* L.). In both common bean (*Phaseolus vulgaris* L.) and soybean, increased inoculum rate of the PGPR *Azospirillum brasilense* co-inoculated with rhizobia decreased both nodulation and yield, what could be related to an excessive production of phytohormone-like substances, inhibiting growth (Hungria et al., 2013). On the other hand, Iswandi et al. (1987) observed that increasing the inoculum rate of PGPR decreased the populations of pathogenic fungi in the rhizospheres of maize

---

M. Hungria, Embrapa Soja, C. P. 231, 86001-970, Londrina, Paraná, Brazil; R. Silva Araujo, Total Biotecnologia Indústria e Comércio S/A. Rua Emílio Romani, 1190, CIC, 81460-020, Curitiba, Paraná, Brazil; E.B. Silva Júnior, Embrapa Agrobiologia, C.P. 74.505, 23891-000, Seropédica, Rio de Janeiro, Brazil, present address: Instituição de Ensino Superior Capixaba, Rua Jacobina 165, 29830-000, Nova Venécia, Espírito Santo, Brazil; J.É. Zilli, Embrapa Agrobiologia, C.P. 74.505, 23891-000, Seropédica, Rio de Janeiro, Brazil. *Corresponding author (mariangela.hungria@embrapa.br).

Abbreviations: BNF, biological nitrogen fixation, CFU, colony forming units; LEM, Luiz Eduardo Magalhães; PGPR, plant growth promoting rhizobacteria.

Published in Agron. J. 109:1–7 (2017)
doi:10.2134/agronj2016.11.0641
Received 5 Nov. 2016
Accepted 15 Feb. 2017

5585 Guilford Road, Madison, WI 53711 USA

(*Zea mays* L.) and barley (*Hordeum vulgare* L.) plants, resulting in improved growth of both species.

The co-inoculation of the PGPR *Serratia proteamaculans* and *S. liquefaciens* with *Bradyrhizobium japonicum* on soybean at optimal doses increased nodule number, plant dry weight, and the efficiency of N_2 fixation (Bai et al., 2002). In addition, Chandra and Pareek (1987) observed that increased rhizobial inoculum rate significantly increased nodule dry weight, grain yield, total nitrogenase activity (acetylene reduction activity), and N uptake by shoots and grains of field grown chickpea (*Cicer arietinum* L.) plants. On the other hand, Yamakawa and Fukushima (2014) observed that the effects of inoculum rate on nodulation and yield of soybean depended on the method of inoculation and on the cultivar used. Previous studies (Brockwell et al., 1989; Thies et al., 1991a, 1991b) had demonstrated that N status in the soil, compatible rhizobial population in the soil and the rate of inoculation affect the establishment of bradyrhizobial populations and the outcome of soybean nodulation and N_2 fixation.

The benefits of inoculation with N_2–fixing nodule bacteria may also be affected by the size of the natural population of bacteria capable of nodulating a given legume host. For example, Smith et al. (1981) investigating inoculum rates applied in-furrow at sowing have determined that inocula of the order of 10^5 bacteria m^{-1} row were necessary for successful soybean nodulation and N_2 fixation in a tropical soil, where no bradyrhizobia existed. In contrast, when soybean was grown in soils with established population of bradyrhizobia, increased inoculum rates applied by different methods were only partially successful in displacing indigenous soil rhizobia (Boonkerd et al., 1978; Vargas and Hungria, 1997). In the case of clover (*Trifolium* spp.), Nazih and Weaver (1994) observed that successful nodulation in the crown region of the plants was highly dependent on the relationship between inoculum rate and the size of the indigenous population of clover rhizobia.

Another interesting aspect concerns soybean genotypes that are restrictive to nodulation. It has been shown that some soybean genotypes restrict nodulation by certain strains of *Bradyrhizobium* spp. when high inoculum rates are employed, but not at low inoculum levels, clearly demonstrating that more may not always be better (Lohrke et al., 2000). In another study, soybean nodulation was completely suppressed by super optimal inoculation with *B. japonicum* (Takats, 1986). Since plant breeding to improve agronomic traits of soybean is a continuous process, such fine plant–bacterium interactions may be carefully studied to maximize the benefits from inoculation and N_2 fixation. This aspect becomes even more important when we consider the development and improvement of inoculant production technology, providing the market with more concentrated products (Deaker et al., 2004; Hungria et al., 2005).

In this study, we have addressed the issue of investigating the adequate number of viable N_2–fixing bacteria that must be applied to soybean seeds in tropical soils, to maximize benefits from inoculation in soils with or without established populations of bradyrhizobia.

MATERIALS AND METHODS

Sites Description and Procedures before Sowing

Ten field experiments were conducted at different sites of four Brazilian States during four crop seasons. The experiments comprised sites (Table 1) with predominance of sand or clay and under different climatic classifications according to Köppen's system (Alvares et al., 2013). In four sites soybean was grown for the first time, and the other six had been previously cropped with inoculated soybean, therefore containing established populations of bradyrhizobia.

At each location, 20 soil subsamples were collected at 0- to 20-cm soil layer about 40 to 50 d before experimental setup, and were joined in a composed sample used to evaluate chemical properties, as described before (Hungria et al., 2015); the results are shown in Table 2. Soil population of soybean bradyrhizobia were estimated by the plant-infection most probable number technique, also as described before (Hungria et al., 2015) and the results are shown in Table 2.

According to the soil analysis of each site, lime was added to reach 70% of base saturation. Still before sowing, all sites received 300 kg ha^{-1} of an N–P–K fertilizer formulation (0–28–20) which contains no N but delivers P and K, applied in-furrow. Therefore, no N fertilizer was applied.

Treatments, Inoculation and Field Management

Two months before each trial, inoculants were prepared in the laboratory, with gamma-sterilized peat and injection of two strains approved for the use in commercial inoculants in Brazil, *B. japonicum* SEMIA 5079 (= CPAC 15) and *B. diazoefficiens* SEMIA 5080 (= CPAC 7). According to the concentration of cells obtained in the inoculant analysis 10 d before sowing, the treatments were prepared to supply zero, 0.6×10^6, 1.2×10^6, and 2.4×10^6 CFU per seed. For inoculation, adherence of peat-based inoculum was achieved by adding a 10% sucrose solution (300 mL per 50 kg of seeds) (Hungria et al., 2015). Soybean cultivars used were chosen from the recommended genotypes for each region in each crop season, and all genotypes were non-transgenic. Seeds were not treated with fungicides or insecticides.

At all locations, row spacing was 50 cm, with the establishment of about 18 plants m^{-1}, resulting in a final population of

Table 1. Geographic coordinates, climate, and soil type of the localities where the experiments were performed.

District (State)	Latitude	Longitude	Climate Köppen†	Soil type	Area‡
Taciba (São Paulo, SP)	22° 23′ S	51° 17′ W	Cfa	sandy	new/old
Luiz Eduardo Magalhães (Bahia,BA)	12° 06′ S	51° 17′ W	Aw	sandy	new
Luziânia (Goiás, GO)	16° 15′ S	45° 48′ W	Aw	clay	old
Londrina (Paraná, PR)	23° 18′ S	51° 09′ W	Cfa	clay	old

† Cfa–humid, subtropical climate; Aw–tropical savannah.

‡ New areas are those which had never been cropped with soybean and showed less than 10^2 cells g^{-1} soil; old areas had been cropped with soybean and inoculated before and showed at least 10^4 cells of *Bradyrhizobium* g^{-1} soil.

approximately 300,000 plants ha^{-1}. All experiments were set in a completely randomized block design with six replicates. Plot sizes had at least 24 m^{2}. At all locations the plots were separated by 0.5 m-wide rows and 1.5 m-wide terraces to avoid cross contamination from surface run-off containing bacteria or fertilizers that may occur in consequence of heavy rainfall.

All plants received leaf sprays of Mo (20 g ha^{-1}) and Co (2 g ha^{-1}) at the V4 stage (scale of Fehr and Caviness, 1977). Weeds were controlled equally with conventional herbicides in all treatments at each site. Pests control was accomplished by means of biological and chemical insecticides and chemical fungicides, according to technical recommendations for each site.

Sampling, Harvest, and Analyses Performed

Plant samples were taken around the V6 (Fehr and Caviness, 1977) stage from each plot, varying from 35 to 42 d after plant emergence (DAE), according to the location, to evaluate the number and dry matter of nodules, shoot dry weight, and total N accumulated in shoots. At the R8 stage (full maturity) (Fehr and Caviness, 1977) stage, grain yield (kg ha^{-1}) was determined. Nitrogen content (%) in the grains (Kjeldahl method) was also determined, except for the experiment in Luiz Eduardo Magalhães (LEM).

Data were analyzed with Sisvar v.4.5 software (Ferreira, 2011), and subjected to variance analysis by the F test at $p = 0.05$. After the analysis of variance, linear and quadratic regression equations were adjusted, and their significance tested at $p = 0.01$and $p = 0.05$, and $p = 0.1$.

RESULTS

Experiments in Fields Grown with Soybean for the First Time

Practically no nodulation could be observed on plants from the treatments with zero CFU seed^{-1} in areas where soybean was grown for the first time (Fig. 1a), reflecting in very low values of nodule dry matter, below 50 mg plant^{-1} in all four experiments (Fig. 1b). On the other hand, when inoculum rates were increased, linear increases in nodule number and dry matter were observed in two experiments, conducted in Taciba (I- 2003/2004) and in LEM (Fig. 1a, 1b). In the other two experiments (Taciba 2002/2003 and Taciba II 2003/2004), increases in the number and dry matter of nodules fit a quadratic model. In addition, it could be noted that the maximum nodulation was around 25 nodules plant^{-1}, with accumulation of dry matter slightly above 100 mg plant^{-1} in all experiments. However, the maximum nodulation in response to the inoculum rate was variable, since in the two experiments that fit a quadratic model the maximum nodulation was obtained with 1.2×10^{6} CFU seed^{-1}, whereas in the other two sites the inoculum rate necessary to reach maximum nodulation was of 2.4×10^{6} CFU seed^{-1} (Fig. 1a, 1b).

As for nodulation, grain yield and N accumulation in the grains in two experiments conducted in Taciba (2002/2003 and II-2003/2004) fit into a quadratic model relative to inoculum rates (Fig. 1c, 1d). In both cases, maximum grain yields (approximately 2000 and 4800 kg ha^{-1}, respectively, in 2002/2003 and 2003/2004) were obtained when the inoculum rate was 1.2×10^{6} CFU seed^{-1}. On the other hand, for the other two experiments, grain yields averaged 2000 kg ha^{-1}, but no model fit for grain yield or N accumulation in the grains was observed (Fig. 1c, 1d); as commented before, N in grains in LEM was not evaluated.

Experiments in "Old" Soybean Fields

Increased seed inoculum rates resulted in increases in nodule number (Fig. 2a) and dry matter accumulation (Fig. 2b) in five out of the six experimental areas established on fields with previous history of soybean cultivation. In three of these areas, nodulation increase fit a quadratic model, with maximum values obtained when the inoculum rate was 1.2×10^{6} CFU seed^{-1}, whereas in the two other areas the best fit was obtained with a linear model. Opposite to what happened in the new areas, plants grown in old fields presented nodulation in the control treatments (zero CFU seed^{-1}), in some cases more than 40 nodules plant^{-1} (Fig. 2a, 2b).

Grain yield in one of the experiments conducted in Taciba (2002/2003) responded positively, presenting an average 6% increase, equivalent to 250 kg grains ha^{-1}, to the increased inoculum rates (Fig. 2c), fitting a linear model with maximum yields

Table 2. Chemical properties and population of soybean rhizobia of the soils (0–20 cm) before liming and fertility correction.

District (site, year)	pH	Al	H+Al	K	Ca	Mg	P	C	CTC	BS†	Rhizobia population
	CaCl$_2$	———— cmol$_c$ dm^{-3} ————					mg dm^{-3}	g dm^{-3}	cmol$_c$dm^{-3}	%	cell g^{-1}
Taciba (new, site 1, 2002/2003)	4.3	0.26	4.9	0.32	1.05	0.51	12.4	14	6.7	27	<100
Taciba (new, site 2, 2003/2004)	4.0	0.24	5.2	0.24	0.90	0.41	7.2	8	6.6	23	<100
Taciba (new, site 3, 2003/2004)	4.2	0.20	5.1	0.28	0.91	0.48	9.4	10	7.0	24	<100
Luiz Eduardo Magalhães (new, 2005/2006)	4.3	0.18	5.0	0.25	0.90	0.54	5.0	15	6.7	25	<100
Taciba (old, site 4, 2002/2003)	4.8	0.07	3.8	0.25	1.81	0.45	18.1	14	6.3	40	1.9×10^{4}
Taciba (old, site 1, 2003/2004)	5.0	0.10	4.2	0.61	1.90	1.10	10.9	12	7.8	46	3.1×10^{4}
Luziânia (old, 2000/2001)	4.9	0.07	6.2	0.19	1.76	0.49	8.5	20	8.7	28	4.1×10^{4}
Londrina (old, site 1, 2002/2003)	4.9	0.00	5.3	0.53	3.21	2.05	14.8	18	11.1	52	1.4×10^{4}
Londrina (old, site 1, 2003/2004)	5.1	0.00	5.0	0.71	4.10	1.98	13.1	18	11.8	58	1.9×10^{4}
Londrina (old, site 1, 2004/2005)	5.2	0.00	4.2	0.20	1.55	1.45	12.8	17	7.4	43	7.9×10^{5}

† Base saturation = (K + Ca + Mg)/T_{cec}) × 100, where T_{cec} = K + Ca + Mg + total acidity at pH 7.0 (H + Al).

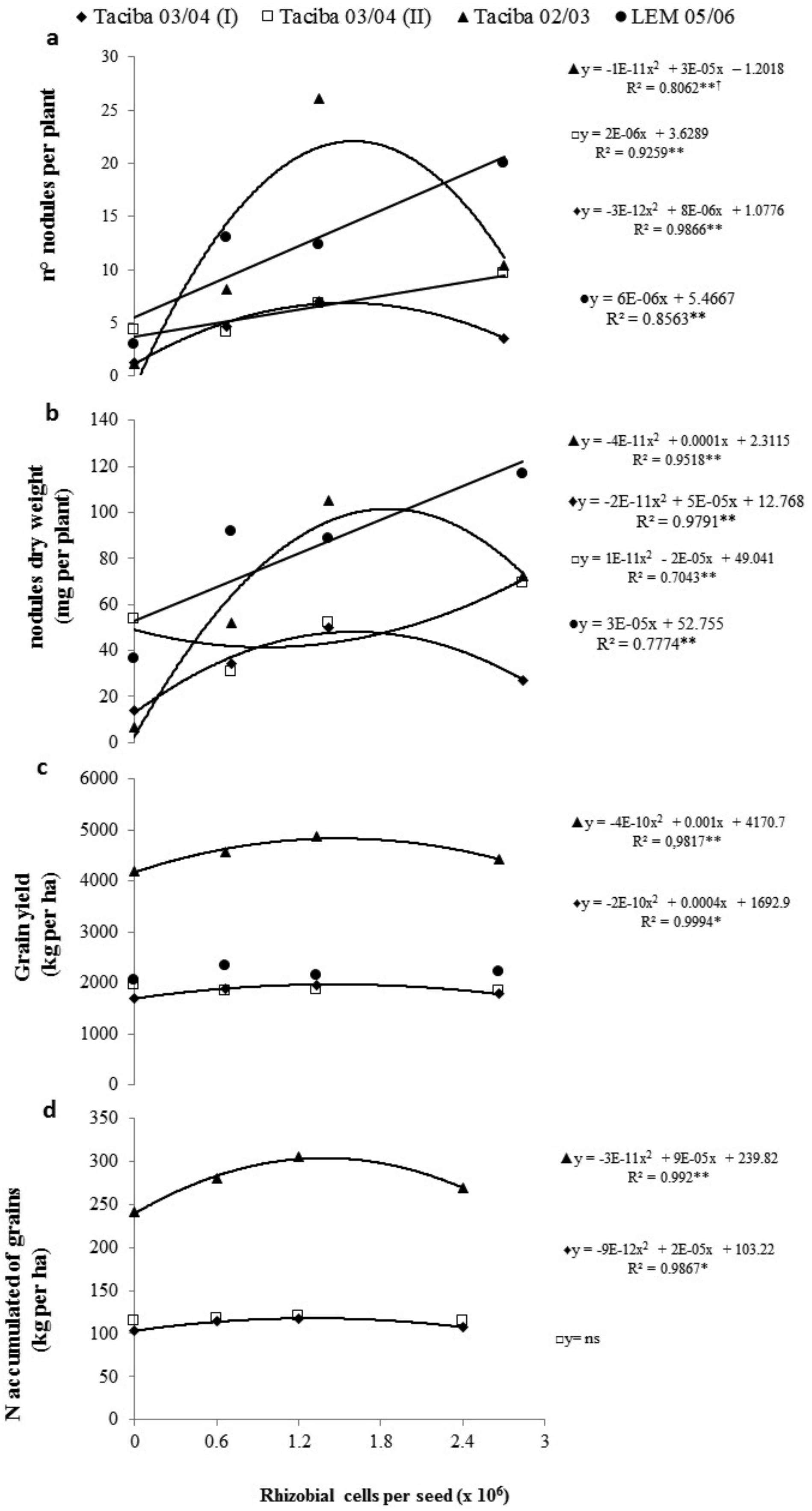

Fig. 1. (a) Nodule number and (b) dry weight, (c) grain yield, and (d) total N in grains of soybean in four field experiments performed in areas without established population of soybean bradyrhizobia. Experiments performed in Taciba-SP, 2002/2003 and 2003/2004 (I and II) and in Luiz Eduardo Magalhães (LEM)-BA, 2005/2006. Data represent the means of six replicates. ns, not significant; * Significant *p*=0.05; ** Significant *p*=0.01; † Transformed data with root X.

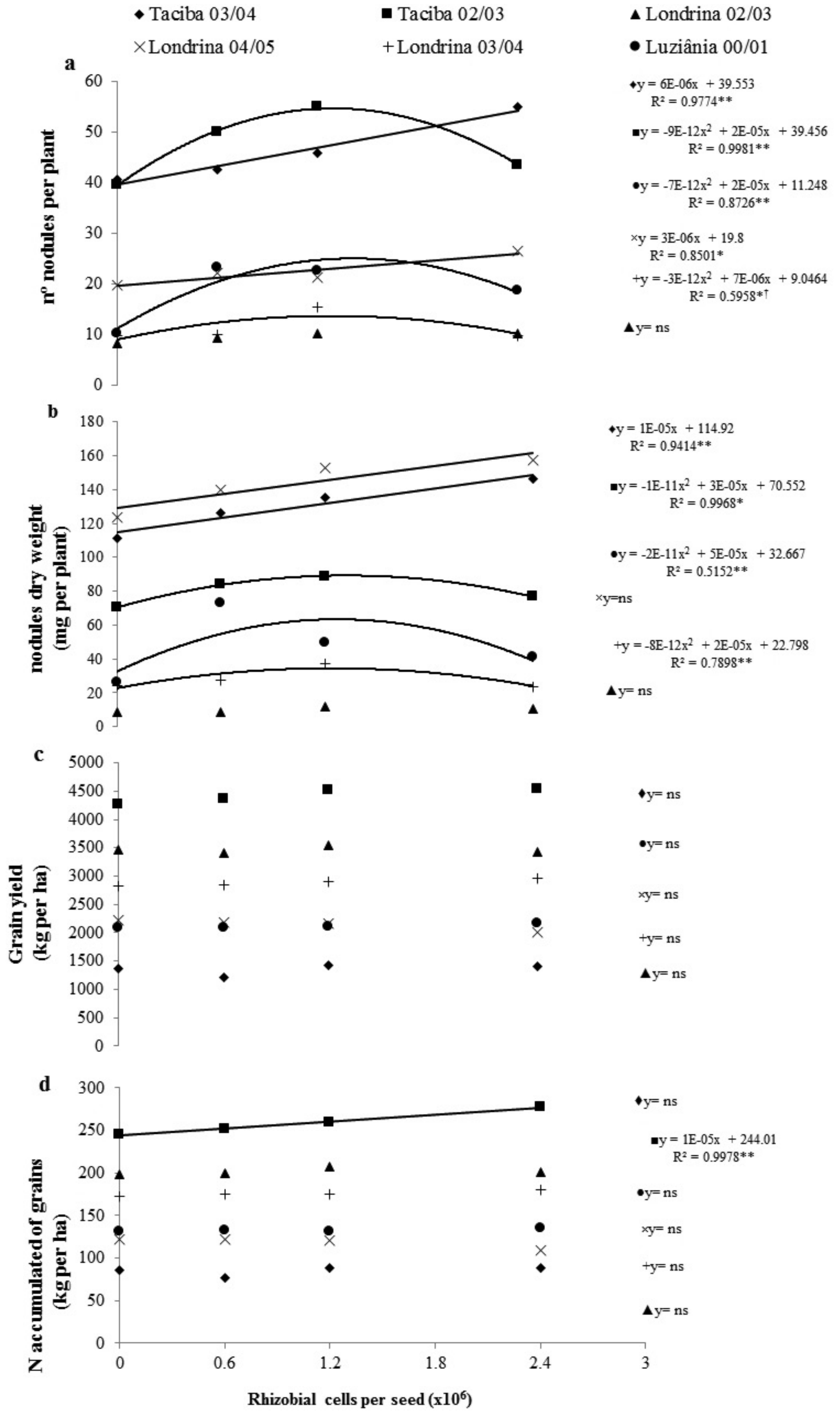

Fig. 2. (a) Nodule number and (b) dry weight, (c) grain yield, and (d) total N in grains of soybean in six field experiments performed in areas previously cropped with inoculated soybean and showing established population of bradyrhizobia. Experiments performed in Taciba-SP, 2002/2003 and 2003/2004, Luziânia-GO, 2000/2001, and Londrina-PR, 2002/2003, 2003/2004, 2004/2005. ns, not significant; * Significant *p*=0.05; ** Significant *p*=0.01; † Transformed data with log X.

(ca. 4500 kg ha^{-1}) when 2.4×10^6 CFU were inoculated per seed. No significant increases in grain yield in response to increased inoculum rates were observed in any of the other areas, with yields ranging from 1000 to 3500 kg ha^{-1} (Fig. 2c). The accumulation of N in the grains followed the same tendency observed for grain yield, and total grain N significantly increased in response to increased inoculum rates only in Taciba (2002/2003) (Fig. 2d).

DISCUSSION

The maximization of the benefits from the interaction between leguminous plants and N_2–fixing bacteria depends on the fate of the bacteria inoculated on the seeds after reaching the soil environment. In other words, if the bacteria that are inoculated on the seeds do not form nodules on the roots of the host plants, inoculation may not be as beneficial as it could be.

In many cases, the poor performance of the inoculated bacteria is related to their lack of competitiveness against soil-inhabiting bacteria that are able to nodulate a given legume host (Graham, 2008). Many studies have addressed the issue of nodulation competitiveness, and factors such as inoculum positioning (López-García et al., 2002), cell-surface characteristics and bacteria multiplication in the rhizosphere (Araujo et al., 1994), genetic determinants (Bittinger et al., 1997), composition of the rhizobial community (Vargas and Hungria, 1997; Batista et al., 2007; Mendes et al., 2004) and inoculum rate (e.g., Brockwell et al., 1989; Thornton, 1929; Weaver and Frederick, 1974) have been implicated in nodulation competitiveness. However, to date, there are no conclusive results about the ideal inoculum rate, and no one knows exactly whether more is better or worse.

In our experiments, we studied the effects of increased inoculum rates on the nodulation, grain yield, and N accumulation in the grains of soybean plants grown in soils with (six field experiments) or without (four field experiments) previous history of inoculation with soybean bradyrhizobia. In soils that received inoculation for the first time, nodulation, grain yield, and accumulation of N in the grains responded positively to increasing inoculum rate, with responses fitting both linear and quadratic models, depending on the location. Smith et al. (1981) have also observed linear responses of soybean to increased inoculum rates, in Puerto Rican soils. Similarly, Albareda et al. (2009) observed the same in soils of southern Spain, but in this case, rhizobial persistence in the soil depended on the type of soil and on the strain employed.

Since no bacteria from previous inoculations were present in the soils of some of our field sites, it is possible that factors beyond inoculum rate were determinant for nodulation and N_2 fixation, especially in the cases where the responses fit a quadratic model. In fact, Brockwell et al. (1989) have demonstrated that the negative effects of N available in the soil on soybean nodulation could only be ameliorated by increased inoculum rates.

In soils with previous history of inoculation, responses to increased inoculum rates were variable. Boonkerd et al. (1978) have previously shown that soybean responses to variations in inoculum rates are highly variable in soils with established population of bradyrhizobia. In our studies, responses fit either linear or quadratic models, or no model fit at all could be obtained. In soils where there is a resident population of bradyrhizobia, regarded as specialized PGPR, competition for limiting resources between the introduced inoculum and the resident microorganisms is determinant of inoculant survival (Strigul and Kravchenko, 2006). In addition, the N status of the soil, which is altered by previous cultivation of legume crops, may affect the outcome of nodulation and N_2 fixation by introduced rhizobia (Albareda et al., 2009; Brockwell et al., 1989). Our results corroborate the observations that nodulation, N_2 fixation, and grain yield are complex responses and may be affected by factors other than merely bacterial numbers in inocula.

In any event, the responses we obtained demonstrated that satisfactory nodulation and grain yields could be obtained in all experiments when inoculum rate was of 1.2×10^6 CFU seed^{-1}. It is noteworthy that there were both "new" and "old" sites where maximum nodulation and/or yields were achieved with inoculum rates of 2.4×10^6 CFU seed^{-1} and that could require even higher concentrations, as shown in the linear model. We should highlight that under tropical conditions, where our experiments were performed, stressful conditions such as high temperature and water deficit usually represent main factors limiting rhizobia survival in soil, nodulation, and N_2 fixation (Hungria and Vargas, 2000; Hungria and Mendes, 2015). Therefore, we propose that the minimum inoculum rate of 1.2×10^6 CFU seed^{-1} be adopted in all cases for soybean planting in the tropics, both in soils with or without previous history of soybean inoculation and cultivation.

ACKNOWLEDGMENTS

To Dr. Rubens José Campo, for his participation in the project and field experiments; he retired in 2009 and declined from being an author of the manuscript. Authors also acknowledge Dr. Marco Antonio Nogueira and Dr. Fernando Augusto Henning (Internal Publication Committee of Embrapa Soja) for suggestions on the manuscript and Dr. Fabio Luis Mostasso, for help with the field data. Project financed by Embrapa (02.13.08.003.00.00) and INCT-MPCPAgro (CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico, 465133/2014-2). M. Hungria and J.E. Zilli are also research fellows from CNPq. Approved for publication by the Editorial Board of Embrapa Soja as manuscript number 276/2016.

REFERENCES

Albareda, M., D.N. Rodríguez-Navarro, and F.J. Temprano. 2009. Soybean inoculation: Dose, N fertilizer supplementation and rhizobia persistence in soil. Field Crops Res. 113(3):352–356. doi:10.1016/j.fcr.2009.05.013

Alvares, C.A., J.L. Stape, P.C. Sentelhas, J.L. de Moraes Gonçalves, and G. Sparovek. 2013. Köppen's climate classification map for Brazil. (In English.) Meteorol. Z. 22(6):711–728. doi:10.1127/0941-2948/2013/0507

Araujo, R.S., E.A. Robleto, and J. Handelsman. 1994. A hydrophobic mutant of *Rhizobium etli* altered in nodulation competitiveness and growth in the rhizosphere. Appl. Environ. Microbiol. 60(5):1430–1436.

Bai, Y., B. Pan, T.C. Charles, and D.L. Smith. 2002. Co-inoculation dose and root zone temperature for plant growth promoting rhizobacteria on soybean [*Glycine max* (L.) Merr] grown in soil less media. Soil Biol. Biochem. 34(12):1953–1957. doi:10.1016/S0038-0717(02)00173-6

Batista, J.S.S., M. Hungria, F.G. Barcellos, M.C. Ferreira, and I.C. Mendes. 2007. Variability in *Bradyrhizobium japonicum* and *B. elkanii* seven years after introduction of both the exotic microsymbiont and the soybean host in a Cerrados soil. Microb. Ecol. 53:270–284. doi:10.1007/s00248-006-9149-2

Bittinger, M.A., J.L. Milner, B.J. Saville, and J. Handelsman. 1997. *rosR*, a determinant of nodulation competitiveness in *Rhizobium etli*. Mol. Plant Microbe Interact. 10(2):180–186. doi:10.1094/MPMI.1997.10.2.180

Boonkerd, N., D.F. Weber, and D.F. Bezdicek. 1978. Influence of *Rhizobium japonicum* strains inoculation methods on soybeans grown in rhizobia populated soil. Agron. J. 70(4):547–549. doi:10.2134/agronj1978.00021962007000040007x

Brockwell, J., R.R. Gault, L.J. Morthorpe, M.B. Peoples, G.L. Turner, and F.J. Bergersen. 1989. Effects of soil nitrogen status and rate of inoculation on the establishment of populations of *Bradyrhizobium japonicum* and on the nodulation of soybeans. Aust. J. Agric. Res. 40(4):753–762.

Brockwell, J., D.F. Herridge, L.J. Morthorpe, and R.J. Roughley. 1988. Numerical effects of *Rhizobium* population on legume symbiosis. In: D.P. Beck and L.A. Materon, editors, Nitrogen fixation by legumes in Mediterranean agriculture. ICARDA, Syria. p. 179–192.

Chandra, R., and R.P. Pareek. 1987. Effect of inoculum rate on the performance of chickpea (*Cicer arietinum* L.) *Rhizobium* strains in the field. Biol. Fertil. Soils 5(1):83–87. doi:10.1007/BF00264351

Deaker, R., R.J. Roughley, and I.R. Kennedy. 2004. Legume seed inoculation technology. Soil Biol. Biochem. 36:1275–1288. doi:10.1016/j.soilbio.2004.04.009

Fehr, W.R., and C.E. Caviness. 1977. Stages of soybean development. Spec. Rep. 80. Iowa State Univ., Ames.

Ferreira, D.F. 2011. SISVAR: A computer statistical analysis system. Ciênc. Agrotec. 35(6):1039–1042. doi: 10.1590/S1413-70542011000600001

Graham, P.H. 2008. Ecology of root-nodule bacteria of legumes. In: M.J. Dilworth, E.K. James, J.I. Sprent, and W.E. Newton, editors, Nitrogen-fixing leguminous symbiosis. Springer, Dordrecht, the Netherlands. p. 23–58.

Hungria, M., R.J. Campo, I.C. Mendes, and P.H. Graham. 2006a. Contribution of biological nitrogen fixation to the N nutrition of grain crops in the tropics: The success of soybean (*Glycine max* L. Merr.) in South America. In: R.P. Singh, N. Shankar, and P.K. Jaiwa, editors, Nitrogen nutrition and sustainable plant productivity. Studium Press, Houston, TX. p. 43–93.

Hungria, M., J.C. Franchini, R.J. Campo, C.C. Crispino, J.Z. Moraes, R.N.R. Sibaldelli et al. 2006b. Nitrogen nutrition of soybean in Brazil: Contributions of biological N_2 fixation and of N fertilizer to grain yield. Can. J. Plant Sci. 86:927–939. doi:10.4141/P05-098

Hungria, M., M.F. Loureiro, I. de Carvalho Mendes, R.J. Campo, and P.H. Graham. 2005. Inoculant preparation, production and application. In: D. Werner and W.E. Newton, editors, Nitrogen fixation in agriculture, forestry, ecology and the environment. Springer, Dordrecht, the Netherlands. p. 223–253. doi:10.1007/1-4020-3544-6_11

Hungria, M., and I.C. Mendes. 2015. Nitrogen fixation with soybean: The perfect symbiosis? In: F.J. De Bruijn, editor, Biological nitrogen fixation. John Wiley & Sons, Hoboken, NJ. p. 1009–1023. doi:10.1002/9781119053095.ch99

Hungria, M., M.A. Nogueira, and R.S. Araujo. 2013. Co-inoculation of soybeans and common beans with rhizobia and azospirilla: Strategies to improve sustainability. Biol. Fertil. Soils 49:791–801. doi:10.1007/s00374-012-0771-5

Hungria, M., M.A. Nogueira, and R.S. Araujo. 2015. Alternative methods and time for soybean inoculation to overcome adverse conditions at sowing. Afr. J. Agric. Res. 10(23):2329–2338. doi:10.5897/AJAR2014.8687

Hungria, M., and M.A.T. Vargas. 2000. Environmental factors impacting N_2 fixation in legumes grown in the tropics, with an emphasis on Brazil. Field Crops Res. 65:151–164. doi:10.1016/S0378-4290(99)00084-2

Iswandi, A., P. Bossier, J. Vandenabeele, and W. Verstraete. 1987. Influence of the inoculation density of the rhizopseudomonad strain 7NSK2 on the growth and the composition of the root microbial community of maize (*Zea mays*) and barley (*Hordeum vulgare*). Biol. Fertil. Soils 4(3):119–123. doi:10.1007/BF00256984

Lohrke, S.M., C.J. Madrzak, H.G. Hur, A.K. Judd, J.H. Orf, and M.J. Sadowsky. 2000. Inoculum density-dependent restriction of nodulation in the soybean-*Bradyrhizobium japonicum* symbiosis. Symbiosis 29(1):59–70.

López-García, S.L., T.E.E. Vázquez, G. Favelukes, and A.R. Lodeiro. 2002. Rhizobial position as a main determinant in the problem of competition for nodulation in soybean. Environ. Microbiol. 4(4):216–224. doi:10.1046/j.1462-2920.2002.00287.x

Mendes, I.C., M.A.T. Vargas, and M. Hungria. 2004. Establishment of *Bradyrhizobium japonicum* and *B. elkanii* in a Brazilian Cerrados oxisol. Biol. Fertil. Soils 40:28–35. doi:10.1007/s00374-004-0739-1

Nazih, N., and R.W. Weaver. 1994. Numbers of clover rhizobia needed for crown nodulation and early growth of clover in soil. Biol. Fertil. Soils 17(2):121–124. doi:10.1007/BF00337743

Papakosta, D.K. 1992. Effect of inoculant rate on nodulation and various agronomic traits of soybean. J. Agron. Crop Sci. 168(4):238–242. doi:10.1111/j.1439-037X.1992.tb01004.x

Patrick, H.N., and W.L. Lowther. 1995. Influence of the number of rhizobia on the nodulation and establishment of *Trifolium ambiguum*. Soil Biol. Biochem. 27(4-5):717–720. doi:10.1016/0038-0717(95)98654-7

Smith, R.S., M.A. Ellis, and R.E. Smith. 1981. Effect of *Rhizobium japonicum* inoculant rates on soybean nodulation in a tropical soil. Agron. J. 73(3):505–508. doi:10.2134/agronj1981.00021962007300030025x

Sparrow, S.D., and G.E. Ham. 1983. Nodulation, N_2 fixation, and seed yield of navy beans as influenced by inoculant rate and inoculant carrier. Agron. J. 75(1):20–24. doi:10.2134/agronj1983.00021962007500010006x

Strigul, N.S., and L.V. Kravchenko. 2006. Mathematical modeling of PGPR inoculation into the rhizosphere. Environ. Model Soft. 21:1158–1171.

Takats, S.T. 1986. Suppression of nodulation in soybeans by superoptimal inoculation with *Bradyrhizobium japonicum*. Physiol. Plant. 66(4):669–673. doi:10.1111/j.1399-3054.1986.tb05597.x

Thies, J.E., P.W. Singleton, and B.B. Bohlool. 1991a. Influence of size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Appl. Environ. Microbiol. 57:19–28.

Thies, J.E., P.W. Singleton, and B.B. Bohlool. 1991b. Modeling symbiotic performance of introduced rhizobia in field-based indices of indigenous populations size and nitrogen status of the soil. Appl. Environ. Microbiol. 57:29–37.

Thornton, H.G. 1929. The influence of the number of nodule bacteria applied to the seed upon nodule formation in legumes. J. Agric. Sci. 19(2):373–381. doi:10.1017/S0021859600011357

Vargas, M.A.T., and M. Hungria. 1997. Fixação biológica do N_2 na cultura da soja. In: M.A.T. Vargas and M. Hungria, editors, Biologia dos solos dos Cerrados. (In Portuguese.) EMBRAPA-CPAC, Planaltina. p. 297–360.

Weaver, R.W., and L.R. Frederick. 1974. Effective of inoculum rate on competitive nodulation of *Glycine max* L. Merr. II Field studies. Agron. J. 66:233–236. doi:10.2134/agronj1974.00021962006600020015x

Yamakawa, T., and Y. Fukushima. 2014. Low inoculum densities of *Bradyrhizobium japonicum* USDA 110 is effective on production of soybean (*Glycine max* L. Merr.) cultivar Fukuyutaka. J. Fac. Agr. Kyushu U. 59(1):45–53.

Zhang, S., M.S. Reddy, N. Kokalis-Burelle, L.W. Wells, and J.W. Kloepper. 2001. Lack of induced systemic resistance in peanut to late leaf spot disease by plant growth-promoting rhizobacteria and chemical elicitors. Plant Dis. 85:879–884. doi:10.1094/PDIS.2001.85.8.879