BIOLOGICAL NITROGEN FIXATION AS A KEY COMPONENT OF NUTRITION FOR THE SOYBEAN CROP IN BRAZIL

Mariangela Hungria[1,2], Ricardo Silva Araujo[1,2,3], Rubens José Campo[1]

[1]Embrapa Soja, Cx. Postal 231, CEP 86001-970, Londrina, Paraná, Brazil; [2]Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-MCT); [3]Total Biotecnologia, Curitiba, Paraná, Brazil. E-mail: hungria@cnpso.embrapa.br; rsaraujo@totalbiotecnologia.com.br; rjcampo@cnpso.embrapa.br

Abstract

The area under soybean cultivation in South America accounts for more than half of the world soybean production, and the high cost of mostly important N fertilizers has necessitated a cropping approach emphasizing biological nitrogen fixation (BNF). Most of the work on BNF in South America has been performed in Brazil, with continuous selection programs for both rhizobial strains and soybean cultivars, aiming at achieving superior symbiotic performance. Yields as high as 5,000 kg ha^{-1} without any supply of N-fertilizer, and rates of BNF exceeding 300 kg of N ha^{-1} have been obtained. Increases in grain yield (~8%) have also been obtained by reinoculation in soils showing up to 10^6 cells g^{-1} soil. Among the main challenges of research are the difficulties of introducing new efficient strains in soils showing high populations of *Bradyhrizobium japonicum* and *B. elkanii* strains established by previous inoculations. The incompatibility between inoculation and seed treatment with fungicides and micronutrients is another main constrain to the maximization of the BNF process under field conditions. There are no rhizobial-friendly products in the market and the in-furrow inoculation comes up as an attractive alternative, although higher doses of liquid inoculants are required. In relation to the seed-applied molybdenum (Mo), often also toxic to the rhizobia, both the foliar fertilization and the production of seeds enriched on Mo help to avoid toxicity. The success of BNF also relies on a well-built legislation to guarantee high-quality inoculants, as well as on a strong extension program to guarantee that good practices of inoculation are annually employed by farmers.

Brazilian rhizobial strain selection program

The first studies of strain evaluation were performed in Brazil in the 1920s, but testing was performed only at experimental levels. When soybean crop expansion started in Brazil, mainly from the 1960s, experiments using disinfected seeds reported zero or close to zero nodulation and yellow plants in N-poor soils, indicating that the soils were void of rhizobial strains able to establish an effective symbiosis with soybean. At this time, a national soybean commission determined that BNF was an important trait and needed to be considered in breeding activities; most important, the identification and selection of strains with good interaction with local soybean genotypes has started and still continues. Commercial inoculants were then brought to the country, mostly from the U.S.A. and Australia, but often failed because of inappropriate storage, delays in custom clearance, and strain/variety interaction. A strain selection program has then started, searching for strains compatible with the locally developed soybean genotypes and also with the soil and environmental conditions of Brazil. Since then every two years the Brazilian rhizobiologists meet to analyze the results of field trials conducted to evaluate strain performance. The strains authorized for the production and/or commercialization of inoculant strains in Brazil are controlled by the Ministry of Agriculture, based on the reports of the rhizobiologists. Nowadays there are four strains authorized for the use in commercial inoculants: *B. japonicum* strains SEMIA 5079 (=CPAC 15) and SEMIA 5080 (=CPAC 7) and *B. elkanii* strains SEMIA 587 and SEMIA 5019 (=29W). Inoculants can carry one or two of those strains, independent of the combination. It has been exhaustingly proved that the four strains can supply all plant's needs of the cultivars available today, allowing yields of up to 5,000 kg ha^{-1} with rates of BNF as high as 300 kg of N ha^{-1} and providing from 69 to 94% of total plant N. Benefits due to the release of N to the following crop have also been reported (Hungria and Vargas, 2000; Hungria et al., 2005, 2006a).

Symbiotic performance of soybean genotypes

Most Brazilian soybean cultivars derive from North American genotypes, and the main achievement of the Brazilian soybean breeding program has been that breeding has always been performed in areas inoculated with *Bradyrhizobium* strains, and commonly in soils that are limited on N. However, there is variability among the Brazilian soybean genotypes in relation to BNF, so understanding better the bases of the symbiotic performance is mandatory. From the identification of contrasting soybean cultivars in relation to the symbiotic performance our research group has then started a crossing program aiming at the identification of molecular markers related to BNF and also of cultivars with higher BNF capacity. Some quantitative trait loci (QTLs) controlling nodulation N_2 fixation capacity have been already identified and mapped in the linkage groups B1 (Satt509, Satt197, Satt332), C2 (Satt307), D1b (Satt296) and H (Satt192), while Satt232 is still not linked in group L (Nicolás et al, 2006; Santos et al. 2006). Progresses towards the saturation of the regions containing these markers are in progress. Our main goal is to identify effective SSR markers to help to assist the soybean selection program, enhancing BNF traits.

Responses of soybean to inoculation and reinoculation

As previously commented, soybean was introduced into South America at the end of the 19th century, but significant commercial crop expansion only began in the 1960s. Nowadays most soils cropped with soybean have high *Bradyrhizobium* populations from previous inoculations. In Brazil, more efficient and competitive strains have been identified and selected from this population, such that the four strains authorized today for the production of commercial inoculants in Brazil can often compete against naturalized population. Therefore, contrary to many results reported in countries as U.S.A. and Australia, positive responses to reinoculation are often reported in Brazil in soils with 10^3 cells g^{-1} of soil or higher. Table 1 shows the results obtained in thirty field trials performed in the two main cropping regions of Brazil in the period of 1996-2000. In another survey of field trials, Hungria et al. (2006a) have shown that in 74 field trials performed in Argentina yield of reinoculated treatments was enhanced by a mean of 14% in comparison to the non-inoculated treatment, while in 29 field experiments performed in Brazil reinoculation increased yield by 8%.

Table 1. Mean and maximum percentage increases in yield (kg ha^{-1}) and total N in grains (kg N ha^{-1}) due to the inoculation with a combination of the strains of *Bradyrhizobium elkanii* SEMIA 587 and *B. japonicum* SEMIA 5080 (=CPAC 7), when compared to the non-inoculated control. The increases were obtained in thirteen experiments performed in two Brazilian Regions, in soils with established population of soybean bradyrhizobia[1]. Source: Hungria et al. (2006a).

Region	Grain yield (% increase)		Total N in grains (% increase)	
	Mean	Maximum	Mean	Maximum
Central-western	7.8	23	8.1	25
Southern	3.8	20	4.3	24

[1] Each experiment was performed with four to six replicates and after the multivariate analysis the data presented in this table were statistically significant for both grain yield and total N accumulated in the grains (Duncan, $P \le 0.05$).

Response to the application of N-fertilizer

Soybean represents a high profit crop in South America with increasing pressure for farmers to purchase N fertilizer. However, breeding of Brazilian soybean has been based on BNF thus soybean does not respond to N-fertilizer. Starter N doses as low as 20-40 kg of N ha^{-1} may decrease nodulation and N_2 fixation rates, with no benefits to yield. Indeed, in more than 50 experiments where inoculation and fertilization with 200 kg of N ha^{-1} have been compared (split application of N at sowing and flowering), no increases in yield due to N-fertilizer use have been observed. Similarly, there were no benefits when N-fertilizer was applied at a rate of 400 kg N ha^{-1}, split across ten applications (Hungria et al., 2005, 2006a).

More recently, a series of experiments was performed to confirm that no extra benefits would be obtained through the application of N-fertilizer. Forty experiments were performed over three years in oxisols containing at least 10^3 cells of *Bradyrhizobium* g^{-1} in the State of Paraná, southern Brazil, to estimate the contributions of BNF and of N fertilizer. The experiments were performed at two sites, Londrina and Ponta Grossa, under conventional (CT) or no-tillage (NT) systems, with two cultivars [Embrapa 48 (early-maturing) or BRS 134 (medium-maturity group)]. Treatments included non-inoculated controls without or with 200 kg of N ha^{-1}, and inoculation without or with N fertilizer applied at sowing (30 kg of N ha^{-1}), or at the R2 or R4 stage (50 kg of N ha^{-1}). Compared with the non-inoculated control, reinoculation significantly increased the contribution of BNF estimated by the N-ureide technique (on average from 79 to 84%), grain yield (on average 127 kg ha^{-1}, or 4.7%) and total N in grains (on average 6.6%). The application of 200 kg of N fertilizer ha^{-1} drastically decreased nodulation and the contribution of BNF (to 44%), with no further gains in yield. Application of starter N at sowing decreased nodulation and slightly the contribution of BNF and did not increase yields, while N fertilizer at R2 and R4 stages decreased the contribution of BNF (to 77%) and also yields. The results highlight the economical and environmental benefits resulting from replacing N fertilizer with inoculation in Brazil, and reinforce the benefits of reinoculation, even in soils with high populations of *Bradyrhizobium* (Hungria et al., 2006b). It is a striking contrast with northern and central China, the center of genetic origin of soybean, where BNF alone apparently cannot meet the N requirement for maximum yield (Gan et al., 2002, 2003). In those areas, best results were obtained with top dressing of N fertilizer (50 kg ha^{-1}) at V2 (the unifoliolate node and the first two trifoliate leaf nodes with unfolded leaflets), and especially at R1 stage (Gan et al., 2002, 2003). These authors mention that the inefficient use of N fertilizer can often result in high costs, while the reported yields are lower than generally obtained in South America.

Compatibility of seed treatment with inoculants and with fungicides and micronutrients

Seed treatment with fungicides has been broadly practiced in South America as cheap insurance against seed- and soil-borne pathogens, but toxicity of most fungicides has often been underestimated. In our laboratory we have studied the compatibility between seed treatment with fungicides in single or mixed applications (including Benomyl, Captan, Carbendazin, Carboxin, Difenoconazole, Thiabendazole, Thiram, Tolylfluanid) and bradyrhizobial inoculants in laboratory, greenhouse and field experiments over six years. Bacterial survival on the seeds was severely affected by all fungicides (Table 2), resulting in mortalities of up to 62% after only two hours and of 95% after twenty four hours. Fungicides also reduced nodule number, total N in grains and decreased yield by up to 17%. The toxic effects of fungicides were more drastic in sandy soils without soybean inoculation and cropping history, reducing nodulation by up to 87%, but were also important in areas with established populations of soybean bradyrhizobia (Campo et al., 2009b). Therefore an important conclusion from these results is that the fungicides available at the market today in Brazil (and also in other countries of South America) should be used only when the seeds or soil are effectively contaminated, or under unfavorable climate conditions, otherwise BNF may be severely affected.

Table 2. Reduction (%) of soybean nodulation (nodule number, n° plant^{-1}) and yield (kg of grains ha^{-1}) caused by seed treatment with fungicides applied as mixture of active ingredients, in comparison to the treatment where seeds were just inoculated with *Bradyrhizobium*. Data obtained from field experiments in the southern, southeastern and central-western regions of Brazil. Numbers in parenthesis mean the number of experiments in which the combination has been tested. From Campo et al. (2009b).

Active ingredients	Old areas		New areas			
			clay soils		sandy soils	
	nodulation	yield	nodulation	yield	nodulation	yield
Carboxin+Thiram	16.5 (02)	0.1 (02)	40.5 (08)	12.8 (05)	53.6 (03)	5.1 (03)
Thiabendazole+Tolylfluanid	14.0 (02)	1.6 (02)	32.7 (10)	9.8 (05)	88.0 (02)	8.2 (02)
Carbendazin+Thiram	10.0 (02)	5.8 (02)	36.2 (10)	14.5 (05)	57.5 (02)	11.0 (02)
Carbendazin+Captan	4.0 (02)	0.5 (02)	42.4 (10)	7.3 (05)	74.5 (02)	8.9 (02)
Difenoconazole+Thiram	26.0 (02)	0.0 (02)	23.4 (09)	3.9 (05)	69.5 (02)	5.8 (02)
Benomyl+Captan	NI[1]	NI	22.0 (02)	5.4 (02)	74.0 (01)	NI
Benomyl+Thiram	NI	NI	42.5 (02)	5.6 (02)	78.0 (01)	NI
Benomyl+Tolylfluanid	NI	NI	36.0 (02)	4.0 (02)	78.0 (01)	NI
Carbendazin+Tolylfluanid	NI	NI	33.0 (02)	0.0 (02)	83.0 (01)	NI
Thiabendazole+Captan	NI	NI	26.0 (02)	3.0 (02)	87.0 (01)	NI
Thiabendazole + Thiram	NI	NI	30.0 (02)	0.8 (02)	70.0 (01)	NI
Fludioxonil + Metalaxyl	NI	NI	NI	NI	57.7 (06)	NI
General mean	14.1	1.6	33.2	6.1	72.6	7.0

[1] Treatment not included in the field experiment.

In Brazil, as soybean is considered an important cash crop, fertilizers containing macronutrients necessary to plant growth are always applied. However, the efficiency of BNF can also be limited by micronutrient deficiencies, and the most common micronutrient limiting production has been the molybdenum (Mo). Soybean generally responds positively to fertilization with Mo in soils of low fertility and in fertile soils depleted of Mo due to long-term cropping. The micronutrient can be supplied by seed treatment however, similarly to what happens with fungicides, toxicity of Mo sources to *Bradyrhizobium* strains applied to seed as inoculants has been observed, resulting in bacterial death, and reductions in nodulation, BNF and grain yield. Therefore, use of seeds enriched in Mo could be a viable alternative to exterior seed treatment, allowing elite inoculant strains of *Bradyrhizobium* to sustain high rates of BNF. We have demonstrated the feasibility of producing Mo-rich seeds of several soybean cultivars, by means of two foliar sprays of 400 g Mo ha^{-1} each, between the R3 and R5 stages, with a minimum interval of ten days between sprays. As a result of this method, considerable increases in seed-Mo content were obtained, of as much as 3,000%, in comparison to seeds obtained from plants which received no Mo. In field experiments performed in soils with low N content and without any N-fertilizer supply, inoculation of Mo-rich seeds produced plants with increased N and Mo contents in the grain and higher yields of total N and of grain (Table 3). In most cases, Mo-rich soybean seeds did not require any further application of Mo-fertilizer (Campo et al., 2009a).

In-furrow inoculation to decrease effects of incompatibility with fungicides and other seed treatments

Inoculation practices which can alleviate the negative effects of agrochemicals (fungicides and micronutrients) must be searched and the in-furrow inoculation comes up as an attractive alternative. We have performed seven field experiments in Brazil for three crop seasons, three in soils previously cropped with inoculated soybean (>10^4 cells g^{-1} soil), and four in areas cropped for the first time (<10^2 cells g^{-1} soil). Compatibility with agrochemicals was compared in seeds inoculated with peat or liquid inoculants, or receiving different doses of liquid inoculant in-furrow. In areas with established populations, in general agrochemicals applied to the seeds did not affect nodulation, but also did not improve yields, while inoculation always increased N accumulation in the grains or yield, and N fertilizer decreased both nodulation and yield. In areas cropped for the first time, seed treatment with agrochemicals affected nodulation when applied together with peat or liquid inoculant on the seeds. In-furrow inoculation alleviated the effects of seed treatment with agrochemicals, and the best performances were achieved with high concentration of cells, of up to 2.5 million cells seed^{-1}.

Table 3. Grain yield (kg ha^{-1}) of soybean cultivar BR 16 grown from seeds with low (poor), medium, high (rich), and very high (very rich) Mo contents, in response to complementation with 0, 10 or 20 g Mo ha^{-1} applied to the seeds at sowing; experiments performed in an oxisol of Londrina, State of Paraná, Brazil. Source: Campo et al. (2009a).

Seed[b]	Mo complementation		
	0	10	20
1997/1998			
Poor (0.0)	2766cB	3075bA	3020bA
Medium (0.3)	3049bA	3217bA	3045bA
Rich (7.6)	3378aB	3508aAB	3641aA
1998/1999			
Poor (0.73)	2314bB	2645bA	2793bA
Medium (7.5)	3167aB	3794aA	3790aA
Rich (13.3)	3602aA	3892aA	3823aA
1999/2000			
Poor (2.4)	2398bB	2684aA	2699aA
Medium (9.8)	2592abA	2596aA	2603aA
Rich (15.6)	2561abA	2670aA	2630aA
Very rich (31.6)	2750aA	2701aA	2753aA

[a] Means (n=6) from a same column (lowercase) or line (uppercase) followed by different letters are significantly different ($p \leq 0.05$, Duncan's test).

[b] Numbers in parentheses denote Mo content in the seeds in µg Mo g^{-1} seed.

Production and quality of the inoculants produced in Brazil

The first production and distribution of inoculants for legumes in Brazil dates from 1930 in São Paulo State, but industrial scale production did not begin until the 1960s. Control of inoculant quality in the country was a main concern of researchers from the very beginning, with official control of inoculant quality established by the Federal Government in 1975. The initial standard was 10^7 cells g^{-1}, a concentration that would supply 7,000 cells seed^{-1}. Later, in 1982, the Ministry of Agriculture (MA) started to require 10^8 viable cells per g of product at the industry and 10^7 at the stores, creating penalties for lower levels.

A very important initiative contributing to the a strong inoculant legislation in Brazil and also to the quality of the commercial inoculants was the creation, in 1985, of a group including researchers, members of the inoculant industry and of the Ministry of Agriculture, the RELARE, "Rede de Laboratórios para Recomendação de Estirpes de *Rhizobium*". This group gives technical support to the official decisions, as recommendation of strains, of inoculant quality, concentration of cells on the seeds, among others.

From 2004, the legislation changed the quality requirement to a minimum concentration of $1.0.10^9$ viable cells per g or mL of the product at the shelf, with a commercial period of at least six months. Furthermore, the inoculant must not contain contaminants at the 1.10^{-5}. Legislation controls number of cells, contaminants, and information to the consumer. The research recommends that the inoculant should be applied to the seeds to allow a population of 1.2 million cells seed^{-1} (Hungria et al., 2006a, 2007; Hungria and Campo, 2007). A strong inoculant legislation has been a key step to guarantee a good contribution of BNF at the field.

Economical, social and environmental importance of biological N_2 fixation to the agribusiness in Brazil,

The process of BNF is crucial for the viability of the agribusiness in Brazil, and the major example is given by the soybean crop. For each 1,000 kg of soybean produced, the plant requires approximately 80 kg of N (65 kg allocated to seeds and 15 kg N left in roots, stems and leaves). Thus, to achieve the national mean yield of ~2,700 kg ha^{-1}, the N needs, the cropped area, the low amounts of N of the soil—supplying only 15 to 30 kg of N crop^{-1}—the N-fertilizer utilization efficiency (50-60%) and the price of the N-fertilizers, an excess of ~US$ 6.6 billion are saved every year (Hungria et al., 2007). However, the research on BNF must continue, because, if today 300 kg de N ha^{-1} are required to achieve yields of 2,500 kg ha^{-1}, 1,000 kg of N ha^{-1} will be required to supply the 8,000 kg ha^{-1} estimated as genetic potential for the crop. It is also noteworthy that annual reinoculation increases yield by an average of 8%, and that a supply of only 30 kg of N ha^{-1} would cost US$ 660 millions per year to the country (Hungria et al., 2007). In addition, release of N to the following crop—wheat, maize—has been reported, and cropping systems including soybean every one or two years are highly sustainable on N (Hungria et al., 2005, 2007). However, the success of BNF also relies on a strong extension program to guarantee that good practices of inoculation are annually employed by farmers.

References

Campo RJ, Araujo RS and Hungria, M, 2009a. Molybdenum-enriched soybean seeds enhance N accumulation, seed yield, and seed protein content in Brazil. Field Crops Research, 110(3):219-224.

Campo RJ, Araujo RS amd Hungria, M, 2009b. Nitrogen fixation with the soybean crop in Brazil: compatibility between seed treatment with fungicides and bradyrhizobial inoculants. Symbiosis, 48(1-3,):154-163.

Gan Y, Stulen I, Posthumus F, van Keulen H and Kuiper PJC, 2002. Effects of N management on growth, N_2 fixation and yield of soybean. Nutr. Cycl. Agroecosyst., 62:163–174.

Gan Y, Stulen I, van Keulen H and Kuiper PJC, 2003. Effect of N fertilizer top-dressing at various reproductive stages on growth, N_2 fixation and yield of three soybean (*Glycine max* (L.) Merr.) genotypes. Field Crops Research, 80:147–155.

Hungria M and Campo RJ, 2007. Inoculantes microbianos: situação no Brasil. In: Izaguirre-Mayoral ML, Labandera C andSanjuan J (ed.). Biofertilizantes en Iberoamérica: Visión Técnica, Científica y Empresarial. Montevideo: Cyted/Biofag, 22-31.

Hungria M and Vargas MAT, 2000. Environmental factors impacting N_2 fixation in legumes grown in the tropics, with an emphasis on Brazil. Field Crops Research, 65:151-164.

Hungria M, Campo RJ and Mendes IC, 2007. A Importância do Processo de Fixação Biológica do Nitrogênio para a Cultura da Soja: Componente Essencial para a Competitividade do Produto Brasileiro**.** Londrina, Brazil: Embrapa Soja, 80 p. (Embrapa Soja. Documentos, 283).

Hungria M, Campo RJ, Mendes IC and Graham PH, 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: Singh RP, Shankar N and Jaiwal PK (ed.), Nitrogen Nutrition and Sustainable Plant Productivity. Houston, TX: Studium Press, LLC, 43-93.

Hungria M, Franchini JC, Campo RJ, Crispino CC, Moraes JZ, Sibaldelli RNR, Mendes IC and Arihara J, 2006b. Nitrogen nutrition of soybean in Brazil: contributions of biological N_2 fixation and of N fertilizer to grain yield. Canadian Journal of Plant Science, 86:927-939.

Hungria M, Franchini JC, Campo RJ and Graham PH, 2005. The importance of nitrogen fixation to soybean cropping in South America. In: Werner W and Newton WE (ed.), Nitrogen Fixation in Agriculture, Forestry, Ecology and the Environment. Dordrecht, Amsterdam: Springer, 25-42.

Nicolás MF, Hungria M; and Arias CAA, 2006. Identification of quantitative trait loci controlling nodulation and shoot mass in progenies from two Brazilian soybean cultivars. Field Crops Research, 95(2-3):355-366.

Santos MA, Nicolás MF and Hungria M, 2006. Identificação de QTL associados à simbiose entre *Bradyrhizobium japonicum*/*B. elkanii* e a soja [*Glycine max* (L.) Merr.]. Pesquisa Agropecuária Brasileira, 41(1):67-75.