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Short communication. Biological fixation of nitrogen and N balance in soybean crops in the pampas region

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Abstract

Biological nitrogen fixation (BNF) is of key importance in the N balance of soybean (*Glycine max*) crops. A number of authors have suggested that a negative balance may occur under high yield conditions. Few studies have measured the contribution of BNF to soil N in the pampas region. The aims of the present study were to compare three BNF determination methods – two isotopic methods using sorghum or a non-nodulating soybean isoline as a reference crop, and one involving the calculation of the difference in N content between the nodulating and non-nodulating soybean isolines – and to estimate the N balance in soybean crops raised under conventional tillage and no tillage practices. The study was performed in 2004-2005; a complete randomised block design was used with three replicates (plot dimensions 3×7 m). The different methodologies estimated BNF to account for 45-58% of total plant N, equivalent to 94 to 123 kg N ha^{-1}. Depending on the methodology for estimating the BNF the soil N balance varied between –7 and 22 kg N ha^{-1}. With an average grain yield of 1,618 kg ha^{-1} and a BNF accounting for approximately 50% of total plant N (i.e.,115 kg N ha^{-1}), the soil N balance was slightly positive (14 kg ha^{-1}) and independent of the tillage practice. The tillage systems had no effect ($P < 0.05$) on the mass or number of nodules, shoot biomass production at the R1 or R6 growth stages, the N content, BNF, or grain yield. Since the present results were obtained using non-commercial soybean isolines, further research is required to determine the soil N balance when high yielding soybean crops are raised.

Additional key words: ^{15}N, *Glycine max*, isotopic dilution, nodulation and non-nodulation isolines, tillage system, yield.

Resumen

Comunicación corta. Fijación biológica y balance de nitrógeno de soja en la región pampeana

La fijación biológica de nitrógeno (BNF) es de importancia central para el balance de N en cultivos de soja (*Glycine max*) y varios autores sugieren un balance negativo en condiciones de alto rendimiento. Pocos estudios han medido a la fecha la contribución de la BNF al N del suelo en la región pampeana. Nuestro objetivo fue el de cuantificar la BNF utilizando tres metodologías de medición diferentes (dos metodologías isotópicas usando como cultivo de referencia sorgo o una isolínea no nodulante de soja y el método de la diferencia en el contenido de N de la isolínea de soja no nodulante y la nodulante) y estimar el balance de N en el cultivo de soja bajo labranza convencional y siembra directa. El estudio fue realizado durante 2004-2005 y los tratamientos se realizaron utilizando un diseño en bloques completamente al azar con tres réplicas en parcelas de 3×7 m. La estimación de la BNF basada en las tres metodologías estudiadas, varió entre el 45% y el 58% del contenido total de N de las plantas, equivalente a 94 a 123 kg N ha^{-1}. Dependiendo de la metodología empleada para estimar la BNF, el balance de nitrógeno varió entre –7 a 22 kg ha^{-1}. Con un rendimiento promedio en grano de 1.618 kg ha^{-1} y una BNF cercana al 50% del total de N en las plantas, el balance de N del suelo resultó levemente positivo (14 kg ha^{-1}) y fue independiente del sistema de labranza utilizado. No se registraron diferencias ($P < 0,05$) entre los sistemas de labranza en el peso o número de nódulos ni producción de biomasa aérea en los estadios R1 ó R6, contenido de N, BNF o producción de grano. Se requiere seguir las investigaciones para analizar el balance de N en el suelo bajo condiciones de alto rendimiento de cultivos de soja.

Palabras clave adicionales: ^{15}N, dilución isotópica, *Glycine max*, isolíneas nodulantes y no nodulantes, sistemas de labranza, rendimiento.

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Biological N fixation (BNF) plays a key role in the sustainability of agriculture. Rhizobia establish symbiotic associations with many leguminous and some non-leguminous plants, forming root nodules in which the fixation of atmospheric N_2 takes place. The fixation of N_2 by legumes greatly facilitates environmentally friendly agriculture and has very positive economic advantages. Currently, some 12-15% of the Earth's arable land is devoted to grain and forage legume production, which accounts for one third of humankind's dietary protein needs – two thirds for populations living under subsistence conditions (Alberton *et al.,* 2006).

High dosages and frequencies of use of herbicides, mainly those containing glyphosate, can negatively affect normal nodulation in soybean (*Glycine max*) and therefore BNF and crop yield (Hernández *et al.*, 1999; Zablotowicz and Reddy*,* 2007). In general, no-tillage (NT) practices require higher glyphosate doses (which also provide higher doses of the accompanying substances present in commercial formulations) (Benbook, 2005) than conventional tillage (CT) systems involving initially the mechanical control of weeds. Tillage systems with soil turnover tend to increase the availability of N since organic matter mineralisation rates are stimulated; however, this can inhibit BNF, reducing the amount of N entering the system. Under such conditions, N extraction from soils is greater and N is exported from the system with the grain. In contrast, the lower soil N content resulting from NT could lead to improved nodulation, increasing the amount of N fixed from the air (Wheatley *et al.,* 1995; Peoples *et al.*, 2001). Tillage systems also affect grain yields as well as the biomass that remains in the soil after harvest.

Practices such as NT and crop rotation with legumes contribute to agricultural sustainability (Ferreira *et al.*, 2000). Due to their ability to fix N_2 from the air in symbiosis with rhizobia, soybean crops around the world hardly ever require N fertilizer. However, high yielding soybean crops are associated with a negative N balance (Di Ciocco *et al.,* 2004). In Argentina, soybean crops are cultivated on 16 million hectares of land in areas with temperate and subtropical environments - the pampas and the Northeastern and Northwestern regions. These regions produce an overall estimated mean yield of 2,900 kg ha^{-1}; total production in 2006-2007 was 47 Mg (SAGPyA, 2007a). According to some estimates, production could increase to 100 million tons over the next decade. This could lead to increased losses of soil nutrients and organic matter, especially in marginal agricultural soils where agro-ecosystem sustainability could be seriously affected in the absence of adequate production practices (SAGPyA, 2007b). Soybean is mainly cultivated under the NT system (SAGPyA, 2007c), which is in fact employed on 72.4% of all Argentina's agricultural land. Since the soils are kept under plant cover, and because of the development of a stable porous system, water use is more efficient. In addition, soil disturbance is slight. Erosion and fertility losses are therefore lower than under CT. However, despite BNF, soybean grain yields over 3,000 kg ha^{-1} produce N losses from soils of over than 100 kg ha^{-1} yr^{-1} (Di Ciocco *et al.,* 2004). These estimates are close to the N extraction rates of high yielding wheat (170 kg N ha^{-1} yr^{-1}) or corn crops (200 kg N ha^{-1} yr^{-1}) (Álvarez *et al.*, 2000). The negative N balance can be explained by the soybean's high harvest index (high grain protein content), which leaves little N to return to the soil. In an earlier study, Di Ciocco *et al.* (2004) estimated that despite fixing approximately 90 kg N ha^{-1} from the air, soybean crops still leave a negative N balance — a mean of —138 kg N ha^{-1} yr^{-1} for current average yields.

The aims of the present work were to compare three different BNF estimation methods and to determine the N balance in dryland soybean crops under different tillage systems —NT and CT— in the pampas region of Argentina. All work was performed in experimental fields belonging to the Universidad Nacional de Luján (Luján, Buenos Aires Province, Argentina). The soil in these fields is a silt loam (Typic Argiudoll soil). Soybean crops were raised under the NT and CT systems established during the two seasons prior to commencement of the study. The main soil properties of the A horizon sampled at a depth of 0-7 cm were: nitrates at sowing 11.0 mg kg^{-1}, organic carbon 24.0 g kg^{-1}, clay 272.7 g kg^{-1}, silt 590.9 g kg^{-1}, sand 136.4 g kg^{-1}, and water pH 5.6. Soil analyses at sowing revealed an N content of 11.0 mg kg^{-1} nitrates and 2.25 g kg^{-1} total N. A completely randomised block design including the two main tillage treatments and three replications was used, resulting in six main plots (dimensions 3×7 m). Conventional tillage was performed with mechanical control of weeds; under the NT system weeds were controlled with glyphosate. Two isogenic lines (isolines) of soybean —D71-9289 (nodulating) and D71-9291

Abbreviations used: BNF (biological nitrogen fixation), CT (conventional tillage), NT (no-tillage), SAGPyA (Secretaría de Agricultura, Ganadería, Pesca y Alimentos), SOM (soil organic matter).

(non-nodulating)— were sown in each plot. Within each of the six plots, two 2×2 m subplots were established. One was sown with the nodulating isoline and the other with the non-nodulating isoline, neither of which are resistant to glyphosate. Both isolines, which belong to maturity group VI, were developed and provided by the USDA-ARS Delta Station at Stoneville, Mississippi, USA (Hartwig, 1994). The borders and remaining area of each plot were sown with the commercial soybean cv. M-Soy 8080 RR (Roundup Ready). Both isolines were manually sown in November 2004 (distance between rows 50 cm) at high density. Before sowing, the seeds were treated with the fungicide Tiram, and inoculated with *Bradyrhizobium japonicum* at an estimated rate of 1×10^5 colony forming units seed^{-1}. To provide a reference value, another two subplots next to the test subplots were sown with sorghum (*Sorghum bicolor*, a commonly used, non-fixing reference crop), one under the CT and one under the NT system. These reference sub-plots were subject to the same conditions as the soybean subplots.

After crop emergence, the plant density was manually adjusted to 20 plants m^{-1}, equivalent to 400,000 plants ha^{-1}. During the growth period, weeds were manually controlled in the CT treatment; in the NT plots glyphosate was applied at a rate of 4 kg ha^{-1}, split into two applications at stages V2 and V6. In both systems chlorpyrifos was applied once for insect control at stage R3. All plots received 20 kg ha^{-1} of P_2O_5 in the form of $Ca_3(PO_4)_2$ at planting.

After plant emergence, one 2 m^2 microplot was established inside each of the subplots and fertilised with labelled $(^{15}NH_4)_2SO_4$ at an equivalent rate of 10 kg N ha^{-1} with 10% atom excess ^{15}N, following the isotopic dilution method (IAEA, 1976). Fertilisation was repeated at the R2 stage. The remaining area of each sub-plot was fertilized at the same rate with non-labelled $(NH_4)_2SO_4$. When the plants reached the R1 stage, three were collected from each subplot to measure their aerial biomass. The root biomass and the weight and number of nodules per plant were determined by introducing 500 cm^3 cylinders into the ground over the roots; the roots were extracted and weighed and the nodules counted and weighed. Final harvesting was performed by hand on April 12, 2005 at stage R6, collecting all plants within a 1 m^2 area at the centre of each microplot. At physiological maturity (R6), soybean plants have achieved 100% of their final biomass and total N, and 95-100% of the grain biomass and N, but defoliation is only incipient (Ritchie *et al.*, 2002).

The harvested plant material was then separated into grains, stems, leaves and pods, dried at 60°C, and weighed. Grain and leaf N was determined by the Kjeldahl method (Bremmer, 1996) and ^{15}N by the Kjeldahl-Rittenberg method (IAEA, 1976). The percentage N derived from the atmosphere (%Ndfa) was calculated as:

$$\%\ Ndfa = [1-(\%AEN\ (\%AER)^{-1}] \times 100$$

where %AEN is the percentage of excess atom ^{15}N in nodulating plants and %AER the percentage of excess atom ^{15}N in reference plants.

Nitrogen biological fixation was calculated using three different approaches: (1) the isotopic dilution method, using a non-nodulating soybean isoline as a control, (2) using sorghum as a non-fixing reference crop, and (3) using the difference in total N for the two soybean isolines (indirect method). The isotopic techniques for BNF estimation used in this study are the only ones considered to offer global BNF estimates and to clearly distinguish between plant N coming from the soil, fertilizer and atmosphere. They also provide BNF values for entire growth cycles for legume-rhizobia systems (Bellone and Carrizo de Bellone, 2006). Of the two isotopic methods used, that involving the fixing and non-fixing isolines employs a control crop (the non-fixing isoline) of identical morphology, physiology and growth pattern. However, due to the difficulty in securing reliable fixing and non-fixing isolines, these techniques often make use of sorghum or other non-fixing plants as controls. In this work, both methods were used.

The N balance was calculated as the difference between the N in the whole plant coming from the air via BNF, and the total accumulated N in the harvested grain (Peoples *et al.*, 2001). Total rainfall during the cropping period (November to May) was 635.4 mm; 58.8 mm fell before sowing (October). All data were analysed by factorial ANOVA, considering the tillage system and isoline type as independent factors (α was set at 0.05).

At R1, the tillage system had no significant effect on aerial and root biomass, or on nodulation (Table 1). Neither did it have any effect at physiological maturity. The data for both systems were therefore combined and used together for determining BNF and the soil N balance. Grain, stem, and leaf weight, but not pod weight, differed significantly in the nodulating and non-nodulating soybean isolines (Table 2).

Soil N showed no significant differences ($P < 0.05$) before and after the crop cycle, with 11.0 and 11.7 mg kg^{-1} of nitrates, and 2.25 and 2.22 g kg^{-1} of total N at sowing and harvest respectively.

Table 1. Soybean aerial and root biomass nodulation under both tillage systems

	Conventional tillage	No tillage
Aerial and root biomass and nodulation at stage R1		
Stems and leaves (g plant^{-1})	8.29 ± 1.09 a	7.68 ± 0.50 a
Root (g plant^{-1})	0.83 ± 0.15 a	0.61 ± 0.01 a
Nodules plant^{-1}	6.78 ± 1.98 a	9.33 ± 1.73 a
Nodules weight (g plant^{-1})	0.12 ± 0.03 a	0.17 ± 0.03 a
Nodules weight/roots weight	0.14 ± 0.01 a	0.27 ± 0.02 a
Soybean yields		
Grain (kg ha^{-1})	959 ± 353 a	1,322 ± 479 a
Stems (kg ha^{-1})	1,639 ± 414 a	1,611 ± 435 a
Leaves (kg ha^{-1})	1,590 ± 304 a	1,917 ± 513 a
Pods (kg ha^{-1})	703 ± 210 a	1,065 ± 264 a
Total aerial biomass (kg ha^{-1})	4,891	5,915

Means ± standard error followed by the same letter in each line do not differ significantly according to the Duncan test ($P < 0.05$).

Table 2 shows the N contents of the different plant parts. Nitrogen biological fixation, estimated in the three different ways, accounted for 45-58% of plant total N (equivalent to 94-123 kg N ha^{-1}) (Table 3). The isotopic dilution method using ^{15}N and sorghum as a non-fixing reference crop estimated 45% N fixation from the atmosphere (equivalent to 94 kg N ha^{-1}). The same calculation using the non-nodulating soybean isoline estimated N fixation to account for 50.2% of total plant N in the fixing line (106 kg N ha^{-1}). The same calculation using the difference between the two soybean isolines estimated 58% of the total N in the fixing plants (123 kg N ha^{-1}) to be provided by BNF. These results indicate a partial N balance for the soil (i.e., considering only BNF and the total N exported by the grain) of close to zero (–7 to 22 kg N ha^{-1}) (Table 3).

These estimates are higher than the 40% estimated by Bonel *et al.* (2005), possibly because in the present work the ^{15}N was delivered in two applications, resulting in smaller losses and greater crop utilization. When taking into account the loss of N in the few leaves shed by the plants prior to harvest plus the N contained in

Table 2. Yields and nitrogen contents for the nodulating and non-nodulating soybean isolines

	Isolines	
	Nodulating	Non-nodulating
Soybean yields		
Grain (kg ha^{-1})	1,618 ± 421 a	662 ± 140 b
Stems (kg ha^{-1})	2,255 ± 138 a	994 ± 153 b
Leaves (kg ha^{-1})	2,269 ± 384 a	1,238 ± 123 b
Pods (kg ha^{-1})	1,014 ± 288 a	755 ± 209 a
Total shoot biomass (kg ha^{-1})	7,156	3,649
Harvest index	0.12	0.18
Nitrogen contents		
Grain (kg ha^{-1})	101.1 ± 26.4 a	37.4 ± 8.4 b
Stems (kg ha^{-1})	21.8 ± 2.9 a	7.0 ± 1.3 b
Leaves (kg ha^{-1})	68.1 ± 12.5 a	29.9 ± 4.8 b
Pods (kg ha^{-1})	20.5 ± 5.8 a	14.4 ± 5.0 b
Total shoot biomass (kg ha^{-1})	211.4 a	88.7 b
N index	47.8	42.2

Means ± standard error followed by the same letter in each line do not differ significantly according to the Duncan test ($P < 0.05$).

Table 3. Biological N fixation and soil N balance results provided by the three different methods of calculation (for details on procedures see the text)

BNF calculation method	N fixed (%)	N fixed (kg ha^{-1})	Grain N content in the modulating isoline (kg ha^{-1})	Soil N apparent balance (kg ha^{-1})
N difference between isolines	58.0	123	101.1	21.6
^{15}N isoline control	50.2	106	101.1	5.0
^{15}N sorghum control	44.5	94	101.1	–7.0

the roots (estimated on the basis of previous work) (Di Ciocco *et al.,* 2004), the quantity of N fixed from the air would appear to be 20% greater than that actually measured in the present work. With this correction, the N soil balance becomes slightly positive for the nodulating isoline (between 11.9 and 46.1 kg N ha^{-1} for a production of 1,618 kg grain ha^{-1}).

The N soil balances estimated in this work agree with those of a previous test under similar conditions (Di Ciocco *et al.,* 2004). In the latter work it was estimated that the break point for the system in terms of soil N balance occurs at a production of about 1,600 kg grain ha^{-1}. With greater grain yields the soil N balance is likely to become increasingly negative and could affect the sustainability of the system, requiring the use of different production practices providing a lower harvest index or the enhancement of BNF. Greater N fixation levels have, however, been reported. Using the ^{15}N isotope dilution technique, Boddey and Chalk (1984) estimated the contribution of BNF to be 250 kg N ha^{-1} after 92 days of crop growth.

The lack of differences between the tillage systems in terms of BNF contrasts with the fact that soil movements under CT lead to an organic matter mineralisation pulse, as well as residue decomposition that generates high nitrate levels during the initial part of the soybean growth period (Álvarez *et al.,* 2000). This N pulse can reduce or even prevent the nodulation process responsible for the higher initial growth of soybean under CT. These differences between tillage systems can be compensated for, however, over the crop cycle (Yusuf *et al.,* 1999). The observed lack of difference is in agreement with the findings of other authors. Harper *et al.* (1989) estimated BFN in an NT system to be 130 kg ha^{-1}, and 92 kg ha^{-1} in a CT system. When present, differences are probably related to environmental conditions; they therefore vary between years (Hughes and Herridge, 1989). Experiments performed over several years have shown that differences in nodulation and therefore soybean yields can occur. Soybeans that can stand water deficits and/or higher glyphosate doses (Dos Santos *et al.,* 2005) may favour one tillage system over the other, a possibility that should be investigated.

In conclusion, the results obtained using the different techniques are similar, and allow N balance estimates to be made. Further, these can be used for a wide range of situations, crop management systems and ecosystems. No significant differences were seen between the tillage systems in terms of the mass or number of nodules or shoot biomass production at the R1 or R6 growth stages, in the N content, BNF or grain yield. This agrees with that reported in similar studies that assign such differences to soil and/or weather conditions rather than to production practices. With average soybean yields in Argentina close to 2,900 kg ha^{-1}, the loss of soil N under no fertilization practices is close to 80 kg ha^{-1}. Since some 16 million ha are devoted to soybean (2600 figures), the overall loss of soil N may amount to one million tons per growth cycle. Similar losses are likely worldwide, at least in places were N fertilization is not common and the use of rhizobia inoculants is inefficient.

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