Plant Soil (2010) 331:413–425
DOI 10.1007/s11104-009-0262-0

REGULAR ARTICLE

Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil

Mariangela Hungria · Rubens J. Campo · Emanuel M. Souza · Fabio O. Pedrosa

Received: 2 September 2009 / Accepted: 10 December 2009 / Published online: 13 January 2010

Abstract Interest in the use of inoculants containing bacteria that promote plant growth is likely to increase in the coming years, due to higher costs of fertilizers, concerns over pollution and emphasis on sustainable agriculture. Although Brazil has a long tradition in research on nitrogen fixation in *Azospirillum*-grass associations, it has not led to recommendations of strains for use in commercial inoculants. In this study, we report the selection and evaluation of *Azospirillum* strains for the maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) crops, following protocols established by the Brazilian legislature, i.e. field experiments have to be performed in at least two different localities representing the crop growing regions, and for at least two seasons. In a first set of nine trials performed at Londrina and Ponta Grossa, southern Brazil, nine *Azospirillum* strains were evaluated after application to seeds as peat-based inoculants. *A. brasilense* strains Ab-V4, Ab-V5, Ab-V6 and Ab-V7 increased grain yields of maize by 662–823 kg ha^{-1}, or 24–30%, in relation to non-inoculated controls. Two *A. lipoferum* strains were tested in two of these experiments and promising results were also obtained. With wheat, *A. brasilense* strains Ab-V1, Ab-V5, Ab-V6 and Ab-V8 were the most effective, increasing yields by 312–423 kg ha^{-1}, or 13–18%. In a second trial set with eight field experiments at Londrina an Ponta Grossa, liquid and peat-based inoculants carrying a combination of *A. brasilense* strains Ab-V5 and Ab-V6 increased maize and wheat yields by 27% and 31%, respectively. Effects of inoculation were attributed to general increases in uptake of several macro and micro-nutrients and not specifically to biological nitrogen fixation. All experiments received only a low N-fertilizer starter at sowing (24 kg and 20 kg of N ha^{-1} for the maize and wheat, respectively) and although

Responsible Editor: Euan K. James.

M. Hungria (✉) · R. J. Campo
Embrapa Soja,
Cx. Postal 231,
86001-970 Londrina, Paraná, Brazil
e-mail: hungria@cnpso.embrapa.br
e-mail: hungria@pq.cnpq.br

R. J. Campo
e-mail: rjcampo@cnpso.embrapa.br

E. M. Souza · F. O. Pedrosa
Depto. of Biochemistry and Molecular Biology, UFPR,
Curitiba, Paraná, Brazil

E. M. Souza
e-mail: souzaem@ufpr.br

F. O. Pedrosa
e-mail: fpedrosa@ufpr.br

M. Hungria · E. M. Souza · F. O. Pedrosa
CNPq Conselho Nacional de Desenvolvimento
Científico e Tecnológico,
Brasília, Federal District, Brazil

yields can be globally considered low, they were compatible with Brazilian mean yields. This study resulted in the identification of the first *Azospirillum* strains authorized for the production of commercial inoculants in Brazil.

Keywords *Azospirillum brasilense* · *Azospirillum lipoferum* · Inoculation · Maize · Plant growth promoting bacteria · Wheat

Introduction

Plant-growth-promoting bacteria (PGPB) comprise a group of microorganisms that are beneficial to plants due to their capacity to colonize root surfaces, the rhizosphere and phyllosphere, and internal plant tissues (Davison 1988; Kloepper et al. 1989). PGPB can stimulate plant growth by means of several processes, including: biological nitrogen fixation (Huergo et al. 2008); by increasing nitrate reductase activity when growing as plant endophytes (Cassán et al. 2008); by the synthesis of hormones such as auxins, cytokinins (Tien et al. 1979), gibberelins (Bottini et al. 1989), ethylene (Strzelczyk et al. 1994), and a variety of other molecules (Perrig et al. 2007); by solubilizing phosphate (Rodriguez et al. 2004); and by exerting biological control of pathogens (Correa et al. 2008). Overall, it is believed that PGPB benefit plant growth by combinations of these mechanisms (Dobbelaere et al. 2003). A broad-range of genera of PGPB has been described, including *Pseudomonas*, *Burkholderia*, *Bacillus*, *Bradyrhizobium*, *Rhizobium*, *Gluconacetobacter*, *Herbaspirillum* and *Azozpirillum* (e.g. Weller and Tomashow 1994; Glick 1995; Probanza et al. 1996).

Azospirillum is a genus of free-living PGPB found almost everywhere on Earth (Döbereiner and Pedrosa 1987; Huergo et al. 2008). *Spirillum lipoferum* was first described by Beijerinck, and in 1978 its classification as *Azospirillum* was proposed, together with the description of two species, *A. lipoferum* and *A. brasilense* (Tarrand et al. 1978). Many reports have shown that *Azospirillum* may promote growth and yield of numerous plant species, many of which are of agronomic or ecological importance (e.g. Okon and Labandera-Gonzalez 1994; Bashan and Holguin 1997; Bashan et al. 2004). The benefits from PGPB probably result from a combination of factors. The bacteria are believed to produce various phytohormones that improve root growth and absorption of water and minerals and increase tolerance of stresses such as salinity and drought, leading to more-vigorous and more productive plants (e.g. Bashan and Holguin 1997; Dobbelaere et al. 2001; Bashan et al. 2004). In addition, *Azospirillum* fixes atmospheric nitrogen associatively with several non-leguminous species (e.g. Döbereiner and Day 1976; Döbereiner and Pedrosa, 1987), another mechanism by which these bacteria may promote plant growth.

Inoculants containing *Azospirillum* have been tested under field conditions with important crops in developing and developed countries, with various degrees of responses (e.g. Dobbelaere et al. 2001). In some of those trials *Azospirillum* was used in combination with other microorganisms, including other PGPB, arbuscular mycorrhizal (AM) fungi, rhizobia and microalgae (e.g. Gonzalez and Bashan 2000; Dardanelli et al. 2008). However, in the great majority of experiments, the inoculants have been prepared solely with *Azospirillum* in trials with cereals, producing a broad range of responses (e.g. Baldani and Döbereiner 1986; Sumner 1990; Okon and Labandera-Gonzalez 1994; Díaz-Zorita and Fernandez Canigia 2008).

A key factor to the success of inoculation with *Azospirillum* is the choice of bacterial strain(s). Even though no specificity between plant species and bacterial strains has been demonstrated, some affinity exists between bacteria and plant species (Penot et al. 1992) or even cultivars (Wani et al. 1985). Effects of plant genotype on the interaction with *Azospirillum* have been demonstrated, for example, for wheat (*Triticum aestiv*um L.) (Kapulnik et al. 1987; Caballero-Mellado et al. 1992), maize (*Zea mays* L.) (Garcia de Salamone et al. 1996), and pearl millet [*Pennisetum americanum* (L.) K. Shum.] (Bouton et al. 1985).

The evaluation and selection of strains for inoculation of specific crops is an important aspect that must be taken into account to deploy the technology of cereal inoculation with strains of *Azospirillum.* Brazil has a long tradition in research with *Azospirillum*, dating back to the pioneering work of Dr. Johanna Döbereiner (Döbereiner and Day 1976; Döbereiner et al. 1976); however, despite advances in basic and applied research, consistent results have not been obtained from field trials evaluating the agronomic efficacy of inoculants carrying *Azospirillum*, therefore no commercial inoculants are available. This study reports field experiments designed to evaluate the performance of selected strains of *Azospirillum*, which resulted in the authorization of the first strains for the production and use of commercial inoculants in Brazil with wheat and maize.

Material and methods

Sites description and field management

In total, seventeen field experiments were performed. The first set comprised nine experiments, five performed in three different cropping seasons with maize (*Zea mays* L.) and four performed in two different cropping seasons with wheat (*Triticum aestivum* L.); these experiments evaluated inoculants containing single strains of either *A. brasilense* or *A. lipoferum* in peat formulation. The second set of experiments comprised eight experiments, four performed with maize and four with wheat; these experiments evaluated a combination of two strains of *A. brasilense* in peat or liquid formulation.

The trials were located in two different localities in the State of Paraná, southern Brazil, in the districts of Londrina and Ponta Grossa. At Londrina, the experimental station of Embrapa Soja (23°11′S, 51°11′W) is located at an altitude of 620 m and the trials were performed on an oxisol (Latossolo Vermelho Eutroférrico, Brazilian classification; Rhodic Eutrudox, USA classification) with a high content of clay (Table 1). The climate is classified as subtropical (Cfa, according to Köppen´s classification), with mean maximum and minimum temperatures of 28.5°C in February and 13.3°C in July, respectively, with a mean annual precipitation of 1,651 mm yr^{-1}, the rainiest month being January (217 mm) and the driest August (60 mm). At Ponta Grossa, the trials were performed at the Service of Production of Basic Seeds of Embrapa (25° 13′S, 50°1′W), on an oxisol (Latossolo Vermelho Distrófico; Typic Haplustox) with a high content of sand (Table 1). The area is located at an altitude of 880 m, with mean maximum and minimum temperatures of 25.9°C in February and 8.4°C in July, respectively, and mean annual precipitation of 1,507 mm yr^{-1}, the rainiest month being January (184 mm) and the driest August (77 mm), and, according to Köppen's classification, the climate is type Cfb.

At the beginning of the experiments, soil samples were randomly taken from 20 spatially distributed points at each site, from the 0–20-cm layer. Every year, after harvesting the experiments, soil samples (0–20 cm) were taken from each treatment, with six subsamples taken from each replicate. Soil samples were dried (60°C for 48 h), pulverized (2.00-mm sieve), and chemical and physical characteristics were determined after Pavan et al. (1992). Chemical parameters evaluated were: soil pH (in 0.01 M $CaCl_2$; 1:2.5, soil:solution), exchangeable Ca, Mg and Al, P, K, Al and C contents (Pavan et al. 1992). The main chemical and physical characteristics of the soils at the time of establishment of the experiments are shown in Table 1. In addition, after harvesting, the contents of nitrate-N and ammonium-N were also evaluated, as described by Alves et al. (1994).

Yearly, 50 days before starting each experiment, soil pH values were determined and lime (dolomitic calcareous) was applied to alleviate acidity. The amount of lime to be applied was estimated for a saturation of bases of 50% to increase the pH to approximately 5.5. Basic fertilization was applied yearly at sowing and consisted of 24 kg ha^{-1} of N (supplied as urea), 84 kg ha^{-1} of P (supplied as super triple phosphate) and 24 kg ha^{-1} of K (supplied as potassium chloride) for the maize and of 20 kg ha^{-1} of N, 70 kg ha^{-1} of P and 40 kg ha^{-1} of K for the wheat. This basic fertilization was applied to all treatments.

Table 1 Chemical and physical properties of the soils (0–20 cm) in the first year of the establishment of the experiments

Site	Chemical												Physical		
	pH $CaCl_2$	Al $cmol_c$	H+Al dm^{-3}	Ca	Mg	K	CEC[a]	T_{CEC}[a]	BS[a] %	N	C g dm^{-3}	P	Clay g kg^{-1}	Silt	Sand
Londrina	5.00	0.00	5.07	4.72	1.17	0.71	11.67	6.60	56.56	0.16	16.00	34.90	710	82	208
Ponta Grossa	5.12	0.00	4.81	3.45	1.67	0.33	10.34	5.45	52.71	0.19	27.60	14.90	238	30	732

[a] Cation Exchange Capacity (H+Al+Ca+Mg+K); T_{CEC} (Ca+Mg+K); Base Saturation (T_{cec}/CEC) × 100

The experimental plots measured 4.0 m (length)× 5.0 m (width) and were separated by rows of 1.0 m and small terraces of 1.6 m to prevent contamination by surface run-off containing bacteria or fertilizer. The treatments consisted of inoculation with *Azospirillum* strains and non-inoculated controls without or with N-fertilizer (in addition to N applied at sowing, 80 kg of N ha^{-1} for the maize and 50 kg of N ha^{-1} for the wheat, spread at flowering time). The experiments had a completely randomized block design with six blocks as replicates (Cochran and Cox 1957).

The maize was sown as the summer crop (October to March at Londrina, November to April at Ponta Grossa), and the wheat was sown as the winter crop. Herbicides were used equally for all treatments, and insects were controlled with biological and chemical insecticides, according to technical recommendations for each crop. None of the experiments was irrigated and, therefore, growth was conditioned by rainfall.

The maize experiments at Londrina were performed with Hybrid 9,486 in the first year and EMBRAPA-HD-28X in the subsequent years, whereas at Ponta Grossa variety BR 201 was used in the first year and EMBRAPA-HD-28X thereafter. Spacing was of 0.80 m between rows, with four plants per linear meter and a population of 50,000 plants ha^{-1}. Wheat varieties used were BR-18 and Embrapa-16 at Londrina and Ponta Grossa, respectively. Spacing was 0.20 m between rows, with 65 plants per linear meter and a population of 325,000 plants ha^{-1}. The main focus of the study was on the *Azospirillum* strains aiming at their use as inoculants therefore we have not emphasized the use of different plant genotypes. We have chosen the genotypes technically recommended for each region and with higher acceptance by the farmers in each crop season.

The experiments were always performed on the same sites, but in different areas for the maize and wheat. Inoculated maize experiments in the summer were followed by regular non-inoculated wheat in the winter, while inoculated wheat experiments were followed by regular non-inoculated maize in the summer.

Azospirillum strains and inoculation procedure

The choice of strains resulted from a selection program of several promising strains evaluated for physiological and biochemical properties in vitro, by the group of Dr. Fabio O. Pedrosa, and more details are given in the discussion section. The strains have been selected in the State of Paraná. Nine strains were identified, seven of *A. brasilense* (Ab-V1, Ab-V2, Ab-V4, Ab-V5, Ab-V6, Ab-V7 and Ab-V8) isolated from maize plants and effective with both maize and wheat and two of *A. lipoferum* (Al-V1 and Al-V2), isolated from maize and effective only with maize. Interestingly, all selected strains have shown better performance in promoting plant growth than the well studied strains Sp7 and 245 of *A. brasilense*. The strains are deposited at the UFPR—Dept. of Biochemistry and Molecular Biology, Curitiba, Paraná, and also at the "Culture Collection of Diazotrophic and Plant Growth Promoting Bacteria" of Embrapa Soja, Londrina, Paraná.

For the first set of experiments (nine field trials) the inoculants were prepared in sterile peat containing additives (protected by industrial agreement) carrying single strains of *A. brasilense* and *A. lipoferum.* In the second set of experiments (eight field trials) the inoculants were prepared with a combination of two *A. brasilense* strains (Ab-V5 + Ab-V6) prepared in sterile peat containing additives or in liquid formulation (also protected by industrial agreements). Inoculants were prepared at a concentration of 2.10^8 cells of *Azospirillum* g^{-1} of peat or $3.10^8 mL^{-1}$ of liquid inoculant. The peat inoculant was applied at a rate of 250 g of inoculant 50 kg^{-1} of seeds, and a 10% sucrose (w/v) solution was used to increase adhesion of the peat, at a rate of 300 mL 50 kg^{-1} seeds. Seed inoculation consisted of applying the sucrose solution to the seeds followed by the peat inoculant and, after mixing, seeds were allowed to air-dry in the shade for 15 min. The theoretical estimates of cells per seed for the peat inoculants were of 300,000 cells $seed^{-1}$ for the maize and of 30,000 cells $seed^{-1}$ for the wheat. For the liquid inoculant, rates applied were of 150 mL 50 kg^{-1} for maize and of 200 mL 50 kg^{-1} for wheat. The theoretical estimates of cells per seed for the liquid inoculants were of 270,000 cells $seed^{-1}$ for the maize and of 36,000 cells $seed^{-1}$ for the wheat.

Plant sampling and harvesting

At flowering, ten plants were randomly selected per replicate (avoiding areas established for harvesting grains), and evaluated for plant growth. At the laboratory, shoots were carefully washed and placed in a forced-air dryer at 65°C until constant weight was obtained (approximately 72 h). Shoot dry weights were recorded, followed by determinations of nutrient concentrations in the shoots. Shoot total N was evaluated by Kjeldahl digestion and N concentration determined using a Tecator-Automatic N Analyzer (Sweden). All other macro and micronutrients were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Perkin Elmer model Optima 3300DZ).

As we have already mentioned, experimental plots measured 4.0 m (length)×5.0 m (width) and yields were evaluated at the final harvest based on an area of 3.0×2.0 m (6.0 m^2) of the central rows of each plot. Seeds were cleaned and weighed and values corrected to 13% moisture content, after determination of the humidity level in a grain-moisture tester (Vurroughf 700). Total-N content of the seeds was also evaluated by Kjeldahl digestion and the Tecator analyzer and the other macro and micronutrients by ICP-AES.

Statistical analyses

The data were analyzed using the SAS for PC statistical package (SAS Institute 2001). All assumptions required for the analysis of variance (ANOVA) were verified. The error normality, according to the experimental model design, was evaluated by Shapiro and Wilk (1965), the variance of homogeneity by Burr and Foster (1972), and the non-additivity of the model by Tukey (1949). Coefficients of skewness and kurtosis were also checked. Data from all experiments were first submitted to the tests of normality of the variables and of homogeneity of variances, and then to the ANOVA; when significant differences were detected by the ANOVA, means were compared by the Duncan test ($p \leq 0.05$) (SAS Institute 2001).

Results

The selected *A. brasilense* and *A. lipoferum* strains were first evaluated in nine field experiments performed with maize and wheat and in all experiments macro and micronutrient contents were analyzed in leaves at flowering and in grains at final harvest.

Effects of inoculation on grain yield

Maize grain yields in the experiments receiving single-strain inoculants are shown in Table 2. Inoculation with *A. brasilense* strains Ab-V4, Ab-V5, Ab-V6 and Ab-V7 proved to improve yields in at least two experiments performed in two different localities. Besides that, all *A. brasilense* strains resulted in significant yield increases in at least two field experiments; overall increases in grain production ranged from 443–823 kg ha^{-1}, or 16–30% in comparison to non-inoculated controls (Table 2). It is noteworthy that two strains of *A. lipoferum* were also effective with maize, and trials with these two strains will continue with the objective of completing the requirements of the Brazilian legislature.

For the wheat, inoculants containing strains Ab-V1, Ab-V5, Ab-V6 and Ab-V8 resulted in increased yields at both sites in both cropping seasons (Table 3). When compared to the control without inoculation, yield increases due to inoculation with all *Azospirillum* strains ranged from 209–423 kg ha^{-1}, or 9–18%.

Considering the average of all strains in all experiments performed with both maize and wheat, it is also noteworthy that yields were statistically superior to those of the non-inoculated controls (Fig. 1).

Effects of inoculation on nutrient contents in leaves and grains

For the maize, nutrient content in leaves were similar for all treatments. Furthermore, similar contents were obtained at both Londrina and Ponta Grossa in all five experiments inoculated with single strains. Therefore, we have chosen to show the results obtained in the experiment performed in the third year at Londrina (Table 4). Some strains increased uptake of more than one macro and micronutrient, resulting in increased contents in the leaves and grains, e.g. the treatments inoculated with *A. brasilense* strains Ab-V5, Ab-V6 and Ab-V7 and with *A. lipoferum* strain Al-V1 (Table 4). In plants inoculated with those strains, increased nutrient contents in the leaves were observed in at least one of those treatments not only for N, but also for K and B, and increases, although not statistically different were observed for P, Zn and Cu. In addition, although no effects on the N content of the grains were observed as a result of inoculation, the content(s) of one or more of the following nutrients was increased: P, K, Mg, S, Zn, Mn and Cu (Table 4).

Also in relation to nutrient content of the tissues, similar results were obtained in the four experiments inoculated with single strains and cropped to wheat,

Table 2 Effects of inoculation (Peat inoculants prepared at a concentration of 2.10^8 cells g^{-1} and applied at a rate of 250 g of inoculant 50 kg^{-1} of seeds, with a 10% sucrose (w/v) solution to increase adhesion of the peat, at a rate of 300 mL 50 kg^{-1} seeds) with *Azospirillum* on the grain yield of maize (In Londrina, varieties used were Hybrid 9,486 in the first year and EMBRAPA-HD-28X in the 2nd and 3rd years; in Ponta Grossa, BR 201 in the 1st year and EMBRAPA-HD-28 in the 2nd year) (kg ha^{-1}) in field experiments performed in Londrina and Ponta Grossa for three and two cropping seasons, respectively

Treatment	Londrina			Ponta Grossa		Mean[a]	Increase	
	1st	2nd	3rd	1st	2nd		kg ha^{-1}	%
C[b]	3,186 d[c]	2,142 c[c]	2,980 e[c]	2,423 d[c]	2999[c] c	2,746 c[d]		
C+N[b]	4,390 a	2,677 ab	4,540 a	3,322 a	4,102 a	3,806 a	1,060	38.6
Ab-V1	3,665 c	2,658 ab	3,664 cd	2,998 abc	3,221 bc	3,241 b	495	18.0
Ab-V2	3,915 bc	2,399 bc	3,420 de	2,990 abc	3,223 bc	3,189 b	443	16.1
Ab-V4	4,482 a	2,356 bc	4,120 abc	2,890 c	3,664 ab	3,502 ab	756	27.5
Ab-V5	3,543 cd	2,975 ab	4,220 abc	3,108 abc	3,998 a	3,569 ab	823	30.0
Ab-V6	3,713 c	2,423 bc	4,289 ab	3,226 ab	4,002 a	3,531 ab	785	28.6
Ab-V7	3,700 c	2,350 bc	4,106 abc	3,119 abc	3,763 ab	3,408 ab	662	24.1
Al-V2	4,460 a		3,810 a–d					
Al-V1	4,244 ab		3,980 a–d					

[a] Mean considering the five experiments performed in Londrina and Ponta Grossa, each with six replicates. Treatments not included in all experiments were not considered in the statistical analysis

[b] Non-inoculated controls: (C) receiving only N-fertilizer at sowing (24 kg of N ha^{-1}); (C+N) N at sowing + 80 kg of N ha^{-1} at flowering

[c] Data represent the means of six replicates and when followed by the same letter, within each column are not statistically different (Duncan, $p \leq 0.05$)

[d] Means of the five experiments, each with six replicates, and when followed by the same letter are not statistically different (Duncan, $p \leq 0.05$)

and Table 5 shows the results obtained in the second year at both sites. Except for a decrease in the B content, no effects were observed at Londrina as a function of inoculation, however at Ponta Grossa good performances of *A. brasilense* strains Ab-V5 and/or Ab-V6 were observed, resulting in higher concentration of several macro and micronutrients in the leaves (Table 5). Similar to the observations with maize (Table 4), the N contents of the wheat grains were not statistically different from those of the non-inoculated control for any of the strains evaluated, in any of the four field trials (data not shown).

Effects of inoculation on soil properties

No differences in nutrient content of the soils were observed in the first two years of experimentation (data not shown) however, in most of the experiments, slight differences were observed in the soil Ca and Mg contents at the end of each experiment. Table 6 shows the results obtained in one of those experiments, i.e. the third trial with maize at Londrina, where slight decreases in Ca and Mg contents were observed in the treatments inoculated with *A. brasilense* strains Ab-V5, Ab-V6 and Ab-V7, the same strains that had been more effective in the uptake of nutrients (Table 4). It remains to be determined if the lower contents of Ca and Mg in the soil were related to higher plant uptake of these nutrients, or to release of rhizospheric compounds that alter their solubility. However, the higher-uptake hypothesis is strengthened by the finding of lower soil-N (N-nitrate and N-ammonium) content in treatments with the same strains (Fig. 2). Furthermore, experiments performed in controlled environments and axenic conditions confirmed that the selected *Azospirillum* strains—with an emphasis on those strains that were more effective under field conditions—were capable of promoting root growth (data not shown), which could increase uptake of

Table 3 Effects of inoculation (Peat inoculants prepared at a concentration of 2.10^{8} cells g^{-1} and applied at a rate of 250 g of inoculant 50 kg^{-1} of seeds, with a 10% sucrose (w/v) solution to increase adhesion of the peat, at a rate of 300 mL 50 kg^{-1} seeds) with *Azospirillum* on the grain yield of wheat (Varieties used in Londrina and Ponta Grossa were BR-18 Embrapa-16, respectively) (kg ha^{-1}) in field experiments performed in Londrina and Ponta Grossa for three and two consecutive years, respectively

	Londrina		Ponta Grossa		Mean[a]	Increase	
	1st	2nd	1st	2nd		kg ha^{-1}	%
C[b]	2,628 b[c]	2,366 c[c]	2,321 c[c]	2,014 c[c]	2,332 c[d]		
C + N[b]	3,193 a	3,038 a	2,921 a	2,524 a	2,919 a	587	25.2
Ab-V1	3,095 a	2,712 b	2,762 ab	2,201 b	2,693 ab	361	15.5
Ab-V3	3,019 a	2,880 ab	2,322 c	2,101 bc	2,581 bc	249	10.7
Ab-V4	2,929 ab	2,680 b	2,441 bc	2,115 bc	2,541 bc	209	9.0
Ab-V5	3,040 a	2,675 b	2,883 a	2,225 b	2,706 ab	374	16.0
Ab-V6	3,222 a	2,775 b	2,771 a	2,252 b	2,755 ab	423	18.1
Ab-V8	3,014 a	2,661 b	2,662 ab	2,238 b	2,644 ab	312	13.4

[a] Mean considering the four experiments performed in Londrina and Ponta Grossa, each with six replicates

[b] Non-inoculated controls: (C) receiving only N-fertilizer at sowing (20 kg of N ha^{-1}); (C+N) N at sowing + 50 kg of N ha^{-1} at flowering

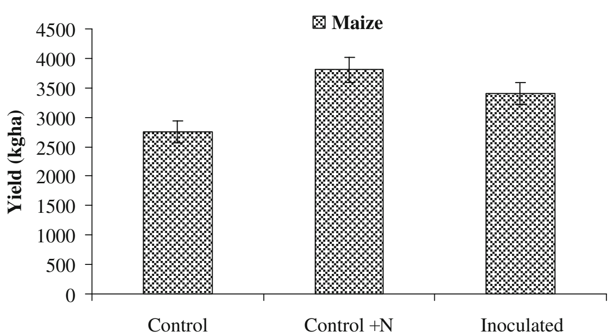
[c] Data represent the means of six replicates and when followed by the same letter, within each column are not statistically different (Duncan, $p \leq 0.05$)

[d] Means of the four experiments, each with six replicates, and when followed by the same letter are not statistically different (Duncan, $p \leq 0.05$)

nutrients, as confirmed in the tissue analyses for both crops (Tables 4 and 5).

Comparison between peat and liquid inoculants

Strains Ab-V5 and Ab-V6 performed well with both maize and wheat, and as farmers have shown preference for liquid inoculants we developed a liquid formulation carrying both strains that was tested in eight field experiments with maize and wheat. The liquid formulation proved to be as effective as the peat formulation, and significantly increased yields in relation to non-inoculated controls (Table 7). On average, treatments inoculated with *A. brasilense* strains Ab-V5 and Ab-V6 increased maize yield by 842 kg ha^{-1} or 27%, while the wheat yield was increased by an average of 638 kg ha^{-1} or 31% (Table 7).



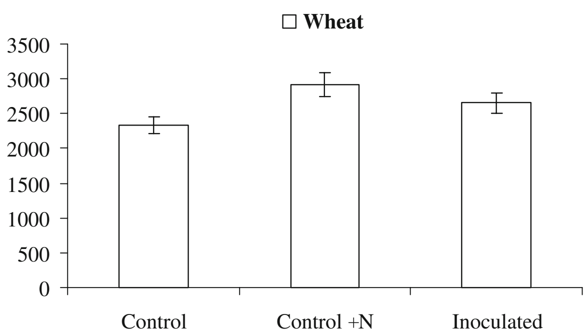


Fig. 1 Effects of inoculation with *Azospirillum* on the grain yield of maize and wheat in field experiments performed in Londrina and Ponta Grossa. Data represent the means and SD values of five (*maize*) and four (*wheat*) field experiments, and the inoculated treatment is composed by the mean of the six strains tested for each crop. More details are given in the footnotes of Tables 2 and 3

Table 4 Effects of inoculation (Peat inoculants prepared at a concentration of 2.10^8 cells g^{-1} and applied at a rate of 250 g of peat inoculant 50 kg^{-1} of seeds, with a 10% sucrose (w/v) solution to increase adhesion of the peat, at a rate of 300 mL 50 kg^{-1} seeds) with *Azospirillum* strains on the nutrient content (g kg^{-1} for macronutrients and μg g^{-1} for micronutrients) of leaves at the flowering stage and grains at the harvest of maize variety EMBRAPA-HD-28X. Experiment performed in Londrina—3rd year

Treatment	N	P	K	Ca	Mg	S	Zn	Mn	Fe	Cu	B
Leaves											
C[a]	9.75 bc[b]	1.10 ab	18.27 b	1.80 a	1.38 a	0.72 a	17.35 ab	49.27 a	356.00 cd	6.17 ab	3.94 bc
C+N[a]	9.72 bc	1.20 ab	19.32 b	2.00 a	1.72 a	0.76 a	17.47 ab	44.64 a	351.58 cd	6.28 ab	4.53 ab
Ab-V1	9.31 c	1.21 ab	19.17 b	1.66 a	1.51 a	0.76 a	18.10 a	49.23 a	345.52 cd	6.68 ab	4.12 abc
Ab-V2	9.55 bc	1.14 ab	18.46 b	1.96 a	1.44 a	0.70 a	18.11 a	50.71 a	343.27 cd	6.07 b	3.81 c
Ab-V4	10.11 bc	1.10 ab	19.32 b	1.76 a	1.40 a	0.71 a	17.47 ab	46.63 a	277.23 d	6.12 b	3.82 c
Ab-V5	11.06 ab	1.25 a	19.65 ab	1.99 a	1.65 a	0.82 a	19.35 a	45.24 a	323.31 cd	6.49 ab	4.53 ab
Ab-V6	11.04 ab	1.40 a	19.66 ab	1.86 a	1.60 a	0.84 a	21.13 a	45.51 a	290.78 d	6.68 ab	4.73 a
Ab-V7	11.86 a	1.29 a	17.92 b	1.96 a	1.56 a	0.81 a	20.22 a	50.23 a	450.45 ab	6.85 a	3.74 c
Al-V1	10.25 abc	1.31 a	22.01 a	2.02 a	1.54 a	0.77 a	19.35 a	52.26 a	383.96 bc	6.55 ab	4.72 a
Al-V2	8.55 c	0.94 b	19.56 b	1.73 a	1.24 a	0.67 a	14.57 b	47.01 a	489.66 a	6.14 b	3.53 c
Grains											
C[a]	13.08 a	2.89 cd	3.13 de	0.09 a	1.14 cd	0.96 c	30.77 c	4.81 bcd	21.47 a	2.16 b	4.62 a
C+N[a]	13.55 a	2.74 d	2.98 e	0.06 a	1.10 d	0.96 c	30.93 c	4.41 d	20.42 ab	2.18 b	3.68 ab
Ab-V1	13.45 a	3.37 ab	3.52 ab	0.06 a	1.27 ab	1.05 a	37.02 a	5.44 ab	22.71 a	2.54 a	3.73 ab
Ab-V2	13.55 a	2.90 cd	3.10 de	0.06 a	1.12 d	0.98 bc	31.56 c	4.60 cd	17.67 b	2.18 b	3.00 ab
Ab-V4	12.98 a	3.07 bc	3.25 cd	0.07 a	1.19 bcd	1.01 abc	32.30 bc	4.94 a–d	20.21 ab	2.44 ab	3.84 ab
Ab-V5	13.32 a	3.20 ab	3.33 a–d	0.09 a	1.26 ab	1.02 ab	35.47 a	5.18 abc	22.18 a	2.52 a	2.92 ab
Ab-V6	13.22 a	3.43 a	3.56 a	0.09 a	1.30 a	1.05 a	37.08 a	5.54 a	22.88 a	2.56 a	3.23 ab
Ab-V7	13.87 a	3.20 ab	3.27 cd	0.06 a	1.23 abc	1.05 a	35.55 a	5.05 a–d	21.18 a	2.56 a	2.37 c
Al-V1	13.32 a	3.28 ab	3.30 bcd	0.07 a	1.24 abc	1.02 ab	35.69 a	5.35 ab	23.16 a	2.64 a	2.42 c
Al-V2	13.23 a	3.31 ab	3.47 abc	0.07 a	1.27 ab	1.04 a	34.66 ab	5.25 abc	23.03 a	2.49 a	2.75 ab

[a] Non-inoculated controls: (C) receiving only N-fertilizer at sowing (24 kg of N ha^{-1}); (C+N) N at sowing + 80 kg of N ha^{-1} at flowering

[b] Data represent the means of six replicates and when followed by the same letter, within each column (for leaves or grains) are not statistically different (Duncan, $p \leq 0.05$)

Discussion

The *A. brasilense* and *A. lipoferum* strains used in this study have been selected in preliminary evaluations of N_2-fixing capacity *in vitro* (data not shown), based on effects on two properties: capacity of plant growth and detection of high rates of acetylene reduction in N-free semi-solid medium (NFb medium, Döbereiner et al. 1976). Strains with an outstanding performance on both parameters were selected. In this study, the strains were first evaluated in nine field experiments performed with maize and wheat in two different localities of the State of Paraná, southern Brazil.

To determine if positive effects of inoculation could be attributed to N_2 fixation, or if they could be attributed to other plant-growth-promoting effects, implying improved uptake of several nutrients, macro and micronutrient contents were analyzed in leaves and grains. For maize, some *A. brasilense* strains, particularly Ab-V5, Ab-V6 and Ab-V7 and *A. lipoferum* strain Al-V1 increased the contents of some nutrients in the leaves and grains, as P, K and Cu; increased N contents due to inoculation were observed in the leaves but not in the grains. In general slight increases in nutrient uptake by wheat were also observed, following the same tendencies observed with the maize, and with a good performance of *A. brasilense* strains Ab-V5 and Ab-V6. A strong indicative of higher uptake of some nutrients by the plants is that we have detected decreased contents of

Table 5 Effects of inoculation (Peat inoculants prepared at a concentration of 2.10^8 cells g^{-1} and applied at a rate of 250 g of inoculant 50 kg^{-1} of seeds, with a 10% sucrose (w/v) solution to increase adhesion of the peat, at a rate of 300 mL 50 kg^{-1} seeds) with *Azospirillum* strains on the nutrient content (g kg^{-1} for macronutrients and μg g^{-1} for micronutrients) of leaves of wheat at flowering stage. Experiments performed in Londrina and Ponta Grossa—2nd year

Treatment	N	P	K	Ca	Mg	S	Zn	Mn	Fe	Cu	B
Londrina—variety BR-18											
C[a]	29.98 a[b]	2.95 a	41.50 a	2.27 a	2.11 a	2.02 a	21.45 a	114.57 a	733.6 a	8.89 a	3.28 a
C+N[a]	29.22 a	3.08 a	40.19 a	2.29 a	2.17 a	2.04 a	21.85 a	119.95 a	1107.39 a	8.86 a	2.30 ab
Ab-V1	29.67 a	2.69 a	36.30 a	1.95 a	2.02 a	1.88 a	19.39 a	101.47 a	696.82 a	7.57 a	1.14 b
Ab-V3	29.57 a	2.92 a	42.10 a	2.19 a	2.14 a	2.01 a	21.00 a	118.01 a	769.28 a	8.56 a	2.16 ab
Ab-V4	32.55 a	2.74 a	36.33 a	1.94 a	1.97 a	1.88 a	18.95 a	108.04 a	550.04 a	7.87 a	1.29 b
Ab-V5	31.83 a	2.84 a	34.50 a	2.07 a	2.19 a	1.98 a	19.21 a	98.72 a	574.51 a	7.94 a	0.97 b
Ab-V6	31.93 a	3.07 a	39.41 a	2.23 a	2.34 a	2.05 a	21.20 a	107.83 a	613.55 a	8.46 a	2.21 ab
Ab-V8	30.03 a	2.96 a	39.54 a	2.08 a	2.22 a	1.93 a	20.04 a	95.54 a	680.59 a	8.08 a	1.08 b
Ponta Grossa—variety Embrapa 16											
C[a]	20.1 b	2.39 a	31.20 b	2.52 a	1.31 ab	1.68 b	38.14 b	96.40 bc	213.17 a	7.16 b	4.95 a
C+N[a]	17.3 c	2.31 a	28.75 c	2.52 a	1.26 abc	1.65 b	32.75 c	100.31 abc	308.88 a	6.81 bc	2.11 cd
Ab-V1	20.1 b	2.08 b	27.55 c	2.22 b	1.16 c	1.60 b	33.49 c	95.60 bc	192.99 a	6.49 c	1.68 d
Ab-V3	19.9 b	2.12 b	29.24 c	2.38 ab	1.20 c	1.68 b	34.48 c	94.47 c	237.38 a	6.72[a] bc	3.74 ab
Ab-V4	20.4 b	2.10 b	27.89 c	2.36 ab	1.22 bc	1.69 b	32.58 c	93.80 c	230.92 a	6.46 c	2.33 cd
Ab-V5	25.2 a	2.42 a	34.15 a	2.55 a	1.33 a	2.18 a	43.46 a	108.44 a	229.51 a	8.03 a	2.71 bcd
Ab-V6	25.1 a	2.25 ab	28.75 c	2.47 ab	1.24 abc	1.64 b	32.12 c	105.43 ab	234.22 a	6.63 bc	3.33 bcd
Ab-V8	19.2 bc	2.12 b	28.05 c	2.33 ab	1.17 c	1.69 b	33.28 c	92.47 c	190.92 a	6.59 bc	1.79 d

[a] Non-inoculated controls: (C) receiving only N-fertilizer at sowing (20 kg of N ha^{-1}); (C+N) N at sowing + 50 kg of N ha^{-1} at flowering

[b] Data represent the means of six replicates and when followed by the same letter, within each column are not statistically different (Duncan, $p \leq 0.05$)

some soil nutrients, as Ca, Mg and N, in the treatments inoculated with the strains showing higher performance. In addition, experiments performed under axenic conditions confirmed increased root growth of inoculated plants. Therefore, although precise techniques involving ^{15}N have not been employed in this study, indications were that the positive effects of inoculation were related to factors other than N_2 fixation, more likely plant-growth-promotion effects. Root growth promotion by the inoculation with *Azospirillum* has been reported in a variety of species, e.g. in tomato (*Lycopersicum esculentum* L.) (Hadas and Okon 1987), and in another experiment performed under axenic conditions with pearl millet Tien et al. (1979) verified that the compounds responsible for root growth promotion released by *A. brasilense* were indole acetic acid (IAA), giberelins and citoquinins. A recent review highlights the mechanisms and importance of the production of phytohormones by *Azospirillum*, emphasizing that about 80% of the strains isolated from the rhizosphere are capable of producing compounds such as IAA (Cassán et al. 2008).

According to the Brazilian legislature, effectiveness of a strain improving grain yields must be proven in at least two experiments performed in two different localities representative of the crop growing regions (Hungria and Campo 2007), and some strains from this study met these requirements: *A. brasilense* strains Ab-V4, Ab-V5, Ab-V6 and Ab-V7 for the maize and *A. brasilense* strains Ab-V1, Ab-V5, Ab-V6 and Ab-V8 for the wheat. Grain yield increases seemed to result from a general improvement in the uptake of several nutrients, and indeed, increases in yield due to inoculation with *Azospirillum* that correlate with increases not only of N but also of other nutrients as P and K have been broadly reported in a variety of species (e.g. Bashan and Holguin 1997;

Table 6 Chemical properties of the soil (0–20 cm) after the third experiment cropped to maize in Londrina

Tratamento	pH	Al	H+Al	Ca	Mg	K	CEC[a]	T_{CEC}[a]
C[b]	4.88 a[c]	0.04 a	0.57 a	4.22 ab	1.47 ab	13.00 a	6.26 a	11.43 a
C+N[b]	4.98 a	0.03 a	0.50 a	4.38 ab	1.51 ab	12.33 a	6.39 a	11.21 a
Ab-V1	4.83 a	0.06 a	0.48 a	4.06 abc	1.40 abc	12.17 a	5.94 ab	11.21 a
Ab-V2	4.87 a	0.04 a	0.45 a	4.20 ab	1.43 ab	12.33 a	6.08 ab	11.17 a
Ab-V4	4.88 a	0.04 a	0.50 a	4.45 a	1.47 ab	12.50 a	6.43 a	11.53 a
Ab-V5	4.89 a	0.06 a	0.49 a	3.50 c	1.23 c	12.50 a	5.22 b	10.26 a
Ab-V6	4.92 a	0.03 a	0.48 a	3.82 bc	1.31 abc	12.60 a	5.84 a b	11.55 a
Ab-V7	4.85 a	0.06 a	0.49 a	3.96 abc	1.33 abc	12.67 a	5.62 ab	10.88 a
Al-V1	4.85 a	0.07 a	0.55 a	4.48 a	1.56 a	12.67 a	6.51 a	10.97 a
Al-V2	4.89 a	0.05 a	0.53 a	4.42 ab	1.46 ab	12.33 a	6.41 a	11.46 a

[a] Cation Exchange Capacity (H+Al+Ca+Mg+K); T_{CEC} (Ca+Mg+K)

[b] Non-inoculated controls, receiving only N-fertilizer at sowing (24 kg of N ha^{-1}), or supplemented with 80 kg of N ha^{-1} at flowering

[c] Data represent the means of six replicates and when followed by the same letter, within each column are not statistically different (Duncan, $p \leq 0.05$)

Steenhoudt and Vanderleyden 2000; Bashan et al. 2004). It should be emphasized that all experiments received only a low N-fertilizer starter at sowing (24 and 20 kg of N ha^{-1} for the maize and wheat, respectively), and although yields can be globally considered low, they were compatible with Brazilian mean yields. In 2007/2008 mean yields in Brazil for the maize and wheat crops were of 3,972 and 1,852 kg ha^{-1}, respectively (CONAB 2009), while considering the strain selection experiments in our study (Tables 2 and 3) the means were of 3,953 and 2,646 kg ha^{-1}, respectively. Furthermore, despite in some experiments the N-fertilization treatment led to higher grain yields than inoculation with *Azospirillum*, this is an expected result and it does not reduce from the potential of using *Azospirillum* in sustainable agriculture. In addition, further experiments applying higher doses of N-fertilizer in addition to *Azospirillum* are now in course and promising results have been obtained, resulting in yields comparable to those of the treatment receiving high doses of N-fertilizer.

A wide range of responses of cereals to inoculation with *Azospirillum* are reported in the literature. In a survey performed by Sumner (1990), 32 experiments were considered to respond positively to inoculation, but there were some negative responses also, mostly

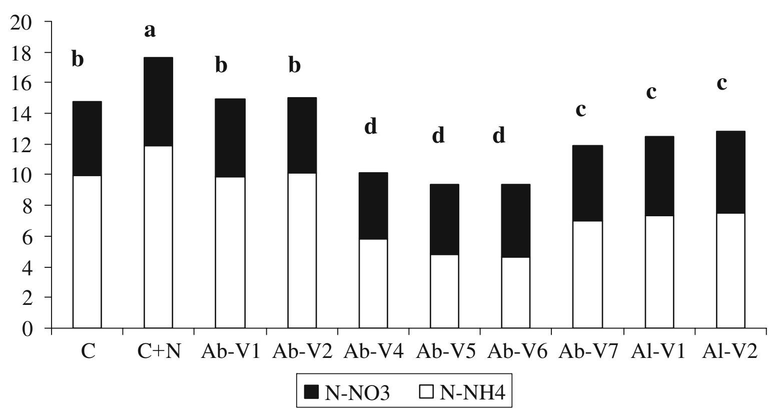


Fig. 2 Ammonium and nitrate contents (mg kg^{-1}) on the soil (0–20 cm) of the plots under control treatments or treatments receiving inoculants containing *Azospirillum*, after the harvest of maize. Data represent the mean of six replicates, and C and C + N represent non-inoculated controls, receiving only N-fertilizer at sowing (24 kg of N ha^{-1}), or supplemented with 80 kg of N ha^{-1} at flowering. Experiment performed in Londrina—3 rd year

in wheat. In a survey of 20 years of experiments, Okon and Labandera-Gonzalez (1994) reported that 60–70% of the experiments showed yield increases due to inoculation, with statistically significant increases in yield from 5–30%. In Argentina, in a survey of 273 cases of inoculation of wheat with *A. brasilense*, 76% resulted in a mean yield increase of 256 kg ha^{-1}; with maize, 85% of the cases were successful, resulting in a mean yield increase of 472 kg ha^{-1} (Díaz-Zorita and Fernandez Canigia 2008). Results from other experiments performed in Argentina and Brazil over three decades were recently compiled, and most showed benefits to plant growth and/or on grain yields from *Azospirillum* inoculation (Cassán and Garcia de Salamone 2008).

In our study, although pioneering results had already demonstrated that inoculation of cereals with *Azospirillum* is likely to result in important contributions to agriculture (Döbereiner and Day 1976), field trials in line with official criteria established by the Brazilian government to authorize the use of strains as commercial inoculants had not been done. We have followed the protocols dictated by the Brazilian legislature—requiring statistically significant differences from the non-inoculated control in field experiments performed in at least two different ecosystems for at least two crop seasons (Hungria and Campo 2007)—and as some *A. brasilense* strains were recognized as very effective in promoting plant growth, they have been authorized for the production of commercial inoculants.

The results obtained in this study resulted in the recommendation of strains Ab-V4, Ab-V5, Ab-V6 and Ab-V7 for maize, which resulted in increases of 662–823 kg ha^{-1}, or 24–30% in relation to non-inoculated controls. For the wheat, strains Ab-V1, Ab-V5, Ab-V6 and Ab-V8 were the most effective, increasing yields by 312–423 kg ha^{-1}, or 13–18%. A liquid inoculant containing a combination of Ab-V5 and Ab-V6 strains proved to be as effective as peat inoculant carrying the

Table 7 Effects of inoculation (Peat inoculant prepared at a concentration of 2.10^8 cells g^{-1} and liquid inoculant at the concentration of 3.10^8 cells g^{-1}. Peat applied at a rate of 250 g 50 kg^{-1} of seeds, with a 10% sucrose (w/v) solution to increase adhesion of the peat, at a rate of 300 mL 50 kg^{-1} seeds. Liquid inoculant applied at the rate of 150 mL 50 kg^{-1} of seeds for the maize, and 200 mL 50 kg^{-1} of seeds for the wheat) with *Azospirillum* in peat or liquid inoculants on the grain yield of maize (Maize variety used in Londrina and Ponta Grossa was EMBRAPA-HD-28 X) and wheat (Wheat varieties used in Londrina and Ponta Grossa were BR-18 Embrapa-16) (kg ha^{-1}) in field experiments performed in Londrina and Ponta Grossa

Maize	Londrina		Ponta Grossa		Mean[a]	Increase	
	1st	2nd	1st	2nd		kg ha^{-1}	%
C[b]	3,200 c[c]	2,980 c[c]	2,999 c[c]	3,112 b[c]	3,073 c[d]		
C+N[b]	4,970 a	4,540 a	4,102 a	4,040 a	4,413 a	1,340	43.6
Ab-V5 + Ab-V6 (peat)	4,125 b	3,999 b	3,600 b	3,980 a	3,926 b	853	27.8
Ab-V5 + Ab-V6 (liquid)	4,028 b	4,102 b	3,590 b	3,899 a	3,905 b	832	27.1
Wheat	1st	2nd	1st	2nd			
C[e]	2,142 b[f]	2,012 b[f]	2,004 b[f]	1,999 b[f]	2,039 b[g]		
C+N[e]	2,677 a	2,866 a	2,324 a	2,884 a	2,688 a	649	31.8
Ab-V5 + Ab-V6 (peat)	2,910 a	2,777 a	2,350 a	2,752 a	2,697 a	658	32.3
Ab-V5 + Ab-V6 (liquid)	2,888 a	2,750 a	2,345 a	2,642 a	2,656 a	617	30.3

[a] Mean considering the two experiments performed with maize and the two experiments performed with wheat in Londrina and Ponta Grossa, each with six replicates

[b] Non-inoculated controls for the maize: (C) receiving only N-fertilizer at sowing (24 kg of N ha^{-1}); (C + N) N at sowing + 80 kg of N ha^{-1} at flowering

[c] Data represent the means of six replicates and when followed by the same letter, for each crop and crop season, are not statistically different (Duncan, $p \leq 0.05$)

[d] Means of the two experiments performed with maize and the two experiments performed with wheat, each with six replicates, and when followed by the same letter are not statistically different (Duncan, $p \leq 0.05$)

[e] Non-inoculated controls: for the wheat (C) receiving only N-fertilizer at sowing (20 kg of N ha^{-1}); (C+N) N at sowing + 50 kg of N ha^{-1} at flowering

same strains, with both maize and wheat. These results show that the selection of *Azospirillum* strains is important and may result in impressive contributions to important crops such as maize and wheat. An increased interest in the use of inoculants containing plant-growth-promoting bacteria has been observed and should increase in the coming years, due to increasing costs of fertilizers, concerns about pollution and emphasis on sustainable agriculture. In Brazil, saving considering only the partial replacement (50%) of the N-fertilizer (for the maize, saving of about 52 kg of N ha^{-1} in 14.1 million ha; for the wheat, saving of about 35 kg of N ha^{-1} in 2.4 million ha^{-1}), required by the maize and wheat crops by the *Azospirillum* strains recognized in this study would result in an economy estimated at US$ 1.2 billion per year. Therefore the use of inoculants containing *Azospirillum* should help worldwide to reach the goal of reducing the use of chemical fertilizers.

Acknowledgments The work was partially supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil)/MCT, PRONEX. The authors thank Dr. Ricardo S. Araujo for helpful discussions and Dr. Allan R. J. Eaglesham for English review. Author thank to Julio C. Franchini and Fabio. L. Mostasso for technical help in several steps of this study and to José Zucca de Moraes, Rubson N. R. Sibaldelli and Rinaldo B. Conceição for agronomic support. This manuscript was reviewed by the internal editorial committee of the Embrapa Soybean Center, by Dr. Ricardo S. Araujo (Total Biotecnologia, Curitiba, Paraná, Brazil) and by Dr. Fabio B. Reis Junior (Embrapa Cerrados, Planaltina, Federal District, Brazil) prior to submission to Plant and Soil.

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