Symbiosis
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Improvement of soybean grain nutritional quality under foliar inoculation with *Azospirillum brasilense* strain Az39

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Abstract

Legumes depend on biological nitrogen fixation through symbiosis with rhizobia to meet their nitrogen requirements. Certain plant growth promoting rizobacteria such as *Azospirillum* may cooperate in the establishment and maintenance of effective legume-rhizobia symbiosis. The aim of this work was to assess if foliar inoculation of soybean with *Azospirillum brasilense* Az39 has advantages over inoculation with this bacterium at sowing in combination with *Bradyrhizobium japonicum* E109 inoculation. To test this, glasshouse and field experiments were carried out. Higher shoot biomass was observed at V6 and R2 stages under foliar inoculation of *A. brasilense* Az39 as compared to coinoculation at sowing. Additionally, increased root dry biomass and higher nodule number and nodule fresh weight per plant were found at V6. Leghemoglobin levels in nodules were significantly greater in foliar-inoculated plants at both stages (V6 and R2) as compared with seed-inoculated plants. In line with these positive effects of foliar inoculation with *A. brasilense* Az39 on soybean growth and nodulation, grains harvested from foliar-inoculated plants had higher nitrogen and protein contents than those harvested from plants coinoculated at sowing. This was corroborated for two soybean varieties cultivated at two different locations. Our findings may open new insights into soybean agricultural technology.

Keywords Foliar inoculation · Soybean · PGPR · Inoculants · Nodules

1 Introduction

It has been largely documented that plant growth promoting rhizobacteria (PGPR), particularly those belonging to genus *Azospirillum*, contribute to the success of rhizobia-legume symbioses (González and Lluch 1992; Andreeva et al. 1993; Itzigsohn et al. 1993; Fabbri and Del Gallo 1995; Burdman et al. 1997; Okon and Vanderleyden 1997; Groppa et al. 1998; Cassán et al. 2009; Benintende et al. 2010; Marks et al. 2013). Positive effects on symbiotic performance of rhizobia by *A. brasilense*, one of the best studied PGPR, have usually been assigned to the biosynthesis of phytohormones (Srinivasan et al. 1996; Molla et al. 2001; Vessey and Buss 2002; Ferri et al. 2017), mainly indole-3-acetic acid (IAA) (Cassán et al. 2014; Puente et al. 2017).

Since Thimann's findings regarding the important role of auxins in nodule ontogeny about eighty years ago (Thimann 1936), many investigations have documented that changes in auxin concentrations (after inoculation with auxin-producing microorganisms or exogenous addition of these chemicals) may impact on nodule organogenesis (Schmidt et al. 1988). Changes at molecular and

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morphological levels, including modifications of nodule amounts, distribution and functionality, have been reported upon inoculation with *Rhizobium* and *Azospirillum* (Yahalom et al. 1990).

Foliar application of active ingredients under intensive crop farming is frequently directed to act on different physiological processes (Castro and Vieira 2001; Vieira and Castro 2002; Taiz and Zeiger 2009). Several reports communicate greater production of non-leguminous crops in response to foliar application of plant growth regulators including citocinins, auxins and gibberellic acid (Palangana et al. 2012; Cato et al. 2013). Salicylic acid and ethylene have been applied on soybeans, resulting in higher vegetative growth and grain yield (Devi et al. 2011). On the other hand, Sudhakar et al. (2000) observed positive effects of foliar inoculation with *Azotobacte*r, *Azospirillum* and *Beijerinckia* in mulberry plants. Portugal et al. (2016) reported increased yields of maize under foliar inoculation with *Azospirillum brasilense*.

Few reports paid attention to the relevance of foliar application of plant growth promoting rizobacteria in legume crops. Seed application is the most widespread procedure to deliver beneficial microorganisms under standard field conditions (Vendan and Thangaraju 2007; Neto et al. 2008), even when environmental hazards such as desiccation or concomitant seed treatments (i.e., fungicides) may result in significant reductions of initial bacterial numbers and, therefore, reduced plant colonization (Penna et al. 2011). Alternative practices to deliver plant growth promoting microorganisms may overcome these constraints.

PGPR effects under foliar application have been largely attributed to changes in the hormonal balance induced by phytohormones released by these bacteria or by the in situ biosynthesis of active metabolites upon bacterial colonization of the phyllosphere (González López et al. 1991).

In a previous work recently published (Puente et al. 2017), we confirmed growth promotion and better nodulation of soybean plants inoculated at sowing with *Bradyrhizobium japonicum* E109 and sprayed at V3 stage with *A. brasilense* Az39 ($1{\cdot}10^8$ cfu.plant^{-1}); the plant growth effect was in direct relationship to the bacterial ability to produce IAA. In this research we evaluated if foliar inoculation of soybean with the same PGPR strain (*Azospirillum brasilense* Az39) has advantages over coinoculation with this bacterium at sowing in combination with *B. japonicum* E109.

2 Materials and methods

Microorganisms *B. japonicum* E109 and *A. brasilense* Az39 from BPCV Colection of IMYZA, INTA (Castelar, Buenos Aires, Argentina) were the bacterial strains used in our experiments. These strains are widely used for inoculant formulations in Argentina and Brazil (Lodeiro 2015; Cassán and Díaz-Zorita 2016); their complete genome sequences have been published (Rivera et al. 2014; Torres et al. 2015).

2.1 Experimental

Glasshouse and field experiments were carried out. All experiments included the following treatments: 1) seed inoculation with *B. japonicum* E109 ($1{\cdot}10^6$ cfu seed^{-1}); 2) seed inoculation with *B. japonicum* E109 ($1{\cdot}10^6$ cfu seed^{-1}) plus *A. brasilense* Az39 ($1{\cdot}10^7$ cfu seed^{-1}); 3) seed inoculation with *B. japonicum* E109 ($1{\cdot}10^6$ cfu seed^{-1}) plus foliar inoculation with *A. brasilense* Az39 ($3{\cdot}10^8$ cfu.plant^{-1}) .

B. japonicum E109 was grown on yeast extract mannitol broth (Vincent 1970), and *A. brasilense* Az39 in Sadasivan and Neyra liquid medium (1985), at 200 rpm and 30 °C for 3 days. The number of viable cells was estimated through standard cultivation on solid media, using yeast extract mannitol agar (YEM), (Vincent 1970) for *B. japonicum* and Congo Red agar for *A. brasilense* Az39 (RC) (Rodríguez Cáceres 1982). Foliar inoculation was achieved by spraying soybean leaves at V3 stage (third node) with 6 ml.plant^{-1} of bacterial suspension prepared by diluting 10 mL of an *A. brasilense* Az39 culture at $1\ {\cdot}10^9$ cfu mL^{-1} in 190 mL of deionized water ($3{\cdot}10^8$ cfu mL^{-1}). For seed inoculation, seeds were immersed for 5 min in cultures containing $1{\cdot}10^6$ cfu mL^{-1} of *B. japonicum* E109 (treatments 1 and 3) or *B. japonicum* E109 ($1{\cdot}10^6$ cfu mL^{-1}) plus *A. brasilense* Az39 ($1{\cdot}10^7$ cfu mL^{-1}) (treatment 2). These inoculation doses and bacterial cultures preparation were selected based on preliminary experiments (Puente et al. 2017; Cassán et al. 2009).

2.1.1 Glasshouse assay

Soybeans (variety A5009 RG, Nidera, Argentina) were cultivated in plastic pots (10-L pots) filled with a mixture of soil, sand, vermiculite and perlite (1:1:1:0.33); capilarity watering with sterile water was performed, and once a week, plants were watered with 25% (*v*/v) nitrogen-free Hoagland solution (Hoagland and Arnon 1950). At V6 (sixth node) and R2 (full flowering) (Fehr and Caviness 1977), 4 plants

Table 1 Physico-chemical properties of the soils in field assays

Location	pH	Nt (%)	P ppm	OM (%)	C (%)	DM (%)	Hum (%)
Barrow	5,59	0,41	38,49	7,99	53,41	15,92	84,05
Bolívar	5,90	0,24	15,84	4,39	2,54	80,11	19,88

N total nitrogen, *P* phosphorus, *OM* organic matter, *C* carbon, *DM* dry matter, *Hum* humidity (0–20 cm)

†. TSP: triple superphosphate applied at sowing

Table 2 Relevant information about soybean crop management in field assays

Location	Variety	Fertilization †	Sowing	Harvest
Barrow	ASP 3911	50 kg·ha-1 TSP	2014/11/28	2015/03/20
Bolívar	Don Mario 3810	60 kg·ha-1 TSP	2014/12/03	2015/04/10

were processed in order to determine fresh and dry biomass, nitrogen content, chlorophyll content, number of nodules (total and attached to main or secondary roots), and leghemoglobin content. Nitrogen content was estimated through de Kjeldahl method (Bremner 1965), and a portable chlorophyllmeter (SPAD-502, Minolta, Spectrum Technologies Inc., Illinois, USA) was used to estimate chlorophyll content. Leghemoglobin determinations were carried out by the spectrofluorometric method, as described by LaRue and Child (1979).

2.1.2 Field experiments

The experimental plots were located at two INTA Experimental Stations of Buenos Aires province: Bolívar (36°15'S, 61°06'W) and Barrow (38°18'S, 60°14'W). Physico-chemical properties of the soils assigned to these experiments are shown on Table 1. Regular crop calendar and standard field practices were followed at both locations. Table 2 summarizes relevant agronomic information such as soybean variety chosen and fertilizations performed. At harvest, yield, plant total nitrogen, 1000-grain weight and nitrogen, assimilable phosphorus and protein contents of soybean grains were determined.

2.2 Statistics

All experiments were carried out by triplicate using a randomized block design, with 12 (glasshouse experiment) or 3 (field trial) repetitions per treatment. Results were subjected to ANOVA; means were compared by Duncan test ($P \leq 0.05$) using INFOSTAT software (Di Rienzo et al. 2014).

3 Results

3.1 Glasshouse assay

At phenological stage V6 (six nodes on the main stem), soybean plants sprayed with *A. brasilense* Az39 showed greater aerial and root biomass than those under single inoculation with *B. japonicum* E109 or those coinoculated with both microorganisms at sowing (Table 3). Nodule number increased under foliar inoculation with *A. brasilense* Az39 as compared to coinoculation, mainly at the expense of nodules attached to secondary roots. Nodule fresh weight and leghemoglobin content were also

Table 3 Results from glasshouse assays

	1		2		3	
	V6	R2	V6	R2	V6	R2
Plant height (cm)	nd	107,92 a	nd	106,67 a	nd	115,33 a
Root length (cm)	nd	53,79 ab	nd	47,18 b	nd	55,40 a
Aerial fresh biomass (g)	20,56 b	33,44 a	19,86 b	32,87 a	30,45 a	37,61 a
Aerial dry biomass (g)	9,28 ab	16,48 ab	8,58 b	15,50 b	11,26 a	17,67 a
Root fresh biomass (g)	nd	19,05 a	nd	15,83 a	nd	19,52 a
Root dry biomass (g)	2,71 ab	nd	2,49 b	nd	3,36a	nd
Nodule number	66,73	107,88	66,27	111,69	88,93	129,62
Nodule primary root	10,70 b	10,94 a	16,1 a	12,44 a	14,85ab	14,44 a
Nodule secondary root	54,03 b	96,94 a	50,17 b	99,25 a	74,08 a	115,18 a
Nodule fresh weight	1,48 ab	nd	1,44 b	nd	2,39 a	nd
Nodule dry weight	0,85 a	1,02 a	1,01 a	0,97 a	1,58 a	1,06 a
Leg Hb ($\mu g\ g^{-1}$) †	83,46 ab	82,63 b	73,52 b	75,45 b	91,20 a	105,56 a
Total N ($mg\ g^{-1}$)	2,31 b	nd	2,64 ab	nd	3,26 a	nd
Total protein ($mg\ g^{-1}$)	14,41 b	nd	16,48 ab	nd	20,36 a	nd
Chlorophyll (SPAD units)	30,88 a	28,58 a	33,48 a	29,68 a	31,55 a	32,88 a

1: *B. japonicum* E109 at sowing; 2: *B. japonicum* E109 and *A. brasilense* Az39; both at sowing; 3: *B. japonicum* E109 at sowing and *A. brasilense* Az39 applied by foliar inoculation at V3 stage. †Expressed on a fresh weight basis. nd: not determined greater under this treatment. At phenological stage R2 (full flowering), plants sprayed with *A. brasilense* Az39 had higher aerial dry biomass and greater leghemoglobin contents in their nodules than those coinoculated with both microorganisms at sowing. Chlorophyll concentrations did not differ among treatments.

3.2 Field experiments

Both methods of *Azospirillum* application on soybean resulted in higher yields than single inoculation with *B. japonicum*, irrespective of soil properties or soybean variety cultivated (Fig. 1a); differences between these treatments were not statistically significant. Although there was a clear trend showing improvement of 1000-grains weight in both locations under foliar inoculation with *Azospirillum*, only in Bolívar plots this yield component was significantly superior (Fig. 1b).

Nitrogen and protein contents of grains were higher in *Azospirillum*-sprayed plants than in single-inoculated or in coinoculated plants at both locations (Fig. 2a and b), but grain assimilable phosphorus was significantly increased only in Barrow plots (Fig. 2c).

4 Discussion

In this research, soybean performance under foliar inoculation of *A. brasilense* Az39 was studied for the first time in Argentina at field scale. *A. brasilense* Az39 is a PGPR widely recognized because of its outstanding phytostimulatory ability, which has been ascribed to its ability to biosynthesize several well-known plant growth regulators. Our results show several improvements on above-ground and below-ground soybean growth after foliar inoculation of *A. brasilense* Az39: higher fresh and dry shoot biomass was observed among the first ones. At the root level, a few positive effects on the nitrogen fixing system were observed: more nodules —which resulted in increased nodule fresh weight per plant— and higher leghemoglobin levels were found under foliar inoculation of *A. brasilense* Az39. Previous experiments of foliar inoculation of soybean using an *ipdC*⁻ mutant of *A. brasilense* Az39 impaired in IAA biosynthesis showed little impact on vegetative and reproductive performance of plants as compared to those inoculated with *B. japonicum* alone or those inoculated with *B. japonicum* and later sprayed with *A. brasilense* Az39 wild type (Puente et al. 2017). Therefore, it may be assumed that bacterial-IAA production plays a key role under foliar inoculation. Plant growth promotion exerted by the inoculation of IAA-producing microorganisms has been largely documented (Cassán et al. 2014; Goswami et al. 2016; and references therein).

When *A. brasilense* Az39 was sprayed on leaves at V3, a greater nodular biomass was observed at V6 and higher leghemoglobin levels were kept until the reproductive period. Changes in soybean hormonal status may account for the increased nodule number here reported. It was established that dual inoculation (rhizobia and *Azospirillum*) of legumes results in increased production of plant flavonoids, which trigger *Rhizobium nod*-gene expression (Okon et al. 2015).

Changes in soybean nodule distribution after coinoculation at sowing with *Bradyrhizobium* and *Azospirilllum* were

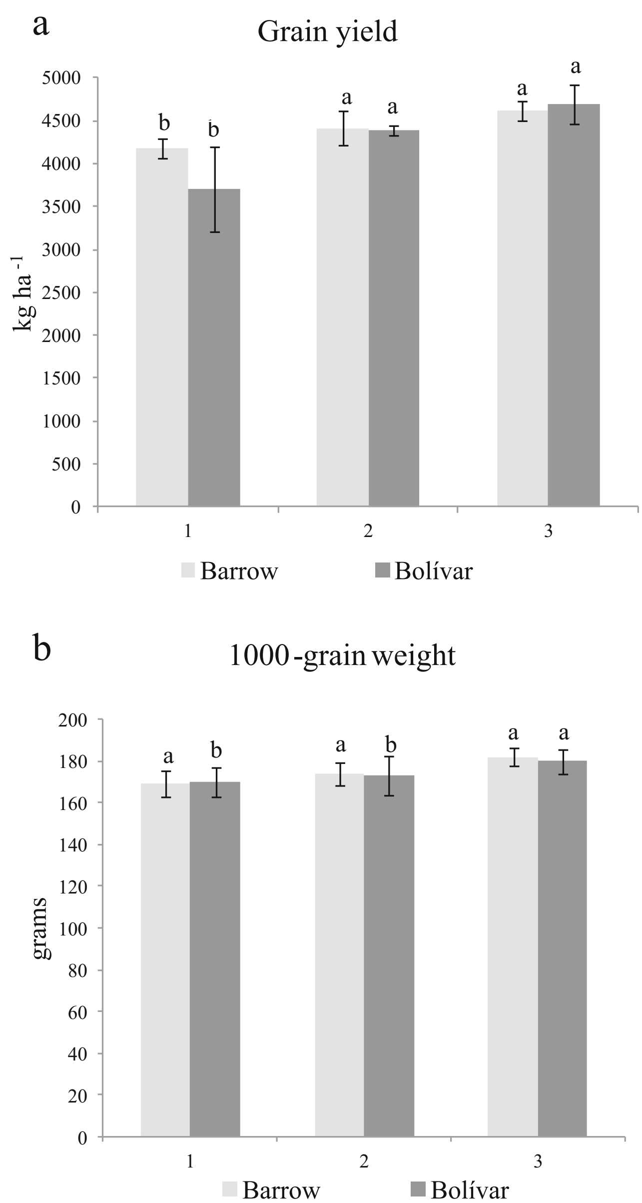

Fig. 1 Yield (**a**) and 1000-grains weight (**b**) of soybean plants subjected to different inoculation treatments. 1: *B. japonicum* E109 (seed inoculation); 2: *B. japonicum* E109 and *A. brasilense* Az39 (seed coinoculation); 3: *B. japonicum* E109 (seed inoculation) and *A. brasilense* Az39 (foliar inoculation at V3 stage). Experiments were carried out by triplicate using a randomized block design, with 3 repetitions per treatment. Different letters in a same location indicate significant differences according to Duncan's test ($P \leq 0.05$)

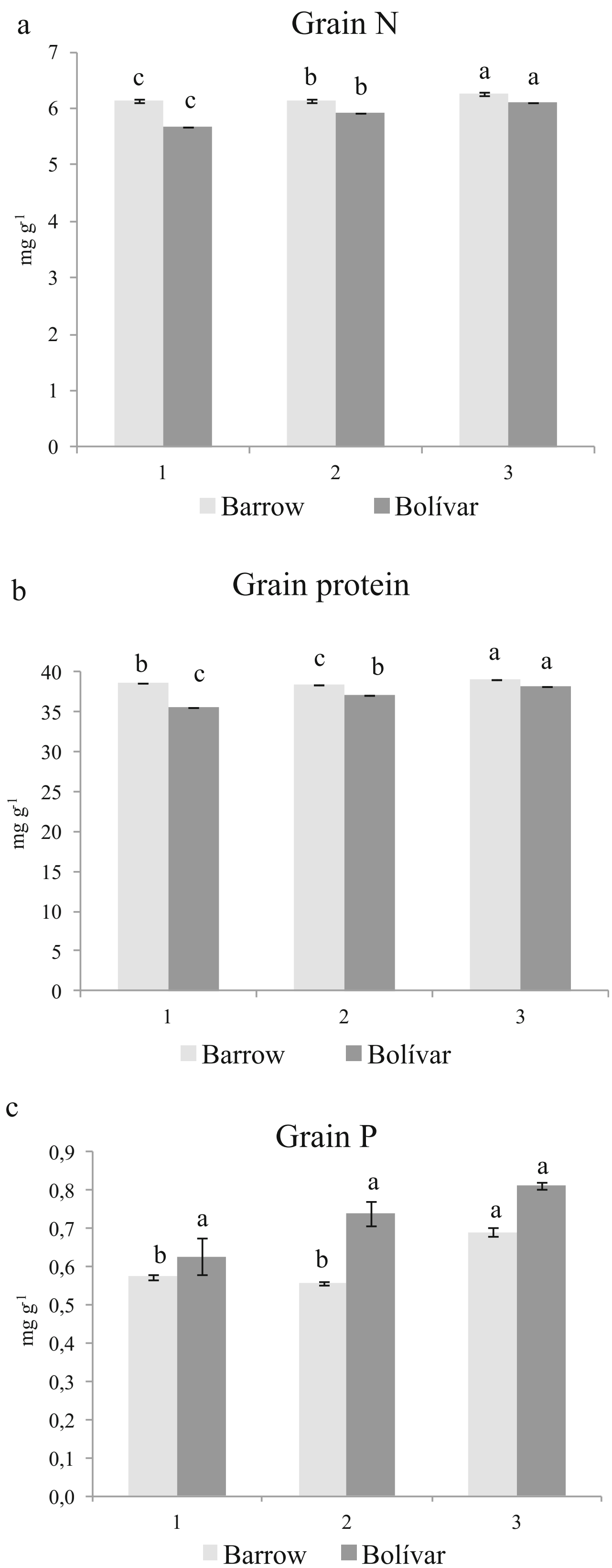

Fig. 2 Nitrogen (**a**), protein (**b**), and assimilable phosphorus (**c**) in soybean grains. 1: *B. japonicum* E109 (seed inoculation); 2: *B. japonicum* E109 and *A. brasilense* Az39 (seed coinoculation); 3: *B. japonicum* E109 (seed inoculation) and *A. brasilense* Az39 (foliar inoculation at V3 stage). Experiments were carried out by triplicate using a randomized block design, with 3 repetitions per treatment. Different letters in a same location indicate significant differences according to Duncan's test ($P \leq 0.05$)

previously reported (Groppa et al. 1998). However, the results obtained in this research suggest that the introduction of the IAA-producer microorganism in an advanced stage of soybean life cycle may prompt the development of more nodules in secondary roots, and that these "later nodules" may have a special role in soybean nitrogen economy at advanced stages of plant life cycle (i.e. grain filling). In fact, we found that foliar inoculation of *A. brasilense* Az39 improved nutritional grain profile: nitrogen and protein contents were significantly higher in grains harvested from foliar-inoculated plots than from coinoculated plots at sowing. This may be linked to a greater and more active nodule mass on soybean roots at the beginning of the reproductive season, which allows continuous nitrogen fixation and proper mobilization of N to developing grains.

Improvements of legume protein and/or nitrogen contents after application of different PGPR have been previously communicated. Zarei et al. (2014) informed that soybean inoculation with *Bacillus* and *Pseudomonas* led to yield rises and greater nitrogen and protein contents in grains. Likewise, Yadegari et al. (2008) informed improvements of yield and grain protein upon inoculation of *Rhizobium* and *Pseudomonas fluorescens* in beans. We did not find superiority of foliar inoculation over coinoculation at sowing in grain yield. However, in one of our field settings (INTA-Bolivar/Don Mario soybean variety), 1000-grain weight (a key yield component) significantly increased. Gathering reproducible results across more soybean campaigns and geographical sites would allow validating present findings.

In summary, we conclude that even when both methods of *A. brasilense* Az39 delivery to soybean (foliar inoculation or coinoculation at sowing, in combination with *B. japonicum*) led to positive responses in terms of nodulation and crop productivity, the nutritional profile of soybean grains improved when this well-known rizospheric microorganism was sprayed on leaves instead of being applied at sowing, as higher protein and nitrogen levels were found. Zuffo et al. (2016) have already stated that application of *Azospirillum brasilense* via spray in an advanced stage of the crop can increase soybean performance. Our findings may open new insights into agricultural technology so that maximum profit from beneficial microorganisms may be obtained.

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Compliance with ethical standards

Conflicts of interest The authors report no conflicts of interest.

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