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RESEARCH ARTICLE

Enhancing soybean yield through co-inoculation of *Bradyrhizobium* spp. and ammonium-excreting *Azospirillum brasilense* HM053

S. C. Araujo · R. M. Etto · E. M. Souza · F. O. Pedrosa · F. Furmam-Cherobim · A. R. Bini · D. R. P. Gonçalves · E. Guerlinguer · P. C. Conceição · A. L. Braccini · D. C. V. Marteli · E. L. Souchie · I. M. B. Torneli · C. W. Galvão

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Abstract
Background and aims The inoculation of *Bradyrhizobium* spp. in soybean is a widespread technology in Brazil and one of the most successful cases of plant-bacteria interaction once all the nitrogen required by the plant is provided through biological nitrogen fixation. Co-inoculation of *Bradyrhizobium spp*. with *Azospirillum brasilense* AbV5/AbV6 was recommended in Brazil in 2013, but its adoption by farmers has been limited due to variable yield gains. In the present work, we hypothesized that additional traits of *A. brasilense*, such as resistance to oxidative stress and ammonium excretion, further enhance its growth-promoting effects in soybean when co-inoculated.
Methods Therefore, an oxidative resistant strain (IH1), two constitutive nitrogen fixing strains (HM053 and HM210) and the commercial strains (AbV5/AbV6) of *A. brasilense* were co-inoculated with *Bradyrhizobium* spp. in soybean. The experiments were carried out in four distinct soil and edaphoclimatic regions of Brazil to evaluate soybean nodulation, growth, and yield.
Results The novel strains of *A. brasilense* enhanced soybean nodulation and grain yield. The co-inoculation with the HM053 strain resulted in the highest increase in soybean grain yield, ranging from 4.3% to

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S. C. Araujo
National Association for the Promotion and Innovation of the Biological Industry (ANPII Bio), Curitiba, PR CEP 80035-010, Brazil

R. M. Etto · F. Furmam-Cherobim · A. R. Bini · D. R. P. Gonçalves · E. Guerlinguer · C. W. Galvão (✉)
Microbial Molecular Biology Laboratory, State University of Ponta Grossa (UEPG), Ponta Grossa, PR CEP 84030-900, Brazil
e-mail: cwgalvao@uepg.br

E. M. Souza · F. O. Pedrosa
Nucleus of Nitrogen Fixation, Federal University of Paraná (UFPR), Curitiba, PR CEP 81531-980, Brazil

P. C. Conceição
Federal University of Technology (UTFPR), Dois Vizinhos, PR CEP 85660-000, Brazil

A. L. Braccini
Department of Agronomy, State University of Maringá (UEM), Maringá, PR CEP 87020-900, Brazil

D. C. V. Marteli
Cooperative for Agronomy Professionals (UNICAMPO), Maringá, PR CEP 87.013-072, Brazil

E. L. Souchie
Goiano Federal Institute (IFGoiano), Rio Verde, GO CEP 75901-970, Brazil

I. M. B. Torneli
São Paulo's Agency for Agribusiness Technology (APTA), Colina, SP CEP 14.770-000, Brazil

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25.4%, or 151.7 to 878.1 kg/ha, compared to single inoculation.

Conclusion This promising technology generates environmental and economic gains, since it promotes plant growth, increases yield and contributes for a sustainable agriculture.

Keywords Biological nitrogen fixation · Sustainable agriculture · Food security · Biotechnology · Biofertilizer

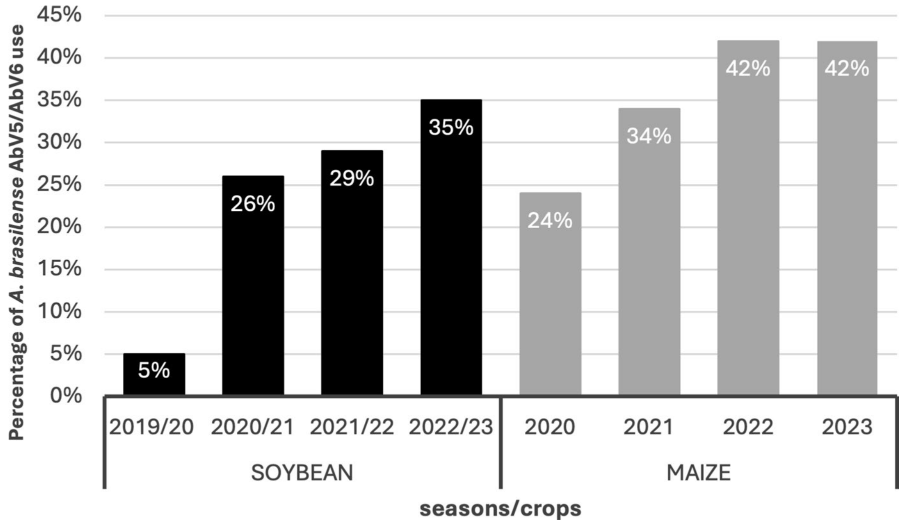
Introduction

The soybean crop is a vital component of Brazilian agriculture, with over 70% of its total production exported, making Brazil the leading exporter of soybeans and the second-largest exporter of soy meal and soybean oil in the world (Ali et al. 2022). The success of soybean cultivation in Brazil can be attributed to the inoculation of seeds with *Bradyrhizobium* bacteria, which can fully satisfy the crop's nitrogen demand, resulting in high grain yields (Hungria et al. 2015). In Brazil, the economic benefits of replacing nitrogen fertilizer (urea) with BNF on soybeans are substantial. In the 2019–2020 crop season, the estimated economic value from this substitution reached 15.2 billion USD. This shift mitigated 183 million Mg CO_2-equivalent emissions, corresponding to over 5.1 billion USD in carbon credits (Telles et al. 2023). The symbiotic relationship between *Bradyrhizobium* and soybean is considered one of the most important interactions utilized in agricultural activities (Zeffa et al. 2020).

In recent years, the use of mixed microbial inoculants, which combine different strains or species of microrganisms, has gained popularity due to their ability to promote plant growth through diverse mechanisms, including BNF, phytohormone production, phosphate solubilization, and biological control (Santos et al. 2019). Since 2013, co-inoculation of *A. brasilense* and *Bradyrhizobium* spp. has been recommended in Brazil's soybean crop, driven by the different mechanisms to improve plant development provided by these bacteria (Hungria et al. 2013). Currently, around 106 inoculants containing *Azospirillum* as the active ingredient are produced in South America, with *A. argentinense* Az39 (formerly *A. brasilense* Az39) being emphasized in Argentina and strains of *A. brasilense* AbV5 and AbV6 in Brazil (Cassán et al. 2020). Besides AbV5/AbV6, other *A. brasilense* Sp7 derivatives strains have shown great potential (Machado et al. 2011; Higuti & Pedrosa 1985). Strains HM210 and HM053 are spontaneous mutants of glutamine synthetase (GS), which can excrete ammonium and fix nitrogen constitutively, even in the presence of high concentrations of NH_4^+ (Machado et al. 2011). IH1 strain is resistant to high concentrations of H_2O_2 due to the presence of high levels of catalase, thus promoting greater resistance to oxidative stress (Higuti and Pedrosa 1985). *A. brasilense* HM053 showed to supply *Setaria viridis* daily demand of nitrogen through Biological Nitrogen Fixation (BNF) under controlled conditions (Pankievicz et al. 2015) and promote root growth in barley and wheat (Santos et al. 2017a, b). Besides, HM053 strain provided an increase in maize grain yield between 4.7 to 29% or 460.5 to 1769.3 kg/ha, showing that this strain is a new alternative for a more sustainable agriculture (Pedrosa et al. 2020). *A. brasilense* HM210 strain was tested in the beet culture, showing superior results for the diameter and length of tuberous roots (Sousa 2022).

The capacity of *Azospirillum* spp. to synthesize phytohormones and stimulate plant growth, especially the root system (Nievas et al. 2023), can favor nodulation and biological nitrogen fixation by *Bradyrhizobium* (Prando et al. 2019). Co-inoculation with *A. brasilense* promotes more abundant nodulation as a result of an increase in the number of sites of infection, which are the root hairs, from where the bacteria enters and starts nodule formation (Timmers 2000). Field experiments conducted in Brazil from 2009 and 2020 demonstrated a 3.2% increase in soybean grain yield with co-inoculation of *Bradyrhizobium* spp. and *A. brasilense* AbV5/AbV6, compared to single inoculation (Barbosa et al. 2021). Despite the recognized benefits of co-inoculation for rural producers, its adoption in Brazil has remained relatively modest, although there has been an upward trajectory in recent years (Fig. 1). For instance, during the 2019/20 season, the application of *A. brasilense* AbV5/AbV6 was only 5%, but this number increased to 26% in the following season (2020/21), reaching 35% in the 2022/23 season (Fig. 1). This inoculation adoption covered an area of 16.1 million hectares, out of a total of 46 million hectares of soybean cultivation in Brazil in 2023. In maize cultivation, its utilization increased from 24% in 2020 to 42% in 2022,

Fig. 1 Percentage of *A. brasilense* AbV5/AbV6 use in soybean and maize crops in Brazil (ANPII Bio 2024)



remaining unchanged in 2023 (Fig. 1). This inoculation adoption covered an area of 10.5 million hectares, out of a total of 25 million hectares of maize cultivation in Brazil in 2023. This demand has led to the sale of more than 36,7 million doses of *A. brasilense* AbV5/AbV6 in 2023 (ANPII Bio 2024).

Considering that local NH_4^+ stimulates lateral root growth (Drew 1975; Hodge 2004), which can create sites for nodule formation, and that root development is associated with the production of reactive oxygen species (ROS) (Mendez-Gomez et al. 2016; Takahashi et al. 2024), we hypothesized that additional traits of *A. brasilense*, such as ammonium excretion and resistance to oxidative stress, further enhance its growth-promoting effects in soybean when co-inoculated. To evaluate the efficiency of co-inoculation in soybeans using *A. brasilense* strains HM053, H210, and IH1, experiments were conducted under four different edaphoclimatic conditions in Brazil. The *A. brasilense* strains AbV5 and AbV6, which has been used as a reference in co-inoculation in Brazil for 12 years, was also included, as it does not exhibit the additional traits found in the tested strains.

Material and methods

Inoculants and experimental design

This experiment was carried out in 2019/20, in the soybean crop, with the participation of ANPII Bio—National Association for the Promotion and Innovation of the Biological Industry. The inoculant *A. brasilense* AbV5/AbV6 were made of strains AbV5 and AbV6 both at 1×10^8 cells mL^{-1}, and the inoculant *Bradyrhizobium* were made of SEMIA 5079 (*B. japonicum*) and SEMIA 5080 (*B. diazoefficiens*), both at 2×10^9 cells mL^{-1}. These strains are used for commercial production of inoculants in Brazil. The novel *A. brasilense* strains, IH1, HM053, and HM210, are deposited at the Federal University of Paraná – UFPR, in the Department of Biochemistry and Molecular Biology (Curitiba, Paraná). Strains HM053 and HM210 are derived from *A. brasilense* strain FP2 (Sp7 ATCC29145, Sm^R Nal^R) and exhibit constitutive nitrogen fixation as a result of cultivation in the presence of Ethylenediamine (Machado et al. 2011). Strain IH1, in turn, which is derived from *A. brasilense* Sp7, displays enhanced catalase activity following cultivation in progressively increasing concentrations of hydrogen peroxide (Higuti and Pedrosa 1985). Inoculants were prepared using these strains (HM053, HM210 and IH1) at the following concentrantions: 3.2×10^8, 7.8×10^8 and 2×10^8 cells mL^{-1}, respectively. ANPII Bio partner companies were responsible for standardizing and production of each novel strain inoculants, as well as for supplying. The recommended dose for both *Bradyrhizobium* and *A. brasilense* inoculants was 100 mL ha^{-1} (2 mL kg^{-1} of seed).

Seed treatment was carried out with pyraclostrobin + methyl thiophanate + fipronil, at a dose of

2 mL kg^{-1}, four hours before inoculation. Subsequently, the seeds were placed in plastic bags and the inoculants were added using autoclaved pipettes. Then, the plastic bags were shaken manually until the seeds were completely covered. They were sown after being air-dried in the shade for 1 h and within 4 h of inoculation to prevent a significant decrease in viability (Takahashi et al. 2022). The entire process was conducted at an ambient temperature of approximately 25 °C. To perform seed co-inoculation, the dose of *Bradyrhizobium* and the dose of *A. brasilense* were mixed in a sterile bottle for subsequent seeds inoculation (Hungria et al. 2015).

Every experiment included two controls, non-inoculated control without N fertilizer (treatment 1), and non-inoculated control with N fertilizer (200 kg N ha^{-1} applied as urea, being 50% applied at sowing and 50% at 35 days after emergence) (treatment 2). The inoculated controls were single inoculation with *Bradyrhizobium* (treatment 3) and co-inoculation of *Bradyrhizobium* + *A. brasilense* AbV5/AbV6 (treatment 4). The novel strains were tested in co-inoculation with *Bradyrhizobium*, as follows: *Bradyrhizobium* + *A. brasilense* IH1 (treatment 5), *Bradyrhizobium* + *A. brasilense* HM053 (treatment 6), *Bradyrhizobium* + *A. brasilense* HM210 (treatment 7) and *Bradyrhizobium* + *A. brasilense* IH1 + HM053 + HM210 (treatment 8). Similar to treatment 1, treatments 3 through 8 also excluded the application of nitrogen fertilizer. The experiments were designed as completely randomized blocks with six replicates.

Sites description and field management

Field experiments were performed in the following Brazilian Edaphoclimatic regions (ECR): ECR-202, ECR-302, ECR-301 and ECR-401. The characteristics of each location are shown in Table 1.

The experimental plots measured 4.0 m (width) × 6.0 m (length) and were separated by 0.5 m rows at ECR-301, ECR-401 and ECR-302 sites, and by 0.45 m at ECR-202 site. At ECR-301 and ECR-401 there were a total population of 440,000 plants ha^{-1} (1056 plants per plot), at ECR-202 site, 311,111 plants ha^{-1} (747 plants per plot) and at ECR-302 site, 300,000 plants ha^{-1} (720 plants per plot). Fungicides and insecticides were used in all treatments and none of the experiments was irrigated.

Before sowing, soil samples were randomly taken at each site, from the 0–10 or 0–20 cm layer. It was dried (60 °C for 48 h) and sieved (2.00 mm) for chemical and physical characterization. The main chemical and physical soil properties for all the four

Table 1 Characteristics of the four experimental sites and field assays

Brazilian Edaphoclimatic Region (ECR)*	ECR-301	ECR-401	ECR-302	ECR-202
City/State of Brazil	Rio Verde/GO	Indiara/ GO	Pindorama/ SP	Mandaguaçu/PR
Geographic Coordinates	17°47′53" S 50°55′41" W	17° 11′ 04" S 49° 59′ 04" W	21° 13′ S 48° 55′ W	23º23′45″ S 52º08′27″ W
Altitude (a.s.l.)	742 m	572 m	546 m	507 m
Soil Type	Oxisol (Latossolo Vermelho)	Oxisol (Latossolo amarelo)	NI	Oxisol (Latossolo Vermelho Distroférrico)
Climate classification	Aw	Aw	Aw	Cfa
Accumulated Precipitation**	1225 mm	1390 mm	766.4 mm	690 mm
NPK# Formulation	00–30-10	00–30-10	00–20-20	04–30-10
Fertilization Dose (kg ha^{-1})	400	400	300	250
Soybean predecessor culture	Maize	Maize	Pasture	NI
Soybean Cultivar tested	Bônus	Bônus	TMG 7062 IPRO	M 6410 IPRO
Soybean harvested area (m^2)	16	16	12	9

* According to Brazil (2012). ** Accumulated Precipitation during the entire experiment. # percentage of each element in the respective formulation

Legend: NI-Not informed; a.s.l-above the sea level sites are shown in Table S1. The base fertilization varied in each location, as well as the soybean cultivar, as each region has different soil and climate conditions (Table 1). The most adopted cultivar in each region was chosen. In all experiments and treatments, two foliar fertilization applications with 150 mL ha^{-1} of cobalt (3.5 g of Co) and molybdenum (35 g of Mo) were carried. The first application occurred between the phenological stages V3 and V4, corresponding to the plant having three and four leaf collars, respectively. The second application was timed for stage R1, when silks are visibly extending beyond the husk.

Plant sampling and harvesting

Nodulation was evaluated 35 days after emergence in the ECR-301 and ECR-401 sites and at flowering in the ECR-302 and ECR-202 sites. Five plants were collected from each experimental plot (avoiding central rows designated for grain yields). In the laboratory, shoots were separated from roots and the latter was carefully washed before removing the nodules. Then, nodules and shoots were placed in an air-forced dryer at 65 °C until achieve constant weight (approximately 72 h) to determine their dry weight.

Grain yield at physiological maturity was determined by harvesting the central area of each plot, the harvested areas are shown in Table 1. Grains were cleaned, weighed and the values were corrected to 13% moisture content, after the determination of humidity in a grain moisture tester.

Indole-3-acetic acid (IAA) biosynthesis

Azospirillum strains (AbV5/AbV6, HM053, IH1, and HM210) were grown at 30 °C with 120 rpm orbital shaking in Nfb lactate medium supplemented with 50 mM of phosphate solution and 20 mM of NH_4Cl (NFbHPN) (Pedrosa and Yates 1984) until they reached exponential growth phase, corresponding to OD600 1.2. Pre-inoculum aliquots (100 µL) were transferred to 50 mL capacity flask containing 10 mL NFbHPN, supplemented with 50 or 100 µg/mL L-tryptophan (Trp) (Ona et al. 2005). Bacteria were incubated at 30 °C with 120 rpm shaking for 24, 48 and 72 h. At each time point, 500 µL of bacterial culture was collected and incubated with chloroform (10µL) and SDS (0.003%) for 5 min at 30 °C to permeabilize the cells and release intracellular IAA. Then, it was centrifuged at 12,000 xg for 10 min to collect the supernatant, which was used to quantify IAA using of the colorimetric Salkowski reaction (Gordon and Weber 1951). Besides, bacterial concentrations were determined by Colony Forming Unit (CFU) micro-drop counting in RC medium (Rodriguez Caceres 1982).

Statistical analyses

The data obtained were submitted to normality test, homocedasticity of variances and analysis of variance (ANOVA), and when significance at 0.05 was reached, the means were compared by LSD test, at 5% significance. After biometric parameters and yield normalization, they were ordinated by principal component analysis (PCA) on the correlation matrix, in R language (R Core Team 2020) with Vegan package (Oksanen et al. 2013).

Results

Effect of co-inoculation with *A. brasilense* strains on nodulation and growth parameters of soybean plants

At ECR-202, all treatments that received co-inoculation with the novel strains of *A. brasilense* showed higher values than the single inoculation with *Bradyrhizobium* and the standard co-inoculation with *A. brasilense* AbV5/AbV6, for the number of nodules (NN) and dry weight of nodules (NDW) (Table 2). All *Azospirillum* strains significantly increased NDW, with improvements from 30% to 154.5% compared to single inoculation with *Bradyrhizobium* (Table 2). Among them, HM053 demonstrated the best performance, doubling the NN and achieving the greatest increase in NDW, with 154.5% enhancement compared to single inoculation with *Bradyrhizobium.* Furthermore, it resulted in 59 more nodules per plant and twice the dry weight of nodules compared to co-inoculation with AbV5/AbV6 (Table 2). Besides, HM053 treatment showed a positive and strong correlation with NN and NDW in the PCA analysis ($r > 0.9$, p value < 0.001) (Fig. 2).

At the ECR-301 and ECR-401, most of the co-inoculation treatments induced the formation of more number of nodules than the single-inoculation with *Bradyrhizobium* (Table 2). When analysing the NDW,

Table 2 Nodulation, growth and yield parameters of the soybean evaluated in the experimental trials performed at ECR-301, ECR-401, ECR-302 and ECR-202 sites

Treatments/Parameters		1 NI	2 NI + N	3 Brady	4 Brady + Azo AbV5/V6	5 Brady + Azo IH1	6 Brady + Azo HM053	7 Brady + Azo HM210	8 Brady + Azo mix
ECR 301 site	**NN**	15.2 d	5.8 c	32.8 c	41.5 a	41.8 a	38.5 ab	36.2 bc	39.0 ab
	NDW	25.5 d	12.3 e	46.5 c	57.3 a	48.6 bc	52.6 abc	51.5 abc	54.3 ab
	SDW	11.8 c	23.3 a	21.1 b	21.7 b	21.3 b	21,5 b	21.0 b	21.2 b
	Yield	2332.2 d	2501.8 c	3551.7 b	3816.0 a	3719.2 a	3703.3 ab	3810.8 a	3708.5 ab
ECR 401 site	**NN**	19.2 e	9.3 f	48.7 d	61.5 a	59.8 ab	56.0 bc	57.2 abc	53.3 c
	NDW	35.5 b	19.8 c	56.5 a	59.0 a	59.1 a	59.8 a	58.5 a	58.7 a
	SDW	13.3 c	25.7 a	23.0 b	24.5 ab	25.2 a	25.2 a	25.3 a	24.3 ab
	Yield	2596.5 d	2431.8 e	3683.7 c	4140.7 a	4101.3 ab	4063.5 ab	4014.8 ab	3977.3 b
ECR 302 site	**NN**	4.8 cd	2.5 d	15.8 b	31.9 a	12.4 b	13.2 b	8.4 c	15.3 b
	NDW	46.4 d	28.1 d	296.8 a	314.3 a	131.3 c	205.4 b	177.7 b	189.8 b
	SDW	11.40 c	21.5 a	13.0 c	14.3 c	13.8 c	11.7 c	12.4 c	18.6 b
	Yield	3264.3 e	4068.6 d	4008.1 d	4338.4 bc	4126.6 cd	4686.9 a	4518.7 ab	4398.4 b
ECR 202 site	**NN**	61.5 g	43.8 f	79.3 e	100.0 d	135.9 b	159.4 a	125.7 c	119.4 c
	NDW	158.9 f	132.1 ef	202.5 e	262.9 d	413.8 b	515.2 a	384.7 bc	332.5 c
	SDW	10.2 g	14.3 f	16.7 ef	18.7 de	26.1 b	36.8 a	23.6 bc	20.7 cd
	Yield	3092.0 g	3326.8 f	3454.0 e	3558.3 e	4008.2 b	4332.1 a	3851.9 c	3677.5 d

Total nodule number (NN), total nodule dry weight (NDW), shoot dry mass (SDW) and yield were evaluated in the following units: nº $plant^{-1}$, mg $plant^{-1}$, g $plant^{-1}$ and kg ha^{-1}. Averages followed by the same letter in the line do not statistically differ from each other by LSD test ($p \leq 0.05$)

Legend: *ECR* Brazilian edaphoclimatic region; *NI* Non-inoculated; *NI + N* Non-inoculated + Nitrogen; *Brady Bradyrhizobium* SEMIA 5079 (*B. japonicum*) + SEMIA 5080 (*B. diazoefficiens*); Azo = *A. brasilense*; Azo Mix = *A. brasilense* IH1 + HM053 + HM210

at ECR-301 site, only the addition of AbV5/AbV6 resulted in a significant increase of 23.3% compared to the single inoculation with *Bradyrhizobium.* In both sites, the inoculated treatments induced higher NN and NDW than the non-inoculated and non-inoculated + N controls (Table 2).

For shoot dry weight (SDW), the non-inoculated and non-inoculated + N controls presented the lowest and the highest values, respectively, except at ECR202, where both controls presented the lowest values. The non-inoculated treatment showed a negative correlation with SDW in the PCA analysis (p value < 0.001) (Fig. 2). Comparing single and co-inoculation treatments, at ECR-401 and ECR-202, the additional presence of *Azospirillum* strains positively influenced SDW as long as the novel strains were present (Table 2). Again, the strain HM053 showed the highest values at ECR-202; its additional inoculation more than doubled the SDW when it is compared to single inoculation.

Effect of co-inoculation with *A. brasilense* strains on soybean yield

In the experiment carried out at ECR-401, all treatments that received co-inoculation with *A. brasilense* strains presented higher yield than the treatment that received single inoculation with *Bradyrhizobium*. The co-inoculation promoted a significant increase from 8 to 12.4% in productivity, leading a yield up to 4,140 kg ha^{-1}.

At ECR-301, ECR-302 and ECR-202, the majority of *A. brasilense* strains induced higher yield than the single inoculation with *Bradyrhizobium*. At ECR-301, the additional inoculation with AbV5/AbV6, IH1 and HM210, promoted a productivity increase of 7.4%, 4.7% and 7.3%, respectively.

At ECR-302 and ECR-202, the highest productivity was obtained with the co-inoculation of *A. brasilense* HM053. It increased the yield in 16.9% and 25.4%, respectively, in relation to the single

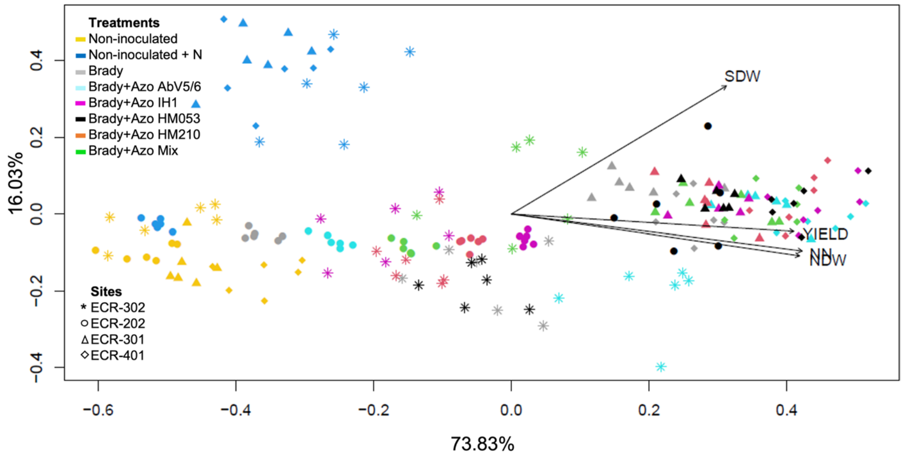


Fig. 2 Co-inoculation of *A. brasilense* HM053 improves soybean nodulation and productivity. Ordination of sites and treatments based upon normalized biometrics parameters and yield by principal components analysis. Variables with correlation r > 0.8, *p* value < 0.001 for significance after 999 permutations are displayed. Each vector points to the direction of increase for a given variable and its length indicates the strength of the correlation between the variable and the ordination scores. Legend: NN = number of nodules; NDW = total nodule dry weight; SDW = shoot dry mass; ECR = Brazilian edapho-climatic region; Brady = *Bradyrhizobium* SEMIA 5079 (*B. japonicum*) + SEMIA 5080 (*B. diazoefficiens*); Azo = *A. brasilense*; Azo Mix = *A. brasilense* IH1 + HM053 + HM210

inoculation with *Bradyrhizobium*. In addition, all the other novel strains showed a productivity superior or equivalent to the AbV5/AbV6 at these two sites. In general, at all four sites, the controls (non-inoculated and non-inoculated + N) presented the lowest productivities and they showed a negative correlation with yield in the PCA analysis (*p* value < 0.001) (Fig. 2).

The overall average grain yield across all evaluated sites and treatments illustrated in Fig. 3, highlights the yield increments associated with the combination of *Bradyrhizobium* and *A. brasilense* compared to the non-inoculated control. The highest yield, reaching 4,196.5 kg ha^{-1} were observed in treatments co-inoculated with HM053 (Fig. 3). This co-inoculation resulted in yield increases of 48.7% compared to the non-inoculated control.

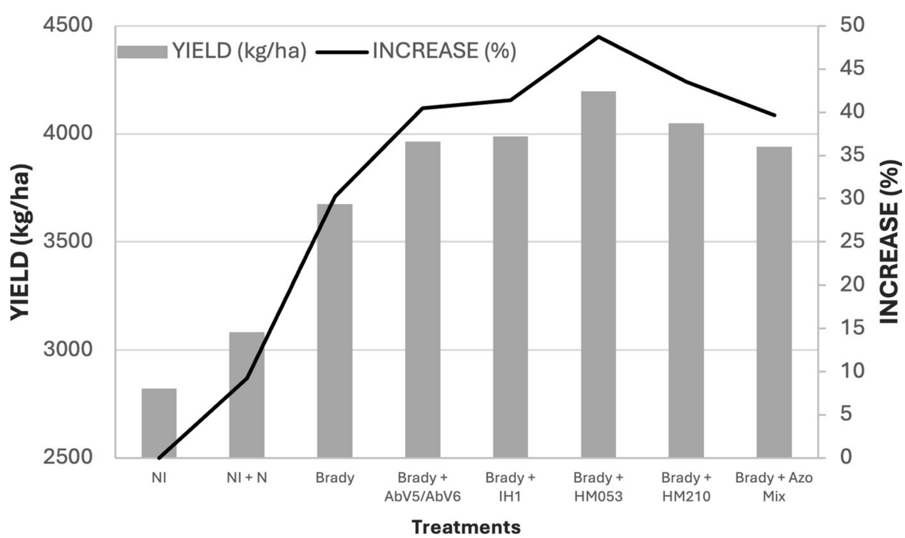


Fig. 3 Effects of *A. brasilense* co-inoculation on the grain yield of soybean. Data represent the means values of all field sites at ECR-301, ECR-401, ECR-302 and ECR-202. More details are given in the Table 2

IAA production by *A. brasilense* strains

Studies on foliar co-inoculation with *A. argentinense* Az39 showed that the *ipdC* mutant did not induce the same effect on soybean nodulation by *Bradyrhizobium* or plant growth as the wild-type strain (Puente et al. 2018). This prompted us to investigate IAA production in the novel strains of *A. brasilense*.

Comparing IAA biosynthesis among *A. brasilense* strains, HM053 exhibited higher production than AbV5/AbV6 over the evaluated period. Additionally, although HM210 strain showed slower production than the other strains, it reached or surpassed AbV5/AbV6 after 72 h (Fig. 4). The IH1 strain exhibited IAA production similar to that of AbV5/AbV6 in the presence of 50 µg/mL of Trp, but it was lower when 100 µg/mL of Trp was added (Fig. 4). This profile was obtained evaluating IAA in µg/mL or µg/logCFU. This result suggests that the effects on plant physiology and nodulation may, in part, be attributed to the distinct patterns of IAA production observed in HM053 and HM210.

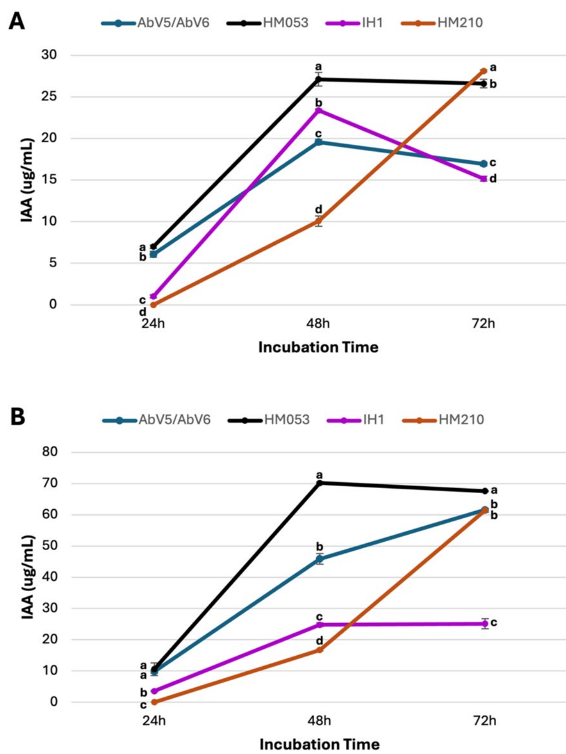


Fig. 4 IAA biosynthesis of *A. brasilense* AbV5/AbV6, HM053, IH1, and HM210 strains along 72 h of growth in NFbHPN medium supplemented with 50 µg/mL (**A**) or 100 µg/mL (**B**) of tryptophan. Data represent the average of three replicates. Averages followed by the same letter in each evaluated time do not statistically differ from each other by LSD test ($p \leq 0.05$)

Discussion

The results of this study highlight two key findings regarding the performance of *Azospirillum* co-inoculation in soybean. First, all five *Azospirillum* treatments significantly increased yield compared to the inoculation with *Bradyrhizobium* alone, with HM053 being the most effective yield enhancer. Second, the yield improvements associated with the different *A. brasilense* strains varied by site and soybean variety.

Regarding the first key finding, *Azospirillum* treatments not only increased yield but also enhanced other growth parameters. Based on our four experiments, co-inoculation with AbV5/AbV6 increased NN by 32% and NDW by 15% compared to single inoculation. Co-inoculation with HM053 produced even greater improvements, boosting NN by 51% and NDW by 38% relative to single inoculation (Table 2). These results exceed those reported in a meta-analysis where co-inoculation of soybean with *Azospirillum* spp. resulted in a 11.05% increase in nodule number and 14.65% in nodule biomass (Zeffa et al. 2020).

In all tested sites, the harmful effect of nitrogen fertilization on the NDW was detected in the treatment non-inoculated + N which received the dose of 200 kg N ha^{-1} in the form of urea. It agrees with Hungria and Vargas (2000) who shows that the application of mineral N in doses greater than 20 kg ha^{-1} at the sowing furrow can reduce nodulation and the efficiency of BNF.

A. brasilense strain HM053 also increased soybean shoot dry weight at ECR-202 (Table 2 and Fig. 2). While specific studies in soybean with HM053 are lacking, studies in other crops have already highlighted HM053 strain efficacy in promoting plant growth and fixing nitrogen. (Santos et al. 2017b) showed in wheat that HM053 strain increased shoot and root dry weight by 30 and 49%, respectively, when compared to non-inoculated plants and by 30 and 31% when compared to the non-ammonium-excreting parent strain FP2.

Regarding specific site responses, in relation to the second key finding, all co-inoculation treatments with novel *A. brasilense* strains at ECR-202 resulted in higher NN, NDW, SDW, and yield compared to both the single *Bradyrhizobium* inoculation and the standard *A. brasilense* strains AbV5/AbV6. Notably, the good development of the nodules with the co-inoculation of HM053 reflected in the plant development and yield at this site (Table 2 and Fig. 2). In contrast, at ECR-302, while co-inoculation with AbV5/AbV6 produced the highest NN and NDW values, it did not translate to higher yield. HM053 provided superior yield performance at both ECR-202 and ECR-302, outperforming all other treatments, including the commercial strains AbV5/AbV6. At the remaining sites, its performance was comparable to that of AbV5/AbV6. IH1 and HM210, outperformed AbV5/AbV6 at one site, ECR-202, and showed similar performance to them at the other sites. These results highlight HM053 as the superior yield enhancer and suggest that IH1 and HM210 may provide benefits under specific conditions, warranting further investigation into their specific contributions to soybean growth and productivity. These findings underscore the importance of considering the environmental when evaluating the effectiveness of co-inoculation strategies.

Meta-analyses of field experiments conducted in Brazil indicated a 3.2% increase in soybean grain yield with co-inoculation of AbV5/AbV6 compared to single inoculation (Barbosa et al. 2021). In the current study, the overall average grain yield across all sites increased by 7.9% with AbV5/AbV6 compared to single inoculation (Fig. 3). Moreover, this yield enhancement was further amplified to 14.2% with the strain HM053, which translates to an additional production of 522.1 kg ha^{-1} compared to the single inoculation treatment (Fig. 3). Considering current soybean price in Brazil (August/24), co-inoculation with the novel *Azospirillum* strains would result in gains ranging from US\$127 to US\$212 ha^{-1}, highlighting HM053 as the most profitable option.

To our knowledge, no studies have yet investigated the inoculation of these novel *A. brasilense* strains in soybeans. Several studies have shown that co-inoculation strategies can enhance precocity of nodulation, abiotic stress tolerance, root system development, and plant growth, thereby improving soybean productivity (Chibeba et al. 2015; Cerezini et al. 2016; Barbosa et al. 2021; Santos et al. 2021). Such effects suggest the involvement of phytohormones produced by *A. brasilense*. Indeed, an *ipdC* mutant of *A. argentinense* Az39, lacking the enzyme involved in indole-3-pyruvate decarboxylation, may not confer the same benefits in co-inoculation (Puente et al. 2018). In agreement with this suggestion, we show that HM053 has a superior ability to produce IAA (Fig. 4) which likely enhances the hormonal effect on the host plant, optimizing root and nodule development (Fig. 2). *A. brasilense* HM053 exhibits constitutive nitrogenase activity and excretes ammonium, accumulating millimolar concentrations in semi-solid medium (Machado et al. 2011). The availabily of ammonium, particularly at early growth stages and in nitrogen-deficient soils, could complement the soybean's symbiotic relationship with *Bradyrhizobium*. Additionally, *A. brasilense* HM053 has been shown to promote increased absorption of iron and manganese in maize (Housh et al. 2021, 2022), which could lead to improvements in stem diameter, leaf thickness, and chlorophyll content, potentially boosting both plant growth and yield. Thus, *A. brasilense* strain HM053 ability to excrete ammonium and produce phytohormones may act synergistically with *Bradyrhizobium* to maximize soybean growth and yield.

Future studies could focus on: quantifying nitrogen fixation and evaluating the effect of IAA production by the HM053 strain in co-inoculation with *Bradyrhizobium* in soybean; assessing the effects of HM053 on nodulation, root development, and soybean yield under more cultivation conditions; tracking and quantifying HM053 across different inoculation methods during soybean development, as done in previous maize studies (Urrea-Valencia et al. 2021; Takahashi et al. 2024). A detailed understanding of this complex interaction could lead to the development of more effective inoculation strategies, harnessing the synergistic potential between *A. brasilense* and *Bradyrhizobium* to optimize soybean production.

Conclusions

Despite the variations in climatic and soil conditions across the study sites, the novel *A. brasilense* strains, particularly HM053, exhibited significant potential to enhance soybean nodulation and grain yield, positioning it as a promising candidate for co-inoculation in soybean cultivation. Our results suggest that the combined traits of ammonium excretion and elevated IAA synthesis in the HM053 strain synergistically enhance the growth-promoting effects of *A. brasilense* when co-inoculated with soybean. Although information on the impact of introducting *Azospirillum* into plant-soil systems and their established microbial communities remains limited, the results obtained with the Poaceae and Fabaceae species of economic importance, highlight the ammonium-excreting *A. brasilense* HM053 as an important Plant Growth-Promoting Rhizobacterium for the sustainable management of tropical agriculture.

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Data Availability All data are available in the main manuscript or in the supplementary material.

Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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