The Interaction between *Bradyrhizobium Japonicum* E109 and *Azospirillum Brasilense* Az39 Improves *Bradyrhizobium*-Soybean Symbiosis: The Secrets Behind Co-Inoculation

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Abstract

Aims

The aim of this work was evaluating the interaction between two of the most widely used strains for soybean inoculation in Argentina, *B. japonicum* E109 (*Bj*E109) and *A. brasilense* Az39 (*Ab*Az39).

Methods

Five treatments were performed: (i) uninoculated seeds; (ii) seeds inoculated with *Bj*E109; (iii) seeds inoculated with *Ab*Az39; (iv) seeds co-inoculated with *Bj*E109 and *Ab*Az39 in a 1:1 proportion (*Bj*E109 + *Ab*Az39) at the seeds sowing and (v) seeds inoculated with a 1:1 proportion of *Bj*E109 and *Ab*Az39 (*Bj*E109-*Ab*Az39) 24 h before seeds sowing. Each treatment was assessed through a seed recovery assay, glasshouse assays and field assays.

Results

The combination between the two strains improved the ability of *Bj*E109 to survive on soybean seeds after inoculation partially explained by *Ab*Az39's capacity to produce diverse biologically active molecules. As a result of the greater rhizobial survival on seeds the nodulation values and symbiosis parameters like nodule number, size and biomass and nodulation percentage also increased. In agreement with these observations, combining *Bj*E109 and *Ab*Az39 at strains the grain yield under field conditions were 13,3 and 17,3% greater than single *Bj*E109 inoculation.

Conclusions

These results here show that the pre-culture combining *Bj*E109 and *Ab*Az39 before the inoculation to the soybean seeds has benefits in plant nodulation and hence production, more than individual inoculation with *Bj*E109 or *Ab*Az39, or the immediate co-inoculation of both strains.

2. Introduction

Rhizobia are diazotrophic bacteria whose symbiotic interactions with plants from the Fabaceae family result in the formation of specific structures where biological nitrogen fixation (BNF) takes place (Stacey et al. 1992). Some rhizobia, mostly belonging to the genus *Bradyrhizobium* such as *B. japonicum*, have been extensively studied in terms of their association with soybean as sustainable alternatives to N-fertilizers (Hungria and Nogueira 2019). On the other hand, *Azospirillum* are plant-growth promoting bacteria that have attracted particular attention over the last four decades because they improve legume growth, development, and yield when co-inoculated with rhizobia (Bashan and de Bashan 2010). These positive effects are mostly related to *Azospirillum*'s ability to produce phytohormones such as auxins (Crozier et al. 1988; Zimmer and Bothe 1988), cytokinins (Horemans et al. 1986; Cacciari et al. 1989), gibberellins (Bottini et al. 1989; Janzen et al. 1992), abscisic acid and ethylene (Perrig et al. 2007), as well as certain polyamines such as cadaverine (Cassán et al. 2009). The production of auxins, mainly indole-3-acetic acid (IAA), is considered the most important plant-growth promoting mechanism in *A. brasilense* (Cassán et al. 2014). However, in the case of *Bradyrhizobium*, the mode of action is still unclear, but many benefits for the plant have been described yet (Jaiswal et al. 2021). The practice of co-inoculation could improve plant performance and the establishment of symbiosis under both controlled and field conditions (Ferri et al. 2017;

Rodelas et al. 1999; Remans et al. 2008; Cassán et al. 2020). Hungria et al. (2013) and Nogueira et al. (2018) reported an increase in soybean [*Glycine max* (L.) Merrill] grain yield of over 15% and 200 kg.ha^{-1}, respectively, through a combination of *Bradyrhizobium* and *Azospirillum* as *A. brasilense*. Studies on soybean coinoculation in Argentina and Brazil also found respective increases in nodulation of over % (Hungria et al. 2015; Fipke et al. 2016; Galindo et al. 2018) and over 1 % (Benintende et al. 2010; Ferraris and Couretot 2011, 2013; Morla et al. 2019). Therefore, *Azospirillum*'s ability to produce phytohormones like auxins and cytokinins, and the morphofunctional changes thus induced in the soybean root system (Rondina et al. 2020) enhance the benefits of *Bradyrhizobium*-soybean symbiosis (Srinivasan et al. 1996; Molla et al. 2001; Vessey and Buss 2002). In the specific case of *B. japonicum* E109, analysis of its genome revealed the existence of three putative pathways for IAA biosynthesis, but the bacterium is unable to produce it, and can instead degrade both natural and synthetic auxins, including IAA and NAA (Torres et al. 2018). Nevertheless, its biomass and exopolysaccharide production increased with the exogenous addition of IAA to the liquid culture medium where it was grown, which in turn modified its symbiotic behavior with soybean: more nodules were created, and a higher percentage of plants were nodulated (Torres et al. 2018). This supports the idea that it is the active compounds synthesized by *Azospirillum* which improve nodulation by *Bradyrhizobium*, yet the mechanisms underlying these effects remain poorly understood. With this in mind, we aimed to elucidate whether symbiosis between *Bradyrhizobium* and soybean is effective at least in part due the release of active molecules by co-inoculated *A. brasilense*, and to investigate some particularities behind this kind of co-inoculation.

3. Methodology

3.1. Bacterial strains

Two selected strains were *Bradyrhizobium japonicum* E109 (*Bj*E109) and *Azospirillum brasilense* Az39 (*Ab*Az39). Both bacteria were provided by the Institute for Agricultural Microbiology and Zoology in Castelar, Buenos Aires, Argentina (IMYZA for its name in Spanish). These strains are two of the most currently used for inoculant formulations in Argentina and Brazil (Lodeiro 2015; Cassán et al. 2020). Their complete genome sequences have been published by Rivera et al. (2014) and Torres et al. (2015), respectively.

3.2. Plant material

Seeds of soybean [*Glycine max* (L). Merrill] var. "Don Mario 3810 RR" were used for these studies. Quality control parameters were established by the International Seed Test Association (ISTA) (http://www.ista.org).

3.3. Bacterial growth and seed inoculation

Seeds were inoculated with individual or combined cultures of *Bj*E109 and *Ab*Az39, according to the treatments described below. The *Bj*E109 titer was adjusted to 5.0 E+9 cfu.ml^{-1} obtained at late exponential growth phase in yeast mannitol broth (YEM), as described by Vincent (1970). The *Ab*Az39 titer was adjusted to 5.0 E+8 cfu.ml^{-1} obtained at late exponential growth phase in Luria broth (LB), as described by Molina et al. (2018). Inoculation doses were adjusted to obtain a final volume of 12 ml.kg^{-1} of soybean seeds in all experiments. Five treatments were performed: *i*) uninoculated seeds treated with phosphate buffer solution (12 ml.kg^{-1}) (control); *ii*) seeds inoculated with equal volume of *Bj*E109 and phosphate buffer solution (1:1) (*Bj*E109); *iii*) seeds inoculated with equal volume of *Ab*Az39 and phosphate buffer solution (1:1) (*Ab*Az39); *iv*) seeds co-inoculated with *Bj*E109 and *Ab*Az39 in a 1:1 ratio (*Bj*E109 + *Ab*Az39), and *v*) seeds inoculated with a mix, in a 1:1 ratio, of *Bj*E109 and *Ab*Az39 (*Bj*E109-*Ab*Az39). In the treatment *iv*) each microorganism was applied separately during the inoculation process, while for the

treatment *v*) equal volumes of both microorganisms were mixed and maintained at room temperature (25°C) during 24 h before the inoculation of the seeds. The incubation time for treatment *Bj*E109-*Ab*Az39 was established following previous results by Torres et al. (2018) about the ability of *Bj*E109 to degrade 40 µg.ml^{-1} IAA and increase its own exopolysaccharide (EPS) synthesis and biomass production in YEM culture medium. We also evaluated each microorganism without dilution as a control. After the application of the treatments, the seeds were maintained under sterile laminar air flow conditions at room temperature (25ºC) until analysis.

3.4. Bacterial count and physiological state

To determine the number of viable cells (cfu.ml^{-1}) in each culture or in the mix of both, the microdrop quantification method was performed (Miles and Misra 1938). Plates containing YEM medium (Vincent 1970) or Congo Red medium (Rodríguez Cáceres 1982) were used for *Bj*E109 and *Ab*Az39, respectively. The plates were analyzed after incubation at 30°C for 7 days in the case of *Bj*E109 and 37°C for 4 days in the case of *Ab*Az39, as described by Cassán et al. (2014) with modifications. In the case of the combined cultures, plates containing both YEM and Congo Red medium were used at temperatures and incubation time mentioned before.

3.4.1. IAA quantification

Quantification of IAA was performed by spectrophotometry (Glickmann and Dessaux 1995) and confirmed by HPLC following Torres et al. (2021). Aliquots of 1000 µl of bacterial culture were centrifuged at 11,300 *g* for 10 min. Next, the samples were filtered (0.2 µm) and 500 µl of the supernatant were mixed with 500 µl of Salkowski´s reagent and gently shaken in an inverted position at least 10 times. The samples were incubated in the dark for 30 min and absorbance was measured at 530 nm. An aliquot of filtered supernatants was injected into the HPLC equipment as per Torres et al. (2021).

3.4.2. Exopolysaccharide (EPS) quantification

Quantification of EPS followed Torres et al. (2018). Briefly, the samples were centrifuged and then the supernatants were filtered and treated with DNase I and proteinase K. The EPSs were precipitated with ethanol and dried at room temperature before being resuspended in deionized water. Total carbohydrate content (EPS.mg^{-1} biomass) was measured through the phenol-sulfuric acid method, with glucose as a standard.

3.5 Seed recovery assays

Soybean seeds that had been either inoculated or co-inoculated (section 3.3) were maintained in aseptic environmental conditions at room temperature (≅ 25°C) for 4 hours before initial analysis and then 6 days before a second analysis, as described by Torres et al. (2018). Bacterial cell count and survival factor percentage (SFP) were determined for *Bj*E109 and *Ab*Az39 following the criteria by Penna et al. (2011). The plates were incubated at 30°C for 7 days in the case of *Bj*E109 and at 37°C for 4 days in that of *Ab*Az39. Additional counts were performed on uninoculated seeds (control). Results are expressed as the number of viable cells recovered from the seeds (cfu.seed^{-1}) and the survival factor percentage (SFP) as the cfu.seed^{-1} 4 h after inoculation, according to Penna et al. (2011).

3.6. Greenhouse assays

The treated soybean seeds from each treatment listed in section 3.3 were sown in plastic pots (300 ml volume capacity) containing vermiculite as a solid substrate. Three seeds per pots were planted in six pots and irrigated with sterile N-free Hoagland's solution (25% v/v) (Hoagland and Boyer 1936) keeping them at field capacity during the

study. The seedlings were maintained inside a growth chamber for 21 days with a photoperiod of 16/8 h light regime, 30/20°C temperature and a relative humidity of 80%. At the end of the experiment, the following parameters were measured: (1) number of nodules located on main root per plant (MRN), (2) number of nodules located on secondary roots per plant (SRN), (3) total number of nodules on roots per plant (RN), (4) percentage of plants with three or more nodules on the main root (BT), following Burton et al. (1976), and (5) shoot and root dry weight (SDW and RDW).

3.7. Field assay

A field experiment was performed during the 2018/19 cropping season close to 9 de Julio, Buenos Aires province, Argentina (38°28'S, 60°57'W). The soil was classified as silty-loam Typic Argiduoll under agriculture with wheat (*Triticum aestivum* L.), soybean and maize (*Zea mays* L) in rotation since more than 20 years. Before the sowing, topsoil samples (0–20 cm) were collected and analyzed in terms of water pH (5,9); organic matter content (25,4 $g.dm^3$); cation exchangeable capacity (CEC) (3,74); C (5,34 $g.dm^3$); extractable P, according to Bray and Kurtz (1945), (3,1 $mg.dm^3$); S-SO_4 (4,3 $mg.dm^3$); extractable cations [Ca (6,42 $cmol_c.dm^3$); Mg (1,28 $cmol_c.dm^3$); K (1,12 $cmol_c.dm^3$) and Na (0,24 $cmol_c.dm^3$)]; extractable Zn (1,24 $cmol_c.dm^3$) and extractable Mn (15,8 $mg.dm^3$) according to Lindsay and Norwell (1978); extractable Cu (0,77 $mg.dm^3$); extractable Fe (68,4 $mg.dm^3$) and extractable B (0,60 $mg.dm^3$). The regular crop calendar and standard field practices were followed. On October 20th, 2018, treated seeds according to the treatments described in section 3.3 were directly sown at a rate of 18 seeds m^{-1} and fertilized in furrow with 60 $kg.ha^{-1}$ of triple superphosphate (0:46:0). The seeds were not chemically treated with fungicides, insecticides, micronutrients or other products. The experiment was placed in randomized blocks with six replicates per treatment. The plots consisted of 4 lines of 8.0 m in length width spaced 0.52 m apart. The crops were kept free of weed, pest and diseases competence using regular integrated management practices when needed. Approximately 7 weeks after the emergence of the crops, in a sub-sample of 20 plants per treatment at the v6 growth stage (Ritchie et al. 1994), the nodule number, its location, size and dry weight were evaluated. Also, the plant biomass (weight) and intensity of the green color in SPAD meter units of the upper leaves (Markwell et al. 1995) were measured. After reaching crop physiological maturity (R8) on March 11th, 2019, the central rows of the plots (1 m^2) were manually harvested. The grain yield was expressed at 140 g kg^{-1} of moisture content. The single grain weight and the nitrogen concentration in the grains was measured to estimate the protein grain content.

3.8. Statistical analysis

The treatments for greenhouse experiments were performed in with six replicates per treatment, while the field experiment was placed in randomized blocks with six replicates per treatment. The values shown represent the mean ± standard error of mean (SEM). The data was analyzed for variance factor differences using ANOVA followed by Tukey's *post hoc* analysis at $p<0.05$. The statistical analyses were made using the PRISM V 4.0 statistical package for Windows®. The matrix correlation was performed using RStudio software (version 1.4.1717, © 2009-2021 Rstudio, PBC) using the packages *factoextra* and *corrplot*.

4. Results

4.1. Bacterial count and physiological state

To define an experimental model based on the interaction between *Bj*E109 and *Ab*Az39, mixes of different proportions of each strain (1:9, 1:1 and 9:1) were evaluated for the EPS production, microbial biomass and IAA concentration in the culture medium after 24 h interaction. The results of this assessment are showed in

Supplementary material (Table S1). An equal proportion of each microorganism (1:1) was selected, since it rendered the highest production of biomass and EPS, the two parameters of our main interest in this experiment according to Torres et al. (2018).

Table 1 summarizes the cell number, EPS content, IAA concentration and biomass production of *Bj*E109 and *Ab*Az39 cultures obtained individually (control), in combination (1:1) with buffer phosphate (PB), or with both bacteria incubated for 15 minutes (T0) or 24 h (T24).

Table 1

Cell number, EPS production, IAA concentration and biomass production of *Bj*E109 and *Ab*Az39 cultures obtained individually (control), in combination (1:1) with buffer phosphate (PB), or with both bacteria incubated for 15 minutes (T0) or 24 h (T24). Different letters represent significant differences according to Tukey test p< 0.05.

Treatment	**log_{10} cell number *Ab*Az39 cfu.ml^{-1}**	**log_{10} cell number *Bj*E109YEM cfu.ml^{-1}**	**EPS µg.ml^{-1}**	**IAA µg.ml^{-1}**	**Biomass production OD_{595}**
*Ab*Az39-Control	8.710± 0.012^{a} **5.12e+8**	*	1.05±0.09^{e}	15.23±1.21^{a}	1.83 ± 0.12^{d}
*Bj*E109-Control	*	9.710± 0.013^{a} **5.12e+9**	3.45±0.10^{b}	*	2.41 ± 0.04^{b}
*Bj*E109 + PB (1:1) - **T0**	*	9.387 ± 0.023^{b} **2.44e+9**	1.87±0.22^{d} **-45.79**	*	1.33 ± 0.18^{e} **-44.81**
*Ab*Az39 + PB (1:1) - **T0**	8.377 ± 0.007^{b} **2.38e+8**	*	0.57±0.12^{f} **-45.71**	8.62±2.03^{b} **-43.40**	1.01 ± 0.2^{f} **-44.80**
*Bj*E109 + *Ab*Az39 (1:1) - **T0**	8.390 ± 0.019^{b} **2.45e+8**	9.399 ± 0.026^{b} **2.51e+9**	2.64±0.14^{c} **+8.19**	7.54±1.38^{b} **-12.52**	2.21 ± 0.09^{c} **-5.55**
*Bj*E109 + *Ab*Az39 (1:1) - **T24**	8.403 ± 0.033^{b} **2.53e+8**	9.416 ± 0.015^{b} **2.61e+9**	5.73±0.17^{a}	*	2.68 ± 0.07^{a}
(*) not identified					

Production of EPS and IAA by *Ab*Az39 was as expected for the biomass and cell count measured in the growth medium, as well as with reference to previous studies under similar conditions (Rivera et al. 2018; Molina et al. 2020). *Bj*E109's production of EPS was three times higher than that of *Ab*Az39 and no IAA was detected in its growth medium, as reported before (Torres et al. 2018). When the pure cultures of each strain were mixed with phosphate buffer (1:1), the values of all the parameters assessed decreased around 50% with respect to the undiluted cultures, so synergic or antagonistic interactions with the buffer should not be ruled out. The 1:1 co-inoculation of both strains at T0 led to a 29.1% and 39.8% increase in EPS and biomass, respectively, with respect to the *Bj*E109 culture diluted in phosphate buffer (1:1), which could be ascribed to the presence of the growth medium for *Ab*Az39. Contrarily, in the co-inoculation the IAA levels were 14.3% lower than in the *Ab*Az39 culture diluted in phosphate buffer (1:1), and this may be attributed to *Bj*E109's ability to degrade the hormone. When the T24 mix was examined,

the values of EPS content and biomass were still increased with three and two times higher than in the *Bj*E109 phosphate buffer culture, respectively, likely due to the interaction between both bacteria. IAA concentration, meanwhile, had fallen below detectable levels because of *Bj*E109's degrading activity.

4.2 Seed recovery assays

Table 2 summarized the values for cell recovered and survival factor on seeds treated with the strains individually or in combination.

Table 2
Cell recovery and survival factor (SFP) of *Bj*E109 and *Ab*Az39 4 hours or 6 days after being inoculated on soybean seeds, either individually (control) or in combination (1:1) after 15 minutes (T0) or 24 hours (T24) of interaction. Different letters represent significant differences according to Tukey test p< 0.05.

Strains and Treatments	***Bj*E109**				***Ab*Az39**			
	4 hours		**6 days**		**4 hours**		**6 days**	
	cfu.seed^{-1} log$_{10}$	**SFP (%)**	**cfu.seed^{-1} log$_{10}$**	**SFP (%)**	**cfu.seed^{-1} log$_{10}$**	**SFP (%)**	**cfu.seed^{-1} log$_{10}$**	**SFP (%)**
Naked seeds	*	*	*	*	*	*	*	*
*Ab*Az39-Control	*	*	*	*	**7,43e+3** 3,870±0,041^{b}	**4,87**	**< 1e+3**	**nd**
*Bj*E109-Control	**2,23e+5** 5,349±0,011^{c}	**14.54**	**1,15e+4** 4,071±0,16^{c}	**0.80**	*	*	*	*
*Bj*E109 + *Ab*Az39 (1:1) - **T0**	**3,05e+5** 5,485±0,009^{b}	**19.88**	**7,20e+4** 4,856±0,004^{b}	**4.69**	**1,04e+4** 4,016±0,054^{a}	**5,93**	**< 1e+3**	**nd**
*Bj*E109 + *Ab*Az39 (1:1) - **T24**	**5,47e+5** 5,349±0,011^{a}	**35.36**	**1,35e+5** 5,013±0,042^{a}	**8.81**	**1,16e+4** 4,062±0,055^{a}	**6,50**	**< 1e+3**	**nd**
(*) not identified; (**) not evaluated; (nd) not calculated; <1e+3: less than detection limit								

No microorganisms compatible with the ones used in this study could be detected on untreated seeds. On seeds inoculated with *Ab*Az39, viable bacteria and the survival factor percentage (4.87%) could be measured only 4 h after inoculation and were lower than those obtained for seeds inoculated with *Bj*E109. The survival factor 4 h post inoculation was 14.54% and 0.8% after six days, i.e., survival was longer despite the significant fall since the 4-day measurement. The 4 h post-inoculation value for *Bj*E109 was ramped up by 36.77% when it was inoculated at the same time as *Ab*Az39 in a 1:1 proportion (T0), while the 6 d post-inoculation value rose almost two times under the same conditions. Co-inoculation at T0 measured 4 h after inoculation also increased *Ab*Az39's survival by 25%. The pattern observed here suggests both strains have better chances of surviving when they can interact among each other on the seeds.

In the 1:1 mix inoculated at T24, the survival rate for *Bj*E109 rose approximately 75% 4 h post inoculation and 90% 6 d post-inoculation with respect to the values obtained at T0. The differences were bigger if these results were

compared to the individual *Bj*E109 inoculation, reaching 2-fold and 10-fold higher values, respectively. The T24 mix, moreover, increased *Ab*Az39's count and survival by around 9% with respect to the T0 treatment, and 33% with respect to the strain inoculated on its own. These results confirmed the mutual benefit relationship between the two strains for longer survival.

Figure 1 shows the recovery at different post-inoculation times of viable *Bj*E109 cells on seeds inoculated with the strain on its own or in combination with *Ab*Az39 (1:1), 15 m after the mix was prepared (T0) o 24 h later (T24). Grey bars represent recovery after treatment with the T24 mix, considered the most successful as regards the survival values up to 15 days after inoculation compared to individual treatment, and 9 days compared to T0. When *Bj*E109 was inoculated on its own, recovery was indeed possible but at very low values and only up to 6 days after inoculation. The difference between the T24 and T0 treatments was at its peak immediately after inoculation, and still observable though ever decreasing between day 3 and day 15 after inoculation.

4.3 Greenhouse assays

The data shown in the Table 3 contains the growth and nodulation values for soybean seedlings 4 hours or 6 days after inoculation with either *Bj*E109 or *Ab*Az39, individually, or the combination of both at T0 or T24.

Table 3
Effects on soybean seedling growth and nodulation 4 hours after being inoculated with *Bj*E109 and *Ab*Az39 individually (control) or in combination (1:1) after 15 min (T0) or 24 h (T24) of interaction. Different letters represent significant differences according to Tukey test p< 0.05.

Treatments	MRN	SRN	RN	SDW	RDW	BT
	4 hours after inoculation					
Naked seeds	*	*	*	0.169±0.005^{d}	0.069±0.005^{e}	*
*Ab*Az39-Control	*	*	*	0.172±0.008^{d}	0.079±0.003^{d}	*
*Bj*E109-Control	13.21±0.42^{c}	11.43±0.33^{c}	24,64±0.75^{c}	0.264±0.007^{c}	0.104±0.009^{c}	84.0%
*Bj*E109 + *Ab*Az39 (1:1) - **T0**	14.58±0.36^{b} **+10.3**	12.75±0.19^{b} **+11.54**	27,33±0.55^{b} **+10.9**	0.289±0.006^{b} **+9.4**	0.127±0.011^{a} **+22.1**	91.0% **+7.0**
*Bj*E109 + *Ab*Az39 (1:1) **T24**	15.44±0.29^{a} **+16.8**	13.92±0.61^{a} **+21.7**	29,36±0.9^{a} **+19.15**	0.301±0.005^{a} **+14.01**	0.119±0.003^{b} **+14.42**	96.0% **+12.0**
	6 days after inoculation					
Natural seeds	*	*	*	0.137±0.006^{d}	0.041±0.011^{e}	*
*Ab*Az39-Control	*	*	*	0.145±0.012^{d}	0.057±0.004^{d}	*
*Bj*E109-Control	6.35±0.39^{c}	13.21±0.52^{c}	19,56±0.91^{c}	0.218±0.004^{c}	0.104±0.003^{b}	58.0%
*Bj*E109 + *Ab*Az39 (1:1) **T0**	7.44±0.19^{b} **+17.16**	16.01±0.39^{b} **+21.19**	23,45±0.98^{b} **+19.88**	0.237±0.008^{b} **+8.71**	0.123±0.09^{a} **+18.26**	67.0% **+9.0**
*Bj*E109-*Ab*Az39 (1:1) **T24**	8.16±0.23^{a} **+28.5**	17.97±0.70^{a} **+36.03**	26,13±0.93^{a} **+22.95**	0.245±0.003^{a} **+12.38**	0.117±0.008^{c} **+12.5**	74.0% **+16.0**

MRN: Main root nodules.plant^{-1}; SRN: Secondary root nodules.plant^{-1}; RN: Root nodules.plant^{-1}; SDW: Shoot dry weight.plant^{-1}; RDW: Root dry weight.plant^{-1}; BT: Percentage of nodulated plants (Burton et al., 1972).

No nodules were formed on the uninoculated controls, which corroborates the absence of *Bradyrhizobium* in the substrate. The significant improvement in both belowground (RDW) and aboveground biomass (SDW) of the plants inoculated with either *Bj*E109 or *Ab*Az39 evidence the positive effects of inoculation, at any of the two times assessed. Meanwhile, the combined 1:1 inoculation at T0 produced 10.3% more nodules on the main root (MRN) and 11.5% more on the secondary roots (SRN) 4 h after inoculation than the individual treatment with *Bj*E109. The plant biomass was also benefited with an increase of shoot (SDW) and root (RDW) dry weight of 9.4% and 22.1%, respectively. At T24, the seed inoculation using the mix of strains also improved the values of other parameters compared to T0: 6.5% in MRN; 10.2% in SRN; 9.8% in root nodules per plant (RN); 4.7% in SDW and 5.0% in the percentage of nodulated plants (BT), whereas the RDW decreased by 8.0%. The results from the comparison of the two co-inoculation treatments demonstrates that allowing the interaction between the two strains during at least 24 hours before inoculation (T24) is beneficial for the nodulation and the plant growth compared to T0 and the treatment only with *Bj*E109. Although all measurements describing the symbiosis and the plant growth significantly dropped 6 days after inoculation (6 dpi), both co-inoculation treatments continued outperforming the one with

*Bj*E109 alone, a further indication of the advantages of co-inoculation over the long term. In a similar manner to the earlier determinations, MRN, SRN and RN were 11.3%, 11.8% and 3.1% higher, respectively, 6 dpi with the T24 mix than at T0. SDW and BT were also higher (3.7% and 7.0%). The co-inoculation at T0 could only outperform the T24 treatment when it came to RDW, both 4 dpi and 6 dpi.

The Fig. 2 shows the nodulation in the roots of soybean seedlings developed from seed inoculated with either *Bj*E109 or *Ab*Az39, individually, or the combination of both at T0 or T24. Both MRN and RN significantly decreased over time regardless of the treatments. Nevertheless, the results were significantly better up to 15 days after inoculation (15 dpi) with the T24 mix than with the treatment at T0 or with *Bj*E109. The co-inoculation at T0 outperformed the seed inoculation with the individual strain, except on day 3 (3 dpi) when there were no statistically significant differences between both treatments. In summary, both mixtures, but particularly the one inoculated at T24, were able to revert the decreasing nodulation trend over time.

4.4 Field assay

The data in the Table 4 show how, under field conditions, the inoculation with either *Bj*E109 or *Ab*Az39, individually, or the combination of both at T0 or T24 modified the growth of soybeans crops and its symbiosis.

Table 4
Effects, under field conditions, on the plant growth and *Bradyrhizobium*-soybean symbiosis (nodulation) of the inoculation with *Bj*E109 and *Ab*Az39, either individually (control) or in combination (1:1) after 15 min (T0) or 24 h (T24) of interaction. Different letters represent significant differences according to Tukey test $p< 0.05$.

	Nodules							**Plants**		
	Biomass	**Size range**			**Number**			**Shoot biomass**	**Root biomass**	**Green color**
		I	**II**	**III**	**MR**	**SR**	**Total**			
Naked seeds	1.038^{d}	10.0^{d}	17.6^{d}	36.9^{d}	36.1^{d}	28.3^{e}	64.4^{e}	20.375^{d}	6.563^{c}	45.3^{a}
*Ab*Az39-Control	1.112^{c}	12.0^{d}	19.0^{d}	42.0^{c}	37.3^{d}	35.7^{d}	73.0^{d}	21.270^{d}	6.923^{c}	45.8^{a}
	+7.1%				**+3.2%**	**+26.3%**	**+13.4%**	**+4.3%**	**+5.4%**	
*Bj*E109-Control	1.425^{b}	24.8^{c}	38.6^{c}	67.1^{b}	59.6^{c}	70.9^{c}	130.5^{c}	26.780^{c}	7.775^{b}	45.9^{a}
*Bj*E109 + *Ab*Az39 (1:1) - T0	1.631^{a}	38.5^{b}	54.8^{a}	63.1^{b}	72.0^{b}	84.4^{b}	156.4^{b}	30.250^{b}	8.575^{a}	45.8^{a}
	+14.4%				**+20.8%**	**+19.0%**	**+19.8%**	**+12.9%**	**+10.2%**	
*Bj*E109 + *Ab*Az39 (1:1) - T24	1.685^{a}	49.4^{a}	50.7^{b}	70.2^{a}	78.5^{a}	91.8^{a}	188.3^{a}	31.501^{a}	8.975^{a}	46.1^{a}
	+18.2%				**+31.6%**	**+29.4%**	**+30.5%**	**+17.6%**	**+15.4%**	

Nodules, **Biomass** (g.plant^{-1}), **size range**: **I**: (> 5 mm); **II**: (2-5 mm); **III**: (< 2 mm); Nodules number: **MR**: main root; **SR**: secondary roots; Plant dry biomass: **shoot biomass** (g. plant^{-1}); **root biomass** (g. plant^{-1}), upper leaves green color intensity (SPAD units)

In the plants developed from seeds inoculated with *Bj*E109, the nodule biomass per plant increased 37.3% compared to uninoculated seeds. When the inoculation was performed with *Ab*Az39 the nodule biomass per plant was 7.1%

greater than in those developed from uninoculated seeds. After the combination of *Bj*E109 and *Ab*Az39, the biomass of the nodules was greater than the observed when single *Bj*E109 or *Ab*Az39 treatments were applied to the seeds. The co-inoculation at T0 also increased 12.9% the soybean shoot biomass compared to the individual inoculation with *Bj*E109 and 17.6% in the case T24 without differences between both combined treatments. The nodule biomass varied from 1.038 g without inoculation to 1.631 g with co-inoculation at T0 and 1.685 g at T24 representing an increase of 14.4% and 18.2% respectively. In the treatment only with BjE109 this value was 1.425 g and represented an increase of 7.1% in comparison to the control without inoculation. In terms of nodule size, the T24 induced the highest number of nodules greater than 5mm (range I) and smaller than 2 mm (range III), while the T0 treatment rendered more nodules from 2 to 5 mm (range II). In both cases, the plants developed from the co-inoculation treatments produced more nodules within all three ranges than the single inoculation with *Bj*E109. At the same time, this last treatment improved the number of nodules in the ranges I and II than those obtained with the single inoculation with *Ab*Az39 but reduced those in the range III. Nodule size after the treatment with *Ab*Az39 was like the uninoculated control in ranges I and II, and this fact suggests the capability of *Azospirillum* to interact with the rhizobia of the soil promoting nodulation.

The nodule number and its location in the root system of the plants was also assessed. The highest MRN, SRN and total number of nodules (TN) was observed after co-inoculation with the 1:1 mix at T24. The SRN and TN were significantly higher after inoculation with *Ab*Az39 on its own than in the control. On the other hand, the relative difference for MRN, was not significant, which might indicate a positive interaction between *Azospirillum* and the native bradyrhizobia in the soil where the assay was carried out. The shoot and the root biomass of the plants was also significantly increased after the co-inoculation (Table 4). However, whereas the SDW was significantly higher in those plants co-inoculated at T24 compared to T0, these differences were not observed for RDW. The intensity of the green color of the upper leaves measured in SPAD index units did not show differences among the treatments.

Insertion Table 5

Table 5
Effects on crop productivity under field conditions of soybean crops developed from seeds individually inoculated (Control) or co-inoculated (1:1) after 15 min (T0) or 24 h interaction (T24) with *Bj*E109 and *Ab*Az39. Different letters represent significant differences according to Tukey test p< 0.05.

	Plant stand at harvest (plants.m^{-1})	Grain moisture at harvest (g.kg^{-1})	Single grain weight (mg.grain^{-1})*	Total grain N (mg.g^{-1})	Total grain Protein (mg.g^{-1})	Grain yield (kg.ha^{-1})*
Naked seeds	14.7^{a}	182^{a}	181.8^{a}	6.24^{c}	34,2^{c}	4027^{d}
*Ab*Az39-Control	14.9^{a}	181^{a}	182.4^{ab}	6.23^{c}	35,1^{c}	4099d
			+0.3%	**+0.9%**	**+2.5%**	**+1.7%**
*Bj*E109-Control	14.1^{a}	182^{a}	181.7^{b}	6.48^{b}	38,2^{b}	4318^{c}
*Bj*E109 + *Ab*Az39 (1:1) - **T0**	14.6^{a}	181^{a}	183.6^{ab}	6.52^{a}	39,1^{ab}	4563^{b}
			+1.0%	**+0.7%**	**+2.4%**	**+13.3%**
*Bj*E109 + *Ab*Az39 (1:1) - **T24**	14.8^{a}	187^{a}	184.9^{a}	6.60^{a}	40,1^{a}	4713^{a}
			+1.7%	**+1.8%**	**+4.9%**	**+17.03%**

*corrected weight at 14.0 g.kg^{-1} of grain moisture

As can be seen in Table 5, the greater nodulation and biomass production observed, under field conditions, with the co-inoculation of *Ab*Az39 and *Bj*E109 at T24 (Table 4) correlated with a higher soybean grain yield. The treatment of co-inoculation at T24 favored a grain yield production 17.0% greater than the control without inoculation, 15.0% than with the single inoculation with *Ab*Az39, 9.1% than only with *Bj*E109, and 3.3% than the co-inoculation at T0. There were no mean differences in the single grain weight and in the concentration of N in the grains suggesting that most of the yield differences were related with better growth conditions among all the growing season and keeping an efficient biological N fixation.

Based on the integrated analysis of the results, we interpret that the co-inoculation with *Ab*Az39 and *Bj*E109 has many advantages in soybean plant growth so improves the quality and the quantity of the nodulation and the crop grain yield. To validate this interpretation of the results we performed a correlation matrix representing the evaluated parameters from all treatments (Fig. 3). As results, we obtained that all the crop growth and yield related parameters, except for the green intensity of the upper leaves, significantly correlated with the nodulation parameters. Also, it is observed that the plant growth has more influence on the number of nodules and its location than in their single size.

5. Discussion

In plants, co-inoculation is performed with a combination of different bacterial genera or species with the aim to modify plant growth and development synergistically. A great number of non-symbiotic or free-living bacteria are known to promote symbiosis in legumes when co-inoculated with rhizobia. A recent analysis of over 300 cases of positive plant responses to *Azospirillum* in 12 countries (Cassán and Díaz-Zorita 2016) found that the rise in its co-inoculation with rhizobia led to a 6.6% increase in legume yield over rhizobia-only treatments. In the last decades,

research in Argentina and Brazil has assessed the benefits of this kind of co-inoculation for the cultivation of soybean (Hungria et al. 2013; Hungria et al. 2015; Queiroz Rego et al. 2018; Deak et al. 2020; Moretti et al. 2020; Rondina et al. 2020). The combined use of rhizobia and *Azospirillum* for the growth of legumes, mainly soybean, goes back to the 1990s. Inoculants containing *Bradyrhizobium* and *A. brasilense* were authorized for commercial use on soybean in Brazil in 2013, and have shown to increase grain yields (Hungria et al. 2013; Hungria and Nogueira 2019; dos Santos et al. 2019). There are about eighty registered products in South America whose active agent is *A. brasilense*, with *Ab*Az39 strain being the most frequent (Cassán et al. 2020). Moreover, in Argentina there are over 15 commercial inoculants for soybean formulated based on *Bj*E109 (the most widely recommended strain for inoculation), for which co-inoculation with *A. brasilense* is also advised. Currently, there is an increasing use of *Azospirillum*-based inoculants in Brazil soybean and beans (*Phaseolus vulgaris*). In the state of Parana, for instance, the use of co-inoculation rose by almost 30% between 2016 and 2018 (Prando et al. 2016; 2018). Despite the data available regarding commercial and agricultural performance, little is known about the exact reasons why this type of co-inoculation improves plant growth. And furthermore, if these responses are do to interactions between *Azospirillum* and rhizobia or because of the interaction of *Azospirillum* and the plants themselves.

In this study, with the aim of finding further evidence that may elucidate the relative importance of the soybean-*Azospirillum* and *Bradyrhizobium*-*Azospirillum* interactions, we evaluated the effect of co-inoculating soybean seeds with *Bj*E109 and *Ab*Az39 at different ratios and interaction times. Both, *in vivo* and *in planta* assays were carried out to compare the performance between co-inoculation and single inoculation treatments with the bacterial strains on their own. The co-inoculation, combining *Bradyrhizobium* and *Azospirillum* strains, in a 1:1 proportion when treating the soybean seeds, represents the frequent co-inoculation treatment applied at farmers' fields. In this case, both strains are mixed and start their interaction with each other at the same time they are inoculated on the seeds. In this scenario, most of the effects of the presence of *Azospirillum* have direct impact on the plant. If the mix of microorganisms is prepared 24 hours before treating the seeds (T24), the strains interact with each other before reaching the seed coat and the derived benefits on nodulation and soybean growth could be interpreted from the effects of *Azospirillum* on *Bradyrhizobium* performance.

Because of its backbone contribution in the nitrogen nutrition of soybean plants, the main microorganism in the co-inoculation mixture is *Bj*E109. Initially, to analyze the physiological behavior of *Bj*E109, we assessed different proportions of the combination with *Azospirillum* (1:9, 1:1 and 9:1). The 1:1 treatment was chosen because it had the greatest values of EPS production, bacterial biomass, and IAA degradation for which *Ab*Az39 is responsible (Table S1). These parameters were selected with reference to previous studies published by our laboratory (Torres et al. 2018; 2021), in which we confirmed that exogenous IAA addition to pure *Bj*E109 cultures triggered physiological changes in the bacterium, such as the ability to increase its biomass and produce EPS, both are advantages on the rhizobia cell survival on soybean seeds and on its symbiotic performance. We also obsserved that *Bj*E109 can degrade IAA when is added to the culture medium through the action of a 3-phenylpropanoate dioxygenase-like enzyme (subunit alpha and beta) within a cluster named *iac* (Torres et al. 2018; 2021). This must be relevant for a better performance of the co-inoculation so the mix at T24 doubled the EPS content and significantly increased the biomass production in comparison to the T0 treatment (Table 1). In turn, T0 outperformed inoculation with *Bj*E109 alone. An increase in the production of EPS by rhizospheric bacteria like rhizobia is related to higher plant tolerance to stress caused by water, oxidation, low temperature, and other factors (Cytryn et al. 2007; Chang and Halverson 2003; Tamaru et al. 2005). The exposure of 1 mM IAA to *B. diazoefficiens* USDA110 increased the production of the EPS content, which also enhanced the plant tolerance to different types of stresses such as heat stress, cold stress, desiccation, among others (Donati et al. 2013).

When the *Bj*E109 survival was evaluated, the co-inoculation allowed recovering of more viable cells compared to the rest of treatments, being better in the T24 mix than in T0 (Table 2). The recovery kinetics for *Bj*E109 on soybean seeds, assessed over time and under the conditions imposed by the different treatments, agreed with our previous results on the greater recovery of *Bj*E109 and *B. diazoefficiens* USDA110 cells in cultures pre-incubated with IAA (Torres et al. 2018; Donati et al. 2013). These findings suggest that the production of IAA by *Ab*Az29 in the culture medium of the mix may be partly responsible for the increase in EPS, which may offer *Bj*E109 a better chance at surviving on the seeds. Auxins have long been posited to play a major role in nodule ontogenesis within legume-rhizobium symbiosis (Thiman 1936), and there are many reports on alterations in nodule organogenesis due to changes in auxin content because of inoculation with auxin-producing bacteria or exogenous hormone addition (Schmidt et al. 1988). The co-inoculation with rhizobia and *Azospirillum* is not an exception. For example, co-inoculation with IAA-deficient *Sinorhizobium meliloti* and IAA-producing *A. brasilense* on alfalfa generated significantly more nodules on the primary root (Schmidt et al. 1988). Remans et al. (2008) also provided direct evidence on the enhancing effects of IAA by co-inoculating beans with *R. etli* and a mutant IAA-deficient *A. brasilense*. The literature, then, might lead us to believe that the improvement observed in legume growth after inoculation with *Azospirillum* is explained by a hypothetical interaction between the bacteria and the plant, in which phytohormone synthesis (primarily IAA) could be crucial.

The co-inoculation could also favor *Ab*Az39 performance that was conditioned by the interaction time. In this regard, it has been observed that the interaction of *Bradyrhizobium* with *Azospirillum* in soybeans seeds highly improved drought tolerance (Rondina et al. 2020) or enhanced the growth and yield of soybean plants under arsenic stress (Armendariz et al. 2019). This must be because the co-inoculation increased the number of nodules on the roots, leading to higher nodule biomass and thus improved BFN (Torres et al. 2018; Rondina et al. 2020). We not only corroborated these results but also demonstrated that an interaction time of 24 h prior inoculation improved the establishment of symbiosis, BNF and, as consequence, plant development under controlled (greenhouse) and uncontrolled growth conditions (field) compared to T0, and much more when the bacteria are used individually (Table 4 and 5). However, we also demonstrate that the number but not the size of the nodule influences the quality and quantity of the plant yield (Fig. 3). In the last decades, several authors have analyzed the contribution of co-inoculation with *Azospirillum sp.* to legume productivity. In the pampas region (Argentina), 21 field trials performed on alfalfa showed that combined inoculation of *Ensifer meliloti* and *A. brasilense* was almost twice as effective as inoculation with rhizobia alone (Díaz-Zorita et al. 2012). Hungria et al. (2013) also reported increased yield for soybean and common bean when complementing seed inoculation with rhizobia with the in-furrow application of *A. brasilense* at four locations in Brazil. They found that inoculation of soybean with *Bradyrhizobium* resulted in an 8.4% increase in yield, against the 16.1% increase achieved by the combination of strains. For common beans, individual inoculation with *R. tropici* boosted yield by 8.3%, but the addition of *A. brasilense* raised this figure to 19.6%. A metadata analysis by Barbosa et al. (2021) revealed that soybean co-inoculation was related to a 2.8% increase in grain yield and a 3.2% increase in N concentration in the grains with respect to the inoculation only with *Bradyrhizobium*. In other 37 field trials, soybean co-inoculation with *Bradyrhizobium* and *A. brasilense* also increased mean yield by 227 kg ha^{-1} with respect to the inoculation with *Bradyrhizobium* alone and by 335 kg ha^{-1} with respect to the uninoculated control (Nogueira et al. 2018). Finally, co-inoculation of soybean was found to raise the nodulation percentage by around 5% in Brazil (Hungria et al. 2015; Fipke et al. 2016; Galindo et al. 2018) and around 12% in Argentina (Benintende et al. 2010; Ferraris and Couretot 2011, 2013; Morla et al. 2019), a difference which may be attributed to the tropical-subtropical and temperate conditions, respectively. Despite this, the limited dataset was insufficient to show a direct relationship between co-inoculation and changes in nodulation and grain yield.

6. Conclusions

The findings presented here show that the pre-culture combining *Bj*E109 and *Ab*Az39 before the inoculation to the soybean seeds has benefits in plant nodulation and hence production, more than the single inoculation with *Bj*E109 or *Ab*Az39, or the immediate co-inoculation of both strains. The higher values for EPS production and rhizobium biomass obtained for this mix in its culture media mean that the rhizobium may be better able to survive on the seeds and establish symbiosis more successfully. This must be due to the beneficial effects on *Bj*E109 growth and survival from the IAA produced by *Ab*Az39. Consequently, the better bacteria performance, and its growth, have positive effects *in planta* with better growth and yield under controlled conditions, and also when are grown at dryland field conditions. The success in the co-inoculation with *B. japonicum* and *A. brasilense*, partially depends on the time that the microorganisms are allowed to interact prior inoculation. Further studies must be done in the future to understand not only the best conditions for co-inoculation of *B. japonicum* and *A. brasilense*, but also with other beneficial bacteria in which the pre-culture must be one of the most relevant points.

Declarations

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Figures

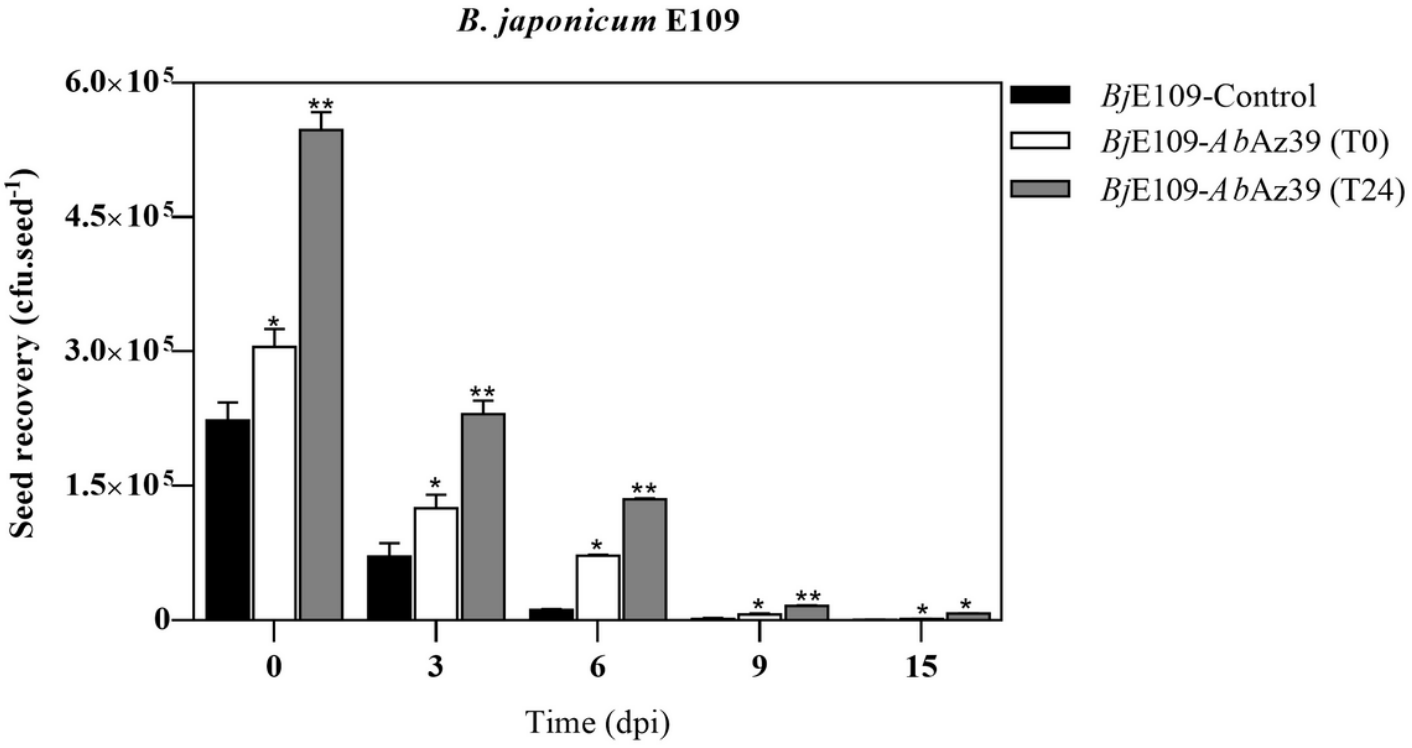

Figure 1

Time-dependent cell recovery (log10 cfu.seed-1) of BjE109 on soybean seeds inoculated individually or in combination with AbAz39 (1:1) after 15 m (T0) or 24 h (T24) of interaction. Asterisks represent significant differences between treatments according to Tukey test p< 0.05.

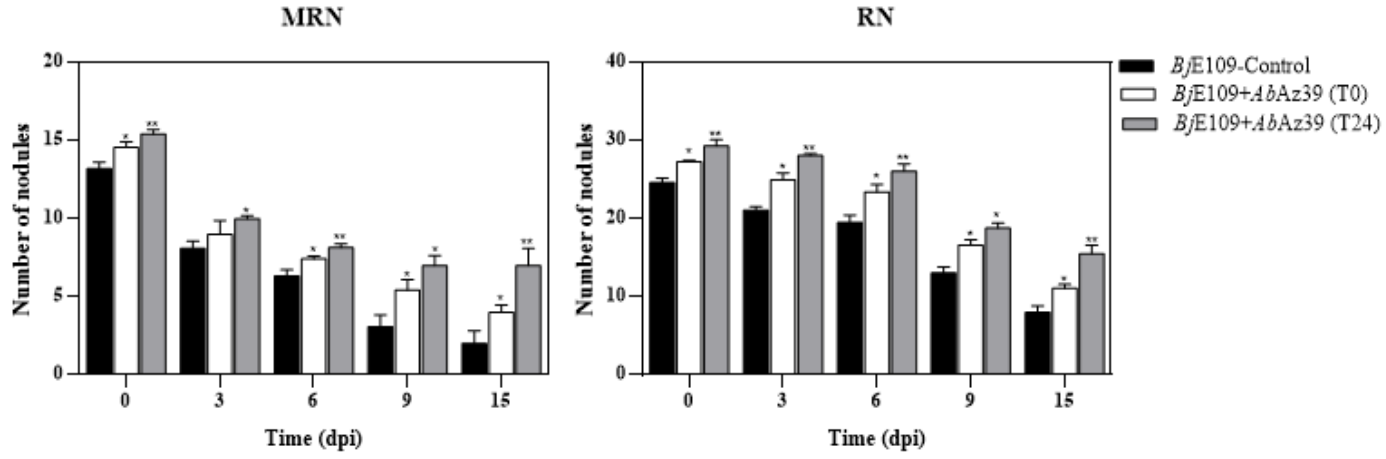

Figure 2

Time-dependent nodulation on soybean roots on different days post-inoculation (dpi) with BjE109 or AbAz39 individually (control) or their combination (1:1) after 15 min (T0) or 24 h (T24) of interaction. Asterisks represent significant differences according to Tukey test p< 0.05. References: MNR: main root nodulation; RN: root nodulation

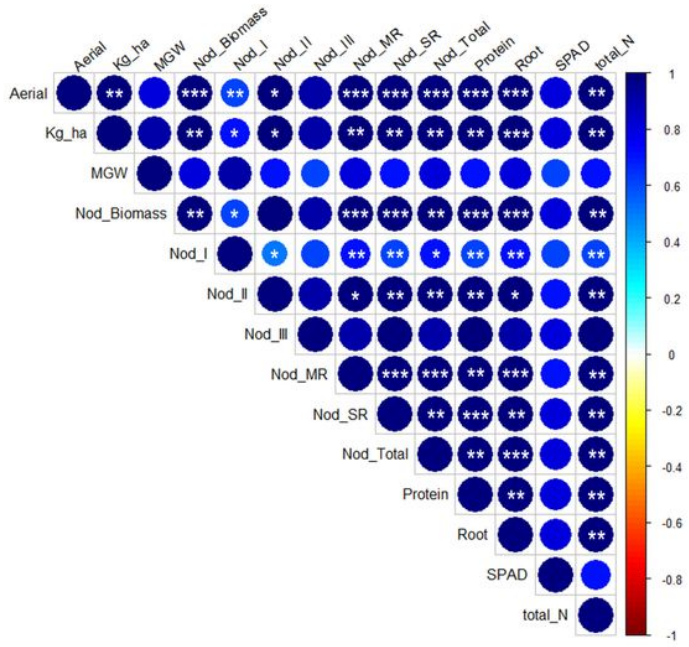

Figure 3

Matrix correlation between nodulation and plant production related parameters of soybean plants from seed uninoculated, inoculated with either BjE109 or AbAz39, individually, or the combination of both at T0 or T24 according to Pearson correlation. Red asterisks indicate significant correlations. P > 0.05 *; P > 0.01 **; P > 0.001 ***

Supplementary Files

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