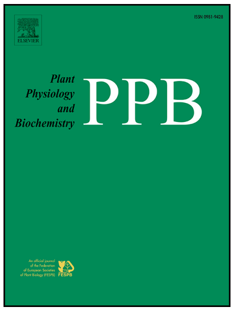

Impact of double inoculation with *Bradyrhizobium japonicum* E109 and *Azospirillum brasilense* Az39 on soybean plants grown under arsenic stress

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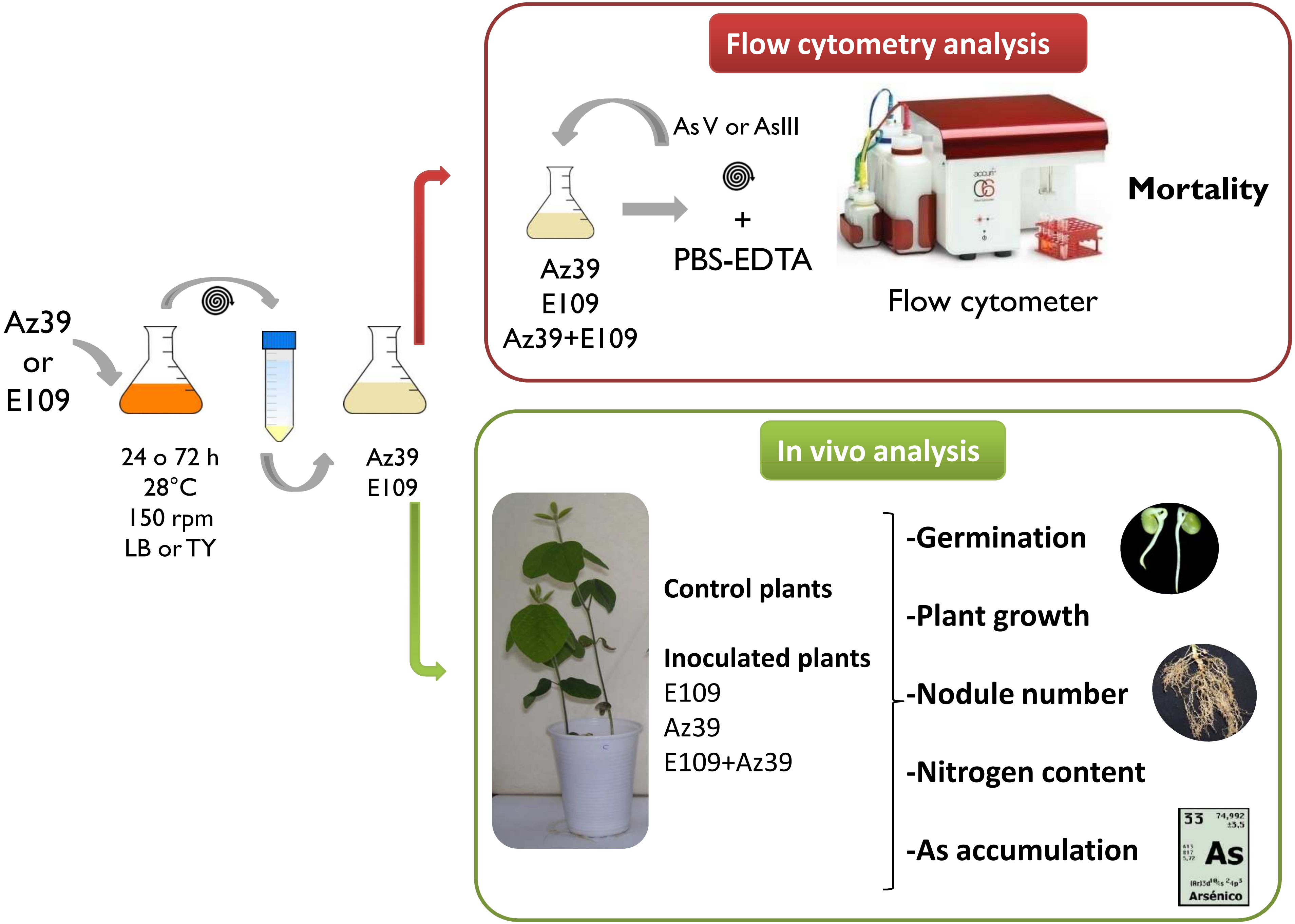

Az39
or
E109

24 o 72 h
28°C
150 rpm
LB or TY

Az39
E109

Flow cytometry analysis

As V or AsIII

Az39
E109
Az39+E109

+
PBS-EDTA

Flow cytometer

Mortality

In vivo analysis

Control plants

Inoculated plants
E109
Az39
E109+Az39

- **Germination**
- **Plant growth**
- **Nodule number**
- **Nitrogen content**
- **As accumulation**

Impact of double inoculation with Bradyrhizobium japonicum E109 and Azospirillum brasilense Az39 on soybean plants grown under arsenic stress

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Abstract

Inoculation practice with plant growth-promoting bacteria (PGPB) has been proposed as a good biotechnological tool to enhance plant performance and alleviate heavy metal/metalloid stress. Soybean is often cultivated in soil with high arsenic (As) content or irrigated with As-contaminated groundwater, which causes deleterious effects on its growth and yield, even when it was inoculated with rhizobium. Thus, the effect of double inoculation with known PGPB strains, *Bradyrhizobium japonicum* E109 and *Azospirillum brasilense* Az39 was evaluated in plants grown in pots under controlled conditions and treated with As. First, the viability of these co-cultivated bacteria was assayed using a flow cytometry analysis using SYTO9 and propidium iodide (PI) dyes. This was performed *in vitro* to evaluate the bacterial population dynamic under 25 µM AsV and AsIII treatment. A synergistic effect was observed when bacteria were co-cultured, since mortality diminished, compared to each growing alone. Indole acetic acid (IAA) produced by *A. brasilense* Az39 would be one of the main components involved in *B. japonicum* E109 mortality reduction, mainly under AsIII treatment. Regarding *in vivo* assays, under As stress, plant growth improvement, nodule number and N content increase were observed in double inoculated plants. Furthermore, double inoculation strategy reduced As translocation to aerial parts thus improving As phytostabilization potential of soybean plants. These results suggest that double inoculation with *B. japonicum* E109 and *A. brasilense* Az39 could be a safe and advantageous practice to improve growth and yield of soybean exposed to As, accompanied by an important metalloid phytostabilization.

Keywords: ARSENIC, PGPB, INOCULATION, PHYTOSTABILIZATION, GLYCINE MAX

1. Introduction

Arsenic (As) is a highly toxic metalloid present in the environment, being arsenate (AsV) and arsenite (AsIII) the predominant inorganic species in soil and water (Farooq et al., 2016). In plants, As interferes with critical metabolic processes such as photosynthesis and can induce water stress by reducing transpiration rate, stomatal conductance, and leaf relative water content along with reduction of xylem vessel size. In addition, this metalloid induces oxidative stress, cellular membrane damage and electrolyte leakage (Stoeva et al., 2004; Gusmán et al., 2013a,b). As consequence, a severe plant growth and reproductive capacity inhibition is often seen (Garg and Singla, 2011; Finnegan and Chen, 2012; Reichman, 2014; Armendariz et al., 2016; Bustingorri and Lavado, 2014).

The use of plants for contaminant removal is named phytoremediation and based on the type of biological mechanism adopted this phytotechnology is classified as phytoextraction, phytostabilization, phytotransformation, phytovolatilization, rhizofiltration or phytostimulation (Abhilash et al., 2009). Generally, plants use a variety of processes that collectively contribute to the overall effectiveness of remediation (Kumar Yavad et col., 2018). For heavy metals, several reviews have been published in the last years, mainly considering phytoextraction, phytostabilization, phytoevaporation and phytotransformation (Gomes et al., 2017; Mahar et al., 2016; Sarwar et al., 2017). Initially, these phytotechnologies focused on heavy metals/metalloids phytoextraction, while phytostabilization received less attention. Recently, phytostabilization has been revalued as a metal immobilization strategy for polluted soils (Sarwar et al., 2017). Even more, high metal/metalloid retention ability in roots takes relevance for edible plants and those which have fruits or grains for food, since the risk of contaminant introduction into the food chain is minimized (Robinson et al., 2009, Sarwar et al., 2017). The use of plant growth promoting bacteria (PGPB) can improve growth of plants exposed to metal/metalloids and even promote phytostabilization through their ability to decrease metal bioavailability. This strategy is named as assisted phytoremediation. Although many PGPB have been isolated and used for metal phytoremediation improvement (Nie et al., 2002; Ullah et al., 2015; Ma et al., 2016; Titah et al., 2013; Ojuederie and Babalola, 2017; Sarwar et al., 2017), few studies have evaluated PGPB potential for As phytostabilization.

Soybean (*Glycine max* L.) is a legume with worldwide economic importance because of its high protein content in grains and other valuable food sub-products. For optimum yields, this crop is inoculated with symbiotic rhizobia, mainly *Bradyrhizobium japonicum* strains. Soybean-rhizobia symbiosis is an important ecological and agronomical association, since plants receive enough Nitrogen (N) supply through biological N-fixation, hence, the use of N fertilizers can be reduced (Sytnikov 2013). The association between soybean roots and *B. japonicum* bacteria results in the formation of specific organs, called nodules, where N-fixation takes place. The main products of N-fixation on soybean nodules, such as ureides (allantoin and allantoic acid), are exported to the rest of the plant where they are incorporated into aminoacids and proteins. Thus, the number of effective nodules (regularly evaluated through its red-pink colour indicative of leghemoglobine presence) is key in those crops in which N content depends mainly on biological N-fixation (Wang and Martinez-Romero 2000; Masciarelli et al., 2014; Pommeresche and Hansen, 2017).

Argentina presents a cultivated area of 20.3 million hectares of soybean, with a production of 58 million tons (2016-2017) (Integrated Agricultural Information System Argentina, 2016). This crop is often cultivated in areas with high As concentration and/or irrigated with groundwater containing this metalloid because of crop expansion to arid and semiarid regions with low rainfall regime (Smedley and Kinniburgh, 2002; Bundschuh et al., 2010). This is of great concern because As toxicity may produce not only animal and human health problems but also negatively affect sustainable crop production. In Argentina, *B. japonicum* E109 is used for soybean inoculation since it is the commercially available strain (Cassán et al., 2009). In a previous work, we showed that under As exposure this bacterium was sensitive, mainly when exposed to AsIII since its growth was reduced a 50% for 10 µM and almost totally reduced for 25 µM AsIII, while for AsV from 25 µM only a minimal reduction in growth was seen (Armendariz et al., 2015). When soybean plants were treated with As, the plant growth was significantly reduced when exposed to 25 µM AsV and AsIII even when they were inoculated with *B.*

japonicum E109 since nodule number was reduced under these conditions (Talano et al., 2013). Other reports have also shown that soybean inoculated with other *Bradyrhizobium* strains was negatively affected by As exposure leading to significant ecological, economic and nutritional losses (Reichman, 2014; Bustingorri and Lavado, 2014). Therefore, in As impacted environments the application of PGPB could not only improve As phytostabilization process, but also alleviate metal toxicity and stimulate plant growth. Hence, it could constitute an economic and effective approach for reducing metalloid impact (Ojuederie and Babalola, 2017). Considering that *B. japonicum* E109 is the commercially available strain and the only one adopted for soybean inoculation schemes in Argentina and taking into account the negative performance when inoculated in As-treated soybean plants, a strategy of combining this with other PGPB could be considered. In this sense, *Azospirillum brasilense* Az39 is a free-living bacterium that when inoculated alone or in combination with *B. japonicum* E109 has shown capacity to promote seed germination, nodule formation, and early development of soybean seedlings in As-free soils (Cassán et al., 2009). *A. brasilense* Az39 is able to produce indole acetic acid (IAA), gibberellins (GA3) and zeatin (Z), which produce morphological and physiological changes in maize and soybean young seed tissues (Cassán et al., 2009; García et al., 2017).

Based on this background, the aims of this work were to evaluate the *in vitro* viability of two rhizospheric strains (*B. japonicum* E109 and *A. brasilense* Az39) under AsV and AsIII exposure in single and co-cultured suspensions and to test *in vivo* the effects of double inoculation (DI) on soybean plants exposed to As. The advantages of DI, in particular on soybean germination parameters, plant growth, nitrogen content, nodule number and As accumulation were evaluated, in order to assess the feasibility of DI strategy for an efficient symbiosis and growth improvement in soybean plants under As stress.

2. Materials and Methods

2.1 Bacterial strain and growth conditions

Two collection strains, *B. japonicum* E109 and *A. brasilense* Az39, were used in the present work. These bacteria belong to a strain collection from the Agriculture Collection Laboratory of the Instituto de Microbiología y Zoología Agrícola (IMYZA) and Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, Argentina. The complete genome sequence of *B. japonicum* E109 is available at NCBI GenBank under the following accession number CP010313 (Torres et al., 2015) while that of *A. brasilense* Az39 is registered as CP007793 for the chromosome and CP007794 to CP007798 for the other replicons (Rivera et al., 2014). Bacterial inocula were obtained by growing *B. japonicum* E109 for 96 h in liquid TY medium containing vancomycin (4 µg mL^{-1}) and *A. brasilense* Az39 for 24 h in LB medium. Both cultures were incubated under agitation at 200 rpm and 28 °C. ~~When necessary, the CFU~~ ~~mL^{-1}~~ ~~of bacterial suspension was calculated by drop count plate method (Somasegaran and Hoben, 1994).~~

2.2. Bacterial in vitro studies

2.2.1. Viability analysis of B. japonicum E109, A. brasilense Az39 and co-cultured strains under arsenic stress using flow cytometry analysis

In order to evaluate rhizospheric strains viability under As stress, a flow cytometry analysis was performed in single or co-cultured bacterial suspensions. For that, bacterial cultures were centrifuged at 10,000 rpm for 20 min at 15 °C, and the pellets were suspended in physiological saline solution (NaCl 0.9%) to reach an OD_{620nm} of 1. Finally, the bacterial suspensions were incubated separately or co-cultured in absence or presence of 25 µM AsV or AsIII for 72 h. After that, bacterial suspensions were harvested by centrifugation and pellets were washed twice with saline phosphate buffer containing 1 mM EDTA, pH 7.4 (Mandal et al., 2008). Viability evaluation was performed using the LIVE/DEAD BacLight Bacterial Viability Kit staining (Invitrogen, ThermoFisher Scientific, CA, USA), according to the manufacturer's instructions. Bacterial viability was carried out by SYTO9 and propidium iodide (PI) dyes which determine cell membrane integrity. SYTO9 dye can be incorporated to live and dead bacterial cells and can be useful to determine the total cells population, while PI dye is commonly used for identify dead cells which present disrupted membranes. Bacterial suspensions were acquired on an ACCURI C6 (BD Biosciences, San Diego, CA, USA) flow cytometer and the data were analyzed using FlowJo software (Tree Star, OR, USA). To evaluate mortality of the strains treated with As, bacteria were detected by forward scatter (FSC), side scatter (SSC), and fluorescence.

2.2.2. IAA produced by A. brasilense Az39 under As stress and its effect on B. japonicum E109 survival

A. brasilense Az39 cultures grown for 24 h in LB medium were harvested by centrifugation and the pellet was suspended in physiological saline solution and adjusted to an OD_{620nm} of 0.5. Those bacterial suspensions were supplemented with stock sodium arsenate ($AsHNa_2O_47H_2O$) (SIGMA) (AsV) and sodium arsenite ($NaAsO_2$) (SIGMA) (AsIII) solutions to reach a final concentration of 25 µM. For the control suspensions the same volume of As stocks was added as distilled water. These suspensions were incubated at 28° C and 180 rpm for 72 h. Then, IAA produced by *A. brasilense* Az39 was tested as described by Glickman and Dessaux (1995) using the Salkowski reagent (H_2SO_4: 37.5 mL; $FeCl_3$ 0.5M: 1.88 mL; H_2O: 62.5 mL for 100 mL). For that, a calibration curve using commercial IAA solutions from 2 to 20 µg mL^{-1} was used and the OD (at 530 nm) values were registered. Finally, the IAA concentration produced by *A. brasilense* Az39 was expressed as µM considering its molecular weight (175.18 g mol^{-1}). As positive control of IAA production, *Azospirillum* sp. Cd strain was included (Kaushik et al., 2000), while non-inoculated physiological saline solution was used as negative control.

For the evaluation of IAA effect on *B. japonicum* E109 viability flow cytometry analysis was performed. For that, *B. japonicum* E109 culture previously grown in TY medium was centrifuged and the pellet was suspended in physiological saline solution to reach an OD_{620nm} of 1. Then, 5 mL-fractions of that suspension were diluted 1/2 to reach a final OD_{620nm} of 0.5 with: a) physiological saline solution with the addition of commercial IAA (final concentration 4 µM), b) cell-free supernatant from *A.*

brasilense Az39 and c) *A. brasilense* Az39 viable cells previously suspended in physiological saline suspension with an OD_{620nm} of 1. As control, *B. japonicum* E109 suspension in physiological saline solution was incubated under the same conditions. The final OD_{620nm} of *B. japonicum* E109 suspensions at all the conditions reached a value of 0.5. All these treatments were exposed to AsV or AsIII (25 µM) adding the proper volume of concentrated stock solutions while those without As were used as control. After incubation for 72 h, bacteria were centrifuged and washed with phosphate saline buffer (PBS) with 1 mM EDTA. Then, cell mortality was evaluated by flow cytometry analysis as previously described in order to discuss the IAA effect on *B. japonicum* E109.

2.3. Inoculation studies in As-treated plants

2.3.1. Plant material, growth and treatment conditions

Seeds of *Glycine max* cv. DM 4670 were used. They were sterilized using 70% (v/v) ethanol for 1 min and then 30% (v/v) sodium hypochlorite for 10 min. They were washed thoroughly with sterile distilled water, submerged in distilled water and incubated at 28 ± 2 °C with agitation for 24 h. Then, they were used for *in vitro* studies (germination test) and *in vivo* inoculation assay in pots as detailed in 2.3.2. and 2.3.3. sections, respectively.

2.3.2. In vitro studies: Effect of inoculation on soybean germination under As stress

To evaluate whether inoculation contributes at the initial development stage of soybean, germination index (*GI*), germination rate index (*S*), root length (cm) and relative radical elongation (*E*) were determined in seeds with single or double inoculation. For that, sterilized seeds (n=10) were placed on Petri dishes containing sterile filter paper. It was impregnated with 6 mL of: sterile water (control condition), *B. japonicum* E109 or *A. brasilense* Az39 suspension made with physiological saline solution (OD_{620nm} 0.5) and equal amount of mixed bacterial suspension for DI condition. For As treatment, water or bacteria suspensions were supplemented with AsV or AsIII solutions to reach 25 µM final concentration. The experiment was repeated three times and each condition was analyzed by duplicate in each independent experiment (n= 60). *GI*, *S*, root length and *E* were determined after incubating the plates for 7 d at 28 ± 2 ° C in darkness.

E and *GI* were calculated according to Barrena et al. (2009): (*E* = [Xf/Xc] x 100) and (*GI* = [(Gf/Gc) x 100] x *E*/100), where: Xf= root length average of AsV or AsIII treated seeds, Xc= root length average of control seeds, Gf= germinated seeds in the presence of AsV or AsIII and Gc= seeds germinated under control conditions. *S* was calculated as described by Ahmed and Wardle (1994): (*S*= [N1/1 + N2/2 + N3/3 + ... + Nn/n] x 100), where: N_1, N_2, N_3 ... N_n is the proportion of seeds germinating on days 1, 2, 3 ... *n* throughout the experiment. In this way, *S* varies from 100 (if all seeds germinate on the first day) to 0 (if the seeds did not germinate at the end of the experiment).

2.3.3. In vivo inoculation assays and responses of soybean plants under As stress

Previously disinfected seeds were placed in sterile flasks and soaked with a necessary volume (28 seeds/4.3 mL) either of physiological saline solution (non-inoculated), or bacterial suspensions obtained as previously described (section 2.2.1) from *B. japonicum* E109 and *A. brasilense* Az39 and both (inoculated and double inoculated (DI)). When soybean seeds were DI, the suspension was prepared from a mixture of both microorganisms in equal parts. Then, seeds were incubated in an orbital shaker (200 rpm) for 2 h at 28 °C to allow the impregnation with bacteria. After draining the seeds from the bacterial suspensions they were kept in a laminar flow hood by 2 h to allow them to dry. Subsequently, 10 seeds (non-inoculated (NI), inoculated with *B. japonicum* E109 or *A. brasilense* Az39 and those DI) were placed in pots containing 50 g of sterile perlite humidified by capillarity with 125 mL distilled water (control) or 25 μM AsV and AsIII solution. Plants were supplemented alternatively with water or free nitrogen ½ Hoagland solution as needed. At 14 and 21 days, plants were repeatedly treated with As, so the treatments were designated as T0, T14 and T21.

The experiments were carried out in a growth chamber set with controlled temperature (28 ± 2 °C) under photoperiod regime [16 h light (200 μmol m^{-2} s^{-1})/8 h dark] and relative humidity of 80%. After 30 d, harvested plants were divided in root, shoot and nodules. First, the nodule number was counted. Dry weight of root and shoot (obtained after drying in an electric heating oven at 70 °C for 5 d) was registered. Root and shoot were frozen, homogenized with liquid N_2 and kept at -80 °C until their use for analytical determinations.

2.3.3.1 Total nitrogen content in soybean plants

Total nitrogen content was determined in shoots by Kjeldahl Method (Reference Method) based on titration of protein and non-protein nitrogen through a digestion with concentrated sulfuric acid (AOAC, 1990).

2.3.3.2 Total As accumulation analysis

Root and shoot of inoculated and NI plants were used for As quantification. Dried tissues were acid digested and total As was determined by atomic fluorescence spectrometry (AFS). For digestion, 0.3 g of sample were weighed and mixed with 10 mL of concentrated HNO_3 (Ultrex® II Mallinckrodt Baker, Phillipsburg, NJ, USA) (30 min at 50ºC and 60 min under boiling). After cooling, 2 mL of H_2O_2 30% (Merck, Darmstadt, Germany) were added and the digestion was continued at constant boiling during 60 min. Each digested sample was left to cool, and then it was filtered and transferred to a 50 mL flask. Subsequently, 5 mL of HCl 37% (v/v) (Merck) and 2 mL of IK 25% (w/v) (JT Baker, USA) were added to the flask. Finally, ultrapure water (18 MΩ cm) (Bedford, MA, USA) was added to reach a volume of 50 mL. Arsenic was detected using a Rayleigh AF-640A atomic fluorescence spectrometer (Beijing Rayleigh analytical Instrument Corp., Beijing, China). Instrumental and experimental conditions were: lamp and wavelength: As High intensity hollow cathode lamp, 197.3 nm; main current: 40mA; auxiliary current: 0 mA; reductant: 0.7% (w/v) $NaBH_4$ (Merck), carrier: 5% (v/v) HCl (Merck); reductant and carrier flow rates: 12 mL min^{-1}, argon flow rate: 800 mL min^{-1} and atomizer temperature: 300ºC. Calibration was performed against aqueous standards and blank solutions. For validation, a Perkin Elmer (Uberlingen, Germany) Model 5100ZL atomic absorption spectrometry equipped with a transversely heated graphite atomizer, an As Electrodeless Discharge Lamp (EDL) and a Zeeman correction system, was used.

2.4. Statistical analysis

Results are the average of at least 3 independent replicates, performed by triplicate. Mean and standard errors of the evaluated parameters were calculated and plotted using the Microsoft Excel 2007 program. To determine the statistical difference between at least one pair of means, analysis of variance test (ANOVA) was used. When the assumptions of homogeneity of variance (*Levene* test) and normality (*Shapiro*-*Wilk* test) were not checked, corresponding transformations were performed using the appropriate functions. To determine significant differences between treatments, *Tukey* test was applied, with a significance level of 0.05 (p <0.05). For some parameters nonparametric analysis was performed by *Kruscal Wallis* test (Software InfoStat versión 2015; from National University of Córdoba, Argentina).

3. Results and Discussion

3.1. Bacterial in vitro studies

3.1.1. Viability analysis of B. japonicum E109, A. brasilense Az39 and co-cultured strains under AsV and AsIII treatment

In order to understand how 25 µM AsV and AsIII affects *B. japonicum* E109 and *A. brasilense* Az39 viability, single or mixed cultures were stained with SYTO9 and PI dyes and analyzed by flow cytometry. As shown in Figure 1A (representative dot plots) and Figure 1B, the metalloid increased *B. japonicum* E109 and *A. brasilense* Az39 mortality in single and DI cultures. In this sense, mortality increase was statistically significant only for AsIII treatment and *B. japonicum* E109 was more affected than *A. brasilense* Az39, since mortality values were 45% and 38%, respectively (Fig 1B). These data are in agreement with previous results obtained using conventional methodology [growth curves (OD_{620nm}) and plate count (log10 CFU mL^{-1})] (Armendariz et al., 2015). As it is shown, As is an important stress factor especially for *B. japonicum* E109, severely affecting its viability. However, co-culture of *B. japonicum* E109 and *A. brasilense* Az39 improved bacteria survival under As treatment compared with single cultures. Furthermore, this effect was more significant under AsIII treatment since co-cultured mortality decreased 21% for AsV and 13-27% AsIII treatment, compared with the mortality of single bacteria suspensions. Hence, flow cytometry was useful for identifying and quantifying viable and dead rhizobacteria in an easy, fast and efficient way as a complement to standard methods (Mandal et al., 2008; Tejerizo et al., 2015; Valdameri et al., 2015). Moreover, flow cytometry assay allowed us analyzing in an accurate and exact manner the behavior of this mixed bacterial population under As exposure. These results suggest that there may be a synergistic/cooperative effect between bacteria, which encourage us to evaluate their effectiveness under *in vivo* conditions for the improvement of soybean plants exposed to As.

3.1.2 IAA produced by A. brasilense Az39 under As stress and its effect on B. japonicum E109 survival

With the purpose of exploring whether IAA produced by *A. brasilense* Az39 is responsible of the increased viability of *B. japonicum* E109 in co-culture under As stress, it was incubated with commercial IAA, *A. brasilense* Az39 cell-free supernatant and *A. brasilense* Az39 bacterial suspension. *B. japonicum* E109 alone was also included as control and incubated under the same conditions. The cell-free supernatant was included to consider the presence of another potential soluble compound in the culture medium responsible of *B. japonicum* E109 survival.

First, IAA produced by *A. brasilense* Az39 was determined under AsV and AsIII exposure. As shown in Table 1, *A. brasilense* Az39 produced around 4-5 µM of IAA, similar to *A. brasilense* Cd, used as a positive control, with no significant effect of 25 µM AsV or AsIII on IAA production. Considering this, 4 µM was chosen as the concentration of commercial IAA added to *B. japonicum* E109.

As it can be seen in Figure 2, when *B. japonicum* E109 was incubated with commercial IAA (4 µM), *A. brasilense* Az39 cell-free supernatant and *A. brasilense* Az39 cells, its mortality percentage was reduced. Although IAA induced a mortality reduction effect in all conditions, the main effect was observed under AsIII treatment (Figure 2). These results indicate that IAA produced by *A. brasilense* Az39 would represent an important component associated to *B. japonicum* E109 viability under As stress. In fact, it has been reported that *B. japonicum* strains can use this compound as a carbon source (Egebo et al., 1991; Jensen et al., 1995). In addition,-there is some evidence that IAA might be a signal able to coordinate bacterial behavior to enhance protection under adverse conditions (Spaepen et al., 2007 and references there in). Using *E. coli*, Bianco et al. (2006a) and (2006b) showed that IAA induces the expression of genes related to survival under stress conditions and others involved in the central metabolic pathways such as the tricarboxylic acid cycle (TCA), glyoxylate shunt and amino acid biosynthesis (leucine, isoleucine, valine and proline). These findings showing IAA as a signaling molecule shed new light on the role of IAA in bacteria-plant interactions, but can also explain bacteria-bacteria interactions in the rhizosphere. Accordingly, in the present work, this phytohormone can play a key role in the protection of the more As-sensitive bacterial partner in the *B. japonicum* E109 and *A. brasilense* Az39 interaction in an As-contaminated environment. In order to evaluate the advantages of double inoculating soybean plants using *B. japonicum* E109 and *A. brasilense* Az39 in an As-contaminated soil, *in vitro* studies of germination parameters as well as *in vivo* studies with plants were performed.

3.2. Inoculation studies in plants treated with As

3.2.1. In vitro studies: Effect of inoculation on soybean germination under As stress

Some parameters related to germination and young stages of soybean growth such as *GI*, *S*, root length and *E* were determined in NI and inoculated seedlings treated with As.

In NI seeds, *GI* was significantly reduced (around 64%) under both As treatments compared to control (Table 2). Similar reduction in germination percentage was shown in our previous work by concentrations from 25 μM AsV or AsIII (Talano et al., 2013). Considering that germination percentage is sometimes a relatively low-sensitive parameter to study the toxicity of a xenobiotic and not enough to predict subsequent effect on tested plant growth (Gong et al., 1999) here we present results from other related parameters such as *S*, root length and *E*. *S* was significantly reduced (23.8%) when seeds were treated with 25 μM AsIII, whereas root length and *E* were significantly affected by both As treatments, with a decrease of 50%. Similar results were found by Kaur et al. (2012) whom reported that As exposure (10 μM) caused a reduction of around 50% of radicle emergence and elongation in *Phaseolus aureus*. The negative effect of As on germination and early development of seedlings has been attributed to the marked decline in amylolitic enzyme activities in rice and wheat endosperms, which produce a delay in mobilization of starch (Jha and Dubey 2005; Liu et al., 2005). Also, As produced a reduction of N-assimilatory enzyme activities (nitrate reductase, nitrite reductase and glutamine synthetase) in germinating rice seeds and seedlings, with the consequent reduced vigor and impaired growth (Jha and Dubey, 2004a; Jha and Dubey, 2004b). Inhibition of proteases has been also reported in As-treated plants, thus it can explain the reduced germination of soybean since proteins are the main reserve material in the grains. Thus, the disturbance of As on sugars, N and protein metabolism of germinating seeds could explain the reduced *GI*, *S*, root length and *E* observed for As-treated soybean seeds.

Regarding inoculation, in the present work, no improvement was observed in seeds inoculated with *A. brasilense* Az39, which was surprising since this strain presented high tolerance to the metalloid as it was previously demonstrated (Armendariz et al., 2015). Contrarily, when As-treated seeds were inoculated with *B. japonicum* E109, all the analyzed parameters significantly increased compared to NI seeds. Similarly, a positive effect has also been reported by Dary et al. (2010) since germination of *Lupinus luteus* seeds was improved when they were inoculated with *Bradyrhizobium* sp. 750 and exposed to contaminated soils with moderated heavy metal concentration (including around 65-70 mg Kg^{-1} of As).

3.2.2. In vivo inoculation assays and responses of soybean plants under As stress

3.2.2.1. Effect on growth and nodulation

Under control condition (without As), inoculation with *B. japonicum* E109, *A. brasilense* Az39 or DI produced a significant increase in shoot and root biomass compared to NI plants (Fig. 3). Plants inoculated with *B. japonicum* E109 showed an increase in root and shoot biomass of 27% and 47%, respectively, while in plants inoculated with *A. brasilense* Az39 the increase was lower (22 and 17%, respectively). However, when soybean seeds were DI no significant differences in plant biomass were found compared to single inoculations. These results agree with pre-existing data, since numerous field studies and laboratory tests have shown that *B. japonicum* E109 significantly increases soybean production (Cassán et al., 2009; Benintende et al., 2010). However, it seems that the beneficial effects of each individual strain would not be additive when they were DI. This could be explained by alteration in microbial ecology of the rhizosphere, probably by natural competition. Some evidences indicate that the production of secondary metabolites and other physiological processes in bacteria depend on population density. Therefore, the benefits that microorganisms produce in plants could not be significant if they do not reach an appropriate number or density (Barnard et al., 2007).

Under As stress, inoculation was an effective strategy to improve plant growth, although with less efficiency. Although there was a negative effect of As on soybean, reflected as biomass reduction, the damage was more severe in NI plants (Fig. 3). Inoculation with *A. brasilense* Az39 or *B. japonicum* E109 separately produced an increase in root and shoot biomass in As-treated plants, but this effect was higher for those inoculated with *B. japonicum* E109. Considering DI, there was a significant growth improvement of As-treated plants, although it was statistically significant only for AsV treatment. Similarly, Reichman (2007; 2014) observed that inoculation with *B. japonicum* CB1809 promoted soybean, wheat and sunflower growth when exposed to AsV compared to those NI plants. In addition, other authors have reported better results in canola and rice growth when inoculated with *Brevundimonas diminuta* and *Enterobacter cloacae* CAL2, respectively, under As stress (Nie et al., 2002; Singh et al., 2016). On the other hand, there are few reports on *Azospirillum* strains inoculated in

As-treated plants. This is not surprisingly because our previous results indicated that *A. brasilense* Az39 did not promote germination parameters in the presence of As. Similarly, Lyubun et al. (2006) neither found significant differences in biomass of wheat plants inoculated with *A. brasilense* Sp245 growing in presence of As compared to NI ones.

Regarding nodulation under control conditions, the number of effective nodules was not modified in DI plants compared to those inoculated with *B. japonicum* E109 (Fig. 4). Under As treatment, the nodule number was significantly reduced compared with control, mainly by 25 μM AsIII. However, in DI plants the nodule number significantly increased compared *B. japonicum* E109 inoculated plants, under AsIII stress. Several authors have described that nodulation of legumes is generally reduced or inhibited in As-contaminated soils (Carrasco et al., 2005; Mench et al., 2006; Talano et al., 2013). For instance, Reichman (2007) reported that the nodule number in soybean plants inoculated with *B. japonicum* CB1809 was reduced by 90% in the presence of 5 μM AsV. In addition, in As-treated plants of *Vigna mugno* and *Medicago* sp. inoculated with highly As-resistant bacterial strains this parameter was also reduced (Pajuelo et al., 2008; Mandal et al., 2011). This decrease would be related to the toxic effect of As on roots, mainly with reduction or damage of radical hairs which would affect the sensitivity, or the low expression level of several nodulin genes, which have a fundamental role in the infection thread formation (Pajuelo et al., 2008; Lafuente et al., 2010). More recently, La Fuente et al., (2015) using the model legume *Medicago truncatula* and *Ensifer* (syn. *Sinorhizobium*) *medicae* MA11, a highly As-resistant bacterium, found a strong reduction of nodule number under AsIII treatment with a median inhibitory concentration (ID_{50}) of 20 μM. The author emphasized that nodulation was the most sensitive process comparing the AsIII-ID_{50} for plant growth, seed germination, shoot and root length, nodulation and other physiological parameters.

In the present work, the lower nodulation in As-treated plants inoculated with *B. japonicum* E109 would be a consequence of root biomass reduction and minor number of root hairs as available infection points. In addition, since *B. japonicum* E109 is highly sensitive to As, mainly AsIII (Armendariz et al., 2015), a smaller number of bacteria are alive for colonization and symbiosis is reduced. Other explanation about As deleterious effect would be related with metalloid injuries on root structure. The toxicity of As would also be related with delicate regulatory events through gene modulation during rhizobia-legume interaction. Recently, La Fuente et al., (2015) studied the effect of As on *M. truncatula-E. medicae* MA11 symbiosis through transcriptomic meta-analysis. In this experimental model, the enhancement of chalcone synthase transcripts (involved in the first step of legume-rhizobia cross-talk) and the repression of 13 subsequent nodulation genes codifying for Nod factors (involved in perception, infection, thread initiation and progression, and nodule morphogenesis) suggests that plants are impaired to establish symbiotic interactions under AsIII stress. This focus involving transcriptomic analysis of As-treated plants inoculated with rhizobia would complement the advances made with 'arsenomic' approach which includes the study of non-legume plants or legume-rhizobia interaction but without stress. Certainly, more studies in this line but under As exposure would allow elucidating the effect of the metalloid on symbiotic interactions from a global perspective.

3.2.2.2 Total N content

In control condition, the total N content in shoots (Fig. 5) was higher when soybean plants were inoculated with *B. japonicum* E109, and also when they were DI. Contrarily, plants inoculated with *A. brasilense* Az39 did not show considerable increase in N content compared to control NI plants. In the presence of As, N content of NI plants did not change while As-treated plants inoculated with *A. brasilense* Az39 showed higher N content although without significant difference. Contrarily, in plants inoculated with *B. japonicum* E109 As treatment produced reduction in N content (around 20-25%), which could be explained by the considerable reduction in nodule number, as it was previously shown (Fig 4), and the reduction of nitrogenase activity in nodules of As-treated plants (data not shown). In addition, soybean root nodules derived from plants treated with both AsV and AsIII showed a pale pink or whitish inner coloration as compared to the intense red color of control plant nodules. This result indicates a lower concentration of leghemoglobin thus, higher O_2 concentration diffuses inside the nodule and nitrogenase activity decreases (Kundu et al., 2003). It is important to remark that, in DI plants, the N content increased under As treatment. These results suggest that *A. brasilense* Az39, a highly As-tolerant strain, would be efficient in N-fixing under As stress, slightly improving N content in As-treated plants when compared with those inoculated only with *B. japonicum* E109. It shows that addition of *Azospirillum* strain to inoculation programs would give better results in plant growth

3.3.3 Effect of inoculation on As accumulation in soybean plants

As shown in Fig 6, the pattern of As accumulation changed depending on the bacterium used. In general, inoculation produced a reduction in As concentration in roots independently of As chemical species, except for DI plants treated with AsIII. In this case, the root accumulated higher As content, constituting a good strategy for an efficient phytostabilization of As, even more when these plants had low As accumulation in shoots. In fact, inoculated plants mainly those with *A. brasilense* Az39 or DI showed reduced As concentration in aerial parts and consequently lower As translocation compared with NI plants.

In a similar way, different plant species inoculated with plant growth promoting bacteria from *Staphylococcus, Bacillus, Acinetobacter* genera and others, have shown reduced As uptake and minor accumulation in aerial parts, grains and/or other edible parts of plants as a result of the bacterial inoculation (Srivastava et al., 2013; Das et al., 2016; Das and Sarkar, 2018). Therefore, those bacteria can be accounted for an efficient As phytostabilization. This finding emphasizes the important role of inoculation strategies to avoid high translocation and As accumulation in aerial parts of plants, mainly those which produce seeds/grains, fruits or are themselves vegetable foods for human and/or animal consumption. In this sense, inoculation could be helpful to avoid transference of As to food chain. However, it is important to consider that depending of bacterial strain and As chemical species, results can differ.

The presence of microorganisms affects the bioavailability of As in soybean rhizosphere. In this sense, it is known that bacteria are able to promote the mobility of metals and metalloids either by acidification and changes in the redox state of the medium, production of chelating agents or siderophores and accumulation and/or adsorption in the biomass or exopolysaccharides (EPS) (Zubair et al., 2016 and references cited therein). Therefore, the different results obtained in the present work can be related with bacteria abilities for As metabolism in the rhizosphere as well as with tolerance mechanisms such as EPS and biofilm production (Armendariz et al., 2015). Joshi and Juwarkar (2009) reported that the ability of *Azotobacter* spp. to chelate Cd and Cr in EPS explained the low adsorption of metals by *Triticum aestivum*. In the present work, the lower content of metalloid in roots of AsIII-treated plants inoculated with individual strains (*B. japonicum* E109 or *A. brasilense* Az39) could be explained by their increased biofilm production under 25 μM AsIII treatment, as shown in Armendariz et al. (2015). Biofilm would retain As and/or adsorbed it on the polymeric matrix frequently formed by EPS, thus leaving lower As concentration available for root (Rajkumar et al., 2012). In addition, other explanation for the lower As concentrations in roots inoculated with single bacterium would be the high As content translocated to aerial parts, which would depend on the metabolism of AsIII in the rhizosphere, uptake transporters and movility in plant tissues.

In the present work, it is important to remark that double inoculation of soybean plants, in particular under AsIII treatment, improved As-phytostabilization, hence reducing not only As lixiviation in soils but also As translocation to aerial parts and consequently, the potential risk of introducing this contaminant into the food chain. In addition, soybean plants treated with AsIII and DI showed a better growth and higher N content compared with NI plants. Also, it seems that the presence of both bacteria in soybean rhizosphere would contribute positively with nodule formation, probably as a result of the protective role of *A. brasilense* Az39 on *B. japonicum* E109 survival through IAA production.

As shown for soybean, legumes often accumulate As (and metals) mainly in root (Pajuelo et al., 2007, 2011; Reichman, 2007; El Aafi et al., 2012), and this fact is adequate for metal phytostabilization (Dary et al., 2010; El Aafi et al., 2012), as it reduces metal/loids mobilization in the plant rhizosphere with a scarce translocation to shoot (Mendez and Maier, 2008). In this sense, autochthonous legumes and resistant rhizobia are the most effective partnerships for many cases of metal-polluted soil restoration (Maynaud et al., 2013). However, when rhizobia are highly sensible to As, its combination with resistant bacteria could be a synergistic way to improve plant and inoculation performance under stressful condition.

4. Conclusion

Combining complementary properties of strains used for inoculation such as N-fixing ability from a poorly As-tolerant symbiotic strain (*B. japonicum* E109) with a highly As-tolerant free-living bacterium (*A. brasilense* Az39) is a good strategy to attenuate the As deleterious effect on soybean plants. A synergistic effect when both bacteria were co-cultured was observed through flow cytometry assays under As exposure. Despite there could be many factors involved in that protection, IAA produced by *A. brasilense* Az39 could be one beneficial metabolic relation that would reduce *B. japonicum* E109 mortality, mainly under AsIII treatment. Independently of the inoculation scheme used, single or combined, it produced positive effects on growth of As-treated plants. It is important to remark that DI plants significantly promoted plant growth, total nodule number and N content under As treatment. Regarding As accumulation, DI inoculation caused a reduction in As content in shoot and root of plants treated with AsV, while those exposed to AsIII showed higher retention of As in roots with low translocation to aerial parts. This would constitute an improvement of plant phytostabilization potential when exposed to AsIII, helping with As immobilization and consequently reducing As entry into the food chain. These results would allow considering DI strategy using *B. japonicum* E109 and *A. brasilense* Az39 as a safe and advantageous practice for the improvement of growth, yield of soybean crops and safe grain consumption for foods.

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6. Figures Legends

Figure 1. Bacterial viability after incubation with or without 25 µM of AsV or AsIII in saline solution for 72 h at 28°C. A) Representative dot plots [SYTO9 green fluorescence intensity (FL1-A) vs. PI red fluorescence intensity (FL3-A)] of the bacterial suspensions analyzed by flow cytometry. *B. japonicum* E109, *A. brasilense* Az39 and co-incubated strains (E109+Az39) in saline solution for 72 h (control), positive death control (Heat-killed), 25 µM of AsV or AsIII. B) Bar graphs show the percentages of cell mortality obtained by flow cytometry and represent the mean ± SE (n = 6). Different letters indicates significant differences (Tukey's test, $p < 0.05$).

Figure 2. Effect of IAA on *B. japonicum* E109. Percentages of cell mortality incubated alone (E109), with commercial IAA (E109+IAA), with supernatant produced by *A. brasilense* Az39 (E109+SNT) or co-incubated with *A. brasilense* Az39 (E109+Az39) analyzed by flow cytometry. All samples were incubated with or without 25 µM of AsV or AsIII in saline solution for 72 h at 28°C. Results represent the mean ± SE (n = 5). Different letters indicates significant differences (Tukey's test, $p < 0.05$).

Figure 3. Effect of As on root and shoot fresh weight of soybean plants non-inoculated (NI), inoculated with *B. japonicum* E109, *A. brasilense* Az39 or double inoculated (DI). The results represent the mean ± SE (n = 40). Different letters indicates significant differences (Test de Kruscal Wallis, $p \leq 0,05$).

Figure 4. Effect of As on nodule number formed after inoculation with *B. japonicum* E109 or with *B. japonicum* E109 and *A. brasilense* Az39 (DI). The results represent the mean ± SE (n = 40). Different letters indicate significant differences (Test de Tukey, $p \leq 0,05$).

Figure 5. Nitrogen content in aerial parts of soybean plants non-inoculated (NI), inoculated with *B. japonicum* E109, *A. brasilense* Az39 or both strains (DI) treated with AsV and AsIII (25 µM). The results represent the mean ± SE (n = 2). Asterisks represent significant difference with the corresponding NI plants under AsV and AsIII treatment.

Figure 6. Arsenic accumulation in roots or aerial parts of soybean plants non-inoculated (NI) or inoculated with *B. japonicum* E109, *A. brasilense* Az39 or with both strains (DI). The results represent the mean ± SE (n = 3).

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Table 1. *IAA production by A. brasilense Az39 incubated in saline solution for 72 h at 28°C under As treatment. Positive control: Azospirillum brasilense Cd. Results represent the mean ±SE (n = 8).*

IAA production (µM)		
	Az39	AzCd
Control	3.9 ± 1.1	4.5 ± 0.3
AsV	4.1 ± 0.5	4.5 ± 0.7
AsIII	5.6 ± 1.1	5.7 ± 1.1

Table 2. *Germination parameters of soybean seedlings treated with 25 µM AsV and AsIII. Effects of inoculation with B. japonicum E109, A. brasilense Az39 and double inoculation.*

	Treatment	*Germination index (IG)*	*Speed of germination index (S)*	*Radical length (cm)*	*Radical relative elongation (E)*
NI	Control	100.0 ± 0.0^{b}	93.8 ± 2.4^{a}	8.3 ± 0.4^{b}	100.0
E109	Control	110.9 ± 1.9^{ab}	94.0 ± 1.7^{a}	9.4 ± 0.5^{ab}	113.0
Az39	Control	109.3 ± 5.7^{ab}	93.0 ± 2.3^{a}	9.5 ± 0.4^{ab}	115.0
E109+Az39	Control	119.1 ± 2.6^{a}	91.0 ± 3.3^{ab}	10.2 ± 0.5^{a}	123.0
NI	AsV	46.2 ± 1.7^{de}	89.7 ± 4.8^{ab}	4.1 ± 0.2^{de}	49.5
E109	AsV	60.7 ± 1.4^{c}	89.6 ± 3.3^{ab}	5.9 ± 0.3^{c}	62.9
Az39	AsV	53.3 ± 5.9^{cde}	90.6 ± 5.1^{ab}	5.1 ± 0.3^{cde}	53.9
E109+Az39	AsV	55.2 ± 2.7^{c}	86.3 ± 6.0^{ab}	5.9 ± 0.4^{c}	58.0
NI	AsIII	45.8 ± 1.0^{de}	71.5 ± 1.7^{bcd}	3.9 ± 0.2^{e}	47.4
E109	AsIII	60.9 ± 1.7^{c}	77.6 ± 2.6^{abc}	5.7 ± 0.3^{cd}	60.8
Az39	AsIII	39.7 ± 2.5^{e}	55.7 ± 3.2^{cd}	3.7 ± 0.3^{e}	39.1
E109+Az39	AsIII	50.6 ± 1.4^{cde}	64.0 ± 3.1^{d}	5.2 ± 0.4^{c}	50.5

NI: non-inoculated seeds.

Fig 1

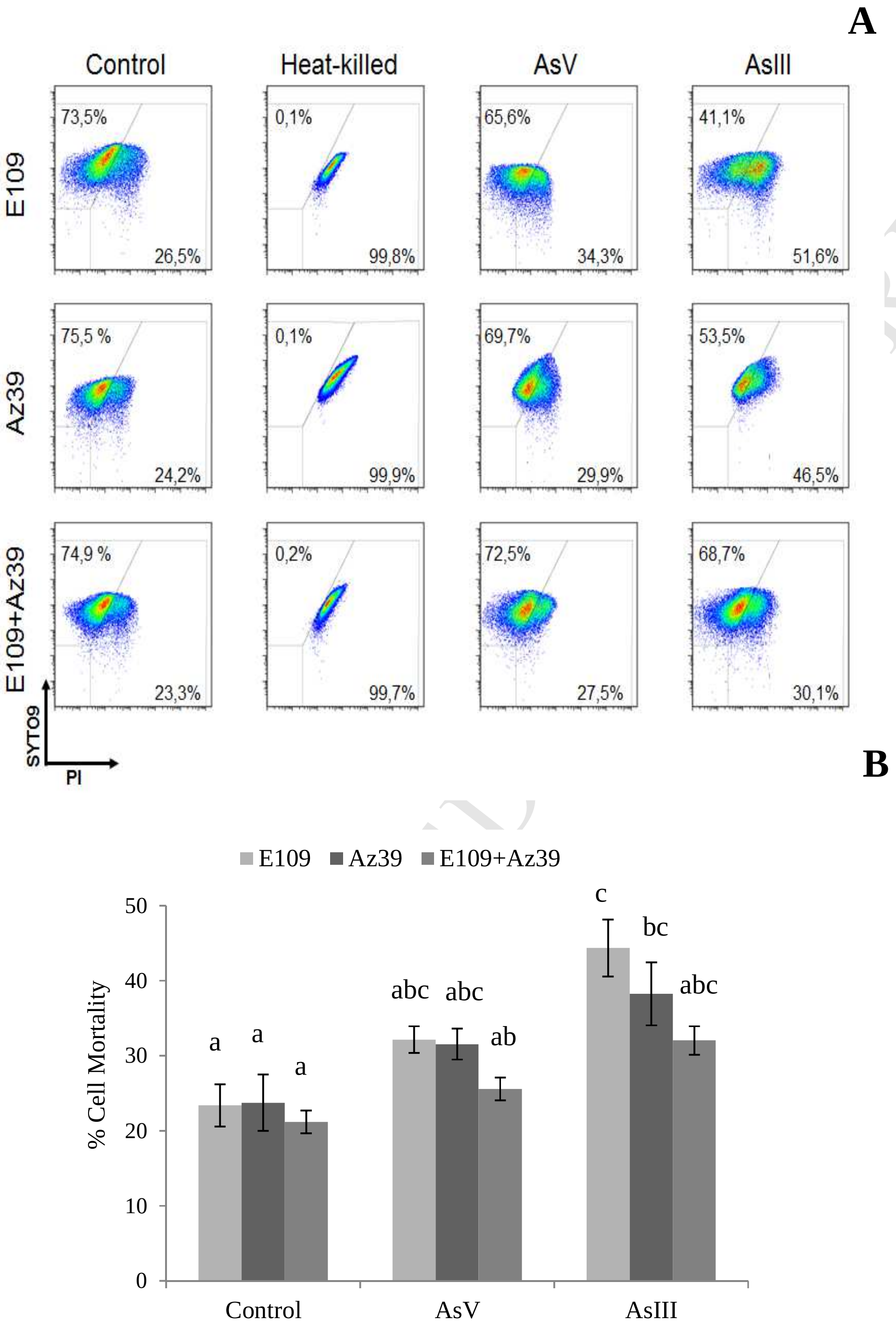

A
Control
Heat-killed
AsV
AsIII
E109
73,5%
26,5%
0,1%
99,8%
65,6%
34,3%
41,1%
51,6%
Az39
75,5 %
24,2%
0,1%
99,9%
69,7%
29,9%
53,5%
46,5%
E109+Az39
74,9 %
23,3%
0,2%
99,7%
72,5%
27,5%
68,7%
30,1%
SYTO9
PI
B
■ E109 ■ Az39 ■ E109+Az39
% Cell Mortality
50
40
30
20
10
0
a
a
a
abc
abc
ab
c
bc
abc
Control
AsV
AsIII

Fig 2

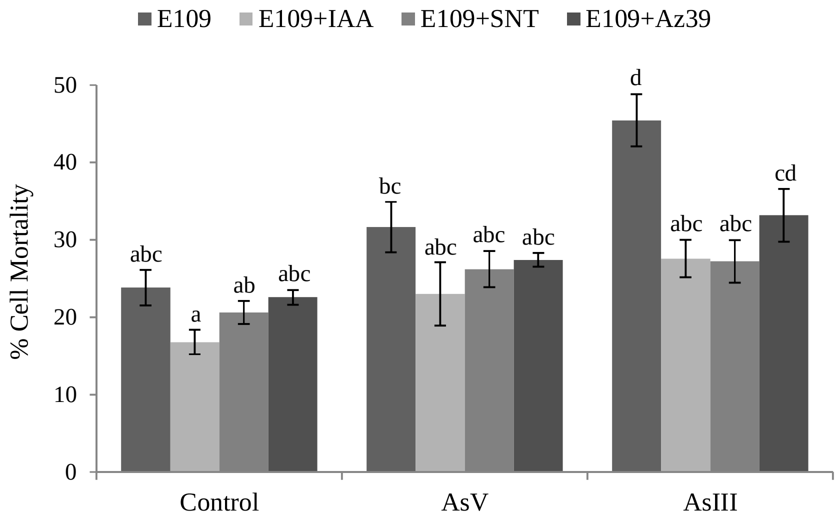

E109
E109+IAA
E109+SNT
E109+Az39
% Cell Mortality
0
10
20
30
40
50
abc
a
ab
abc
bc
abc
abc
abc
d
abc
abc
cd
Control
AsV
AsIII

Fig 3

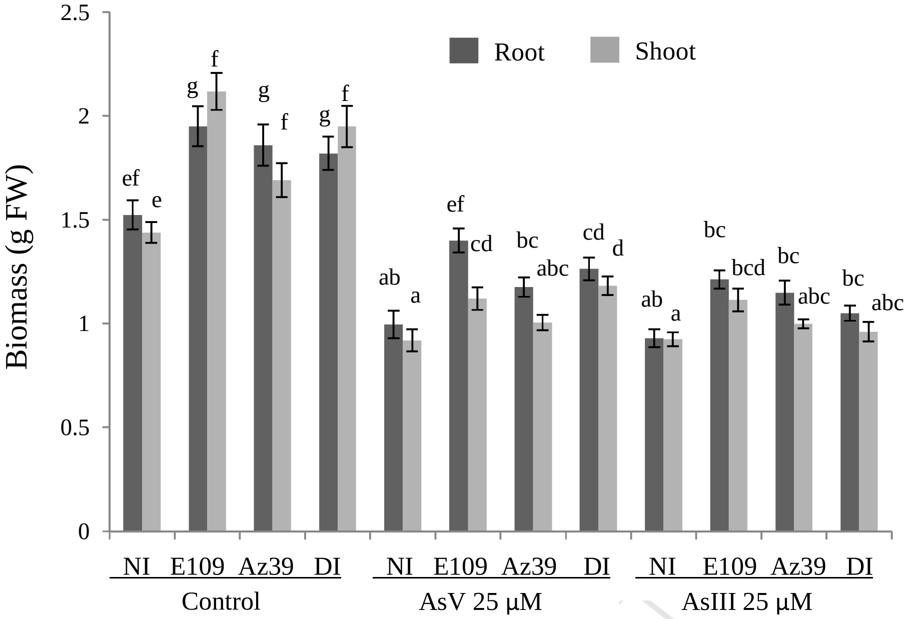

2.5
2
1.5
1
0.5
0
Biomass (g FW)
Root
Shoot
ef
e
g
f
g
f
g
f
ab
a
ef
cd
bc
abc
cd
d
ab
a
bc
bcd
bc
abc
bc
abc
NI
E109
Az39
DI
NI
E109
Az39
DI
NI
E109
Az39
DI
Control
AsV 25 µM
AsIII 25 µM

Fig 4

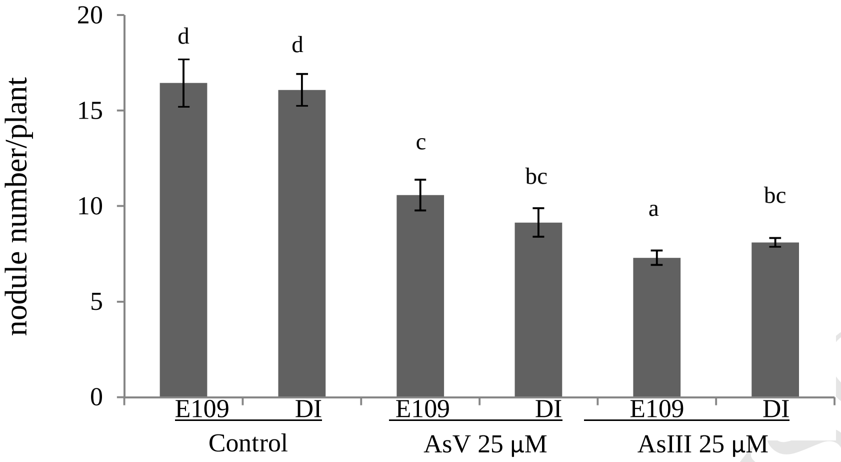

nodule number/plant
20
15
10
5
0
d
d
c
bc
a
bc
E109
DI
E109
DI
E109
DI
Control
AsV 25 µM
AsIII 25 µM

Fig 5

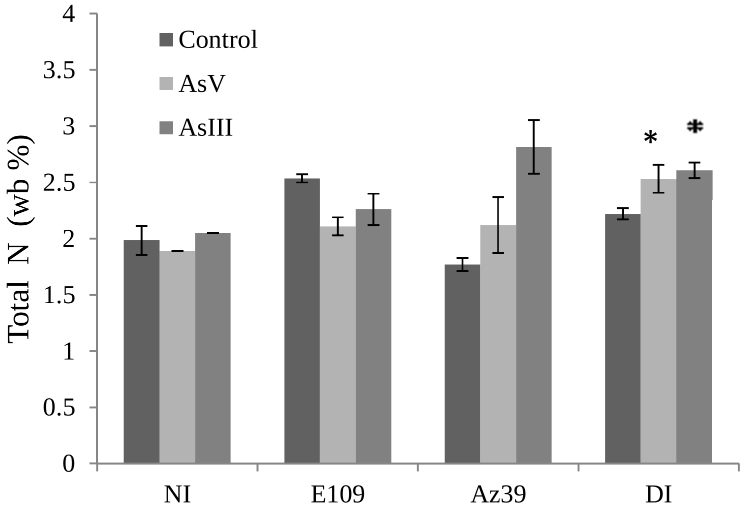
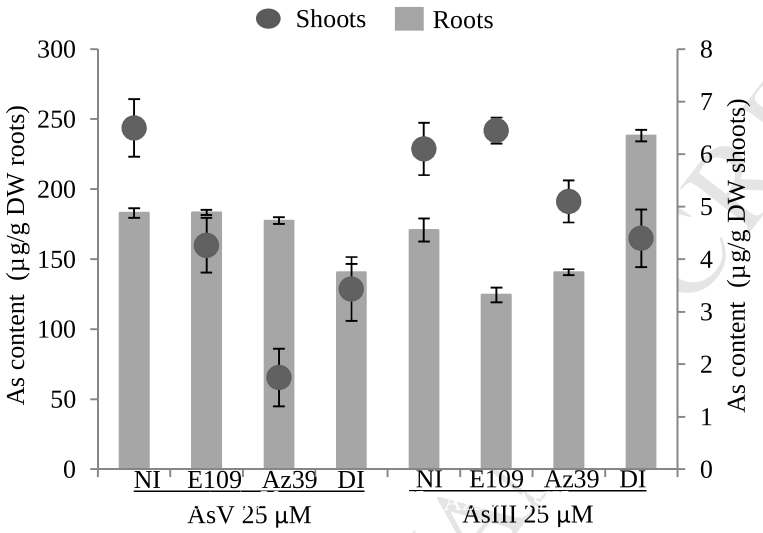


Fig 6


Shoots
Roots
300
250
200
150
100
50
0
As content (µg/g DW roots)
8
7
6
5
4
3
2
1
0
As content (µg/g DW shoots)
NI
E109
Az39
DI
AsV 25 µM
NI
E109
Az39
DI
AsIII 25 µM

Highlights

-Flow cytometry revealed synergysm between two rhizospheric bacteria when exposed to As.

-Indole acetic acid produced by *A. brasilense* Az39 would protect *B. japonicum* E109 when exposed to As.

-Plant growth improvement, increase of nodule number and N content was observed in double inoculated plants treated with As.

-Double inoculation strategy promoted As phytostabilization potential of soybean plants.

Contributions

MAT and EA conceived and planned the experiments. ALA y MAT carried out the inoculation experiments, MFON and MLB carried out cytometry assays and LE made the arsenic quantification. ALA, MAT, MFON, MLB, CP and EA contributed to the interpretation of the results. MAT wrote the manuscript with input from all authors. EA, LE and CP provided critical feedback and helped shape the research and manuscript analysis.