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Research article

Arsenic effect on the model crop symbiosis *Bradyrhizobium*–soybean

Melina A. Talano*[,1], Romina B. Cejas [1], Paola S. González, Elizabeth Agostini

Departamento de Biología Molecular, FCEFQyN, Universidad Nacional de Río Cuarto, Ruta Nacional 36 Km 601, CP 5800 Río Cuarto, Córdoba, Argentina

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ABSTRACT

Soybean (*Glycine max*) is often being cultivated in soils with moderate to high arsenic (As) concentrations or under irrigation with As contaminated groundwater. The purpose of this study was to determine the effect of As on soybean germination, development and nodulation in soybean-*Bradyrhizobium japonicum* E109 symbiosis, as a first-step approach to evaluate the impact of As on soybean production. Semi-hydroponic assays were conducted using soybean seedlings inoculated and non-inoculated with *B. japonicum* E109 and treated with arsenate or arsenite. Soybean germination and development, at early stage of growth, were significantly reduced from 10 μM arsenate or arsenite. This also was seen for soybean seedlings inoculated with *B. japonicum* mainly with arsenite where, in addition, the number of effective nodules was reduced, despite that the microorganism tolerated the metalloid. This minor nodulation could be due to a reduced motility (swarming and swimming) of the microorganism in presence of As. Arsenic concentration in roots was about 250-times higher than in shoots. Transference coefficient values indicated that As translocation to aerial parts was low and As accumulated mainly in roots, without significant differences between inoculated and non-inoculated plants. The presence of As restricted soybean–*B. japonicum* symbiosis and hence, the efficiency of most used commercial inoculants for soybean. Thus, water and/or soils containing As would negatively impact on soybean production, even in plants inoculated with *B. japonicum* E109.

1. Introduction

Arsenic (As) is an ubiquitous and toxic metalloid that can be found in different oxidation states, being inorganic arsenate [As(V)] and arsenite [As(III)] the main species present in the environment. Sources of pollution include both natural processes (e.g., weathering reactions, biological activity, volcanic emissions, etc.), as well as anthropogenic activities (mining, use of pesticides and wood preservatives, fossil fuels combustion, etc.). Arsenic presence in soils and groundwater in Latin America was discovered a long time ago, and particularly Argentina is one of the most affected regions due to the volcanic activity of Andes mountains [1].

The major human health hazard of As is related to consumption of contaminated water, nevertheless the presence of this metalloid in water and soils, and mainly the use of groundwater with high As content in agriculture, can be a source of As incorporation into the food chain.

It is assumed that plants take up As(V) through phosphate transporters due to their similarity with phosphorous, while As(III) is incorporated by aquaporin channels [2]. The ability to accumulate As differs among plant species, therefore As concentration transferred from soil to plant, represented by the As transference coefficient (As TC), ranges from 0.01 to 0.1 [3]. High As uptake by edible plants constitutes a serious problem and further when plants translocate high levels of As to aerial portion and to the grains. In this sense, rice plants (*Oriza sativa*) are being thoroughly studied because they accumulate the metalloid in grains to hazardous concentrations for human consumption [4,5].

Since legumes have been found as natural pioneers in contaminated sites allowing re-vegetation of soils, there is an increasing interest in their use for remediation purposes [6–8]. In this sense, leguminous plants such as *Lupinus albus* L. and more recently leguminous woody species, such as *Mimosa caesalpiniaefolia, Erythrina speciosa* and *Schizolobium parahyba* has been postulated as potential species to remediate and also for the re-vegetation of contaminated areas containing high concentration of Zn and lead (Pb) [9,10]. In addition, rhizobial bacteria have been found in soils with high As and other metals concentrations [11,6] which have allowed proposing

Abbreviations: As, Arsenic; As(V), arsenate; As(III), arsenite; CFU, colony-forming unit; DW, dry weight; FW, fresh weight; OD, optical density; TC, transference coefficient; YEM, yeast mannitol medium.

* Corresponding author. Tel.: +54 358 4676537; fax: +54 358 4676232.
E-mail addresses: mtalano@exa.unrc.edu.ar, melinatalano@hotmail.com (M.A. Talano), rominacejas@hotmail.com (R.B. Cejas), pgonzalez@exa.unrc.edu.ar (P.S. González), eagostini@exa.unrc.edu.ar (E. Agostini).

[1] Should be considered as first authors, because they have equally contributed to this work.

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the *Rhizobium*-legume interaction as an interesting strategy for soil remediation. Pajuelo et al. [12] demonstrated that alfalfa-*Rhizobium* partnership exposed to arsenite showed a reduction in nodule number due to the establishment of minor infection events. In this case, once established the nodulation, nodules continued a normal development and they were effective for nitrogen fixation. The authors concluded that the reduction of rhizobial infections was mainly associated with root damage produced by As. Considering all these antecedents, the use of PGPR (Plant growth promoting rhizobacteria) has been proposed as an alternative for rhizoremediation due to their contribution with the improvement of plant growth and development in contaminated regions as it was the case for wheat–*Azospirillum* interaction [13]. In addition, Lyubun et al. [13] also found that wheat plants inoculated with *Azospirillum brasilense* Sp245, a natural nitrogen(N)-fixing endophyte which produce indole acetic acid, accumulated less As than non-inoculated plants. Based on those studies, it is proposed that legumes tolerance to metals/metalloids could depend on their ability to establish and maintain effective partnerships with beneficial bacteria/rhizobacteria. However, rhizospheric microorganism survival, root colonization ability and metabolism should also be considered since they can produce changes in metal/metalloids mobility and availability, affecting their speciation and toxicity for plants [14].

Soybean (*Glycine max*) is a legume with worldwide economic importance. The Southern region of South America is one of the major producer and exporter of grains, flour and oil. Actually, Argentina has a cultivated area of 18.8 million hectares of soybean, with a production of 49 million tons (2011–2012) [15]. Since soybean has high protein content in grains, it becomes the crop with the greatest demand for N [16]. In this context, soybean-rhizobia symbiosis is an important ecological and agronomical association because through biological N-fixation process, plants receive enough N supply and hence, the use of nitrogen fertilizers could be reduced. In particular, for soybean, application of inoculants based on *Bradyrhizobium japonicum* has been widespread since this symbiont was absent in soils when this crop was introduced in Argentina [17]. Moreover, *B. japonicum* E109 strain was selected as the most suitable for soybean inoculants formulation in Argentina, by the Strains Collection Laboratory of the Agricultural Zoology and Microbiology Institute (IMYZA) and the Agricultural Technology National Institute (INTA).

Soybean is often cultivated in soils with high As levels as well as in regions where As containing groundwater is being increasingly used for irrigation schemes. However to our knowledge, there is no enough research about soybean tolerance to As exposition and the effect of this toxic metalloid on the soybean–*Bradyrhizobium* interaction. Therefore, the specific objectives of this study were to examine: (a) the effect of sodium arsenate and arsenite on early stages of soybean growth (germination and seedling development), (b) *B. japonicum* E109 resistance to As and the effect of the metalloid on bacterium swarming and swimming motility, (c) the effect of As on legume-rhizobium symbiosis and, finally (d) As accumulation pattern in roots and aerial parts of inoculated and non-inoculated plants. The implication for food chain of high As accumulation in soybean tissues is discussed.

2. Results

2.1. Effect of As on soybean germination and development parameters at early stage of growth

Germination percentages of seeds exposed up to 10 μM of As salts did not showed significant differences compared with controls (Fig. 1A). However, soybean germination was negatively affected by 25 and 50 μM sodium arsenate and arsenite treatments. The highest arsenate and arsenite concentration tested (50 μM) produced a reduction in germination of around 46% and 76%, respectively, being these differences statistically significant.

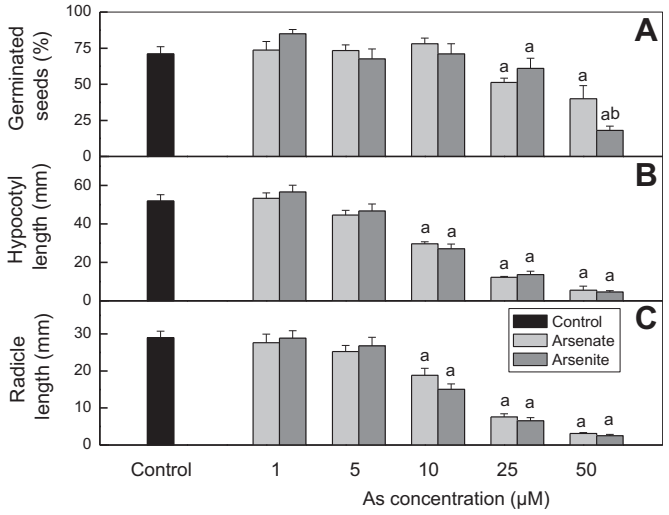

Fig. 1. Effect of different arsenate or arsenite concentrations on (A) seed germination, (B) hypocotyl length and (C) radicle length of soybean seedlings after 8 d. Data represents means ± standard error. (a) Indicative of significant differences from control. (b) Significant differences between arsenate and arsenite treatments (Duncan's test, $p < 0.05$).

Hypocotyl and radicle lenght was not affected with 1 and 5 μM arsenate and arsenite treatment after 8 d of development (Fig. 1B, C). However, treatments with As salts from 10 μM produced a significant inhibition of hypocotyl and radicle length compared with control seedlings. This inhibitory effect was As concentration dependent, reaching almost a completely inhibition of soybean seedling development at 50 μM of arsenate or arsenite.

2.2. Resistance and growth of B. japonicum E109 under As treatments

In order to establish *B. japonicum* E109 resistance to arsenate and arsenite, its ability to grow in yeast extract mannitol agar (YEMA) plates containing different As concentrations was evaluated. The results showed that *B. japonicum* was able to grow in all arsenate concentrations tested until 5 mM; however, the microorganism resisted only up to 10 μM arsenite (data not shown). Similarly, in liquid medium growth was not affected with arsenate until 500 μM, but it was considerably reduced with arsenite (Fig. 2). Growth of *B. japonicum* E109 was strongly reduced with 25 and 50 μM arsenite, reaching lower values of optical density (OD_{600nm}) at 120 h (1.08 for 25 μM and 0.38 for 50 μM), compared to control treatment. This effect was more pronounced with increasing arsenite concentration showing complete growth inhibition for 100 μM arsenite.

2.3. Effect of As on B. japonicum E109 motility: swarming and swimming

Both swarming and swimming are involved in early steps of plant colonization and nodule formation. To the best of our knowledge, little is known about the effect of As on the motility of rhizobia, thus, different concentrations of sodium arsenate and arsenite were assayed to study swarming and swimming motilities of *B. japonicum* E109. On control 0.5% agar plates (without As), *B. japonicum* E109 exhibited swarming behavior, showing the typical colonies with irregular edges and filamentous extrusions (Fig. 3A1). When arsenate or arsenite were added to the medium

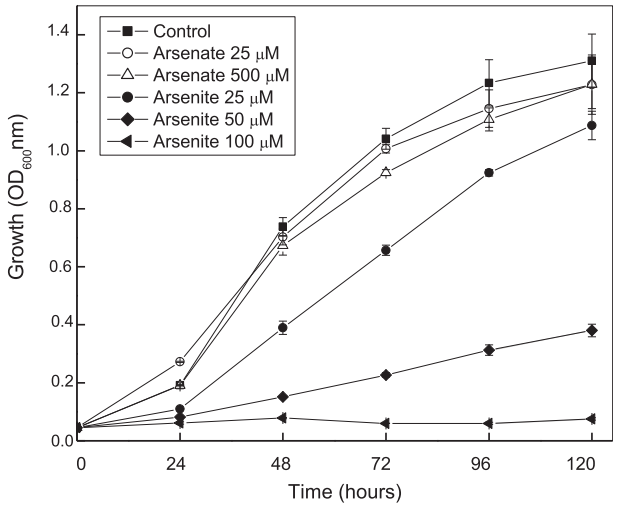

Fig. 2. Growth of *B. japonicum* E109 in YEM liquid medium containing 0, 25 and 500 μM sodium arsenate or 25, 50 and 100 μM sodium arsenite.

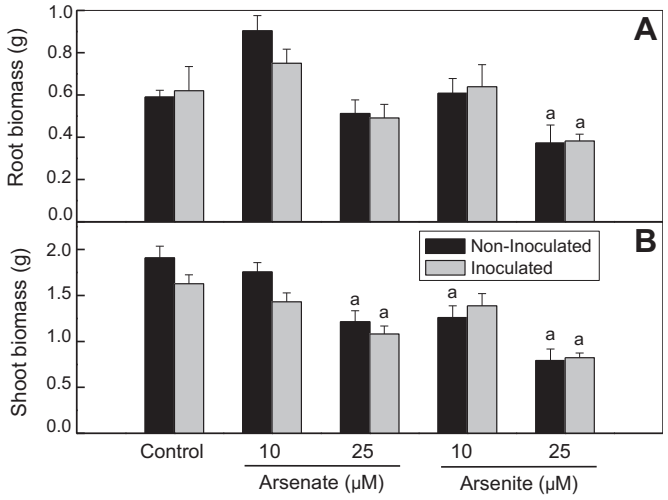

Fig. 4. Effect of 10 and 25 μM sodium arsenate or arsenite on (A) root and (B) shoot biomass of soybean plants non-inoculated and inoculated with *B. japonicum* E109. Data represents means ± standard error. (a) As treatments significantly different from the control (Duncan's test, $p < 0.05$).

the normal consolidation was not observed (Fig. 3A2–A5), and the diameter of swarming ring decreased for all tested concentrations (Fig. 3C). Treatments with arsenate 10–500 μM or arsenite 10 μM produced an inhibition of around 70% of swarming ring compared with control conditions, while arsenite 25 μM was the concentration that mostly affected *B. japonicum* E109 swarming motility (Fig. 3C). Swimming was not affected by any of the sodium arsenate concentrations tested (Fig. 3D, B2 and B3), while the diameter of swimming ring decreased with arsenite treatments being completely inhibited at 25 μM. At this concentration the strain was unable to swim (Fig. 3D, B4 and B5).

2.4. Effect of inoculation on soybean growth and nodulation under As treatment

Based in previous results, two arsenate and arsenite concentrations (10 and 25 μM) were selected to evaluate the effect of As on soybean growth and nodulation, in plants inoculated with *B. japonicum* E109.

Regarding soybean growth, root biomass did not showed significantly differences for 10 and 25 μM arsenate and 10 μM arsenite compared with control plants (without As), whereas it decreased significantly with 25 μM arsenite (Fig. 4A), showing no statistically significant differences between non-inoculated and inoculated plants. Shoot biomass was significantly affected by 25 μM arsenate and from 10 μM arsenite compared with plants grown without As, except than inoculation attenuated the reduction in shoot biomass for 10 μM arsenite treatment (Fig. 4B).

In order to evaluate and compare the physiological status of non-inoculated and inoculated plants in response to As, chlorophyll *a*, *b* and carotenoids were quantified. Chlorophyll *a*, *b* and carotenoid levels did not change with arsenate and arsenite treatments neither in inoculated nor in non-inoculated plants compared with

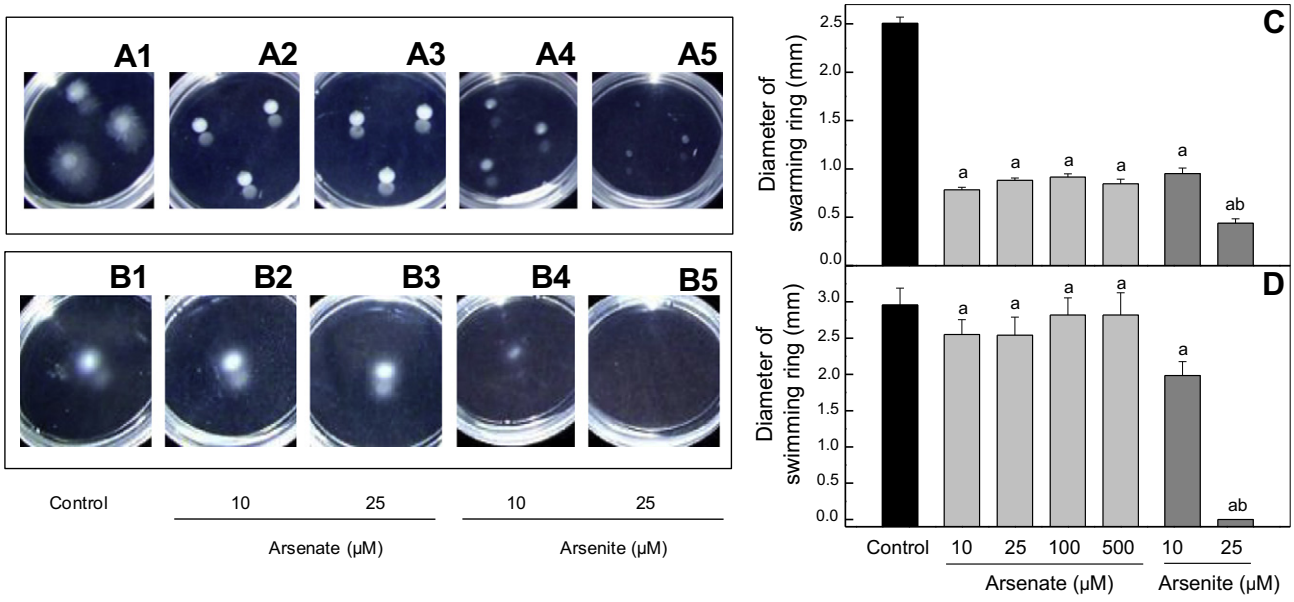

Fig. 3. Effects of different As concentrations on swarming (A1-A5) and swimming (B1–B5) behavior of *B. japonicum* E109 under control conditions (A1, B1) or supplemented with 10 and 25 μM of sodium arsenate (A2, A3, B2, B3) or arsenite (A4, A5, B4, B5). Colony diameter of swarming (C) and swimming (D) of *B. japonicum* E109 exposed to different As concentrations. Data represents means ± standard error. (a) As treatments significantly different from the control. (b) Treatment significantly different from the other As treatments (Duncan's test, $p < 0.05$).

control conditions except for plants treated with 25 μM arsenite. Under this condition, inoculated plants showed significantly higher values of chlorophyll *a*, *b* and carotenoids compared with non-inoculated plants (Table 1).

Nodulation of soybean plants inoculated with *B. japonicum* E109 was affected by As. The number of total nodules per plant decreased approximately 40% and 60% for 25 μM arsenate or arsenite, respectively, but this difference was not statistically significant (Fig. 5A). The number of active nodules per plant only decreased significantly for 25 μM arsenite (Fig. 5B).

2.5. Content of As in root and shoots of soybean

Arsenic content in soybean tissues of non-inoculated and inoculated with *B. japonicum* E109 plants growing on pots supplemented with 25 μM arsenate or arsenite was quantified. In addition, As TC values in root and shoot of soybean plants were calculated.

As it could be seen in Table 2, As concentration was significantly higher in roots than in shoots and As TC values in roots were around 1000-fold higher than in shoots. There were no significant differences in As content of non-inoculated and inoculated soybean plants and between arsenate and arsenite treatments both in root and shoots.

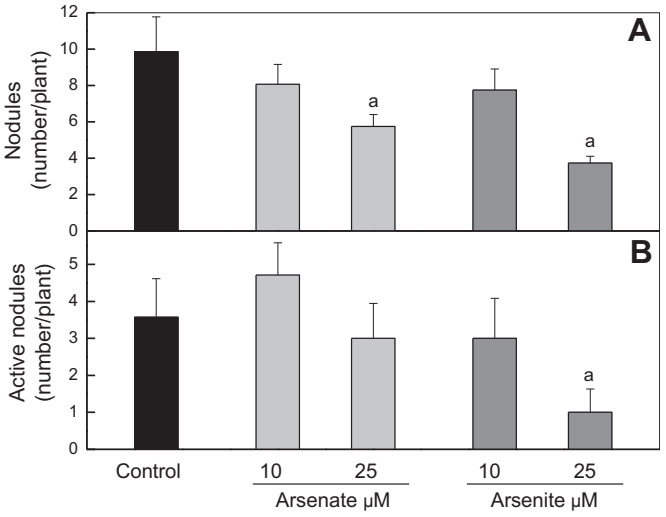

Fig. 5. Effect of 10 and 25 μM sodium arsenate or arsenite on the number of (A) total root nodules per plant and (B) effective nodules of soybean plants inoculated with *B. japonicum* E109. Data represents means ± standard error. (a) As treatments significantly different from the control (Duncan's test, $p < 0.05$).

3. Discussion

Legumes have been found as pioneer species on As contaminated sites [6]. These plants can establish interactions with rhizobial bacteria, which could attenuate toxic effects of metalloids in plants through higher N supply or other plant growth promoting property. However, root nodulation of legumes is generally reduced or absent in As contaminated soils [18]. To our knowledge, there are no enough studies about the effect of both As salts, arsenate and arsenite, on soybean development and hence, on production yield estimation, as well as whether soybean–*B. japonicum* E109 symbiosis is affected by As. In the present work, the effect of rhizobia on tolerance and accumulation of this metalloid in soybean tissues has also been addressed.

As it was shown, germination was significantly reduced with concentrations from 25 μM of both As salts. In particular, 50 μM arsenite produced a significantly higher germination reduction (76%) compared with arsenate at the same concentration (46%), which showed remarked arsenite higher toxicity. Germination of *Medicago sativa* seeds was similarly reduced under 40 μM arsenite treatment since a germination inhibition of about 60% was observed [12]. Contrarily, wheat germination was less affected by As salt treatments, since 57 μM arsenate and arsenite only inhibited 13% and 20% germination, respectively [19]. However, these authors found that other parameters, such as wheat shoot and root length were reduced more significantly than germination rate with increasing As concentrations, thus they might be used as indicators of As toxicity and/or vegetative response endpoints. In similar way, soybean root and shoot length was more sensitive than germination to As treatments, not only at early stages of growth but also for 25-d-old seedlings.

As mentioned before, concentrations from 10 μM of both salts reduced significantly hypocotyl and radicle length of soybean seedlings at early stage of growth. Similar inhibitory effect of As treatments was seen for seedlings grown in volcanic sand after 25 d. In this case, root biomass was significantly reduced by arsenate and arsenite concentration from 25 μM, whereas shoot biomass was reduced significantly under 25 μM arsenate and 10 μM arsenite treatments. These results, clearly demonstrated the high As sensitivity of this plant species. Reichman [20] studied the behavior of another cultivar of soybean plants under arsenate treatment. In those assays, soybean showed higher inhibition of growth than in the present work since arsenate concentration from 1 μM produced a significant reduction in root (38%) and shoot (48%) biomass. However, no data related to arsenite effects were shown. This difference could be due to the system and conditions selected by Reichman [20] for soybean growth because the assay was carried out in hydropony, promoting continuous contact of roots with contaminated solution with the consequent higher As accumulation and producing major deleterious effect on soybean plants than in the present work.

Since As treatments had deleterious effects on soybean growth, we were interested in elucidate whether soybean–*B. japonicum* E109 association could improve As plant tolerance. First, *B. japonicum* resistance to arsenate and arsenite was evaluated and moderate arsenate resistance was found in solid and liquid medium. However, the microorganism only tolerated 10 μM arsenite in agar plates and its growth rate was mildly diminished under 25 μM arsenite treatment in liquid medium. Despite that, soybean seedlings inoculated with *B. japonicum* did not show significantly differences in growth, neither in shoot nor in root biomass, compared with non-inoculated plants. However, chlorophyll and carotenoid contents were significantly higher in shoots of inoculated plants compared with non-inoculated ones under 25 μM arsenite treatment. Even though it would indicate a slight advantage of inoculation, more studies are needed to confirm these findings by measuring additional biochemical and/or physiological parameters. On the contrary, soybean plants inoculated with *B. japonicum* CB1809 from Australia improved until 38% biomass

Table 1
Chlorophyll *a*, *b* and carotenoid levels of non-inoculated and inoculated plants treated with 25 μM arsenite during 25 d.

(mg g^{-1} FW)	Non-inoculated	Inoculated
Chlorophyll *a*	1.02 ± 0.04	1.26 ± 0.01*
Chlorophyll *b*	0.04 ± 0.01	0.081 ± 0.01*
Carotenoids	70.26 ± 2.38	83.39 ± 1.18*

*Significant differences ($p < 0.05$).

Table 2
As accumulation and effect of *B. japonicum* E109 inoculation on soybean tissues treated with 25 μM arsenate (As V) and arsenite (As III).

			Dry weight (g)	As content (mg kg^{-1})	As TC
Root	Non-inoculated	As (V)	0.092 ± 0.012	357.4 ± 30.8	0.160 ± 0.014
		As (III)	0.067 ± 0.015	371.1 ± 12.7	0.094 ± 0.002
	Inoculated	As (V)	0.089 ± 0.011	290.1 ± 5.5	0.159 ± 0.005
		As (III)	0.069 ± 0.006	423.2 ± 104.1	0.141 ± 0.035
Shoot	Non-inoculated	As (V)	0.129 ± 0.013	0.63 ± 0.01	$3.9 \times 10^{-4} \pm 9.2 \times 10^{-6}$
		As (III)	0.084 ± 0.013	1.11 ± 0.002	$9.6 \times 10^{-4} \pm 7.7 \times 10^{-5}$
	Inoculated	As (V)	0.115 ± 0.009	2.35 ± 0.19	$6.2 \times 10^{-4} \pm 1.0 \times 10^{-6}$
		As (III)	0.087 ± 0.005	1.93 ± 1.37	$8.2 \times 10^{-4} \pm 5.8 \times 10^{-4}$

Data represents means ± standard error. Duncan's test ($p < 0.05$).

yield comparing inoculated and non-inoculated plants treated with 10 μM arsenate [20]. This increase in biomass of inoculated plants was attributed to additional growth promoting properties of *B. japonicum* CB1809 such as phytohormone production instead of improved N nutrition or As absorption. Considering the obtained results, *B. japonicum* E109 seems not to be a proper partner in order to attenuate negative effects of As on soybean growth. Thus, water and/or soils containing As would negatively affect soybean production, even more when As contaminated water is being increasingly used for soybean irrigation in regions with low rainfall records [21].

Treatments with As produced a significant reduction in total and effective nodule number, similarly to that found by Reichman [20], which has been by root disorders caused by the metalloid. In this sense, Pajuelo et al. [12] found important injury evidences by As on roots as well as decrease of root hairs number, which was related with a decrease of around 90% in rhizobia infection number in *M. sativa.* For legumes exposed to other metals (Cu2+, Cd2+, Hg2+) the reduction in nodulation was also attributed to the injury of metals on root hairs [22,23]. However, the minor nodulation of soybean plants could be also the result of reduced motility of the microorganism under As presence.

Before nodulation, an effective bacterial colonization of root surface is needed. This colonization depends, at least partially, on bacterium motility. It is assumed that *B. japonicum* has two groups of flagella, one of them is frequently used for swimming motility to reach plant roots, while the other group is used for swarming to form biofilms in root surface previous to nodule formation [24]. As shown in the present work, *B. japonicum* E109 swarming motility was considerable reduced under 25 μM of both As salt treatments, and swimming motility was inhibited with 25 μM arsenite. Therefore, the reduced motility showed by *B. japonicum* E109 under As exposition could be at least one of the reasons for the minor nodulation, since the metalloid would affect root colonization or biofilm formation with a consequent alteration of infection process. Although the most frequent mechanisms for As resistance involves As(III) reduction and/or efflux through proteins encoded by *ars* genes [25], As(III) oxidation and/or As methylation [26,27], some bacterial species develop biofilms as another mechanism for As resistance. For example, *Thiomonas arsenivorans* developed biofilms as physical barrier to decrease As(III) accessibility to sessile cells [28]. Thus, if bacteria motility is reduced under metal toxicity and preferably microorganisms promote biofilm formation to avoid the toxicity of As, minor number of bacteria would be available for the establishment of effective root infections. More studies related to the effect of As in biofilm formation and exopolysaccharides production in *B. japonicum* E109 are currently being carried out in our laboratory to contribute with our understanding of the minor nodulation observed under As treatment.

Bacterial metabolism can affect As speciation and bioavailability in the rhizosphere through different mechanisms [14], affecting positive or negatively plant development as well as uptake and/or translocation of the metalloid in plants. As concentration in soybean roots was about 250 times higher than in shoots. However, no significant differences were found in As content in inoculated soybean plants treated with 25 μM arsenate or arsenite, compared with non-inoculated plants. Contrarily, the inoculation of greengram (*Vigna radiata*) with *Bradyrhizobium* sp. (vigna) strain RM8 reduce the content of nickel and zinc in grains obtaining increased seed yield and seed protein compared with non-inoculated plants [29] which would imply higher crop yield and economical advantages in field production. Regarding As TC, values found for roots were about 1000 times higher than TC of shoots, which indicated that As translocation to aerial portion of soybean plants is low and that the metalloid was mainly accumulated in roots. Similarly, poor translocation of nickel and zinc from roots to shoot system were seen in greengram plants under both inoculated and non-inoculated treatments [29]. This data clearly reflects the low possibility that As could be accumulated in seeds, and thus soybean crops irrigated with contaminated water would not be a potential entry of As into the food chain with the consequent risk for human and/or animals health. Since soybean plants were able to accumulate As in their roots with scarce translocation to aerial portions, these plants would constitute a natural system of As phytoremediation/phytostabilization *in situ,* even when they were inoculated with *B. japonicum* E109. This additional As rhizoremediation capacity of soybean is important, due to that crop covers a substantial extension of soils and in some cases, as it was pointed out before, it is being cultivated in regions under irrigation with water containing high As levels, which is progressively incorporated in soils.

4. Conclusion

All these findings showed that soybean growth was significantly reduced by 25 μM As, mainly arsenite, even on inoculated plants, in which the number of total and effective nodules was diminished. The presence of this metalloid in soils could negatively affect soybean–*B. japonicum* E109 symbiosis and, hence, the efficiency of most used inoculants. Consequently, high levels of As in water/soils would negatively impact on the production of soybean crop.

5. Materials and methods

5.1. Plant material

Soybean (*G. max*) cv. Don Mario DM 4670 seeds were used. Aseptic soybean seedlings were obtained as follow: seeds were disinfected with 70% ethanol during 10 min and with 30% diluted commercial bleach solution for 10 min. Then, they were washed three times with sterile distilled water. After this procedure, seeds were kept overnight in dark under agitation in an Erlenmeyer flask

with sterile water. Then, seeds were used for different assays as described below.

5.2. Effect of As on soybean germination and development parameters in early stages of growth

For germination evaluation, 10 aseptic seeds were placed in each plate containing moistened filter paper with 6 mL of distilled water (control) or solutions with 1, 5, 10, 25 and 50 μM sodium arsenate heptahydrate ($AsHNa_2O_4$ $7H_2O$) [As(V)] and sodium arsenite ($NaAsO_2$) [As(III)] respectively, both from SIGMA. The plates were placed at 27 ± 2 °C, in the dark. After 8 d, germinated seeds, considering those with radicles of 3 mm or longer, were counted and length (mm) of both radicle and hypocotyl were registered. The assay was repeated three independent times by quadruplicate.

5.3. Bacterial strain

B. japonicum E109 strain was gently provided by our colleague Dr. F. Cassán from the Agriculture Zoology and Microbiology Institute (IMYZA) and the Agricultural Technology National Institute (INTA), Castelar, Argentina. This microorganism was cultivated in liquid YEM medium [30] and/or solid medium (YEMA) supplemented with Congo red (25 mg L^{-1}) and vancomycin (4 μg mL^{-1}) to avoid contamination.

5.4. Resistance and growth of B. japonicum E109 under As treatments

Resistance of *B. japonicum* E109 to different sodium arsenate and arsenite concentrations was evaluated. For that, bacterium was spread in YEMA agar plates without As (control) and others supplemented with 5, 10, 50, 100 and 500 μM and 5 mM of each As salt. Plates were incubated at 30 °C. The maximum tolerated concentration was established as the highest concentration of the contaminant at which bacterial growth could be observed after 7 d.

Growth of *B. japonicum* E109 in YEM liquid medium with As was also evaluated. Culture flasks containing 20 mL of YEM with vancomycine (4 μg mL^{-1}) and others supplemented with sodium arsenate 25 and 500 μM and sodium arsenite 25, 50 and 100 μM, respectively, were inoculated with a proper volume of a *B. japonicum* E109 culture previously grown in YEM liquid medium without As to reach an initial OD_{600nm} of 0.05. Cultures were incubated in an orbital shaker at 200 rpm and 30 °C. OD_{600nm} was monitored each 24 h, for a period of 120 h. The assay was repeated three independent times by triplicate.

5.5. Effect of As on B. japonicum E109 motility: swarming and swimming

YEMA plates for swimming and swarming motility assays contained 20 mL of 1/10 YEM supplemented with antibiotic and 0.3 or 0.5% agar (SIGMA), respectively. Plates were supplemented by a proper volume of stock solutions of sodium arsenate and arsenite to reach concentrations of 25, 50, 100 and 500 μM of arsenate and 10 and 25 μM of arsenite. Agar plates were dried for 30 min in a laminar flow hood before being inoculated with 7-d-old *B. japonicum* E109 colonies grown in a YEMA plate at 30 °C. The plates were then inverted and incubated at 30 °C during 8 d. The distance from the point of inoculation to the border of the swimming or swarming colony was measured and results were photographed using a Kodak K Z712 digital camera.

5.6. Inoculation assays

Five disinfected pre-germinated soybean seeds were placed in pots containing 190 g of sterile volcanic sand. Previously, pots were moistened with 110 mL of Hoagland solution or modified Hoagland without nitrogen, with or without 5, 10 or 25 μM of sodium arsenate or arsenite. For inoculation assay, bacterial inoculum was obtained by growing *B. japonicum* E109 during 6 d at 30 °C and 200 rpm in liquid YEM medium containing antibiotic. The culture was centrifuged at 10,000 rpm during 20 min and the pellet was suspended in saline solution (NaCl 0.9%) to reach an OD_{600nm} of 1.56. The CFU mL^{-1} of this bacterial suspension was calculated by drop count plate method [31]. Aliquots of 0.5 mL of this suspension (10^9 CFU) were used to inoculate seeds, which were grown in pots containing modified Hoagland medium. Plants grown in Hoagland medium with nitrogen were used as controls and they were not inoculated. Pots were incubated in a chamber with controlled temperature (27 ± 2 °C) under photoperiod regime (16 h light/8 h dark). The plants were irrigated with Hoagland (1/4) and modified Hoagland (1/4) solution as corresponds during the first 6 d; then, water was used for irrigation.

Soybean plants were harvested after 30 d and fresh weight (FW) of roots and shoots was registered for each treatment. In inoculated plants, nodules were dissected and counted. The red ones were classified as active nodules, which are indicative of leghemoglobine presence and potential N-fixation capacity, whereas those white, were classified as inactive nodules [32]. Shoot and root tissues were frozen in liquid nitrogen, homogenized in a mortar and kept at −80 °C. Then, these samples were used for chlorophyll and carotenoids determination as described in Dere et al. [33] as well as As quantification, as it is described later.

5.7. As quantification in soybean roots and shoots

Root and shoot samples of inoculated and non-inoculated soybean plants exposed to 25 μM of arsenate and arsenite were used for As quantification. For that, they were dried until constant weight at 80 °C. Then, dried tissue was digested with 3 mL of nitric acid and 2 mL of H_2O_2 (30%) and finally, total As was determined by flame atomic absorption spectrometry (AAS) technique, as described in AOAC [34]. Results of total As were expressed as mg kg^{-1} of dry weight and values of As TC were calculated as follow:

$$\text{As TC} = \text{mg As plants/mg As in the pot}$$

$$\text{As TC} = (\text{DW(g)}/1000 \times \text{C As (mg kg}^{-1})/0.11\ L \times 1.87\ \text{mg L}^{-1}$$

where DW (g) = average of dry weight per plant; C As (mg kg^{-1}) = As concentration in the tissue (root or shoot); 0.11 L = volume of 25 μM As solution added per pot; 1.87 mg L^{-1} = equivalence in mg L^{-1} of a solution 25 μM As.

5.8. Statistical analysis

All experiments were performed three or four times in independent assays. Results were analyzed with STATISTICA (version 6.0) software. Variance analysis (ANOVA) and post hoc *Duncan* test were applied to determine significant differences between groups. Results were considered statistically significant when $p < 0.05$.

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