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**Symbiosis**

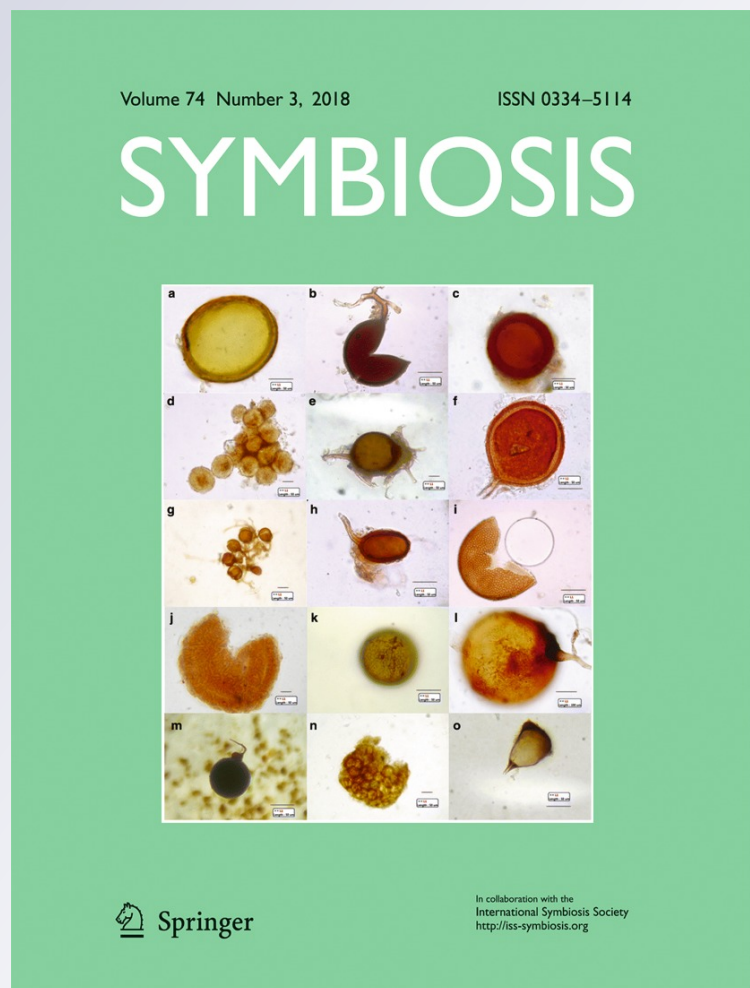
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# Arsenic toxicity in soybean alleviated by a symbiotic species of *Bradyrhizobium*

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**Abstract** Arsenic (As) is a toxic metalloid that has gained special interest in the past years as a global environmental problem. Groundwater in Córdoba province (Argentina) presents high As concentrations which can be absorbed by plants or be used for artificial irrigation. The aim of this research was to elucidate the differential responses of symbiotic interactions established with three bacterial strains and soybean plants to realistic doses of arsenic. The reference strain *Bradyrhizobium diazoefficiens* USDA110 and the native isolate *Bradyrhizobium* sp. Per 3.64 were able to grow up to 13 mM As(V) whereas the native strain *Bradyrhizobium* sp. Per 3.61 grew up to 9.5 mM As(V). Metalloid addition did not modify the soybean plant growth at 6  $\mu$ M As(V). Nevertheless, it was enough to induce oxidative stress as observed by an increase on lipid peroxidation. The soybean-*Bradyrhizobium* sp. assay at 6  $\mu$ M As(V) showed no changes in growth variables (shoot and root dry weight) in plants inoculated with the reference microsymbiont or *Bradyrhizobium* sp. Per 3.61. Regarding As uptake by plants, metalloid accumulation followed the same distribution pattern among strains. Remarkably, at 6  $\mu$ M As(V), soybean inoculation with *Bradyrhizobium* sp. Per 3.61 revealed a significantly lower translocation factor (TF) in comparison to other

inoculated strains promoting As phytostabilization. At the highest As(V) concentration tested, only *Bradyrhizobium diazoefficiens* USDA110 was able to nodulate the legume, however, a significant decrease in the number and dry weight of nodules and nitrogen content was observed. In conclusion, the inoculation of soybean plants with the reference strain *Bradyrhizobium diazoefficiens* USDA110 exposed to high As(V) concentration represents an effective and promising symbiotic interaction that allows the development of the legume due to the minimal effects on plant growth. However, in low As(V) concentration environments, the native isolate *Bradyrhizobium* sp. Per 3.61, is shown to be the best inoculant among the tested strains, owing to the limitation of metalloid translocation and accumulation to edible parts of the legume, avoiding fruit contamination and human poisoning.

**Keywords** Arsenic · Legume · *Bradyrhizobium* sp. · Symbiotic interaction

## 1 Introduction

Arsenic (As) is a well-known toxic element that is of special interest in Córdoba province (Argentina) since around 90% of its groundwater has As concentrations coming from sedimentary depositions that exceed the maximum level allowed for drinking water (0.1  $\mu$ M) (FAO 2016). This is aggravated due to anthropogenic activities that increase metalloid levels in the environment (Trueb 1998; Reimann and de Caritat 1998). In some regions, including cropping areas, As concentration can reach up to 24  $\mu$ M (Cabrera et al. 2005; Francisca et al. 2006). As toxicity is related to its chemical form and oxidation state, it is present both in organic and inorganic forms, the latter being the most toxic (Hughes 2002). Arsenate ( $\text{AsO}_4^{3-}$ , As(V)) and arsenite ( $\text{AsO}_2^-$ ,

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As(III)) are the prevalent inorganic chemical species of As and are highly soluble and stable in water under a wide range of pH and redox conditions. Under oxidizing conditions, the predominant arsenic form is arsenate (Blarasin et al. 2014). Arsenate is an analog of phosphate ( $\text{PO}_4^{3-}$ ) and can be absorbed by cells via  $\text{PO}_4^{3-}$  transporters (Tripathi et al. 2007). Once in the cytoplasm As(V) is rapidly transformed by different arsenate reductase enzymes to As(III) which causes oxidative stress due to high sulphhydryl reactivity (Dhankher et al. 2006; Duan et al. 2007; Verbruggen et al. 2009). Finnegan and Chen (2012) demonstrated that the oxidative stress induced by the overproduction of reactive oxygen species (ROS) in plants is one of the most harmful constraints induced by heavy metals and metalloids as observed in *Phaseolus vulgaris* and *Arachis hypogaea* L. (Talukdar 2013; Bianucci et al. 2017). Moreover, metal(-loid)s cause well-known physiological disturbances associated with growth inhibition, depletion of photosynthesis and respiration rates, root darkening, chlorosis and in some cases death (Poschenrieder et al. 1989; Sanitá di Toppi and Gabbrielli 1999; Benavides et al. 2005; Sobrino-Plata et al. 2009; Bianucci et al. 2012, 2013, 2017).

Soybean (*Glycine max* L.) is a legume with a high nutritional quality due to its high protein and fat levels and low carbohydrate content, representing an important crop in terms of the global economy. Argentina, United States, Brazil and China are the world's major producers of soybean. Crop yield relies on nitrogen (N) supply that can be absorbed from soils by plants as nitrate ( $\text{NO}_3^-$ ) and ammonia ( $\text{NH}_4^+$ ) or being transformed from the atmosphere by microorganisms in a process known as biological nitrogen fixation (BNF). One of the most efficient nitrogen fixation systems is constituted by the symbiotic interaction established between soybean and members of the rhizobia family, in which a new organ able to fix atmospheric N is formed in the root: the nodule. Thus, BNF represents the main source of N for soybean, being an economically and ecologically sustainable alternative to the use of nitrogen fertilizers (Wang and Li 2003). Environmental stresses have an important impact on BNF since they can reduce or inhibit the process, which can lead to decreasing crop yield (Zahran 1999). Therefore, considering As levels found in Córdoba's groundwater, the direct absorption by plants constitutes not only a serious agricultural problem but also a risk to humans through contamination of the trophic food chain. In addition, Córdoba province is prone to water scarcity and so crops require artificial irrigation where As may be present, otherwise root systems could elongate to find water and reach groundwater with high As concentration (Sharp and LeNoble 2002). In order to select the best inoculant for alleviating As toxicity in soybean plants we studied the response of the symbiotic interaction established with three bacterial strains and the plant, subject to realistic doses of As(V) supply as found in Córdoba's groundwater.

## 2 Material and methods

### 2.1 Bacterial strains

*Bradyrhizobium diazoefficiens* USDA110, reclassified from *Bradyrhizobium japonicum* USDA110 by Delamuta et al. (2013), was obtained from Belstville, USDA. *Bradyrhizobium* sp. Per 3.61 and *Bradyrhizobium* sp. Per 3.64 are native isolates obtained from soils cultivated with soybean plants in Pergamino (Buenos Aires, Argentina) by Fernández et al. (2008). Bradyrhizobial strains were grown on yeast extract mannitol (YEM) culture media (Vincent 1970) devoid of As (control) or containing varying concentrations (6–16 mM) of  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$  and incubated on an orbital shaker (150 rpm) at 28 °C during 96 h. The viability of each strain was determined by the number of viable cells at different time points of bacterial growth as colony forming units (CFU)  $\text{ml}^{-1}$  by the drop-plate method (Somasegaran and Hoben 1994a) on YEM solid medium (15% agar w/v).

### 2.2 Plant material and growth condition

Soybean seeds cv. Don Mario 4200 RR (IVC group) widely used in Córdoba province (Argentina) were surface sterilized as described by Vincent (1970) and when the radicle reached 3–5 cm, individual seedlings were aseptically transferred into sterile pouches (hydroponic system) containing 30 ml of Hoagland medium (Hoagland and Arnon 1950). Plants were grown either in the absence or presence of As(V) in ascending concentrations. The arsenic concentrations tested were selected in order to be similar to the realistic doses found in groundwater in Córdoba province (6 and 20  $\mu\text{M}$ ) (Cabrera et al. 2005) and a third concentration above those mentioned (100  $\mu\text{M}$ ). Plants were grown in a controlled environment chamber (light intensity of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 16 h day/8 h night cycle, a constant temperature of 28 °C and a relative humidity of 50%) for 10 days. At harvest, plant length was measured and the roots and shoots were dried to constant mass at 65 °C for 72 h to determine their dry weight (DW).

### 2.3 Stress index

Lipid peroxidation was analyzed in soybean roots by determining the concentration of thiobarbituric acid-reactive substances (TBARs) as described by Heath and Packer (1968). Plant material (0.1 g) was homogenized in 20% (w/v) of trichloroacetic acid (TCA) and then mixed with 0.5% (w/v) of TBA. The extract was heated for 25 min at 95 °C and then the reaction was stopped on ice. Finally, samples were centrifuged at 6200 g for 6 min. TBARs were quantified in the supernatant by measuring absorbance at 535 nm, corrected for non-specific turbidity by subtracting the absorbance at 600 nm using a UV-visible light spectrophotometer.



## 2.4 Nodulation assay

Pre-germinated soybean seeds were aseptically transferred into the above mentioned hydroponic system (sterile pouches) containing 6 or 20  $\mu\text{M}$  As(V) and 5-day post-emergence plants were inoculated with 3 ml of *Bradyrhizobium diazoefficiens* USDA110, *Bradyrhizobium* sp. Per 3.61 or *Bradyrhizobium* sp. Per 3.64 ( $1 \times 10^9$  CFU  $\text{ml}^{-1}$ ). Inoculated plants were cultivated in a greenhouse under the same controlled environment conditions as described above. Thirty days after inoculation, plants were harvested and growth (shoot and root dry weight) and nodulation variables were determined. Nodules were cut transversely and an internal color scored as brownish red was assumed to be indicative of the presence of leghemoglobin, serving as an index of the potential N-fixation (Wittenberg et al. 1974; Angle et al. 1993; Ott et al. 2005). The nitrogen content of the soybean shoots was determined by the method described by Nelson and Sommers (1973).

### 2.4.1 Arsenic accumulation in plant tissues

Metalloid concentration was determined in soybean shoots, roots and nodules digested with an acid oxidizing mixture  $\text{H}_2\text{O}:\text{HNO}_3:\text{H}_2\text{O}_2$  (5:3:2) as described by Sobrino-Plata et al. (2009). Arsenic concentration was measured using inductively coupled plasma mass spectrometry (ICP-MS). To evaluate the transport of As from plant roots to shoots under metalloid treatment, the translocation factor (TF) was calculated (Singh and Agrawal 2007) using the following formula:  $\text{TF} = \text{As concentration of shoots} / \text{As concentration of roots}$ .

### 2.4.2 Statistical analysis

Experiments were conducted in a completely randomised design and repeated three times. The data were analyzed using ANOVA and Duncan's test at  $P > 0.05$ . Prior to the test of significance, normality and homogeneity of variance were verified using the modified Shapiro-Wilk and Levene tests, respectively. If homogeneity of variance was not given, data were transformed using an appropriate function.

## 3 Results

### 3.1 Effect of arsenic on bradyrhizobial viability

*Bradyrhizobium diazoefficiens* USDA110 and *Bradyrhizobium* sp. Per 3.64 were able to grow up to 13 mM As(V), showing a markedly extended lag phase but reaching a viability above  $1 \times 10^9$  UFC  $\text{ml}^{-1}$  at the end of the exponential phase. Moreover, both strains showed a similar growth rate at 6.5 mM As(V) in comparison with control

conditions showing a decrease of growth at 9.5 mM As (Fig. 1a, c). *Bradyrhizobium* sp. Per 3.61 grew up to 9.5 mM As(V) showing a reduction of growth even at the lowest As concentration tested, being inhibited at higher arsenate concentrations (Fig. 1b).

### 3.2 Arsenic impact on soybean plant growth

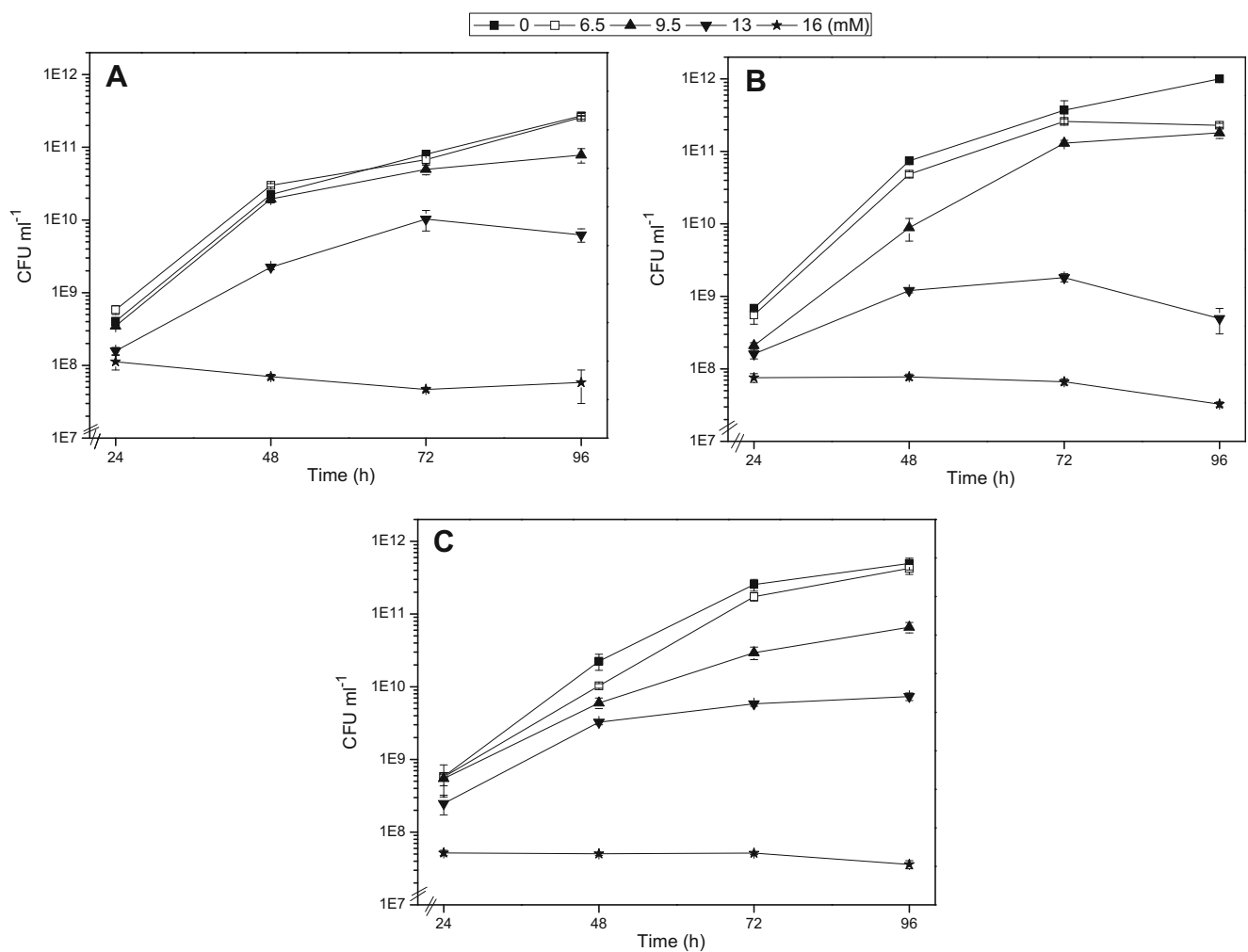
Soybean seedlings exposed to realistic doses of As(V) for 10 days did not show appreciable changes in their shoots; nevertheless, the roots had a reduction in the size of the primary root and an increase of the lateral root development up to 20  $\mu\text{M}$  As(V) (Fig. 2). These observable changes were confirmed by the measurement of growth variables. At 6  $\mu\text{M}$  As(V) no modification of shoot length and dry weight was detected; however, the primary root length was significantly reduced. At 20  $\mu\text{M}$  As(V) most of the growth variables were reduced, except for the root dry weight. At the highest As concentration tested all growth variables measured were significantly reduced. Taking into account the total dry weight of the plant, reductions of 25 and 60% were observed at 20 and 100  $\mu\text{M}$  As(V), respectively (Table 1).

### 3.3 Soybean lipid peroxidation

The addition of As(V) increased lipid peroxidation (estimated by TBARs content) of soybean roots from 6  $\mu\text{M}$  As(V), and in a significant and progressive way in accordance with increments of the metalloid concentration, reaching a plateau for the last two doses (Fig. 3).

### 3.4 Arsenic impact on the symbiotic interaction between soybean and *Bradyrhizobium* sp. strains

Soybean root length was affected at the minimal As(V) concentration tested irrespective of the inoculated bradyrhizobial strain. At 20  $\mu\text{M}$  As(V), only soybean plants inoculated with *Bradyrhizobium diazoefficiens* USDA110 maintained the root dry weight as in the control conditions (Table 2). The addition of 6  $\mu\text{M}$  As(V) caused a reduction of the shoot length and dry weight of soybean plants inoculated with *Bradyrhizobium* sp. Per 3.64, while plants inoculated with *Bradyrhizobium* sp. Per 3.61 showed a decrease of these variables at 20  $\mu\text{M}$  As(V). The shoot length was maintained at control condition levels in the interaction soybean- *Bradyrhizobium diazoefficiens* USDA110 at all As(V) doses tested, but the shoot dry weight was reduced at the highest dose. Remarkably, at 6  $\mu\text{M}$  As(V), plants inoculated with *Bradyrhizobium diazoefficiens* USDA110 showed a higher shoot dry weight than plants inoculated with *Bradyrhizobium* sp. Per 3.61 or Per 3.64 (Table 2). All tested strains were able to form nodules on soybean roots exposed to 6  $\mu\text{M}$  As(V) without significant differences to control conditions. Regardless of the



**Fig. 1** Bradyrhizobial viability at different arsenate concentrations. **a** *Bradyrhizobium diazoefficiens* USDA110, **b** *Bradyrhizobium* sp. Per 3.61, **c** *Bradyrhizobium* sp. Per 3.64. Data are the means  $\pm$  SE of four independent experiments



**Fig. 2** Impact of different arsenate concentrations on the exomorphology of soybean plants. **a** control; **b** 6  $\mu$ M; **c** 20  $\mu$ M; **d** 100  $\mu$ M

**Table 1** Effect of arsenic on soybean growth

As(V) Addition ( $\mu\text{M}$ )	Root length (cm)	Root dry weight (mg)	Shoot length (cm)	Shoot dry weight (mg)	% of total dry weight reduction
0	14.73 $\pm$ 0.66 <sup>A</sup>	34 $\pm$ 1.42 <sup>A</sup>	15.10 $\pm$ 0.63 <sup>A</sup>	94.47 $\pm$ 6.16 <sup>A</sup>	0
6	12.85 $\pm$ 0.67 <sup>B</sup>	37 $\pm$ 1.45 <sup>A</sup>	14.04 $\pm$ 0.49 <sup>A</sup>	92.07 $\pm$ 4.26 <sup>A</sup>	0
20	12.49 $\pm$ 0.43 <sup>B</sup>	33 $\pm$ 1.20 <sup>A</sup>	10.83 $\pm$ 0.43 <sup>B</sup>	64.94 $\pm$ 4.83 <sup>B</sup>	25
100	10.38 $\pm$ 0.54 <sup>C</sup>	23 $\pm$ 1.21 <sup>B</sup>	5.37 $\pm$ 0.40 <sup>C</sup>	29.21 $\pm$ 2.96 <sup>C</sup>	60

Data represent the mean  $\pm$  SE ( $n = 10$ ). Different letters in each column indicate significant differences ( $P < 0.05$ ) according to the Duncan's test

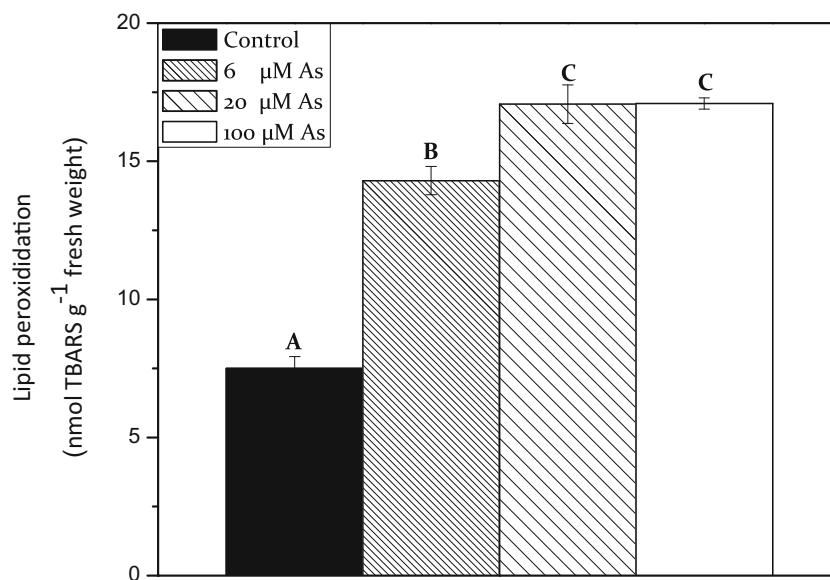
inoculation treatment, nitrogen content of soybean plants exposed to this As concentration showed no significant difference when compared with control plants. However, a comparison among strains revealed that inoculation with *Bradyrhizobium diazoefficiens* USDA 110 showed higher nitrogen content under control and treated conditions (6  $\mu\text{M}$  As(V)) compared to native isolates at the mentioned conditions (Table 3). At 20  $\mu\text{M}$  As(V), only *Bradyrhizobium diazoefficiens* USDA110 was able to nodulate the legume, although nodule number, dry weight and nitrogen content were significantly decreased with respect to the control treatment (Table 3). All of the soybean nodules exposed to 20  $\mu\text{M}$  As(V) presented a brownish-red deposit in the infection zone due to the presence of leghemoglobin, indicating active nodules.

### 3.5 Accumulation of arsenic in soybean plants inoculated with *Bradyrhizobium* sp. strains

Arsenic accumulation by soybean plants exposed to 6  $\mu\text{M}$  As(V) was mainly detected in roots, followed by nodules

and finally shoots, regardless of the inoculated strain (Table 4). Arsenic content in shoots of plants inoculated with the three distinct strains was unchanged. However, plant roots inoculated with *Bradyrhizobium diazoefficiens* USDA110 showed a lower As content than those inoculated with *Bradyrhizobium* sp. Per 3.61, while plants inoculated with *Bradyrhizobium* sp. Per 3.64 did not show different As levels in roots, compared with the other two strains. It is noteworthy that nodules obtained from plants inoculated with the native strains showed greater As accumulation than nodules occupied with the reference strain (Table 4). Regarding As translocation from roots to shoots, inoculation of soybean plants with *Bradyrhizobium* sp. Per. 3.61 showed a significant lower TF in comparison with the other tested strains. At 20  $\mu\text{M}$  As(V), a similar As distribution was observed in plants inoculated with *Bradyrhizobium diazoefficiens* USDA110 (shoot: 9.31  $\pm$  1.56; root: 193.61  $\pm$  36.61; nodules: 45.36  $\pm$  3.65) showing significant differences among organs. Nodule As accumulation was similar to those of plants inoculated with native strains and exposed to the minimum As concentration tested.

**Fig. 3** Effect of arsenate on lipid peroxidation in soybean roots. Data represent the mean  $\pm$  SE ( $n = 10$ ). Different letters denote significant statistical differences ( $P < 0.05$ ) according to Duncan's test



**Table 2** Impact of arsenate on soybean growth inoculated with bradyrhizobial strains

Bradyrhizobial strains	Root length (cm)		Root dry weight (mg)		Shoot length (cm)		Shoot dry weight (mg)			
	Control	6 $\mu$ M As	20 $\mu$ M As	6 $\mu$ M As	20 $\mu$ M As	6 $\mu$ M As	20 $\mu$ M As	6 $\mu$ M As	20 $\mu$ M As	
USDA110	24 $\pm$ 0.7 <sup>A1</sup>	19 $\pm$ 0.7 <sup>AB2</sup>	18 $\pm$ 0.5 <sup>A2</sup>	56 $\pm$ 4.7 <sup>A1</sup>	48 $\pm$ 2.7 <sup>A1</sup>	23 $\pm$ 1.2 <sup>A1</sup>	26 $\pm$ 1.5 <sup>A1</sup>	150 $\pm$ 12 <sup>AB1</sup>	169 $\pm$ 13 <sup>A1</sup>	125 $\pm$ 4.0 <sup>A2</sup>
Per 3.61	24 $\pm$ 0.7 <sup>A1</sup>	21 $\pm$ 1.1 <sup>B2</sup>	17 $\pm$ 0.9 <sup>A3</sup>	56 $\pm$ 4.4 <sup>A1</sup>	38 $\pm$ 2.8 <sup>B2</sup>	28 $\pm$ 1.3 <sup>B1</sup>	24 $\pm$ 1.0 <sup>A12</sup>	124 $\pm$ 6.0 <sup>A1</sup>	127 $\pm$ 5.0 <sup>B1</sup>	85 $\pm$ 6.0 <sup>C2</sup>
Per 3.64	23 $\pm$ 0.6 <sup>A1</sup>	17 $\pm$ 0.6 <sup>A2</sup>	17 $\pm$ 0.9 <sup>A2</sup>	54 $\pm$ 2.9 <sup>A1</sup>	37 $\pm$ 2.3 <sup>B2</sup>	29 $\pm$ 1.2 <sup>B1</sup>	23 $\pm$ 1.5 <sup>A2</sup>	170 $\pm$ 5.0 <sup>B1</sup>	119 $\pm$ 8.0 <sup>B2</sup>	103 $\pm$ 6.0 <sup>B2</sup>

Data represent the mean  $\pm$  SE ( $n = 10$ ). Different letters in each column indicate significant differences among strains for each treatment and different numbers on each row indicate significant differences among treatment for each strain according to the Duncan's test ( $P < 0.05$ )

**Table 3** Impact of arsenate on soybean nodulation

Bradyrhizobial strains	Nodule number plant <sup>-1</sup>		Nodules dry weight (mg) plant <sup>-1</sup>		Nitrogen content (mg N plant <sup>-1</sup> )			
	Control	6 $\mu$ M As	20 $\mu$ M As	6 $\mu$ M As	20 $\mu$ M As	6 $\mu$ M As	20 $\mu$ M As	
USDA110	9.17 $\pm$ 1.68 <sup>A1</sup>	7.00 $\pm$ 1.56 <sup>A12</sup>	2.75 $\pm$ 1.03 <sup>2</sup>	6.50 $\pm$ 1.10 <sup>A1</sup>	4.20 $\pm$ 1.20 <sup>A12</sup>	4.58 $\pm$ 0.21 <sup>A1</sup>	5.31 $\pm$ 0.18 <sup>A1</sup>	2.84 $\pm$ 0.2 <sup>2</sup>
Per 3.61	6.60 $\pm$ 1.63 <sup>A1</sup>	6.33 $\pm$ 1.69 <sup>A1</sup>	0	4.30 $\pm$ 0.10 <sup>A1</sup>	2.00 $\pm$ 0.50 <sup>A1</sup>	3.18 $\pm$ 0.10 <sup>B1</sup>	3.54 $\pm$ 0.31 <sup>B1</sup>	nd
Per 3.64	7.31 $\pm$ 1.33 <sup>A1</sup>	5.2 $\pm$ 1.46 <sup>A1</sup>	0	4.80 $\pm$ 0.80 <sup>A1</sup>	2.20 $\pm$ 0.20 <sup>A1</sup>	4.17 $\pm$ 0.27 <sup>B1</sup>	3.86 $\pm$ 0.12 <sup>B1</sup>	nd

Data represent the mean  $\pm$  SE ( $n = 10$ ). Different letters in each column indicate significant differences among strains for each treatment and different numbers on each row indicate significant differences among treatment for each strain according to the Duncan's test ( $P < 0.05$ ). nd: not determined



**Table 4** Arsenic accumulation in soybean plants exposed to 6  $\mu\text{M}$  As(V) and inoculated with distinct bradyrhizobial strains

Bradyrhizobial strains	As accumulation ( $\mu\text{g g}^{-1}$ dry weight)			
	Shoot	Root	Nodules	TF
USDA110	2.28 $\pm$ 0.31 <sup>A3</sup>	58.09 $\pm$ 5.51 <sup>B1</sup>	29.96 $\pm$ 1.85 <sup>B2</sup>	0.035 $\pm$ 0.006 <sup>B</sup>
Per 3.61	1.99 $\pm$ 0.24 <sup>A3</sup>	82.84 $\pm$ 7.02 <sup>A1</sup>	50.70 $\pm$ 2.89 <sup>A2</sup>	0.022 $\pm$ 0.001 <sup>A</sup>
Per 3.64	2.72 $\pm$ 0.49 <sup>A3</sup>	68.68 $\pm$ 4.01 <sup>AB1</sup>	46.03 $\pm$ 3.40 <sup>A2</sup>	0.040 $\pm$ 0.002 <sup>B</sup>

Data represent the mean  $\pm$  SE ( $n = 10$ ). Different letters in each column indicate significant differences among strains for each organ and different numbers on each row indicate significant differences among organs for each strain, according to the Duncan's test ( $P < 0.05$ )

## 4 Discussion

Arsenic is a widely known toxic metalloid that can cause growth reduction in prokaryotic cells (Mandal et al. 2008; Panigrahi et al. 2013; Talano et al. 2013) and its toxicity is related with ROS accumulation that induces oxidative stress (Hughes 2002; Verbruggen et al. 2009; Sharma 2012). However, microorganisms possess an effective defense system to overcome this oxidative challenge. Specifically, the As resistance in bacteria can come from metalloid complexation with glutathione (GSH), efflux systems that expulse the metalloid outside the cell, arsenite oxidation and/or anaerobic arsenate reduction and methylation (Bhattacharjee and Rosen 2007). Several arsenic resistance (*ars*) genes that code for As(III) repressors, efflux permease and/or ATPase to extrude As(III) have been widely studied in a range of bacterial species (Bhattacharjee and Rosen 2007; Yang and Rosen 2016). Our findings showed that As(V) affected the bradyrhizobial viability in a strain-specific manner. Therefore, the contaminant transport out of the cell and/or an enzymatic or non-enzymatic response could underlie the differential rhizobial defense mechanisms.

Arsenic uptake by plants resulted in growth reduction or inhibition (Finnegan and Chen 2012). In correspondence, Päivöke and Simola (2001) observed that addition of As(V) affected shoot and root dry weight of *Pisum sativum*, growing on sand as an inert substrate. However, root and shoot length were not altered. Similar results were reported for *Phaseolus vulgaris*, *Triticum aestivum* and *Oryza sativa* exposed to different As(V) concentrations (Stoeva et al. 2005; Liu et al. 2005; Shri et al. 2009). In support of our results, Vázquez-Reina et al. (2005) and Bianucci et al. (2017) showed a significant reduction in soybean and peanut growth when exposed to 18 and 20  $\mu\text{M}$  As(V), respectively, in hydroponics. Hydroponic systems have been widely used to analyze the effects of metal(loids) on plants (Cho and Park 2000; Gothberg et al. 2004; Cargnelutti et al. 2006; Moreno-Jiménez et al. 2007; 2008; Sobrino-Plata et al. 2009, 2013; Bianucci et al. 2017). In this system, the root is completely immersed in the growth culture media and directly exposed to the contaminant. This eliminates the problem of As forming complexes with particles of different substrates, enabling

collection of the clearest results of the As effect on the whole plant (Finnegan and Chen 2012).

It is known that arsenic induces ROS generation leading to oxidative stress in plants (Hartley-Whitaker et al. 2001; Mascher et al. 2002; Molina et al. 2008; Shri et al. 2009; Finnegan and Chen 2012; Bianucci et al. 2017). In general, all lipid molecules, and in particular unsaturated lipids, are sensitive to ROS oxidation (Sharma 2012). Our results showed an increase of TBARs content, an estimation of lipid peroxidation upon metal stress, in soybean roots exposed to 6  $\mu\text{M}$  As(V). In concordance with this observation, an augmented TBARs content was also found in *Phaseolus vulgaris*, *Oryza sativa*, *Arachis hypogaea* and in the hyperaccumulator plant *Pteris vittata* growing under As exposure (Stoeva et al. 2005; Srivastava et al. 2005; Singh et al. 2006; Shri et al. 2009; Bianucci et al. 2017).

In our work, soybean root length was reduced showing an increase of the lateral root development and revealing oxidative stress symptoms at 10 days of growth when exposed to 6 and 20  $\mu\text{M}$  As(V). At the highest As concentration tested all growth variables were reduced, accompanied by a decrease of lateral root development. Considering that the increment of lateral roots might lead to more sites for rhizobial infection and therefore improve nodulation (Mandon et al. 2009; Ibáñez et al. 2009), we decided to evaluate the impact of As on nodulation of plants exposed to 6 and 20  $\mu\text{M}$  As(V).

The legume-rhizobia symbiotic interaction is important since it fulfils a plant's N demand via the BNF process. Some of the most common effects of metal(loids) on this symbiotic interaction are associated with low rhizobia viability due to increasing ROS (Ortega-Villasante et al. 2005, 2007; Bianucci et al. 2013), reduced nitrogen assimilation (Balestrasse et al. 2001, 2003), alteration of nodule redox state and organogenesis (Carpena et al. 2003; Bianucci et al. 2013). A transcriptomic analysis performed by Lafuente et al. (2010) demonstrated that As negatively impacts the early stages of symbiosis by reducing the expression of early nodulins (*nork*, *NIN*, *N6*, *Enod2*), without differences in the expression of markers for primordium initiation and differentiation (*Enod40* and *ccs52*, respectively). In accordance with these findings, Pajuelo et al. (2008) showed that once the symbiotic interaction is established, the development of the nodule

continues as normal. Neumann et al. (1998) found a decrease in the number of nodules of inoculated *Medicago sativa* exposed at 5.5 and 21.6  $\mu\text{M}$  As(V). Reichman (2007) and Vázquez et al. (2009) revealed a reduction in the number of nodules of the soybean-*B. japonicum* symbiotic interaction when exposed to 10 or 18  $\mu\text{M}$  As(V). Our findings showed that at 6  $\mu\text{M}$  As(V) all studied strains were able to nodulate soybean plant and maintain an unchanged nitrogen content with respect to control conditions. However, at 20  $\mu\text{M}$  As(V) only the symbiotic interaction established between soybean and *Bradyrhizobium diazoefficiens* USDA110 was able to nodulate. At this As(V) concentration, the number of nodules was significantly lower than at control conditions and a reduction of nitrogen content was also observed. Previous research demonstrated the presence of leghaemoglobin in nodules by an internal color scored as brownish-red, serving as an indicator of the potential N-fixation (Wittenberg et al. 1974; Angle et al. 1993; Ott et al. 2005). Furthermore, the N-content represents a good estimate of N-fixation efficiency as proposed by Somasegaran and Hoben (1994b). Taking all these data into account and considering that in our work the only source of N was provided by the BNF we suggest that the reduction in N content could be related to a decrease in the number of developed nodules under As(V) treatments. Nitrogen is a major element for crops and its low availability restrict plant growth. Thus, nitrogen supply achieved by the BNF process can satisfy this high demand, enhancing yields and crop quality. Under metal(loid) conditions, legume inoculation with rhizobia could improve plant growth, increasing not only N nutrition but also solubilization of phosphate and production of siderophores (Abril et al. 2007; Reichman 2007; Wani et al. 2008; Dary et al. 2010). Moreover, the symbiotic interaction established between rhizobia and legumes growing in metal(loid)-contaminated areas has been proposed for bioremediation (Reichamn 2007; Pajuelo et al. 2011). However, special care has to be taken since the remediation process is not advisable if parts of the plants are to be eaten by humans or animals. Specifically, in the symbiosis model *Medicago truncatula* –*Ensifer medicae* MA11 exposed to As(V), the inoculation of an As-tolerant rhizobia strain alleviated As toxicity in the plant (Lafuente et al. 2015). Furthermore, these researchers found an enhancement in the relative expression of chalcone synthase (an enzyme involved in the first steps of the legume-rhizobia cross-talk), indicating that plants prefer to establish symbiotic interactions under As stress. Since all the tested strains in this work were able to grow at As(V) concentrations almost 1000-fold higher than found in the plant, all of them can be considered as tolerant strains. Therefore, bradyrhizobial tolerance to As is not the determining factor in the nodulation of soybean grown under enhanced levels of As. Our results revealed that soybean plants were subjected to oxidative stress under As treatment. Thus, considering that microorganisms are able to prevent or counteract the plant

defense system inhibiting the signalling pathway (Herouart et al. 2002; Pauly et al. 2006). It is suggested that the antioxidant capacity of each strain plays a fundamental role in infecting the legume and allowing a successful symbiotic interaction of plants exposed to realistic doses of As.

Regarding As uptake by plants, metalloid accumulation follows the same distribution pattern of the most studied non-hyperaccumulator legumes: lupin, *Vigna*, alfalfa and peanut (Pajuelo et al. 2008; Mandal et al. 2008; Panigrahi and Randhawa 2010; Panigrahi et al. 2013; Bianucci et al. 2013). Remarkably, at 6  $\mu\text{M}$  As(V), inoculation of soybean with *Bradyrhizobium* sp. Per 3.61 showed a lower TF compared to other tested strains, demonstrating that this microsymbiont promotes As phytostabilization and therefore limits metalloid translocation and accumulation into edible parts of the legume. Consequently, we propose the use of the native isolate *Bradyrhizobium* sp. Per 3.61 as a potential inoculant of soybean crops growing under exposure to low As concentrations.

Finally, all tested strains were able to nodulate soybean at 6  $\mu\text{M}$  As(V), overcoming the plant's oxidative stress and leading to an effective symbiosis. Nevertheless, the inoculation of the native isolate *Bradyrhizobium* sp. Per 3.61 contributed to limiting metalloid translocation to edible parts of the plants. At higher As concentrations, as found in many crop areas of Córdoba province, soybean plants exhibiting high oxidative stress damage and could only be nodulated by the reference strain *Bradyrhizobium diazoefficiens* USDA110.

## 5 Conclusion

Inoculation of soybean plants exposed to high As(V) concentrations with the reference strain *Bradyrhizobium diazoefficiens* USDA110 represents an effective and promising symbiotic interaction that allows legume development in terms of minimal effects on plant growth. However, at low As(V) concentrations the native isolate *Bradyrhizobium* sp. Per 3.61 is presented as the best inoculant, among the tested strains, for limiting metalloid translocation and accumulation to edible parts of the legume, avoiding fruit contamination and human poisoning.

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