Environmental Management (2020) 66:930–939
https://doi.org/10.1007/s00267-020-01351-z

Assessing the Plant Growth Promoting and Arsenic Tolerance Potential of *Bradyrhizobium japonicum* CB1809

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Received: 8 January 2020 / Accepted: 21 August 2020 / Published online: 11 September 2020

Abstract

Accumulation of heavy metals in soil is of concern to the agricultural production sector, because of the potential threat to food quality and quantity. Inoculation with plant growth-promoting bacteria (PGPR) has previously been shown to alleviate heavy metal stress but the mechanisms are unclear. Potential mechanisms by which inoculation with *Bradyrhizobium japonicum* CB1809 affected the legume soybean (*Glycine max* cv. Zeus) and the non-legume sunflower (*Helianthus annus* cv. Hyoleic 41) were investigated in solution culture under 5 μM As stress. Adding As resulted in As tissue concentrations of up to 5 mg kg^{-1} (shoots) and 250 mg kg^{-1} (roots) in both species but did not reduce shoot or root biomass. Inoculation increased root biomass but only in the legume (soybean) and only with As. Inoculation resulted in large (up to 100%) increases in siderophore concentration but relatively small changes (±10–15%) in auxin concentration in the rhizosphere. However, the increase in siderophore concentration in the rhizosphere did not result in the expected increases in tissue N or Fe, especially in soybean, suggesting that their function was different. In conclusion, siderophores and auxins may be some of the mechanisms by which both soybean and sunflower maintained plant growth in As-contaminated media.

Keywords Plant growth-promoting rhizobacteria (PGPR) · Arsenic · Bioremediation · Soybean · Sunflower · *Bradyrhizobium japonicum*

Introduction

Contamination of soil by the metalloid arsenic is a worldwide problem resulting from its widespread and versatile uses in industry. The major form of arsenic in soil is As(V), which is much less toxic than As(III) (Abbas et al. 2018). Arsenic contamination of existing soils is difficult to bioremediate (Malik et al. 2009). Mechanical solutions (removal and burial off-site) are expensive (Australian State of Environment Committee, Australia 2001) and only remove the problem elsewhere, for it possibly to reappear as contamination of waterways and the food chain (Madejón et al. 2010).

Interest in alternative solutions to As contamination has focused on plant-based remediation of As-containing soils by utilising rhizosphere bacteria (Hinsinger et al. 2006; Wenzel 2009; Kopittke et al. 2010). This is because the rhizosphere (the soil around plant roots) is a zone rich in soil-borne bacteria due to the numerous compounds leaked from the roots (Bakker et al. 2013; Berendsen et al. 2012), which greatly enhance the growth and activities of the bacteria (Mendes et al. 2013). Prominent among these are plant growth-promoting rhizobacteria (PGPR), which stimulate plant growth using a variety of mechanisms (Gouda et al. 2018; Sekar and Kandavel 2010; Majeed et al. 2015; Vejan et al. 2016; Vessey 2003). The best known example of PGPRs are rhizobia, which improve N nutrition in the legume–rhizobium symbiosis (Kennedy et al. 1997; Marroqui et al. 2001; Perez-Montano et al. 2014). However, PGPRs more generally synthesise compounds used by plants (e.g., phytohormones and siderophores) (Beneduzi et al.

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2012; Glick et al. 2007; Kloepper et al. 1980). They can also reduce the toxicity of heavy metals to plants (Mishra et al. 2017; Zhuang et al. 2007). PGPR therefore hold considerable promise for the transformation of toxic metals in soils (Cardon et al. 2010; Ojuederie and Babalola 2017).

The soil-borne rhizobia have practical potential for use in the remediation and stabilisation of As-contaminated land. Rhizobia have been isolated from As-contaminated sites (Macur et al. 2001; Carrasco et al. 2005). Due to the major role of rhizobia in nodulation and nitrogen fixation in legumes, leading to increased plant production, there are established production facilities and well-known methods of application to soils and seeds in most countries with developed agriculture (Gopalakrishnan et al. 2015). There is direct evidence that some rhizobia are tolerant of arsenic. Reichman (2014) showed that suspension in up to 50 μM As(V) had no effect on respiration of *Bradyrhizobium japonicum* CB1809. Rhizobia take up As(V) and reduce it metabolically to As(III), which is extruded to the environment and is more toxic to plants (Suhadolnik et al. 2017); this could potentially increase rather than stabilise the toxicity of As-contaminated soils. However, inoculation with *B. japonicum* improved the growth of soybean (Reichman 2007) and sunflower (Reichman 2014) in As-contaminated liquid growing medium. The distribution of As was primarily in roots rather than shoots, a desirable trait for use in phytostabilisation of As in contaminated soil.

The reasons why inoculation with this strain of rhizobia increased plant growth in the presence of As are not clear. Inoculation did not change the plant tissue content of As or N and so growth improvements could not be ascribed to nitrogen fixation in soybean (a legume) (Reichman 2007) or sunflower (a nonlegume) (Reichman 2014). Rhizobia extrude many other compounds into the rhizosphere of legumes, e.g., plant hormones (growth regulators), that affect their growth (Vincent 1980). Reichman (2014) showed that inoculation did not change the concentration of indoles (which include the auxin phytohormones) in the rhizosphere. The reason for the improved growth in both soybean and sunflower thus remains unknown. A possible factor is that rhizobia also extrude siderophores that sequester metals, especially Fe (Geetha and Joshi 2013), and so may be involved in preventing detrimental effects on plant growth.

The aim of this study was to find what changes occurred with inoculation by bradyrhizobia, specifically if inoculation increased soybean and sunflower tolerance of As and if rhizobial production of auxin phytohormones and siderophores in the rhizosphere resulted in increased growth and plant tissue concentrations of Fe. As previous studies used soybean and sunflower grown hydroponically, these were chosen again because they enabled direct comparisons with previous work under controlled conditions.

Materials and Methods

Experimental Design and Treatments

The plant species (soybean and sunflower) and the experimental design were largely based on those used previously: soybean (Reichman 2007) and sunflower (Reichman 2014) and details are summarised briefly below, with changes noted in detail.

There were two variables in a factorial design: firstly inoculation with *Bradyrhizobium japonicum* CB1809 (obtained courtesy of New-Edge Microbials, Australia) and secondly the addition of 5 μM As (provided as $Na_2HAsO_4{\cdot}7H_2O$), with four replicates per treatment. Their combination gave four treatments: without either inoculation or As (control (−)); with inoculation but without As (control (+)); without inoculation and with As (As−); and lastly with both inoculation and As (As+). The As concentration was chosen as the concentration in solution culture that produced mild toxicity in these plant species previously (Reichman 2007; Reichman et al. 2001). The species As(V) was chosen over As(III) because As(V) is the most common species in well aerated soil (Smedley and Kinniburgh 2002).

Two plant species were tested: *Glycine max* cv. Zeus (soybean) and *Helianthus anuus* cv. Hyoleic 41 (sunflower); seeds of both were obtained courtesy of the Department of Primary Industry, New South Wales. These were different cultivars from those tested previously; 'Zeus' is a dark-hilum soybean cultivar grown for crushing for oil and livestock feed and 'Hyoleic' is a mono-unsaturated (high oleic acid) cultivar grown for crushing for margarine and cooking oil. Seeds were surface-sterilised with 10% NaOCl, germinated on moist paper towelling and 10-day-old healthy seedlings transferred on day 0 of the experiment to four 2 L polypropylene light-proof vessels at four seedlings per vessel for each treatment (Reichman 2007). Plants were grown with vessels completely randomised in a growth chamber (Conviron Adaptis A1000) with a 12 h photoperiod in a 28/25 °C temperature regime for soybean and a 25/15 °C temperature regime for sunflower. Mineral nutrients were supplied (Reichman 2007), and the nutrient solution was buffered at pH 6.0, adjusted daily.

On day 2, As treatments began. On day 3, inoculation treatments began (approximately 1.3×10^{10} colony-forming units mL^{-1} per vessel). On day 6, seedlings were thinned to two per vessel. There were thus four vessels (each with two plants per vessel) in each treatment; these two plants per vessel were harvested and analysed as one replicate for each parameter reported, giving four replicates per treatment. Nutrient solutions were changed at days 8, 15, 24, 31, 34 and 39 for soybean and days 8, 15, 22, 29, 33 and 38 for sunflower. On days 8 and 15, inoculation was repeated after nutrient solutions were refreshed. Soybean plants were harvested on day 44 and sunflower plants on day 42.

At harvest, each plant was separated into roots and shoots, rinsed once in 10% Decon® solution and then three times in ultrapure water. Roots and shoots were oven-dried separately for 48 h at 70 ± 10 °C. For soybean, the number of root nodules per plant was counted and nodule nitrogen-fixing potential assessed by halving each nodule longitudinally and scoring as pink (active) or white/green (inactive) (Angle et al. 1993; Ott et al. 2005; Wittenbe et al. 1974).

Rooting Zone Tests—Auxin and Siderophore Concentrations

On the day of harvest, samples of the nutrient solution were collected near the roots before plants were removed, filtered through a Minisart 0.45 μM filter and frozen at −20 °C. Auxin concentration was measured using the Salkowski reagent method, with absorbance of the samples measured at 544 nm (Glickmann and Dessaux 1995). Siderophores were analysed using a revised iron-binding assay, with absorbance of the samples measured at 562 nm (Reichman and Parker 2007). Because of evapotranspiration, concentrations may have been elevated uniformly by up to 20%.

Plant Analysis—Tissue Concentrations

Oven-dried shoot and root samples were weighed and ground before aliquots were hot-block digested with 5 mL of 70 % concentrated HNO_3 at 115 °C for 4 h, before cooling to room temperature and diluting with ultrapure water (Reichman 2007). A LECO CNS 2000 analyser was used to determine the concentrations of nitrogen. Inductively coupled plasma-mass spectrometry (Agilent Technologies 7700× analyser) was used to determine concentrations of As, Fe, and cations.

Statistical Analysis

Statistical analyses were performed using Minitab (www.minitab.com) to compare treatment effects. Means were compared by Analysis of Variance (ANOVA) and multiple comparison tests (Fisher's Least Significant Differences). A p-value of ≤ 0.05 was regarded as significant.

Results

Biomass

Adding As to the nutrient solution had no effect on the dry weight (DW) of either soybean or sunflower roots ($p = 0.308$ and $p = 0.173$, respectively) or shoots ($p = 0.927$ and $p = 0.138$, respectively) (Fig. 1). Inoculation of soybean increased root DW with As but not without As ($p = 0.024$) (Fig. 1a), whereas there was no effect of inoculation on sunflower root DW with or without As ($p = 0.308$) (Fig. 1c). There was no effect of inoculation on the shoot DW of either soybean ($p = 0.151$) (Fig. 1b) or sunflower ($p = 0.225$) (Fig. 1d).

Nodulation

Adding As decreased the number of nodules per inoculated soybean plant ($p = 0.019$) (Fig. 2a) but increased the proportion of pink root nodules ($p < 0.001$) (Fig. 2b). No uninoculated soybean plant developed nodules, nor did any sunflower plant in any treatment.

Rooting Zone Tests

Siderophores

The concentrations of siderophores in the rooting zones of soybean were at least twice that in the rooting zone of sunflower in all As treatments (Fig. 3a, c). In soybean, adding As increased the concentration of siderophores ($p = 0.045$) but only in inoculated plants (Fig. 3a). Inoculation increased siderophore concentration ($p < 0.001$), more with As than without As (Fig. 3a). Similarly, in sunflower adding As increased siderophore concentration ($p = 0.037$) but inoculation without As had no effect on siderophore concentrations ($p = 0.386$) (Fig. 3c).

Auxin

The concentration of auxin in the rooting zones of soybean and sunflower were similar (Fig. 3b, d). Adding As increased auxin concentration in the rooting zone of soybean ($p = 0.002$) but only in inoculated plants (Fig. 3b) as there was also a significant As-by-inoculation interaction ($p = 0.007$). By contrast, adding As in sunflower resulted in a significant decrease in auxin concentration but only in uninoculated plants ($p = 0.118$) (Fig. 3d) and there was also a significant As-by-bacterial inoculation interaction ($p = 0.044$).

Tissue Concentrations

Tissue arsenic

Adding As increased tissue As concentrations of roots and shoots in both soybean ($p < 0.001$ for both) and sunflower ($p < 0.001$ and $p = 0.033$, respectively) (Fig. 4). The concentration of As in the roots of both plants was greater than in the corresponding shoots; the translocation factors (Singh and Agrawal 2007) were 0.017–0.026 for soybean and

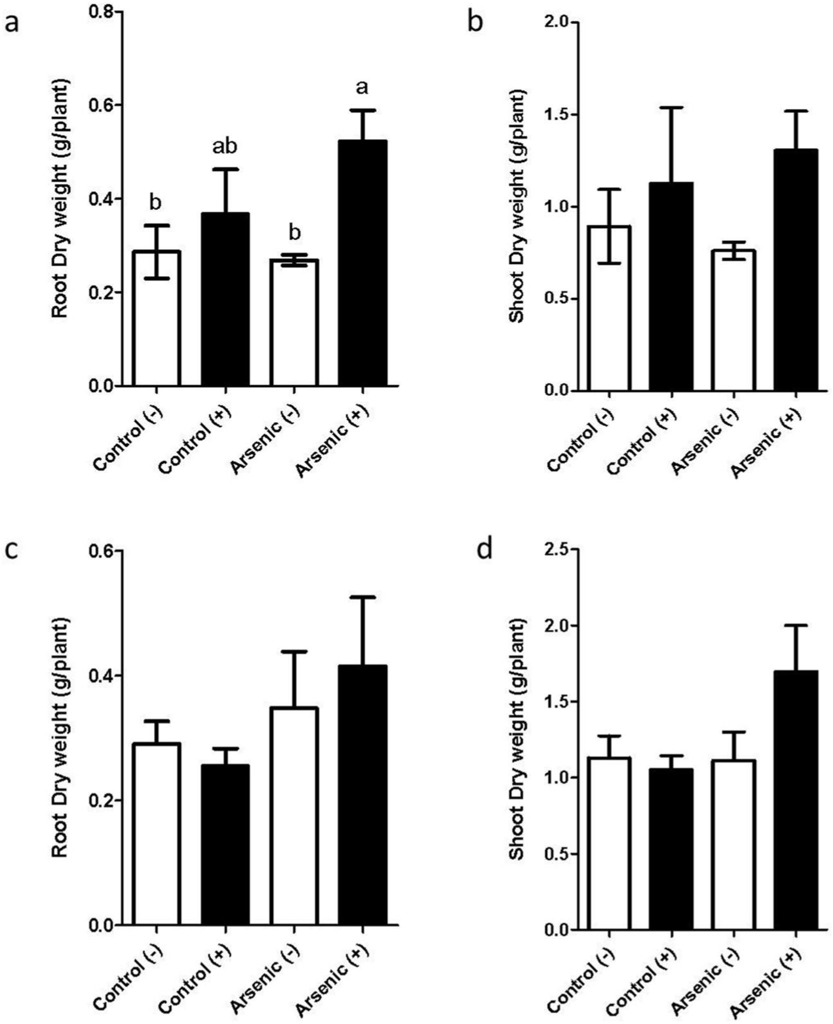


Fig. 1 Effects of As in the nutrient solution on the dry weight of roots and shoots of soybean (**a**, **b**) and sunflower (**c**, **d**) respectively grown without (white) or with (black) the addition of *Bradyrhizobium japonicum* CB1809 to the solution. Values are means ($n = 4$) ± 1 standard error. Where significant differences were found, columns with the same letter within a graph are not significantly different (Fisher's least significant difference, $p > 0.05$)

0.037–0.057 for sunflower. There was no effect of inoculation on the As concentration of soybean roots ($p = 0.279$) and shoots ($p = 0.405$) (Fig. 4a, b) or of sunflower roots ($p = 0.725$) and shoots ($p = 0.690$) (Fig. 4c, d).

Tissue nitrogen

Adding As had no effect on tissue N concentration of shoots of either soybean ($p = 0.146$) or sunflower ($p = 0.395$). There was also no effect of inoculation on shoot tissue N concentration of either soybean ($p = 0.265$) or sunflower ($p = 0.814$), and there was no significant interaction between As and inoculation with soybean ($p = 0.372$ or sunflower ($p = 0.198$) (Table 1).

Tissue iron

In soybean roots, adding As had no effect on tissue Fe concentrations ($p = 0.303$) (Fig. 5a). In shoots, adding As reduced Fe concentrations, although only in uninoculated plants ($p = 0.363$) (Fig. 5b), as there was a significant interaction between As and inoculation ($p = 0.002$). In sunflower roots, there was no overall effect of either As ($p = 0.368$) or inoculation ($p = 0.530$) on Fe concentration in roots. Inoculation increased Fe concentration only without As and there was a significant As-by-inoculation interaction ($p = 0.026$) (Fig. 5c). In shoots, there was no overall effect of As ($p = 0.115$) or inoculation ($p = 0.452$) and no significant interaction (Fig. 5d).

Discussion

This is the first study to show that inoculation with bradyrhizobia in the presence of As benefitted a legume (soybean) more than a non-legume (sunflower) via a mechanism other than improved N nutrition, as inoculation with *B. japonicum* CB1809 increased the root dry weight in soybean (a legume) but not in sunflower (a non-legume) in 5 µM As. This suggests specific mechanisms in the legume

Fig. 2 Effects of As in the nutrient solution on (**a**) the number of root nodules per plant and (**b**) the proportion of root nodules with active N fixing capability of soybean grown in the presence of *Bradyrhizobium japonicum* CB1809 in the absence (white) or presence (black) of 5 μM As to the rooting zone solution. Values are means ($n = 4$) ± 1 standard error. Where significant differences were found, columns with the same letter within a graph are not significantly different (Fisher's least significant difference, $p > 0.05$)

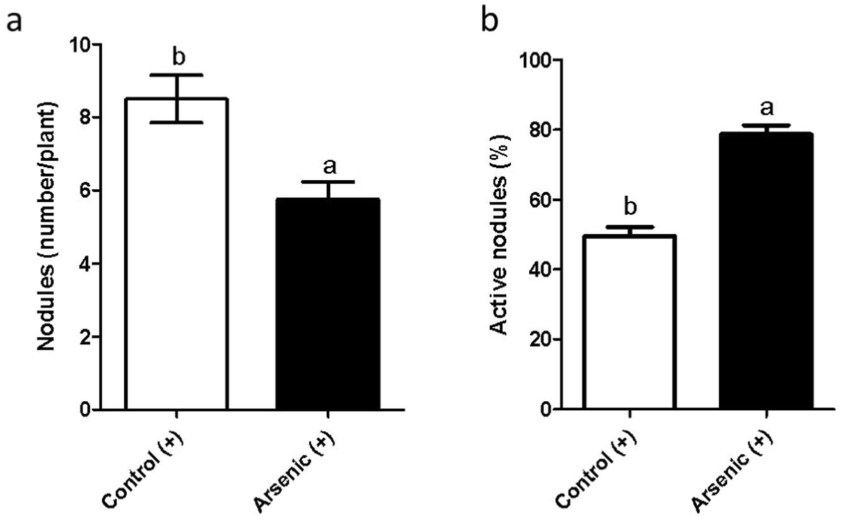
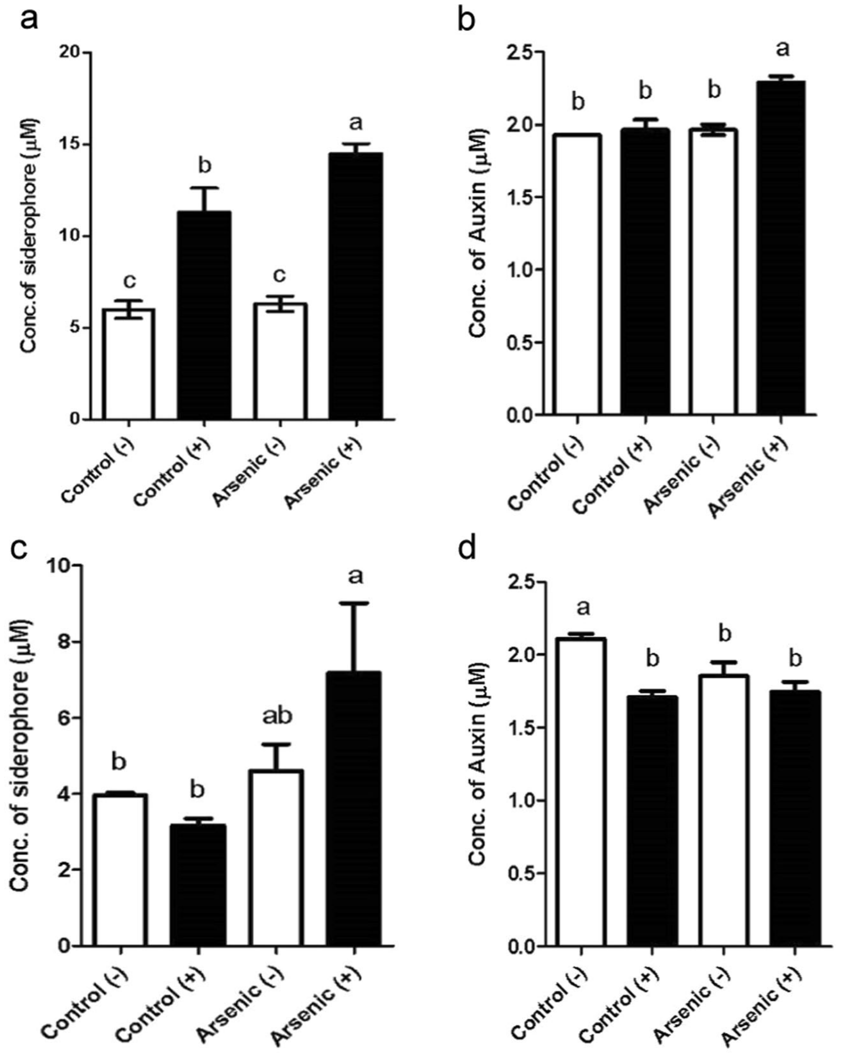


Fig. 3 Effects of As in the nutrient solution on the concentrations of siderophore (**a**, **c**) and auxin (**b**, **d**) in the root zones of soybean (**a**, **b**) and sunflower (**c**, **d**) respectively grown without (white) or with (black) the addition of *Bradyrhizobium japonicum* CB1809 to the rooting zone solution. Values are means ($n = 4$) ± 1 standard error. Where significant differences were found; columns with the same letter within a graph is not significantly different (Fisher's least significant difference, $p > 0.05$)



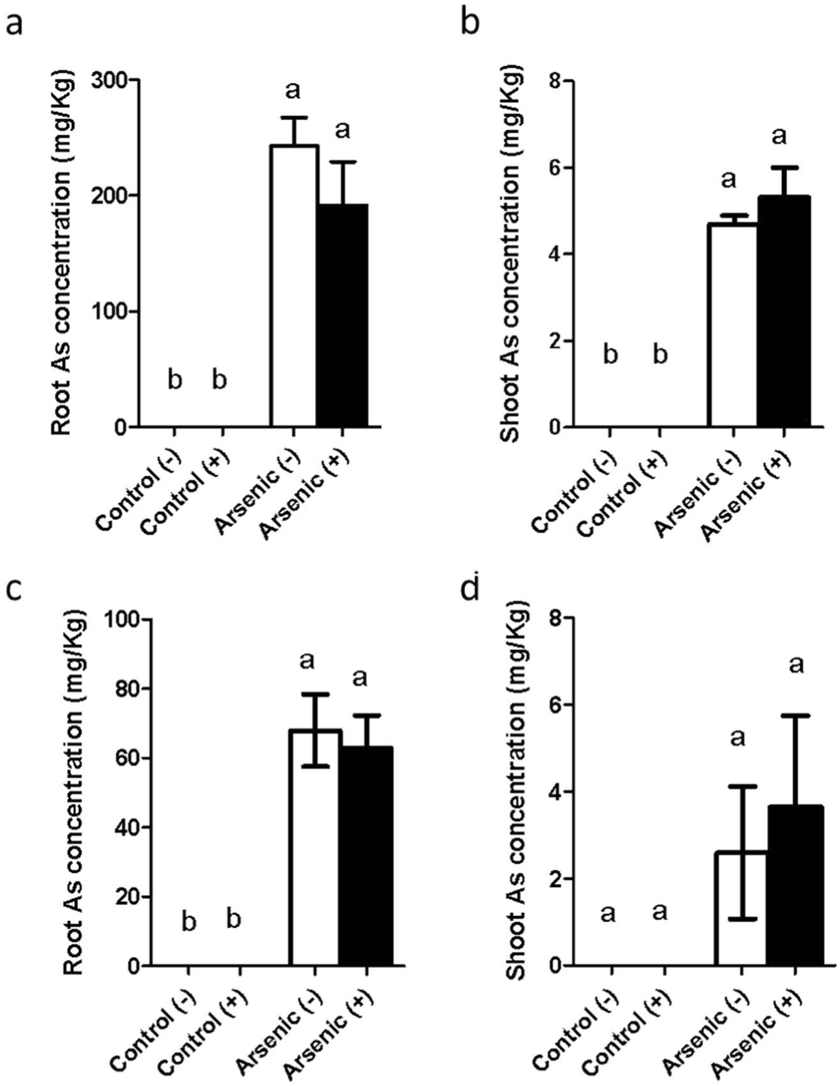


Fig. 4 Effects of As in the nutrient solution on the root and shoot concentrations of As in soybean (**a**, **b**) and sunflower (**c**, **d**) grown without (white) or with (black) the addition of *Bradyrhizobium japonicum* CB1809 to the rooting zone solution. Values are means ($n = 4$) ± standard error. Where significant differences were found, columns with the same letter within a graph are not significantly different (Fisher's least significant difference, $p > 0.05$)

that allow it to benefit more from inoculation than the non-legume in the presence of As. This is also the first study to suggest that two of these mechanisms may be siderophores and auxins produced by bradyrhizobia in the rooting zone, as the concentrations in the rooting zone of both siderophores and indoles increased in the presence of As, and the concentration of siderophores was greater with soybean than with sunflower. In addition, this is the first indication that different cultivars of soybean and sunflower differ in reactions to As under comparable controlled conditions. These points are discussed in more detail below.

Soybean

In soybean, bradyrhizobial inoculation increased the plant's As tolerance, as root dry weight increased in the presence of As but had no effect on tissue As concentration, suggesting that the mechanism of protection was not to reduce As uptake into the tissues. These results are similar to those obtained in similar hydroponic experiments at up to 6 µm As(V) with soybean cv. Don Mario 4200 RR (IVC group), which showed no changes in root and shoot dry weight or in tissue As after inoculation by *Bradyrhizobium* sp. Per 3.61, which was isolated from a soil high in As in Argentina (Bianucci et al. 2018). These results differ from those obtained in a different cultivar (Curringa) of soybean (Reichman 2007) in that there was no effect of inoculation on the dry weights of shoots. As harvest times, growth conditions, As concentration and bradyrhizobial inoculation were comparable, the differences may be due to cultivar effects. "Curringa" (Reichman 2007) is a white-hilum cultivar grown for human consumption whereas "Zeus" (used here) is a dark-hilum cultivar grown for crushing for oil and livestock feed and is tolerant of high levels of the heavy metal Mn (GRDC—Grains Research and Development Corporation 2016). Testing of different cultivars of soybean with different rhizobial strains under uniform conditions is likely to reveal further such differences among cultivars and

Table 1 Tissue concentrations (mg kg^{-1}) of nitrogen in soybean and sunflower grown with and without arsenic and without Bradyrhizobium japonicum CB1809

Treatment	Control (–)	Control (+)	As (–)	As (+)
As	–	–	+	+
Bradyrhizobia	Uninoculated	Inoculated	Uninoculated	Inoculated
Soybean	1.80 ± 0.12	1.78 ± 0.09	2.06 ± 0.09	1.84 ± 0.12
Sunflower	0.25 ± 0.01	0.26 ± 0.02	0.28 ± 0.02	0.26 ± 0.02

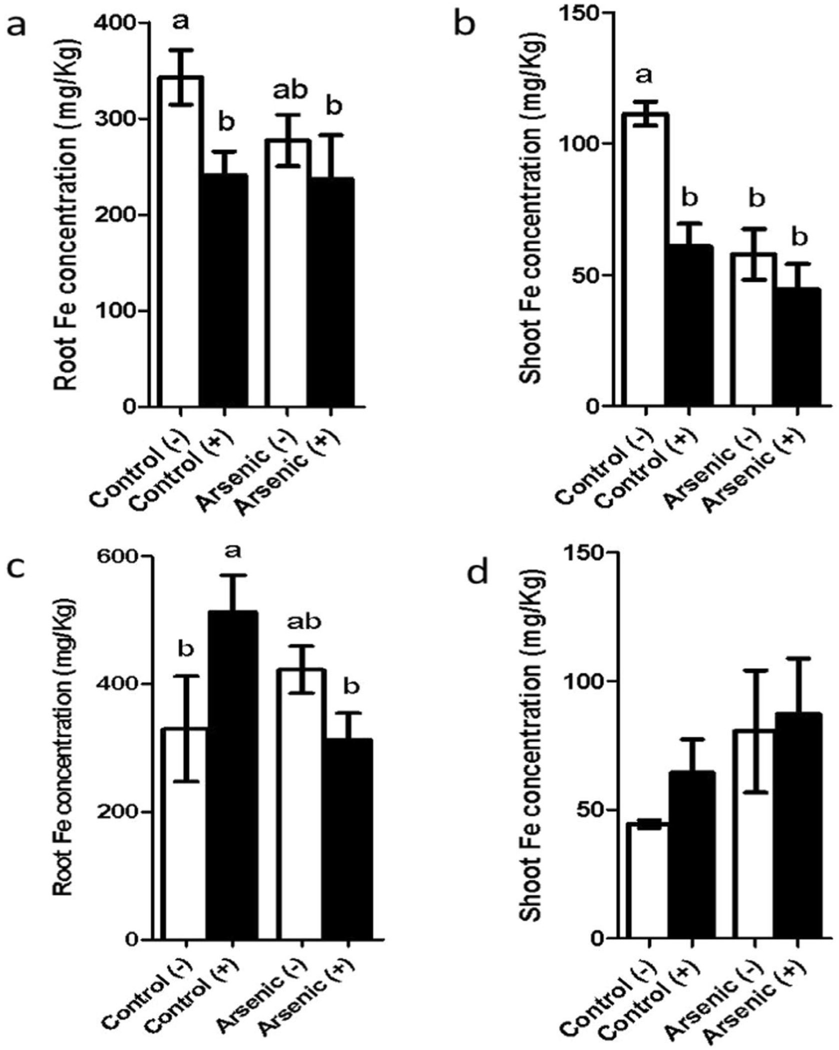


Fig. 5 Effects of As in the nutrient solution on the concentrations of Fe in the root (**a**, **c**) and shoot (**b**, **d**) in soybean (**a**, **b**) and sunflower (**c**, **d**) grown without (white) or with (black) the addition of *Bradyrhizobium japonicum* CB1809 to the rooting zone solution. Values are means ($n = 4$) ± 1 standard error. Where significant differences were found, columns with the same letter within a graph are not significantly different (Fisher's least significant difference, $p > 0.05$)

strains in tolerance of As, as in soybean with Al (Ramirez et al. 2019), alfalfa (*Medicago sativa*) with As (Pajuelo et al. 2008) and *Vicia faba* with Cd (Tang et al. 2019).

The increased tolerance to As was also not due to improved N nutrition, as all plants had the same shoot N concentration irrespective of inoculation or As status. Nitrogen in the tissues is likely to have come from both N_2 fixation and uptake of N from the nutrient solution, as plants grown in the As treatment formed active root nodules, as also noted previously by others (Reichman 2007; Bianucci et al. 2018). Treatment with As reduced the number of nodules per plant, as in previous studies with soybean (Reichman 2007; Vázquez et al. 2009; Bianucci et al. 2018) and alfalfa (Neumann et al. 1998; Pajuelo et al. 2008) but unlike the increase noted in black gram (*Vigna mungo*) with rhizobial isolate VMA301 (Mandal et al. 2008). The decrease in nodulation is probably due to a reduction in infection sites (Pajuelo et al. 2008; Reichman 2007, 2014). The percentage of active N_2-fixing nodules increased with As, unlike a previous report of no change until 10 µM As (Reichman 2007), suggesting that the bacteria were tolerant of the As in solution. This fits with rhizobia typically being tolerant of As at concentrations 1000 times those of the corresponding plant associations (Pajuelo et al. 2008;

Reichman 2014; Bianucci et al. 2018). Similarly, in the *Medicago-Ensifer* (*Sinorhizobium*) system, nodulation and the expression of early nodulin genes were depressed but not the expression of later nodulin genes or nodule functioning (Pajuelo et al. 2008; Lafuente et al. 2010).

Bradyrhizobia excrete several compounds into the rhizosphere, including the auxin phytohormones, which are indoles that increase the growth of roots (Souza et al. 2015). The increases noted in indoles in the rooting zone may explain the growth stimulation in inoculated soybean plants in the presence of As, suggesting that auxins produced by bradyrhizobia may have assisted in As tolerance of soybean. Similar results were found when *Rhizobium leguminosarum* bv. *trifolii* increased auxin concentration in roots of rice, a non-legume (Yanni et al. 2001) but contrast with the lack of response observed here in sunflower and in the non-legumes sunflower and wheat (Reichman 2014). Caution is required in interpreting these responses, as differences may be plant cultivar-specific or bacterial strain-specific.

Also, the increases noted in siderophores produced by rhizobia in the rooting zone may explain the growth stimulation in the roots of inoculated soybean plants. Growth enhancement of roots was also found when siderophores were produced by *Pseudomonas azotoformans* in As-contaminated soil (Nair et al. 2007) and by *B. japonicum* in the rhizosphere of the non-legumes sorghum and finger millet in non-metal-contaminated growth media (Matiru et al. 2005). However, the increase in siderophores in the inoculated treatments here did not result in greater concentrations of Fe in the roots and shoots, as the Fe concentration in roots and shoots was greatest in uninoculated plants not treated with As and fell in inoculated plants. Rhizobia in culture tolerate As partly by reducing As(V) to As(III) and then excreting As(III) through an aquaglyceroprotein (AqpS) channel (Yang et al. 2005; Panigrahi and Singh Randhawa 2010). Inoculation with rhizobia may thus have increased the concentration of As(III) in the rooting zone (Yang et al. 2005; Panigrahi and Singh Randhawa 2010). Plants are more sensitive to As(III) than As(V) (Finnegan and Chen 2012) and so this increase in As(III) would be expected to increase the oxidative stress within the plant (Panigrahi and Singh Randhawa 2010; Finnegan and Chen 2012; Bianucci et al. 2018). This may have resulted in less uptake of Fe and possibly other metals into the plants.

Sunflower

In sunflower, there was no effect of inoculation on the dry weight and tissue As or N concentration of plants with or without As. Similar observations were reported with several PGPRs in sunflower in Brazil (Ambrosini et al. 2012). These results contrast with increases in plant biomass (but not tissue N) reported previously by inoculation of sunflower in the presence of As (Reichman 2014) but plants in that study were grown only half the time of this study and a different cultivar was used (‘Dwarf Sensation’).

Inoculation with *B. japonicum* decreased auxin concentration in the rooting zone but only without As. This contrasts with the lack of effect of both As and inoculation in sunflower ‘Dwarf Sensation’ (Reichman 2014), suggesting cultivar effects. Inoculation also increased siderophore production in As-treated plants without resulting in increased tissue Fe concentration. This fits with sunflower, and other non-grass species, utilising the Strategy I iron acquisition that does not utilise phytosiderophores (Römheld and Marschner 1986).

Soybean vs. Sunflower

Bradyrhizobium japonicum had more growth-promoting potential in its standard legume host, soybean, than in the non-legume, sunflower. Soybean plants had significantly increased root biomass than control plants in the presence of As, whereas no significant effect on root biomass was observed in sunflower. The major difference between the two species may be soybean’s greater ability to respond to molecular signals from the bradyrhizobia, eventually forming symbiotic root nodules.

A combination of As and inoculation increased auxins in soybean but not in sunflower. As auxins are vital in the formation of root nodules in legumes by rhizobia, this may be a critical difference. Why this should be is not clear, given that previous studies (Antoun et al. 1998; Garcia-Fraile et al. 2012; Mehboob et al. 2009) have shown a growth-promoting effect of rhizobia on non-legumes. *Bradyrhizobium japonicum* colonises the epidermis and internal root regions on non-legumes, but only legumes form root nodules, for which the role of auxin may be vital (Schloter et al. 1997; Yanni et al. 2001). Similarly, the concentrations of siderophores increased significantly in the presence of As and inoculation but neither plant responded by increased Fe uptake, suggesting that siderophore concentration was not a limiting factor.

Acknowledgements Thanks to Mr Cameron Crombie for his technical assistance with instruments and chemicals. Thanks also to laboratory staff: Dr Sandro Longano, Dr Muthu Pannirselvam and Mrs Peggy Chang for technical support. Thanks to Paul Morrison for assisting with the ICP-MS analysis. Thanks also for donations of soybean seeds and sunflower seeds by Luke Gaynor and Loretta Serafin respectively, Department of Primary Industries, New South Wales, Australia, and of bradyrhizobial inoculum by Joe Millar from New-Edge Microbials, Australia.

Funding The authors received an internal seed grant from the then School of Civil, Environmental and Chemical Engineering, RMIT University, to undertake this work.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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