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Original article

Probing the plant growth-promoting and heavy metal tolerance characteristics of *Bradyrhizobium japonicum* CB1809

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ABSTRACT

Bradyrhizobium japonicum strain CB1809 was recently identified as a plant growth-promoting rhizobacteria in high arsenic substrate. However, it is not known if *B. japonicum* has growth promoting properties in plant species other than its leguminous host or the bacterium's tolerance to arsenic and metals. Solution culture was used to test the response of sunflower (*Helianthus annus* L.) and wheat (*Triticum aestivum* L.) to *B. japonicum* inoculation under elevated arsenic. The resazurin assay was used to determine the tolerance of *B. japonicum* to bioavailable heavy metals in solution. Inoculated sunflower and wheat were more tolerant of arsenic than uninoculated treatments. Thus, the growth promoting attributes of *B. japonicum* are not limited by the legume-rhizobium symbiosis. The concentration of indolic compounds did not differ between inoculated and uninoculated treatments suggesting the growth-promoting mechanism is not mediated by auxin. The effective concentrations for a 50% decrease in activity were arsenic >50 μM, cadmium 5.8 μM, copper 0.86 μM, manganese 83 μM, nickel 7.4 μM and zinc 29 μM. These results suggest *B. japonicum* has potential as a plant growth-promoting rhizobacterium for a range of plant species in arsenic contaminated sites and possibly for sites contaminated with other heavy metals.

1. Introduction

Heavy metal contamination of natural resources is a global environmental problem. Contamination of land with heavy metals is a particular issue because of the long residence times in soils and the largely irreversible nature of the contamination [1,2]. In addition, current options for remediation are expensive [3] and often offer little guarantee of a long-term sustainable solution as changes in environmental conditions could result in increased bioavailability and release of heavy metals over time [4,5].

There is a growing awareness that practical and sustainable solutions for the remediation of heavy metal contaminated sites will need to involve natural stabilisation processes that allow for *in situ* remediation [6]. Such natural remediation of contaminated land requires a greater understanding of biogeochemical and rhizosphere processes than we currently have [7–9]. In addition, it is only in the last decade that research has begun to focus on the use of rhizosphere processes to assist in the remediation of contaminated sites.

One avenue of research that is showing promise is the use of plant-growth promoting rhizobacteria (PGPR). Plant growth promoting rhizobacteria live in the vicinity of plant roots and improve plant growth via a variety of mechanisms including via hormonal and nutritional pathways [2]. Many PGPR have the capacity to improve plant growth under normal nutritional conditions and a small subset of PGPR taxa are being identified with the ability to improve plant growth under exposure to excess heavy metals. On contaminated sites, inoculation with appropriate PGPR can greatly assist revegetation so that plants can stabilise the site by locking heavy metals in the soil and below ground tissues thus sustainably reducing the movement of metals into groundwater and surrounding ecosystems.

Bradyrhizobium japonicum strain CB1809 was recently identified as a new PGPR that improved the biomass production of its leguminous host, soybean (*Glycine max*), grown in an As contaminated growth substrate [10]. The growth-promoting capabilities of

Abbreviations: DI, deonised; EC_{50}, effective concentration for a 50% reduction in biological activity e.g. growth or resazurin reducing activity; PGPR, plant growth promoting rhizobacteria.

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B. japonicum were not due to improved N nutrition and occurred via an as yet unknown mechanism. Other studies have also recently demonstrated plant growth promoting capabilities of rhizobium species for legumes grown under conditions of excess heavy metals [e.g. Refs. [11,12]]. However, to the author's knowledge, rhizobial PGPR that increase the tolerance of plants to heavy metals are yet to have demonstrated a growth promoting effect on non-legume species and thus demonstrate that the effect is not dependant on the legume-rhizobium symbiosis.

A range of further testing is required before *B. japonicum* CB1809 can be used in the field for the stabilisation of heavy metal contaminated sites. In particular, it is not yet known if the growth promoting capabilities of *B. japonicum* CB1809 extend beyond its legume host. In addition, little is known about the tolerance of *B. japonicum* to heavy metals and thus, its potential for growth promotion in soils contaminated with As and other heavy metals.

The following study examined the effects of As (V) and *B. japonicum* CB1809 on wheat (*Triticum aestivum* cv. 1862) and sunflower (*Helianthus annus* L. cv. Dwarf Sensation) biomass production and rhizosphere indolic compound concentrations. Dilute solution culture was used as a simplified and controllable model of the soil solution [9]. In addition, the tolerance of *B. japonicum* CB1809 to a range of heavy metals (As, Cd, Cu, Mn, Ni and Zn) was tested using the resazurin assay [13,14].

The research explored three main questions. Firstly, do the plant growth promoting properties of *B. japonicum* CB1809 extend to non-leguminous species? Secondly, is there evidence for the hormone auxin being the mechanism via which *B. japonicum* CB1809 improves plant growth? Thirdly, how tolerant is *B. japonicum* CB1809 to bioavailable concentrations of As and other heavy metals?

2. Materials and methods

2.1. Experiment 1 plant growth promoting properties of *B. japonicum* with nonleguminous species

Wheat (*T. aestivum* L. cv. 1862) and sunflower (*H. annus* L. cv. Dwarf Sensation) were used as the test species. Seeds were surface sterilized in 0.3% NaOCl for 10 min before rinsing four times with deionised (DI) water. Seeds were then placed upon germination towels (backed by waxed paper) soaked in DI water. The towels were rolled and placed upright in a container approximately ¼ filled with DI water in a growth chamber set at 23 °C day/18 °C night (12 h/12 h), 65% humidity and day time photosynthetically active radiation of 428 ± 54 μmol m^{-2} s^{-1}. After five days for wheat and eight days for sunflower, seedlings were transferred to 4000 mL polypropylene light-proof vessels with five seedlings per vessel (wheat) and 4 seedlings per vessel (sunflower) (=Day 1). The vessels were situated in a growth chamber with the same environmental conditions as for germination. The vessels were filled with nutrient solution and continuously aerated and stirred by use of airstones (Kordon, Novalek Inc, Hayward, CA, USA) linked to an aeration line run from an aquarium pump. The basal nutrient solution used was developed for plant-rhizobium investigations with nutrients supplied as (μM): NO_3^- 500, NH_4^+ 100, P 15, K 250, Ca 1000, Mg 100, S 1235, Mn 0.25, Zn 0.5, Cu 0.1, Fe 20 (as Fe(III)-N-(2-Hydroxyethyl) ethylenediamine triacetic acid (HEDTA)), B 3, Mo 0.05, and Co 0.04 [10]. To buffer the nutrient solution at pH 6, the solution contained 1 mM morpholinoethanesulfonic acid and 0.5 mM NaOH. Regular measurements of pH were made and the pH adjusted as necessary with 0.1 M NaOH or HCl.

Seedlings were allowed to establish in the nutrient solution before inoculum and As treatments were added on Day 3. The inoculum broth was made by dissolving 0.50 g of concentrated freeze-dried *B. japonicum* strain CB1809 (Easyrhiz, New-Edge Microbials, Albury, Australia) with 100 mL of autoclaved (120 °C, 10 min) deionised water to produce a broth of ~1.3×10^{10} colony forming units mL^{-1}. A 2 mL aliquot of inoculum broth was added to each plus inoculum vessel while minus inoculum vessels each received a 2 mL aliquot of autoclaved DI water. Arsenic was added as Na_2HAsO_4 (As(V)) to produce concentrations of 0 or 5 μM As in the appropriate vessels. The 5 μM As treatment was representative of As concentrations found in the soil solutions of contaminated soils [e.g. Refs. [15,16]]. Each inoculum-by-As treatment was replicated five times.

Nutrient solutions were refreshed on Days 10 and 18. After the nutrient solutions had been changed on Day 10, the plants were inoculated with *B. japonicum* or DI water, as per Day 3. Plants were not re-inoculated on Day 18.

On Day 21, indolic compounds (as a measure of auxin production) in the plant rhizosphere were measured using the PC method [17]. Briefly, duplicate 4 mL aliquots of nutrient solution were collected from within the root ball of each plant-growth vessel. Under sterile conditions the aliquots were combined with the same volume of PC reagent [17], incubated at ambient room temperature in the dark for 30 min and then the absorbance measured at 544 nm. Indoleacetic acid (auxin) was used as a standard.

Plants were harvested on Day 22. All plants were separated into roots and shoots, rinsed in 2% Decon 90 (Decon Labs Ltd, East Sussex, England) then three rinses of DI water before being dried for 48 h at 60 °C. Shoot and root samples were digested in 70% nitric acid and hydrogen peroxide at 90 °C before analysis for As by inductively coupled plasma – optical emission spectrometer (Varian, Mulgrave, Victoria, Australia). Shoot samples were analysed for N via a LECO N analyser (LECO Australia, Castle Hill, NSW, Australia).

Statistical analyses were performed using the SPSS package [18] to compare treatment effects. Means were compared by Analysis of Variance (ANOVA) and multiple comparison test (*post hoc* Fishers Least Significant Differences). Where necessary, data were square root or $\log_{10}$ transformed to stabilize the variance of the residuals and significance was based on analysis of the transformed data. Differences were considered significant at $P < 0.05$.

2.2. Experiment 2 tolerance of *B. japonicum* to bioavailable concentrations of arsenic and heavy metals in the growth medium

The tolerance of *B. japonicum* to heavy metals, *viz*., As, Cd, Cu, Mn, Ni and Zn, was tested at a range of concentrations (Table 1) of relevance to soil solutions in contaminated soils [e.g. Ref. [9]]. All solutions and suspensions were made using autoclaved (121 °C, 10 min) 0.85% NaCl solutions. Bacterial stock suspensions were made by suspending concentrated freeze-dried *B. japonicum* strain CB1809 (Easyrhiz, New-Edge Microbials, Albury, Australia) in 0.85% NaCl solutions. An equivalent suspension of autoclaved (121 °C, 10 min) i.e. non-viable, *B. japonicum* was used as a control. Aliquots of each metal solution (4.0 mL) plus viable or nonviable *B. japonicum* suspension (4.0 mL) were added to acid-washed and sterilised glass vials (three replicates for each treatment

Table 1
Form and final concentrations of heavy metal treatments in sample vials used for testing the tolerance of *Bradyrhizobium japonicum* strain CB1809 to heavy metals.

Arsenic	Cadmium	Copper	Manganese	Nickel	Zinc
$Na_2HAsO_4 \cdot 7H_2O$	$3CdSO_4 \cdot 8H_2O$	$CuCl_2 \cdot 2H_2O$	$MnCl_2 \cdot 4H_2O$	$NiCl_2 \cdot 6H_2O$	$ZnCl_2$
0 μM	0 μM	0 μM	0 μM	0 μM	0 μM
1 μM	1 μM	0.5 μM	10 μM	5 μM	5 μM
5 μM	5 μM	1 μM	100 μM	10 μM	10 μM
10 μM	25 μM	10 μM	500 μM	50 μM	50 μM
50 μM					