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Research article

"*In situ*" phytostabilisation of heavy metal polluted soils using *Lupinus luteus* inoculated with metal resistant plant-growth promoting rhizobacteria

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ABSTRACT

The aim of this work is the evaluation of metal phytostabilisation potential of *Lupinus luteus* inoculated with *Bradyrhizobium* sp. 750 and heavy metal resistant PGPRs (plant-growth promoting rhizobacteria), for *in situ* reclamation of multi-metal contaminated soil after a mine spill. Yellow lupines accumulated heavy metals mainly in roots (Cu, Cd and especially Pb were poorly translocated to shoots). This indicates a potential use of this plant in metal phytostabilisation. Furthermore, As accumulation was undetectable. On the other hand, zinc accumulation was 10–100 times higher than all other metals, both in roots and in shoots. Inoculation with *Bradyrhizobium* sp. 750 increased both biomass and nitrogen content, indicating that nitrogen fixation was effective in soils with moderate levels of contamination. Co-inoculation of lupines with a *consortium* of metal resistant PGPR (including *Bradyrhizobium* sp., *Pseudomonas* sp. and *Ochrobactrum cytisi*) produced an additional improvement of plant biomass. At the same time, a decrease in metal accumulation was observed, both in shoots and roots, which could be due to a protective effect exerted on plant rhizosphere. Our results indicate the usefulness of *L. luteus* inoculated with a bacterial consortium of metal resistant PGPRs as a method for *in situ* reclamation of metal polluted soils.

1. Introduction

Soils polluted by heavy metals represent an important environmental problem due to the toxic effects of metals, their accumulation throughout the food chain and the additional risk of groundwater contamination. The main options for reclamation of soil or sediments polluted by metals are *in situ* and *ex situ* techniques. The *in situ* remediation of soil aims at increasing the stabilisation of metals either on soil particles, or by other methods such as plants, so the potential mobility or bioavailability of the toxic metals to environment are lowered (e.g. immobilisation). On the other hand, *ex situ* techniques aim at extracting or separating metals from soil through a series of chemical, physical, or biological methods in a specially designed reactor [1].

Metal remediation through common physico-chemical techniques is expensive and unsuitable in the case of extensive areas. Therefore, biotechnological approaches have received a great deal of attention in recent years. Phytoremediation, the use of plants for metal reclamation, includes phytoextraction, rhizofiltration, phytostabilisation and phytovolatilisation [2,3]. Phytoextraction is based on the use of hyperaccumulator plants, which can tolerate and accumulate high concentrations of metals. Ideal hyperaccumulators require the characteristics of deep rooted, rapid growth and a high amount of biomass. In fact, many hyperaccumulators are slow in growth, produce low biomass and cannot grow in every particular soil or climate. Furthermore, most metal polluted sites contain marginal, aged soil polluted with mixtures of metals. An optimal phytoremediation strategy would be to use plants with enhanced phytoextraction capacity for an array of metals [4]. The safe disposal of metal enriched plant residues is another disadvantage concerning phytoextraction [2]. A different possibility is *in situ* metal phytostabilisation. This technique uses metal tolerant plants for mechanical stabilisation of polluted land in order to prevent bulk erosion, reduce air-borne transport and leaching of pollutants. In contrast to phytoextraction, plants are required that take up only small amounts of metals in order to prevent transfer into the wildlife food chain [2,5]. Furthermore, phytostabilisation may be the most cost-effective treatment for metal polluted soils, especially in the case of extensive pollution.

Rhizoremediation, a specific type of phytoremediation that involves both plants and their associated rhizosphere microbes, can occur naturally, or can be actuated by deliberately introducing specific microbes. These microbes can be contaminant degraders and/or can promote plant growth under stress conditions [6–8]. For a long period, plant-growth promoting rhizobacteria (PGPR) were mainly used for assisting plants to uptake nutrients from the environment or preventing plant diseases. Recently, the applica-

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tion of PGPR has been extended to bioremediation of both organic and metal pollutants [9,10]. The *Rhizobium*-legume symbiosis has been proposed as a tool for rhizoremediation of As and heavy metals in soils [11–14]. This naturally occurring symbiotic interaction has an extra advantage, the soil nitrogen enrichment due to dinitrogen fixation in plant root nodules [15].

Despite the genetic potential of plants to remove many toxic metals from the soil, phytoremediation is yet to become a commercially available technology. Contradictory results have been reported upon application of laboratory or green house systems in the field [8,16].

The aim of this work is the evaluation of the *in situ* metal phytostabilisation potential of *Lupinus luteus* plants in association with native metal resistant PGPRs in a polluted site affected by the toxic mine spill at Aznalcóllar (Seville, Spain) [17].

2. Materials and methods

2.1. Legume plant and bacteria for rhizoremediation

L. luteus cv. Aurea plants have been used for the *in situ* rhizoremediation experiment. *Bradyrhizobium* sp. 750 [18] was used as the inoculant for lupines, since no native strain isolated from contaminated soils was available. Other PGPRs were isolated from the rhizosphere of legume plants grown at contaminated area of Aznalcóllar [11], including, *Pseudomonas* sp. Az13 and *Ochrobactrum cytisi* Azn6.2 [19,20].

2.2. Determination of metal resistance in bacteria

The resistance of bacteria to As and heavy metals was evaluated on agar plates containing TY (tryptone-yeast extract) agar medium supplemented with increasing metal concentrations, according to [11]. Arsenic was provided as sodium arsenite; cadmium and zinc were added in the form of cadmium and zinc chloride, respectively; copper was added as copper sulphate, and lead was added as lead nitrate (including 5 mM EDTA in those plates in order to avoid lead precipitation). After incubation at 28 °C for 48–96 h, the growth was observed. The maximal tolerable concentration (MTC), defined as the maximal concentration of an element not affecting bacterial growth, is used to evaluate the resistance.

2.3. Determination of the concentration of arsenic and heavy metals in soil

Field experiments were carried out at the experimental plot El Vicario in the zone affected by the toxic spill of the Aznalcóllar mine [17], where a high level of contamination remains after several years [21].

Samples of soil (approx. 500 g) from the surface down to 30–35 cm were collected using a 5 cm diameter cylinder, transported to the laboratory and dried in an oven for 48–72 h at 60 °C. They were homogenised in a mortar, and sifted consecutively through three sieves from a pore size of 5 mm to a final size of 0.21 mm. A final sample of 1 g soil was used for metal determination. Toxic elements in soil samples were determined by inducted coupled plasma optical spectrophotometry (ICP-OES) after *aqua regia* treatment in a microwave according to [22].

2.3.1. Evaluation of metal contamination and experimental setup

The experimental plot had an area of 1000 m^2 (20 m × 50 m). Since local levels of contamination by As and heavy metals could be very different, an exhaustive study of contamination was performed at the experimental plot. The concentration of the five most persistent elements (As, Cd, Cu, Pb and Zn) was determined in 18 locations following a random procedure within the experimental plot. Data are shown in Table 1. The concentration of As were 3–10 times higher than the permissible levels for agricultural soils. It is interesting to note that the contaminated area was an agricultural zone. Cd concentrations were as well in all cases 4–6-fold above the threshold. The concentration of Cu was 1.1–2 times higher than threshold. On the contrary, the concentration of Pb and Zn were in general below the limits established by local environmental regulations (Pb was slightly over the limit in four spots). Since the contamination is due to several metals, a pollution index has been calculated as a parameter for pollution evaluation at each point. The pollution index is computed by averaging the ratios of metal concentration to the hazard criteria, which is the permissible level [23], i.e. maximum concentration of metals allowed by Andalusian regulations for agricultural soils. The pollution index is defined as: pollution index = Σ(metal concentrations in soils/permissible level for each metal)/number of metals (more information in legend of Table 1). Values of the pollution index < 1 indicate average levels of metals below the selected standards, according to local regulations. A pollution index > 1 indicates that, on average, metal concentrations are above the permissible levels. In all the positions tested the pollution index is above 1, indicating that the zone was still polluted. Results allowed us to draw a map of contamination (Fig. 1), in which three different zones of the plot can be delimited, with increasing PI values (1.0–2.0, 2.0–3.0, and over 3.0).

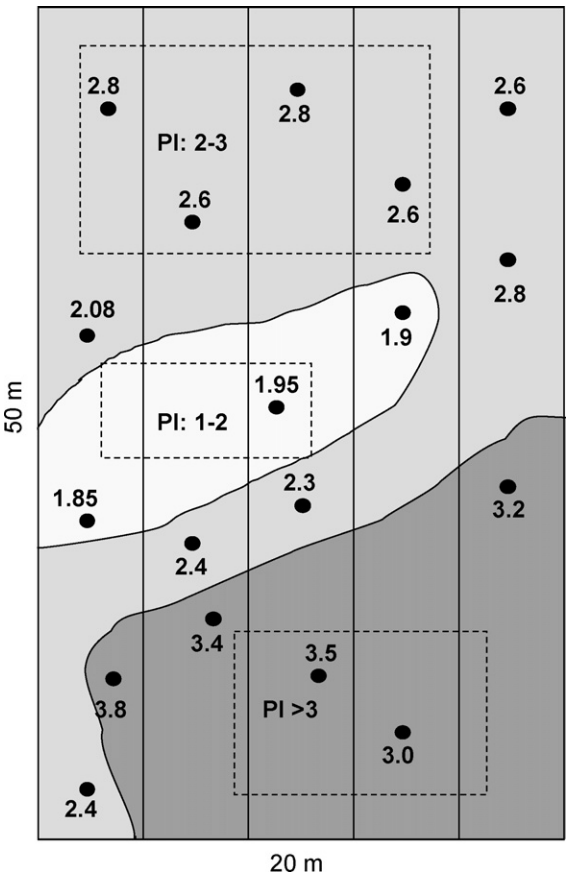

Fig. 1. Map of contamination of the experimental plot. A map of contamination was drawn by grouping the sampling points with similar PIs, whose value is indicated at every position. Three zones with increasing contamination level were obtained, which comprised PI values within the intervals 1.0-2.0, 2.0-3.0, and over 3.0. The plots where the different experiments were conducted are indicated by dot lines squares.

2.3.2. Experimental setup

Two experiments we carried out. The first one consisted in growing *L. luteus* plants inoculated only with *Bradyrhizobium* sp. 750 at

Table 1
Concentration of the most recalcitrant toxic metals and metalloids found at eighteen random positions within the experimental plot. Maximal allowed concentrations for agricultural soils according to Andalusian regulations. Numbers in bold indicate concentrations above the threshold. The pollution index was calculated from the following formula: PI = ([As]/20 + [Zn]/300 + [Pb]/200 + [Cd]/2.5 + [Cu]/100)/5, according to [23].

Sampling point	Concentration of toxic metals					Pollution index
	mg kg^{-1} As	mg kg^{-1} Zn	mg kg^{-1} Pb	mg kg^{-1} Cd	mg kg^{-1} Cu	
B2	**200.00**	159.96	**203.67**	**11.54**	**171.95**	3.4
SR9	**69.45**	223.55	105.02	**10.29**	**110.70**	1.85
A1	**97.83**	178.36	129.15	**12.65**	78.75	2.4
B4	**134.06**	220.64	180.33	**11.05**	**117.63**	2.6
C3	**114.03**	201.26	135.96	**12.01**	**153.71**	2.3
SR19	**60.75**	127.83	168.21	**13.40**	**138.40**	2.08
SR40	**67.35**	266.02	95.60	**11.34**	**114.01**	1.95
SR37	**89.02**	166.03	128.99	**15.04**	**118.49**	2.6
E3	**106.63**	137.46	132.71	**14.15**	**185.44**	2.6
A5	**132.27**	176.10	**207.94**	**14.35**	**117.04**	2.8
SR29	**158.37**	196.60	**220.32**	**15.94**	**172.01**	3.5
SR38	**167.08**	164.62	165.18	**13.05**	**135.49**	2.8
D1	**165.04**	155.45	114.98	**12.78**	**137.99**	3.0
SR39	**102.67**	155.11	108.62	**11.09**	**101.04**	2.3
SR27	**73.90**	145.82	105.28	**13.45**	**178.68**	2.4
B2	**68.66**	231.62	108.03	**11.80**	**123.02**	1.9
E2	**147.78**	141.27	179.56	**14.17**	**193.72**	3.2
SR25	**192.55**	100.58	**241.49**	**16.01**	**147.72**	3.8
Maximum level for agricultural soils	**20**	**300**	**200**	**2-3**	**100**	1

three different positions corresponding to increasing PI. The second experiment was carried out at the zone with intermediate levels of pollution (PIs within the range 2.0–3.0), since no nodulation was observed at the highest pollution index. In this experiment, four inoculation treatments were done: (a) non-inoculated plants, used as control, (b) plants inoculated only with *Bradyrhizobium* sp. 750, (c) plants inoculated with *Bradyrhizobium* sp. 750 and *O. cytisi* Azn6.2, and (d) plants inoculated with *Bradyrhizobium* sp. 750, *Ochrobactrum* sp. Azn6.2 and *Pseudomonas* sp. Az13. In all cases we analysed plant biomass, nodulation efficiency and metal accumulation.

2.3.3. Preparation of soil for field rhizoremediation experiments

Field experiments were carried out at the season between November 2005 and May 2006. After tillage, small plots of 4 m^2 (2 m × 2 m) were prepared for seed sowing at zones with increasing PIs, with a safety distance of 2 m between plots to reduce potential inter-plot contamination. The experiment was laid out with 3 replicates, using a randomized block design. No nitrogen fertilization was applied to the soil.

2.4. Preparation of inoculants for field rhizoremediation experiments

For the inoculation of the seeds, peat base inoculants were prepared using peat adjusted to neutrality by adding $CaCO_3$ and sterilized by autoclaving [24]. *Bradyrhizobium* sp. 750 was grown for 7–9 days at 28 °C in YEM (yeast extract manitol) medium with continuous shaking at 200 rpm. Liquid cultures of other metal resistant PGPRs (*Pseudomonas sp.* Az13 and *O. cytisi* Azn6.2) were grown on TY (tryptone yeast extract) medium for 24–48 h at 28 °C with continuous shaking at 200 rpm. Cells were pelleted by centrifugation at 5000 × *g* for 15 min and resuspended in sterile water. Eight ml of bacterial suspensions were mixed aseptically with 40 g of sterile peat and immediately stored at 4 °C until use (as much one week of storage at 4 °C). For the co-inoculation treatment, equal amounts of bacterial inoculums were mixed. For uninoculated control, equal volume of sterile water was added to peat. Bacterial density of the inoculants using this procedure was previously reported to be around 10^9 u.f.c./ml [25].

At the time of sowing, seed of legumes without any previous treatment were mixed with the peat containing the appropriate inoculants and a few drops of an 8% sucrose solution were added. Inoculated seeds were planted in rows and irrigated with water. The whole experiment was covered with nylon net during plant germination. Then, the mesh was removed and plants were allowed to grow for an additional five months.

2.5. Plant harvesting

Six months after sowing, five plants were harvested at random from the central part of each plot. Plants were brought to the laboratory, and roots and shoots cut separately. Shoot and root biomass were determined and the number of nodules was recorded. Shoots and roots were rinsed several times in water and dried with paper towel. Whole shoots (the above-ground biomass) and roots were dried in an oven at 60 °C for 48–60 h, cut in small pieces and mixed. Three samples of 100 g of shoot or root tissue were triturated and homogenised. A final sample of 1 g was further homogenised with mortar and pestle, and used for determination of As and heavy metals accumulation, as well as nitrogen content as described below.

2.6. Evaluation of nodulation and nitrogen fixation

Nodules were counted in five plants for each of the three replicates of each treatment. Since no nitrogen fertilizer was added to the soil, the determination of total nitrogen in dry matter of plants is usually taken as an indication of the effectiveness of dinitrogen fixation in the nodules of inoculated plants. Nitrogen content in shoots was determined using a Technicon 300B InfraAlyzer (Tarryton, NY, USA), as described in [11].

2.7. Determination of metal accumulation in plants

With regard to plants, final representative samples of 1 g (dry weight) of shoots or roots, prepared as described in Section 2.5, were used for determination of arsenic and heavy metal accumulation. Toxic elements in soil and plant samples were determined by inducted coupled plasma optical spectrophotometry (ICP-OES) after *aqua regia* treatment in a microwave according to [22].

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed using the software program SPSS 13.0 (2004) for Windows. Results given are

means ± standard deviations of three independent replicates. Analysis of variances was performed using the ANOVA post hoc test at $P < 0.1$. Previously, normality and homogeneity of variances was checked by using a Levene test. We have chosen 0.1 as the level of significance since several authors [8] suggest that, due to the inherent variability of field experiments, compared to laboratory experiments, it may be useful for members of the scientific community, industry, and regulatory bodies to establish a value of 10% ($P < 0.1$) as the acceptable level of significance to demonstrate a statistically significant effect of phytoremediation.

3. Results

3.1. Evaluation of metal resistance in bacterial inoculants

The resistance to As and heavy metals of the three bacteria used as inoculants has been evaluated on agar plates. Results are shown in Table 2. *Bradyrhizobium* sp. 750 showed low level of resistance to As and heavy metals, as compared to the other bacteria. On the contrary, *O. cytisi* Azn6-2 was resistant to the highest concentrations of As, Cd, Pb and Zn. Concerning *Pseudomonas* sp. Az13, it showed the highest resistance to Cu (up to 4.5 mM) together with an intermediate resistance to the rest of elements.

3.2. Potential of the L. luteus-Bradyrhizobium symbiosis for metal rhizoremediation

In a first experiment, *L. luteus* plants were cultivated at three different positions, each one with a significantly different value of PI. Seeds had been previously inoculated with *Bradyrhizobium sp.* 750. Data of germination, biomass, and nodulation could be seen in Table 3. Germination did not seem to be affected at PI 1.0–2.0, plants looked healthy, with no apparent toxicity symptoms and a large biomass (Fig. 2A). Furthermore, nodules were red-coloured (not shown) and plants showed a high content of nitrogen in shoots (over 3.9%). In the zone with intermediate level of contamination, 64% of seeds were able to germinate and biomass was diminished by 30%. Functional nodules were present at lower number (approx. 40%) and the nitrogen content of shoots was intermediate, indicating that the bacteria was still able to fix nitrogen under moderate pollution levels. Furthermore, plants were able to set flowers and seeds (Fig. 2B), which is important concerning self-propagation. Finally, lupine plants were also cultivated at PI over 3.0. Under these conditions, germination decreased down to 47%. Plant growth was extremely impaired; plant biomass was about ten percent of biomass of yellow lupines grown in the less contaminated area (PI: 1.0–2.0). Furthermore, plants could not complete their life cycle, being unable to set flowers and seeds (Fig. 2C). Plants did not show nodules under these conditions, so the nitrogen content in shoots decreased to 1.7%.

Table 2
Evaluation of the resistance to As and heavy metals in bacterial inoculants. The resistance to the different elements was determined on TY (tryptone-yeast extract) agar plates supplemented with increasing concentrations of the corresponding element. The resistance was expressed as MTC (maximal tolerable concentration) which is the maximal concentration of an element that does not affect bacterial growth. Data corresponding to *Ochrobactrum cytisi* Azn6.2 have been submitted for publication [20].

Bacteria	Maximal tolerable concentration (mM)				
	As	Cd	Cu	Pb	Zn
Bradyrhizobium sp. 750	2	<0.5	1.5	2	<1
Ochrobactrum cytisi Azn6-2	8	1.5	3.5	6	10
Pseudomonas sp. Az13	4	1	4.5	5	3

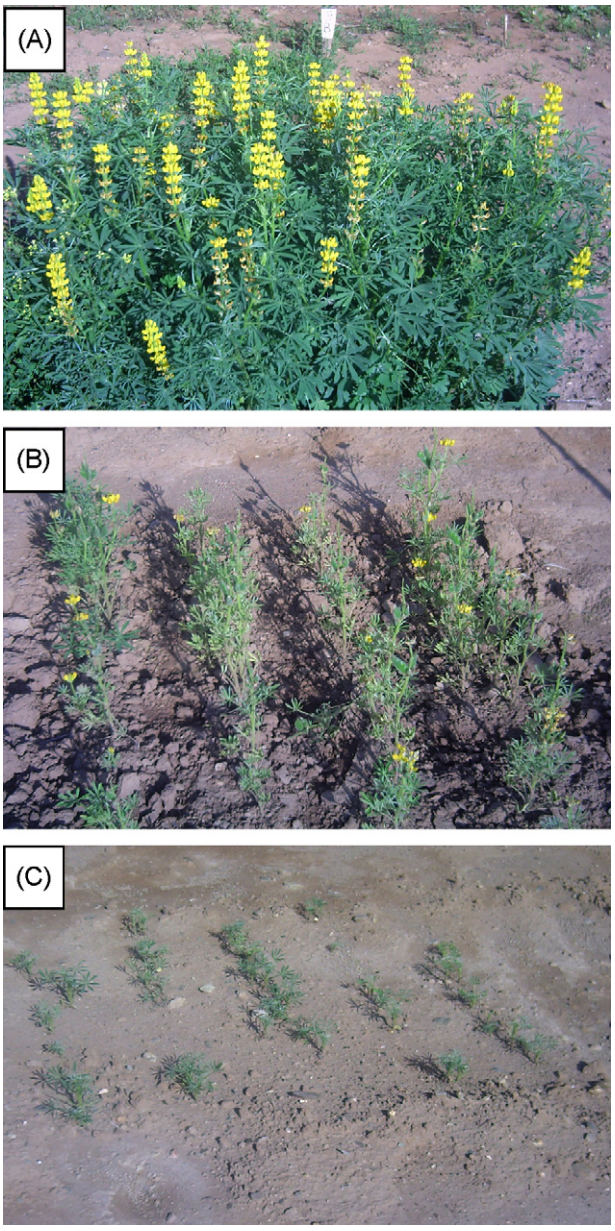

Fig. 2. Growth of yellow lupines on soil with increasing Pollution Indexes. *Lupinus luteus* plants were inoculated with *Bradyrhizobium* sp. 750 and cultivated in zones at different PIs. Aspect of plants after 6 months of growth. A. Lupinus luteus plants grown at low level of metal pollution (PI 1.2-2.0). B. *Lupinus luteus* grown on moderately contaminated soil (PI: 2.0 -3.0). B. *Lupinus luteus* grown on the most contaminated zone of the plot (PI >3.0).

Metal accumulation has been determined in roots and shoots of plants grown at different PIs (Table 4). With regard to arsenic accumulation, yellow lupine behaved as an excluder species, since no arsenic accumulation was detected even in the more contaminated area. Lupines accumulated Cd, Cu and Pb mainly in roots, with very low levels of translocation to shoots. These results indicate that these plants can be used for metal phytostabilisation. Nevertheless, zinc accumulation was much higher (10 times higher than Cu or Pb accumulation and up to 100 times as compared to the content of Cd). In fact, this species can be considered as a Zn accumulator, since the concentration of metal in plant tissues is superior to the concentration of this element in the soil (bioconcentration factor >1). Thus this plant–bacteria combination could be used for phytoextraction of Zn from the soil.

Table 3
Growth and nodulation of yellow lupines in soils with increasing levels of contamination by heavy metals. Plants were cultivated in soils with different pollution indexes and inoculated with the strain *Bradyrhizobium* sp. 750. Growth and nodulation parameters were evaluated after five months. Data are means ± standard deviations in three independent plots. Different letters (a), (b), (c), etc., indicate significant differences at the level $P < 0.1$, as suggested for field experiments [8].

PI	Seed germination (%)	Plant tissue	Biomass (g/plant)	Nodules per plant	Nitrogen content (%)
1–2	95%	Roots	4.2 ± 0.6a	5.4 ± 2.1a	
		Shoots	38.2 ± 7.7a		3.90 ± 0.8a
2–3	64%	Roots	2.4 ± 0.7b	2.1 ± 0.7b	
		Shoots	11.4 ± 3.6 b		2.40 ± 0.4b
>3	47%	Roots	0.9 ± 0.2c	0c	
		Shoots	3.7 ± 0.5c		1.70 ± 0.6c

Table 4
Metal accumulation of yellow lupines in soils with increasing levels of contamination by heavy metals. Plants were cultivated in soils with different pollution indexes and inoculated with the strain *Bradyrhizobium* sp. 750. Aerial parts and roots were harvested after five months for heavy metal determination by ICP-OES. Data are means ± standard deviations in three independent plots. Different letters a, b, c, etc., indicate significant differences at the level $P < 0.1$, as suggested for field experiments [8].

PI	Tissue	As (mg kg^{-1})	Cd (mg kg^{-1})	Cu (mg kg^{-1})	Pb (mg kg^{-1})	Zn (mg kg^{-1})
1–2	Roots	<1.5	1.2 ± 0.2a	27.5 ± 4.3a	11.0 ± 2.2a	165.1 ± 17.9a
	Shoots	<1.5	0.6 ± 0.1a	12.6 ± 1.9a	<1.5	135.1 ± 10.0a
2–3	Roots	<1.5	4.1 ± 0.9b	64.7 ± 9.8b	26.6 ± 7.7b	642.0 ± 144.3b
	Shoots	<1.5	1.6 ± 0.4b	21.5 ± 3.6b	3.5 ± 1.5b	472.0 ± 156.8b
>3	Roots	<1.5	4.8 ± 1.7b	150.7 ± 17.9c	80.7 ± 23.0c	806.3 ± 24.4c
	Shoots	<1.5	2.0 ± 0.6b	52.1 ± 14.7c	35.3 ± 14.6c	748.3 ± 167.8c
		30*	10*	40*	100*	500*

* Maximal concentration of metal recommended for domestic animal consumption [32].

The contents of heavy metals in plant tissues increased as metal soil concentrations did, especially in the roots. In the most contaminated area (PI > 3.0), metal accumulation was around 4–10 times higher than those of plants grown on the less contaminated area. Moreover, plants grown at PI > 3.0 showed a 90% biomass reduction. It must be pointed out that due to their toxic levels, only in the most contaminated area, the levels of Cu and Zn in shoots are over the limit recommended for livestock (Table 4).

3.3. *Effect of metal resistant PGPRs on growth and metal accumulation of L. luteus plants*

In the second experiment we studied the effect of inoculation with a consortium of PGPR resistant to heavy metals, on the phytoremediation capacity of *L. luteus*. This experiment was done at soils with an intermediate level of pollution, since nodulation was not observed at higher PI. Results are shown in Figs. 3 and 4. Inoculation with only *Bradyrhizobium* increased biomass yield by 29% (Fig. 3). Furthermore, inoculation with the consortium of metal resistant PGPRs increased biomass production by 109%, and the yield and the aspect of inoculated plants was much better than those without the PGPR inoculation treatment in adjacent plots (Fig. 4). Nitrogen content also showed a 40% increase with regard to uninoculated plants, due to nitrogen fixation in nodules of *Lupinus* plants, indicating that nodulation was still effective under a moderate level of contamination. The accumulation of heavy metals in those plants was determined, both in shoots and roots. Results are also presented in Fig. 3. We could observe a decrease in the accumulation of all the metals (between 25% and 40% depending on the metal), both in the root tissue as well as in shoots when plants were inoculated with *Bradyrhizobium*. This decrease in metal accumulation was enhanced upon co-inoculation with *Bradyrhizobium* and *Ochrobactrum*. Furthermore, co-inoculation with a consortium of the three bacterial species resistant to heavy metals produced a very significant diminution of the accumulation of all metals, especially in roots. More than 50% reduction in the accumulation of Pb, Cd and Zn in roots was observed. Furthermore, there was also a diminution of the amount of metal translocated to the shoot, which was between 30% and 60% depending on the metal, in plants inoculated with the consortium of three bacteria. In the case of Pb, shoot accumulation was undetectable upon inoculation with the metal resistant PGPR consortium. The ratio root/shoot of metal concentrations (Table 5, supplementary information) is increased upon inoculation with the bacterial consortia.

4. Discussion

Contamination of soils by heavy metals is a widespread problem that poses a great risk for environment, wild-life and human health. Phytoremediation emerges as a cost-effective, environmentally friendly biotechnology approach to clean local areas affected by contamination [1–5].

The toxic spill occurred at the Aznalcóllar mine in 1998 released over 5000 tons of sludge and acidic waters contaminated with extremely high concentrations of heavy metals and metalloids along the Guadiamar river, which runs 20 km away from Sevilla city [17] and is considered as one of the greatest environmental tragedies to happen in Europe. Residual contamination by As, Cd, Cu, Pb and Zn has been reported [21]. The regional government established an experimental plot for the development of research projects aimed to bring the levels of toxic elements below the limits established in Andalusian regulations.

Legume plants have been found among the first colonisers after the toxic spill of Aznalcollar [11,26]. In particular, *Lupinus angustifolius* was profusely found at the contaminated area (unpublished results). In agreement, it has been reported that several legumes are able to grow on heavy metals polluted soils [27]. Legumes in association with *Rhizobium* are getting increasing attention in metal phytoremediation [11–14,28,29]. Besides their capacity to tolerate heavy metals, legumes are able to establish symbiotic interaction with rhizobia, being a source of combined nitrogen for the biosphere and a model for microbe–plant interaction studies [15,30]. In particular, *Lupinus* species have been proposed for phytoremediation of metals [29,31] and organics [32].

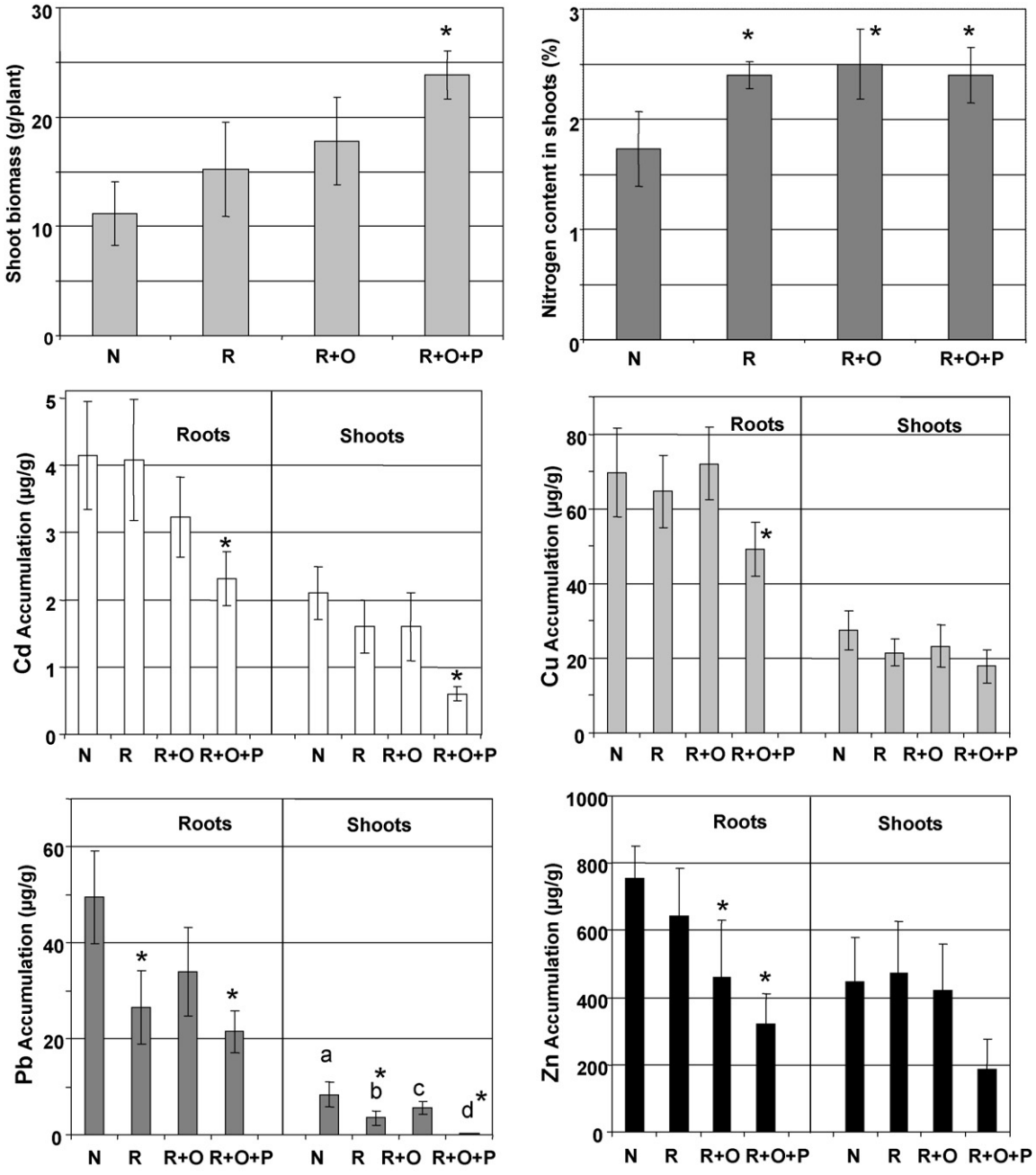

Fig. 3. Effect of inoculation with heavy metal resistant PGPRs on shoot biomass, nitrogen content and metal accumulation of *Lupinus luteus* plants grown on metal polluted soils (PI: 2.0-3.0). N: non inoculated, R: *Bradyrhizobium* sp. 750, R+O: *Bradyrhizobium* sp. 750 + *Ochrobactrum* sp. Azn6.2, R+O+P: *Bradyrhizobium* sp. 750 + *Ochrobactrum* sp. Azn6.2 + *Pseudomonas* sp. Az13. Data are means of three independent determinations. Asterisks indicate significant differences at the level P<0.1 with regard to non-inoculated plants. This level of significance has been proposed for field experiments according to [8]. Arsenic accumulation was in all cases below the detection limit of the technique (<1.5 mg kg^{-1}), both in roots and shoot samples. (The exact value of p (significance) is provided as supplementary information).

An *in situ* rhizoremediation experiment has been performed at the contaminated area, cropping yellow lupines inoculated with *Bradyrhizobium*. With regard to As, no accumulation of this element was detected, so yellow lupines behaved as an arsenic excluder. Furthermore, these plants accumulate heavy metals (Cd, Cu and Pb) mainly in roots, with low rates of metal translocation to the aerial part of the plants. These results indicate the usefulness of *L. luteus* for heavy metal phytostabilisation. The restoration of a dense vegetation cover is possibly the most useful and widespread method to physically stabilize mine wastes [33,34]. Nevertheless, Zn was the only heavy metal whose translocation to shoots was high, especially in severely contaminated soils. In this case, *Lupinus* actively accumulates Zn in plant tissues, even in shoots, slightly over the limits recommended for domestic animal consumption. *Lupinus albus* has also been reported to be a zinc accumulator [29,31]. This should be taken into account when using this plant if phytostabilisation is the choice for soil reclamation [33]. Lupines were also grown at the most contaminated area. Our results suggest that lupines are not adequate for soil reclamation in severely contaminated soils (over 200 mg kg^{-1} As, 16 mg kg^{-1} Cd, 150 mg kg^{-1} Cu and 240 mg kg^{-1} Pb), since plant viability is compromised under these conditions. It could be possible that the utilisation of a more metal resistant *Bradyrhizobium* sp. strain could improve nodulation in the most polluted soils, as it has been reported for other legumes [13]. Furthermore, metal accumulation increased 4–10 times and it can exceed the limit recommended for livestock, although slightly.

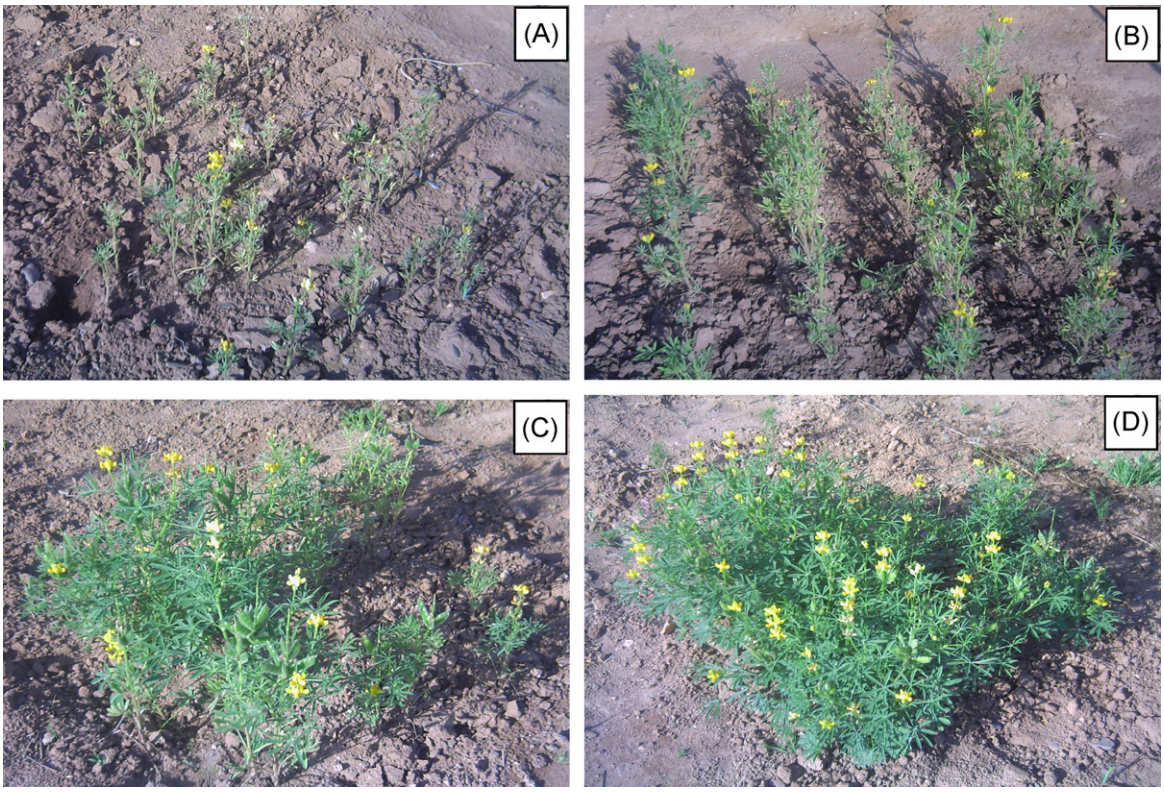

Fig. 4. Effect of inoculation with *Bradyhizobium sp.* 750 and a consortium of PGPR resistant to As and heavy metals on the growth of *Lupinus luteus* on contaminated soil (PI 2.0-3.0). A: non inoculated, B: inoculated with *Bradyrhizobium* sp. 750, C: inoculated with *Bradyrhizobium* sp. 750 + *Ochrobactrum cytisi* Azn6.2, D: inoculated with *Bradyrhizobium* sp. 750 + *Ochrobactrum cytisi* Azn6.2+ *Pseudomonas* sp. Az13.

This could be a risk of other phytoremediation techniques, such as phytoextraction [5].

One of the objectives of our work was to study the effect of inoculation with metal resistant PGPRs on the phytoremediation capacity of yellow lupine. Many reports have been generated recently on the use of plants assisted by metal tolerant PGPRs for assisting metal phytoremediation (revised in [9,35]). The combined use of this legume with *Bradyrhizobium* and metal resistant rhizobacteria, could improve the phytoremediation capacity of the plant due to the rhizosphere activity of the bacteria [6–10]. In particular, the efficiency of phytoremediation can be enhanced by using the appropriate heavy metal resistant PGPRs, including symbiotic fixing organisms [35]. Nevertheless, most of these experiments have been done in pots, and not very many results from field experiments are available. Our results showed that under moderate contamination, inoculation with a bacterial consortium including *Bradyrhizobium* sp., *O. cytisi* and *Pseudomonas* sp. strongly increased plant yield and nitrogen content. A significant diminution of accumulated metals in plant tissues was observed in roots and in shoots. *O. cytisi* is capable of accumulating up to 2500 ppm Cu, 30,000 ppm Zn and 32,000 ppm Cd mainly bound to cell surface [20]. Recently, many reports were released on the use of bacterial biomass as an efficient metal biosorbent [36,37]. This could be a possible explanation for the protection effect exerted on the plant root, since metals can be bound to bacterial surface, rendering them unavailable for plant uptake. In fact, biotechnology approaches have been addressed by expressing mammalian metallothioneins on the bacterial cell surface in order to bind metals and protect plant roots from metal pollution [38]. Other mechanisms different from biosorption have been described for microbial resistance to heavy metals, including redox changes, metal complexation, metal precipitation, metal efflux or metal volatilisation following reduction in the case of Hg [39,40].

An additional problem concerning PGPR-assisted phytoremediation is the survival and the competitiveness of the inoculants against native populations [9]. The selection of native bacterial strains resistant to As and heavy metals isolated from the same area, as it is the case for *O. cytisi* Azn6.2 and *Pseudomonas* sp. Az13, could help the survival and competitiveness of these inoculants, although this point has not been addressed in the present study.

5. Conclusions

L. luteus are adequate for metal stabilisation of soils with moderate level of heavy metal pollution. Our results suggest a positive effect of co-inoculation with *Bradyrhizobium* and metal resistant PGPRs for phytostabilisation of metal polluted soils using this plant–rhizobacteria system, since it increases plant yield and nitrogen, and decreases plant metal accumulation, thus preventing the impact of metals in the food chain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2009.12.035.

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