Comparative analysis of two phytohormone and siderophores rhizobacteria producers isolated from heavy metal contaminated soil and their effect on *Lens esculenta* growth and tolerance to heavy metals

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Due to the sensitivity and the sequestration ability of the microbial communities to heavy metals, the application of plant growth-promoting rhizobacteria (PGPR) for the bioremediation of this kind of contaminants has been done. This study compared the relationships between two rhizobacteria isolated from two plant species collected from contaminated soil with heavy metals by their indole acetic acid and siderophores production and the growth response and tolerance to arsenic and lead of *Lens esculenta* inoculated with these rhizobacteria. The results of Tolerance Index for *L. esculenta*, obtained for arsenic and lead for both rhizobacteria strains, showed that the development of plants inoculated with the rhizobacteria *Pseudomonas* sp. Sp7d strain favored the radical growth of *L. esculenta* seedlings exposed to Pb and *Pseudomonas* sp. Sp7e strain in seedlings exposed to As; both strains keep the safe development of this species in the presence of the heavy metals at higher concentrations.

**Keywords** plant growth-promoting rhizobacteria; phytohormones; siderophores; heavy metals; *Lens esculenta*

1. Introduction

According to Glick [1], heavy metal contamination can be a consequence of industrial activities that eliminate residues in the soil that in long terms, promote their accumulation. Among the metals found more frequently there are cadmium, lead, cobalt, copper, mercury, nickel, selenium and zinc. For cadmium, lead, copper and zinc, their toxicity increases as follows: lead < zinc < copper < cadmium. Plants can be defined as accumulators, excluders and indicators, according to the concentration of metals found in their tissues [2]; plant tolerance to metals is being related to the production of peptides named phytochelatins that sequestrate metals, binding them and thus protecting the enzymes sensitive to metals [3-5] and the species with ability of surviving in soils contaminated by heavy metals are those who are metal tolerant [6]. Not all the metals are evenly retained in the roots, suggesting that tolerance of a species to a certain metal not always guarantees tolerance to another [7]. In order to choose the best species, there are relevant factors that should be considered such as plant age [8], metabolism and the microorganisms that are ubiquitous to them [9].

The plant root activities potentially increase metal/metalloid solubility and may change speciation include acidification/alkalinisation, modification of the redox potential, exudation of metal chelants and organic ligands (in particular low molecular organic acids and phytosiderophores) that compete with anionic species (e.g. arsenate) for binding sites [10-12]. Microorganisms can increase solubility and change speciation of metals/metalloids through the production of organic ligands via microbial decomposition of soil organic matter, and exudation of metabolites (e.g. organic acids) and microbial siderophores that can complex cationic metals or desorbed anionic species (e.g. arsenate) by ligand exchange [13]. Idris et al. [14] characterized the indigenous bacterial communities associated with the nickel hyperaccumulator *Thlaspi goesingense* using cultivation and cultivation-independent techniques. They showed that the majority of bacterial strains had been able to produce siderophores, indicating that—provided the metal–siderophore complex—soil interaction is repulsive [15,16]. Rhizosphere microorganisms, which are closely associated with roots, have been termed plant growth promoting rhizobacteria (PGPR) [17]. These bacteria are capable of promoting plant growth by colonizing the plant root [18]. PGPR can divide into two groups according to their relationship with the plants; symbiotic bacteria and free-living rhizobacteria. Generally PGPR function in three different ways: synthesizing particular compounds for the plants, facilitating the uptake of certain nutrients from the environment and preventing the plants from diseases [19]. An extension of PGPR technology is the emerging use of the bacteria with plants for environmental applications. Recent studies in this area include many different uses; such growth promotion of soil stabilizing plants, to counteract flooding stress of plants, aid plant growth in acidic conditions, counter high temperature stress and the use of PGPR in phytoremediation technologies, so adding PGPR can aid plant growth [20, 21]. The aim of this study was to compare the relationships between two rhizobacteria isolated from two plant species collected from contaminated soil with heavy metals by their indole acetic acid and siderophores production and the growth response and tolerance to arsenic and lead of *Lens esculenta* inoculated with these rhizobacteria.
2. Methods

2.1. Isolation of siderophore producers’ rhizobacteria and inoculum preparation

The siderophore producers rhizobacteria were isolated from the rhizosphere of two plant species that grown in a metal contaminated soils located in Villa de la Paz in the state of San Luis Potosi, Mexico, from a soil with higher concentration of As and Pb [22]. Both rhizobacteria strains were maintained and preserved on agar Luria-Bertani (LB) medium plates for the conventional bacterial analyses such as cell form and size, Gram-staining and colony pigmentation performed for the rhizobacteria strains isolated and identified by the determination of gene 16S rRNA sequences. Colony PCR was performed from live cell cultured on agar LB medium plates. Cells were harvested after 24 h and processed for DNA isolation using the Allers and Lichten procedure [23]. Using the purified genomic DNA, the molecular target gene 16S rRNA was amplified using universal primer set fD1 and rD1 designed by Weisburg et al. [24]. Aliquots of PCR reaction products were electrophoresed in 1% agarose gel and then stained with ethidium bromide. These PCR products were purified and sequenced by the Unidad de Biotecnología y Prototipos of FES-Iztacala (UNAM). The sequences were then compared to similar sequences in the databases using BLAST analysis (Basic Logical Alignment Search Tool, BLAST at NCBI). The rhizobacteria isolated, were analyzed by their Indole Acetic Acid (IAA) production [25, 26] using the Salkowsky reagent according to the method of Bric et al. [27] and the production of siderophores was evaluated by the universal method of Schwyn and Neilands [28] using blue agar plates with LB medium containing the dye Chrome azurol S (LB-CAS) [29]. The siderophore levels produced by the strains were recorded as the diameter of the yellow halo produced around the colonies [30].

2.2 Plant root elongation promoting (PREP) activity of the isolated rhizobacteria strains

The plant root elongation promoting (PREP) activity [31, 32] of the isolated rhizobacteria strains was determined using the modified root elongation assay of Belimov et al. [31].

The bacterial inoculum consisted of both rhizobacteria strains, these were grown on plates with agar LB medium for 48 h at 28°C and resuspended to 5×10⁷ cells mL⁻¹ in sterile distilled water. Bacterial suspensions or sterile water (uninoculated control) were added separately to glass Petri dishes with sterile filter paper and another treatments supplemented with the appropriated As (Na₂H₂AsO₄·7H₂O) and lead (Pb(CH₃COO)₂·3H₂O) concentrations: 0.05, 0.5 and 1 mM, the control experiments without the addition of the metal were done.

Twenty seeds of Lens esculenta were surface-sterilized with 10% sodium hypochlorite solution, rinsed with deionized sterile water and placed in Petri dishes with sterile filter paper, with the respective experiments. All the experiments were performed by triplicate for each treatment and maintained at 30 ºC in a growth chamber in dark for 4 days.

Root length of L. esculenta seedlings was measured after incubation and the tolerance index (TI) were obtained expressed as the ratio of the root lengths of seedlings grown in the presence and absence of the specific added metal [33, 34]; TI = RLm / RLc where RLm is the root length of plants grown in the presence of a specific added metal and RLc is the root length of plants grown in absence of metal (control). And the TI = RLmrb / RLrh Where RLmrb is the root length of plants grown in the presence of a specific added metal and rhizobacteria, and RLrh is the root length of plants grown in absence of metal but inoculated with the rhizobacteria strain.

2.3 Statistical analysis

All the results were analysed by ANOVA test, and Tukey-Kramer Method using the statistics program Graph Pad Instat Ver. 2.03 [35].

3. Results and Discussion

The rhizobacteria isolated were identified as Pseudomonas sp. strains based on its 16S rDNA sequence homology analysis. These two rhizobacteria were classified as high auxin producers among the other rhizobacteria isolated, with 11.76 and 14.58 µg ml⁻¹ of IAA in Pseudomonas sp. strain Sp7d and Pseudomonas sp. Sp7e strain, respectively. Both were higher siderophores producers, where the measurement of the yellow halo diameter around the colonies was 0.3 cm for Pseudomonas sp. Sp7d strain and 0.7 cm for Pseudomonas sp. Sp7e strain.

Plants and bacteria can form nonspecific associations in which normal plant processes stimulate the microbial community; these biochemical mechanisms increase the remediation activity of bacteria associated with plant roots [36]; although both rhizobacteria strains were higher IAA producers, they do not promote the root elongation of the L. esculenta seedlings compared with the control, (Figures: 1A, B and C).

The plant growth promoting rhizobacteria could be developed as inoculants to increase plant biomass and thereby to stabilize and remediate metal polluted soils. Such soils can be made nutrient rich by applying metal-tolerant microorganisms, especially the plant growth promoting rhizobacteria, which would provide not only the essential
nutrients to the plants growing in the contaminant sites but would also play a major role in detoxifying heavy metals [37] and thus help plants capable of remediating heavy metals [1].

The response of L. esculenta roots to As and Pb showed this species was susceptible to both metals, and this response was more evident as the metals’ concentration increase; particularly to arsenic, more than lead in Figure 1A. The results of the root lengths of L. esculenta plants inoculated with both rhizobacteria strains exposed to As (Figures 1B and 1C) showed that the effect of adding the rhizobacteria favored the growth of the roots at the highest concentrations of this heavy metal (0.5 and 1.0 mM) compared with the roots grown without inoculum. For Lead, the plant growth response of L. esculenta inoculated with the rhizobacteria showed a slightly decrease response in the roots grown with metals alone; although the both rhizobacteria inoculum maintained the root growth at the three lead concentrations assayed (Figures 1B and 1C).

The presence of the rhizobacteria strains do not show a significant difference of the radical growth in the control and exposed to heavy metals L. esculenta seedlings. The lead concentrations tested for this species and the presence of the rhizobacteria strains, kept the development of the plants but not the increment in their growth response, as the results reported by Burd et al. [34] with the plant growth promoting rhizobacterium Kluyvera ascorbata SUD165 isolated from metal contaminated wetland, when applied to soil amended with lead. Similarly, metal resistant K. Ascorbata protected Lycopersicon esculentum L., Brasica campestris, and Brasica rapa plants when grown in soils supplemented with lead and other heavy metals [20].

The effect of adding the rhizobacteria strains to L. esculenta before the seeds germination, in presence of the different concentrations of the heavy metals assayed was examined (see Table 1). The results of these experiments are presented as TI (Tolerance Index), making easier to compare the effects of the different experimental conditions. A TI of 1.0 indicates that the treatment was not inhibitory, while a TI 0.1 indicates that the growth of treated plants was only 10% of the growth of the control plants [34]. The results of TI for L. esculenta, obtained for arsenic and lead for both rhizobacteria strains, showed that the development of plants inoculated with the rhizobacteria Pseudomonas sp. Sp7d strain favored the radical growth better than seedlings exposed to lead alone, compared with Pseudomonas sp. Sp7e strain; but this rhizobacteria strain promoted the root length of L. esculenta seedlings grown with arsenic better than Sp7d strain. Both strains keep the safe development of this species in the presence of metal at higher concentrations, compared with the growth plants with heavy metals and bacteria alone; this response could be related with their high ability to produce siderophores in both rhizobacteria strains.

In this study the higher concentrations of As (0.5 and 1.0 mM) were toxic to the root development alone, but when de L. esculenta seeds were inoculated with the rhizobacteria Pseudomonas sp. Sp7e strain, the root length of L. esculenta seedlings were promoted. The presence of As at 0.05mM concentration, favoured also the growth of the plants and in the experiments with bacteria and the metal, the difference of the root growth show a significant difference between them (p < 0.05).
Fig. 1: Root length measurement of *Lens esculenta* seedlings: A) exposed to As and Pb, B) exposed to As and Pb and inoculated with rhizobacteria *Pseudomonas sp.* Sp7e strain, C) exposed to As and Pb and inoculated with rhizobacteria *Pseudomonas sp.* Sp7d strain. Mean values ± S.D. from three replicates. The different lower-case letters show the significant differences between experiments (p < 0.001).
Some plant growth-promoting rhizobacteria can significantly increase the growth of plants in the presence of heavy metals [20,34]. When plant growth promoting rhizobacteria used as seed inoculants, applied to soil either treated/amended intentionally with metals; have shown a substantial reduction in the toxicity of metals and concomitantly improved the overall growth and yield of plant species [38-40]. Besides their role in protecting the plants from metal toxicity, metal tolerant growth promoting rhizobacteria shown a substantial protection to plants against metal toxicity, and consequently improved the growth, symbiosis and seed yield of plants [40-42], so the increase in the growth of agronomically important crops grown in metal-stressed soils by applying metal tolerant rhizobacteria could be attributed to the ability of rhizobacterial strains to mitigate the toxic effects of metals using mechanisms besides providing plants with the sufficient amounts of growth promoting substances [43]. Roots of L. esculenta seedlings grown in presence of the heavy metals: As and Pb and inoculated with Pseudomonas sp. Sp7d strain and Pseudomonas sp. Sp7e strain, do not shown a significant promotion with the rhizobacteria alone; but this work demonstrated that the presence of heavy metals plus the PGPR assure the survival of plants and even protected them; suggesting that the plant response is related with the metal concentration and the exposition time to the contaminants also the intrinsic tolerance of the same one. The presence of both rhizobacteria strains allowed the growth maintenance of the roots of L. esculenta seedlings and its tolerance to the heavy metals assayed, suggesting a synergic effect between this species and the rhizobacteria, particularly in the case with Pseudomonas sp. Sp7e strain in the response to the contaminants effect. Plant growth-promoting rhizobacteria like the analyzed, could be recommended to treat plants for the increase of plant biomass for the stabilizing and remediating metal-polluted soils.

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References

| Table 1: Tolerance index of *Lens esculenta* to arsenic and lead |
|--------------------------|-------|------------------|
| Experiment | TI | % radical growth |
| Pb 0.05 mM | 0.89 | 89.2 |
| Pb 0.5 mM | 0.81 | 81.3 |
| Pb 1.0 mM | 0.75 | 75.0 |
| No inoculated seeds | | |
| Pb + Pb 0.05 mM | 0.87 | 87.1 |
| Pb + Pb 0.5 mM | 0.84 | 84.1 |
| Pb + Pb 1.0 mM | 0.8 | 80.6 |
| Inoculated seeds with *Pseudomonas* sp. Sp7d | | |
| Pb + Pb 0.05 mM | 0.79 | 79.3 |
| Pb + Pb 0.5 mM | 0.88 | 88.1 |
| Pb + Pb 1.0 mM | 0.68 | 68.0 |
| No inoculated seeds | | |
| As 0.05 mM | 1.09 | 109.8 |
| As 0.5 mM | 0.54 | 54.4 |
| As 1.0 mM | 0.52 | 52.45 |
| Inoculated seeds with *Pseudomonas* sp. Sp7d | | |
| As + As 0.05 mM | 1.1 | 110.8 |
| As + As 0.5 mM | 0.84 | 84.1 |
| As + As 1.0 mM | 0.8 | 80.6 |
| Inoculated seeds with *Pseudomonas* sp. Sp7e | | |
| As + As 0.05 mM | 1.2 | 120.1 |
| As + As 0.5 mM | 0.91 | 91.7 |
| As + As 1.0 mM | 0.85 | 85.5 |


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