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Annals of Biological Research, 2013, 4 (11):39-47
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

Release of metabolites and enzymatic activity as physiological response to cadmium by *Sedum praealtum* roots inoculated with a siderophore producing bacteria

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ABSTRACT

The employment of many synthetic chelators like ethylenediamine tetraacetic acid, increase the mobility and bioavility of the heavy metal uptake by plants and favoring their accumulation in the aerial parts of phytoextracting plants, also the particularly participation of plant growth-promoting bacteria prevent the deleterious effects of environmental stresses by the stimulation of plant growth through the synthesis of phytohormones and by the secreted siderophores that may improve the metal bioavailability. The aim of this study was analyze the effect of the inoculation of Sedum praealtum roots with a siderophore producing bacteria (SPB) and EDTA in the release of metabolites and root enzymatic activity as a physiological response to cadmium. In Sedum praealtum roots, Cd doesn´t inhibit the guaiacol peroxidases activity, considering it as protecting mechanism against the heavy metal. The correlation between the IAA and CAS released and quantified in the medium showed the effect of the rhizobacteria Pseudomonas sp. Sp7E employed as inoculant and the chemical chelator to enhance a protecting response of Sedum praealtum roots to Cd.

Keywords: *Sedum praealtum*, roots, plant growth-promoting rhizobacteria, EDTA, cadmium

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INTRODUCTION

Pulford and Watson [1] showed that the bulk of soil heavy metal concentrations present in contaminated soils are commonly found as insoluble compounds and are unavailable for been absorbed and transported into roots, but the employment of many synthetic chelators like ethylenediamine tetraacetic acid (EDTA), diethylene triamine penta-acetic acid (DTPA), ethylenediaminedisuccinic acid (EDDS) and nitrilotriacetic acid (NTA), increase the mobility and bioavility of the heavy metal uptake by plants and favoring their accumulation in the aerial parts of phytoextracting plants [2,3,4].

Ouzounidou and Ilias [5] also recommended as an alternative strategy to increase the efficiency of the assisted phytoextraction, the management of plant growth regulators (PGR) to counteract the negative effects of heavy metal stress in growing plants. Plant growth regulators or phytohormones are organic substances that regulate and modify physiological process in plants growth [6]. The majority of the studies have examined and performed in hydroponic cultures the combined effects of PGRs and heavy metals [5,7]. Leinhos and Bergmann [8] and Lippmann *et al.* [9]

reported that the addition of indole-3- acetic acid (IAA) to soil is similar to the soil's inoculation with some Plant Growth Promoting Rhizobacteria (PGPR) that produces this auxin and it enhanced the uptake of some elements like Zn, Mg, Ca, K and P by plant roots. Kamnev *et al*. [10] mention that bacterial siderophores could be potential environmentally friendly iron (III) carriers (instead of synthetic chelators like EDTA, NTA, etc.). Beneficial interactions between phytoremediation crops and bacteria have been demonstrated to alleviate metal toxicity and nutrient deficiency [11.12]. Sinha and Mukherjee [13] consider that among the different heavy metal pollution, cadmium (Cd) pollution needs special attention because this metal provokes a high toxicity and solubility in water [14]. High Cd concentrations inhibit the plant growth and cause toxic symptoms [15,16] therefore, efforts are being taken to have an effective measure to control Cd pollution.

It is known that the oxidative stress is induced with the overproduction of Reactive Oxygen Species (ROS), which can react with lipids, proteins, pigments, and nucleic acids and cause lipid peroxidation, membrane damage, and enzyme inactivation, thus affecting cell viability [17]. Li *et al*. [18] mentioned that plants have evolved an effective scavenging system composed by an enzymatic antioxidant system: superoxide dismutase, guaiacol peroxidase, catalase and ascorbate peroxidase, that are useful for the plants response in stressful environment and there is an increase in the activities of these enzymes reported in plants exposed to metals [17, 18, 19]. Sinha and Mukherjee [13] and Burd *et al*. [20] noted that the use of various heavy metal resistant bacteria having plant growth-promoting feature has been an ecofriendly management of the heavy metal pollution in soils and the particular use of Cd accumulating and siderophore-producing bacterial strains in the rhizosphere can be a source of benefits to plants counteracting the deleterious effects of Cd contamination in soils, taking the knowledge about the important roles of siderophores in plants regarding to their growth promotion [21]. The aim of this study was analyze the effect of the inoculation of *Sedum praealtum* roots with a siderophore producing bacteria (SPB) and EDTA in the release of metabolites and root enzymatic activity as a physiological response to cadmium.

MATERIALS AND METHODS

Plant growth and roots culture of *Sedum praealtum* and the assessment of their inoculation with the selected siderophores producing bacteria (SPB)

Plantlets of *Sedum praealtum* were cultured for 30 days in hydroponic cultures, after this time, the roots were cut and each of them were surface sterilized with sodium hypochlorite (10%) for 3minutes, rinsed with sterile distilled water and placed separately in sterile baby food flasks with Magenta SIGMA caps with 70mL of ¼ of concentrate mineral medium (0.20 M $NH_4H_2PO_4$, 0.50 M NH_4NO_3, 1.15 M $Ca(NO_3)_2$, 0.26 M $CaCl_2$, 0.2 M $MgCl_2 \cdot 6H_2O$, 0.20 M $Mg(NO_3)_2 \cdot 6H_2O$, 0.40 M $MgSO_4 \cdot 7H_2O$, 0.20 M KH_2PO_4, 1.2 M KNO_3, 0.5 M K_2SO_4, 0.04 M $FeCl_3 \cdot 6H_2O$, 1.2×10^{-2} M H_3BO_3, 1.2×10^{-4} M $CuCl_2 \cdot H_2O$, 2.3×10^{-3} M $ZnCl_2$, 4.4×10^{-4} M $MnCl_2 \cdot 4H_2O$, 6×10^{-6} M $Na_2MoO_4 \cdot H_2O$, EDTA and $FeSO_4 \cdot 7H_2O$, pH = $\pm$ 6.0).

The treatments consider roots inoculated with the rhizobacteria *Pseudomonas* sp. strain Sp7E, isolated from the rhizosphere of *Viguiera dentata* (Cav.) Spreng grown in a metal contaminated soil located in Villa de la Paz in the state of San Luis Potosí, México. Bacteria inoculum was obtained by culturing the rhizobacteria strain on plates with Luria-Bertani (LB) agar medium for 48 h at 28ºC and re-suspending in sterile distilled water to adjust by optical density to $5x10^7$ UFC/mL; 0.2mL of the bacterial suspension were added separately to the property experiments.

The established experiments with and without the rhizobacteria inoculum's, were supplemented with cadmium ($3CdSO_4 \cdot 8 \cdot H_2O$) 1mM and EDTA-$Na_2$ 5mM as a synthetic chelator. The control experiments only had mineral medium. All the experiments were performed by quadruplicate and maintained at 28°C in a growth chamber in dark for 25 days.

Root GPX activity

The root biomass was recuperated and the root fresh weight was determined. The root surface activity of guaiacol peroxidases (GPX) was quantified according to the method of Guerrero [22]; roots were deposited in tubes that contained 3mL of phosphate buffer 100mM pH = 6.5 with 200µL of guaiacol 0.2M and 200µL of H_2O_2 0.03M, this reaction mixture were incubated at room temperature for 60 minutes and the absorbance was read at 436nm. The activity of GPX consider the extinction molar coefficient of guaiacol (ε_{436}= 6,400 M/cm) and expressed as nM of oxidize guaiacol/min/cm^2 root.

Released metabolites: siderophores and indol acetic acid quantity in root culture medium

The siderophores released to the medium were quantified according to the universal method of Schwyn and Neilands [23] modified by Alexander and Zuberer [24] employing the dye Chrome Azurol S (CAS). 1.5mL of the culture medium were taken from each experiment and 500µL of CAS solution were added, the mix reaction was incubated at room temperature for 45 minutes and the absorbance was read at 630nm, the siderophores concentration was estimated by the interpolation of the absorbance on standard curve of CAS solution. The siderophores produced were visually observed by the change of the CAS solution (blue to yellow, orange or purple).

The indol acetic acid (IAA) excreted to the medium culture was quantified using the Salkowski reagent according to the method of Bric *et al.* [25] and Melo *et al.* [26] taking 1mL of the medium culture of each experiments and mixed with 1mL of Salkowski's coloring reagent, the reaction was incubated at room temperature for 60 minutes and the development of a pink color indicates IAA production, this was quantified reading its absorbance at 535 nm. The concentration of IAA was estimated by a standard curve of IAA reagent.

Statistical analysis

All the results were analysed by ANOVA test, and Tukey-Kramer Method using the statistics program Graph Pad Instat Ver. 3.10.

RESULTS AND DISCUSSION

Plant growth promoting effect and root enzymatic activity

The effect of root's inoculation with *Pseudomonas* sp. strain Sp7 showed that the presence of the SPB do not notably increase the root growth; and it was maintained under this conditions, but is important to note that the presence of the chelator (EDTA) favored the protection and growth of *Sedum praealtum* roots with and without the inoculum, against the direct effect of Cd (Table 1). The root surface GPX activity increase in the experiments with the inoculated rhizobacteria (MM + Rb), comparing to the control roots (Fig. 1), but this response increase with the presence of MM +EDTA and MM + EDTA+ Cd. It is important to mention that the presence of the rhizobacteria diminished the effect of the heavy metal to the roots, with the results observed regarding to the GPX activity as a physiological response against the oxidative damage induced by Cd, this was evident in the experiments MM + Rb + EDTA+ Cd (4.86 nM) and MM + Rb + Cd (4.05nM). Li *et al.* [18] reported the antioxidant responses of *Sedum alfredii* roots subjected to elevated Zn for 12 days and found the fact that high activities of antioxidant enzymes were associated with a high plant biomass, suggesting their role in maintaining plant growth. Particularly the antioxidant enzymes play an important role in adaptation and ultimate survival of plants during periods of stress [27], where the enhance of GPX activities was observed in plants exposed to toxic levels of heavy metals, such as Cu, Mn, and Fe [28]; Tian *et al.* [29] also observed that treatments with Cd strongly inhibited activities of GPX in the roots of *Sedum alfredii*. In the case of *Sedum praealtum* roots, Cd doesn´t inhibit the GPX activity, this response was according to the mention by Li *et al.* [18] considering it as protecting mechanism against the heavy metal.

Table 1: Fresh root weight of *Sedum praealtum* roots exposed to cadmium

Without Rhizobacteria	**Root Fresh Weight (g)**
Mineral Medium (MM)	0.91 ± 0.45
Mineral Medium + EDTA 5mM (MM + EDTA)	1.279 ± 0.24
Mineral Medium + Cd 1mM (MM + Cd)	1.264 ± 0.48
Mineral Medium + EDTA 5mM + Cd 1mM (MM + EDTA+ Cd)	1.329 ± 0.07
With Rhizobacteria *Pseudomonas* sp. strain Sp7	**Root Fresh Weight (g)**
Mineral Medium + Rhizobacteria (MM + Rb)	0.966 ± 0.2
Mineral Medium + Rhizobacteria + EDTA 5mM + (MM + Rb + EDTA)	1.231 ± 0.25
Mineral Medium + Rhizobacteria + Cd 1mM + (MM + Rb + Cd)	1.354 ± 0.16
Mineral Medium + Rhizobacteria + EDTA 5mM + Cd 1mM (MM + Rb + EDTA+ Cd)	1.256 ± 0.12

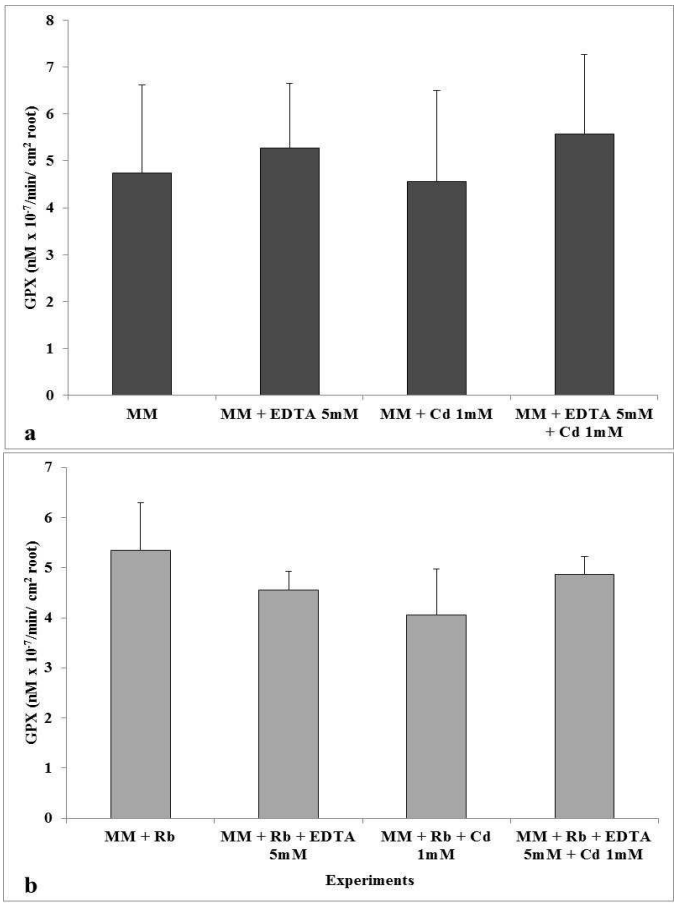

Figure 1: GPX root surface activity of *Sedum praealtum*: a) without the rhizobacteria *Pseudomonas* sp. strain Sp7 and b) with the rhizobacteria *Pseudomonas* sp. strain Sp7 (no statistical differences founded between treatments).

Siderophores and IAA metabolites released to the root medium culture as a chemical response to cadmium presence

The CAS concentration determined in the root medium culture presented an inverse correlation between the siderophores production and CAS concentration; more CAS concentration quantified less production of siderophores released. The results in Figure 2 generally showed that the experiments without the inoculated rhizobacteria presented higher concentrations of CAS; the experiments MM + Rb + EDTA and MM + Rb + Cd were also higher, but it was clearly that the solely rhizobacteria and the experiment of roots exposed to Cd with the biological (rhizobacteria) and chemical (EDTA) chelators increased the siderophores released to the medium with the diminish of the CAS concentration. Qurban *et al.* [30] and Rajkumar *et al.* [31] showed that metal-resistant siderophore-producing bacteria (SPB) plays an important role in the successful survival and growth of plants in contaminated soils by the fact that bacterial siderophores are able to bind metals other than iron and thus enhance their bioavailability in the rhizosphere of plants. Singha and Mukherjee, [18] founded that Cd-induced production of siderophore may be explained taking views of other authors [32] where Zinc-induced siderophore production was reported to be regulated in *Pseudomonas aeruginosa* [33]. These authors consider that Cd is a divalent cation like Zn and the control of siderophore induction was presumed to be regulated through a similar way. Therefore, siderophore production due to Cd exposure might be cell mass independent to a certain extent. Kamnev [34] and Kamnev and van der Lelie [12] *founded* in the metal-tolerant bacterium *Ralstonia eutropha* CH34, a novel siderophore (named alcaligin E) that binds, immobilizing and excluding Cd from metabolism.

In this study, even the siderophores production was observed in only two experiments, the presence of the rhizobacteria and EDTA in roots exposed to Cd suggest a particularly response between the roots of Sedum praealtum and bacteria that induce the release of siderophores and with it as mention the earlier authors, react with Cd and protect the roots.

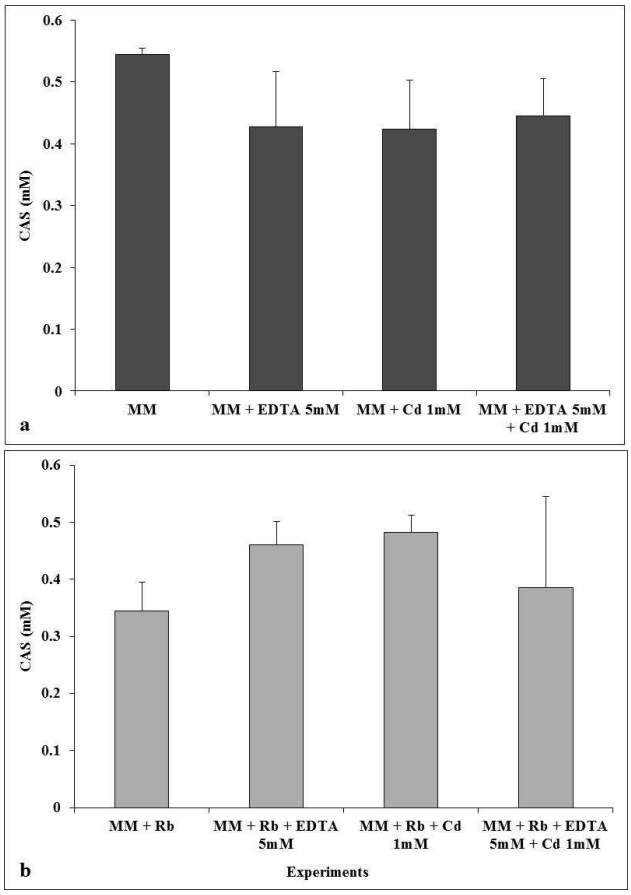

Figure 2: Siderophores production by CAS quantified from the roots medium culture of *Sedum praealtum*: a) without the rhizobacteria *Pseudomonas* sp. strain Sp7 and b) with the rhizobacteria *Pseudomonas* sp. strain Sp7 (no statistical differences founded between treatments).

Even the concentration of the IAA quantified was high in the experiments with solely the mineral medium compared with the rest of the experiments of *Sedum praealtum* roots (Fig. 3), the relationship between the root fresh weight and this released metabolite showed a diminish in its concentration. This can be regarding to the concentration of IAA that is adequate to induce a elongation and grown of roots compared with the rest of the experiments giving an increase in root biomass; the experiments with the inoculation with the rhizobacteria *Pseudomonas* sp. strain Sp7E showed an increase in the IAA quantified according to next order: MM + Rb + EDTA + Cd (2.95mg/mL) > MM + Rb + Cd (2.85mg/mL) > MM + Rb + EDTA (2.5mg/mL).

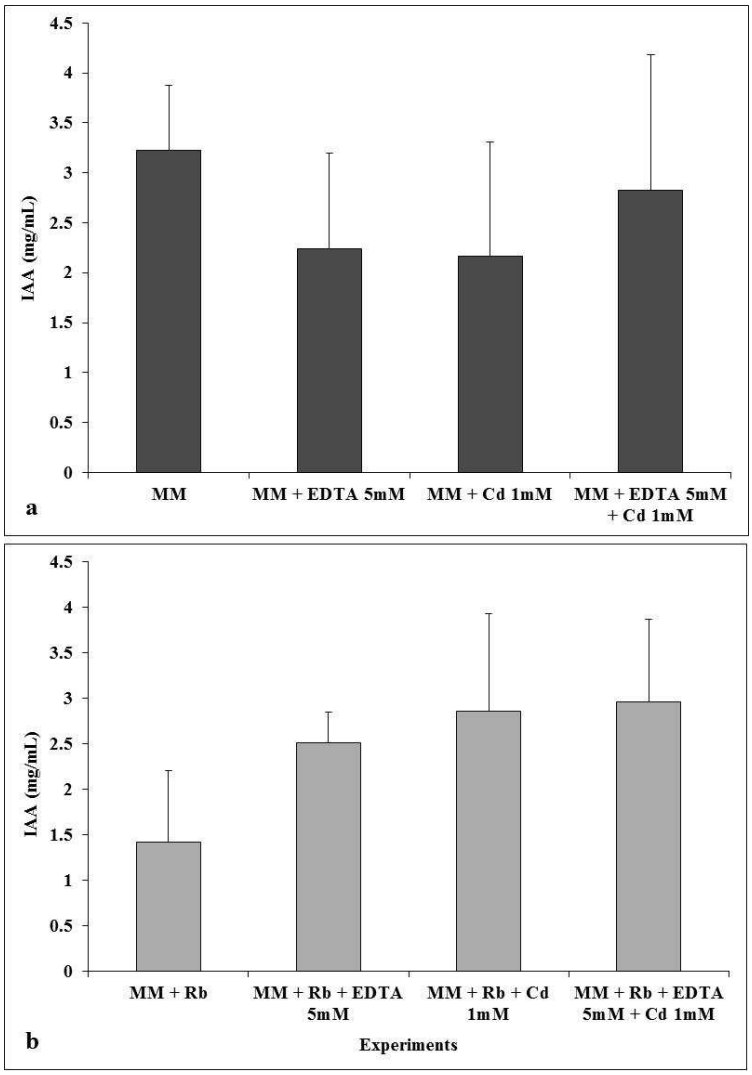

Figure 3: IAA quantified from the roots medium culture of roots of *Sedum praealtum*: a) without the rhizobacteria *Pseudomonas* sp. strain Sp7 and b) with the rhizobacteria *Pseudomonas* sp. strain Sp7 (no statistical differences founded between treatments).

It is known that the IAA production by rhizobacteria plays an important role in plant-bacterial interactions; thus, any direct influence on IAA production by bacteria may affect their phytostimulating efficiency Jing *et al.* [35]. Glick and Stearns [36] mention that the soil bacteria help plants to either avoid or partially overcome many of environmental stresses; and particularly the plant growth-promoting bacteria prevent the deleterious effects of environmental stresses by the stimulation of plant growth through the synthesis of IAA and by the secreted siderophores and/or organic acids that may improve also the metal bioavailability. Lopez *et al.* [37] and Tassi *et al.* [7] comment that other amendments like the plant growth regulators have been used to increase the heavy metal uptake as well as to maintain better plant growth during chelate-assisted phytoextraction technologies; regarding to these authors, in this study maybe the relationship between the IAA and siderophores released to the root medium culture were both involved in the root growth and response to Cd.

Israr *et al.* [38] found that in the presence of EDTA, the plant growth was significantly inhibited when compared to control plants and no significant negative effect on plant growth was observed in the presence of IAA or NAA alone; but when IAA or NAA was added in medium in the combination of EDTA; in the presence of EDTA at lower

concentrations and IAA or NAA up to 10 mM, plant growth was significantly inhibited, while at higher concentrations of IAA or NAA (100 mM), plant growth was as good as the control.

In this study the presence of EDTA and IAA quantified increase the growth of roots and the GPX root surface activity according to the results reported by Israr *et al.* [38], in this case the IAA concentration released to the medium was the adequate to enhance this kind of response in roots of *Sedum praealtum*. The correlation between the IAA and CAS released and quantified in the medium (Fig. 4) showed the important localization of the response of the rhizobacteria *Pseudomonas* sp. strain Sp7E employed as inoculant; particularly in the experiments MM + Rb and MM + Rb + EDTA + Cd (5 and 8, respectively) apart from the other experiments, the presence of rhizobacteria and the chemical chelator enhance the protective response of *Sedum praealtum* roots to Cd.

Some authors [39,40,41] consider that plant root activities could potentially increase metal/ metalloid solubility and may modify the redox potential, exudation of metal chelants and organic ligands (in particular low molecular organic acids and phytosiderophores) that compete with anionic species for binding sites. Mench and Martin [42] have demonstrate *in vitro* the mobilizing effect of root exudates and some authors in resin buffered nutrient solutions and soil experiments [43,44]. It is known that microorganisms can also increase the solubility and change speciation of metals/metalloids through the production of organic ligands via microbial decomposition of soil organic matter, and the exudation of metabolites and microbial siderophores can complex cationic metals or desorb anionic species by ligand exchange [11,45].

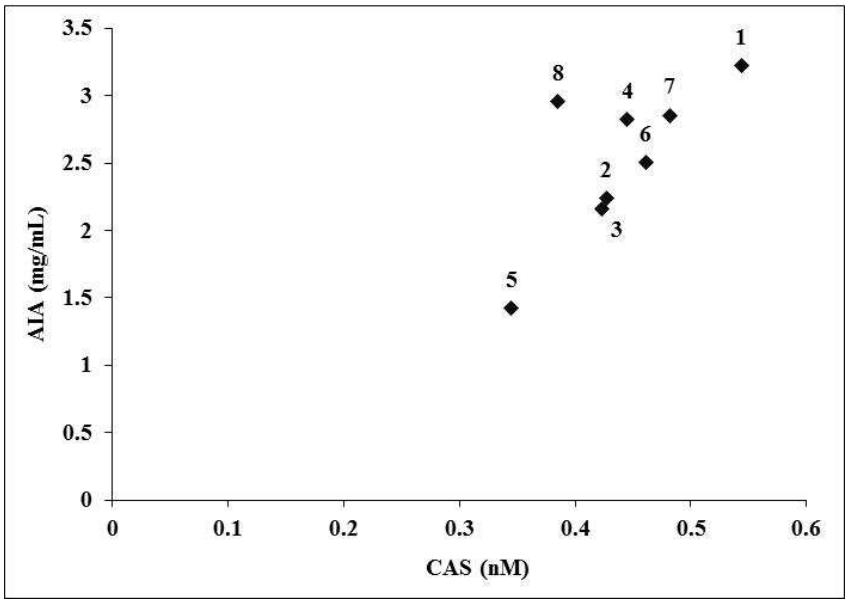

Figure 4: Linear regression curve showing the relationship between IAA production (mg/mL) and CAS (nM): 1) MM, 2) MM + EDTA, 3) MM + Cd, 4) MM + EDTA + Cd, 5) MM + Rb, 6) MM + Rb + EDTA, 7) MM + Rb + Cd and 8) MM + Rb + EDTA + Cd (r= 0.73).

CONCLUSION

Finally, the results obtained from this study do not only demonstrated that the relationship between the roots, microorganisms and chemical chelators are associated with the mechanisms that could be related with the phytoextraction of metals, particularly in the assisted phytoextraction employing rhizobacteria; these results also recommend to treat plants with the SPB like the employed for the stabilization and removal of metals in polluted soils.

Acknowledgements
Authors are grateful to the Research Projects SIP: 20121516 and SIP: 20131494 of the Secretaría de Investigación y Posgrado del I.P.N. and for the COFAA-IPN, EDI-IPN and SNI-CONACYT fellowships.

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