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In vitro SIMULTANEOUS ACCUMULATION OF MULTIPLE HEAVY METALS BY *Prosopis laevigata* SEEDLINGS CULTURES

ACUMULACIÓN SIMULTÁNEA DE MÚLTIPLES METALES PESADOS POR CULTIVOS *In vitro* DE PLÁNTULAS DE *Prosopis laevigata*

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Abstract

Experiments were conducted to investigate the capability of *Prosopis laevigata* to individually or simultaneously uptake four heavy metals (HM; Cr, Ni, Cd, and Pb). To this end, *P. laevigata* seedlings were cultured during 50 days on modified MS medium supplemented with 30 g L^{-1} of sucrose and added with 1, 2, 3 or 4 HM (50 mg L^{-1} of each HM). When the four HM were added simultaneously, the medium was supplemented with or without ethylenediaminetetracetic acid (EDTA). In the MS media contained only one HM, the seedlings tolerance to HM was as follows: Pb≥Cr>Ni≥Cd. The accumulation of HM from higher to lower concentration in shoots was Pb≥Ni>Cd≫Cr and in roots Cr≫Pb≫Ni>Cd. When the media contained more than one HM, the accumulation in shoots was the highest for Ni and the lowest for Pb, whether EDTA was added or not. EDTA supplementation increased 61, 39, 22, and 3 fold uptake of Cr, Ni, Pb, and Cd in roots, respectively.

Keywords: *Prosopis laevigata*, phytoremediation, *In vitro* culture, heavy metals, simultaneous accumulation.

Resumen

El presente trabajo fue conducido para investigar la capacidad de *Prosopis laevigata* en acumular simultáneamente hasta cuatro metales pesados (HM; Cr, Ni, Cd y Pb). Para ello, plántulas de *P. laevigata* fueron cultivadas durante 50 días en medio MS modificado, suplementado con 30 g L^{-1} de sacarosa y adicionado con 1, 2, 3 o 4 HM (50 mg L^{-1} de cada HM). Cuando los cuatro HM fueron adicionados simultáneamente, el medio fue suplementado con o sin ácido etilendiaminotetraacético (EDTA). En los tratamientos conteniendo solo un HM, la tolerancia de la plántulas a los HM fue la siguiente: Pb≥Cr>Ni≥Cd. La acumulación de HM, de la concentración más alta a la más baja, en la parte aérea fue Pb≥Ni>Cd≫Cr y en las raíces Cr≫Pb≫Ni>Cd. Cuando el medio contenía más de un HM, la concentración más alta determinada en la parte aérea, fue para el Ni y la más baja para el Pb, independientemente de la adición o no de EDTA. Mientras que el enriquecimiento del medio con EDTA, incrementó en raíz 61, 39, 22 y 3 veces la acumulación de Cr, Ni, Pb y Cd, respectivamente.

Palabras clave: *Prosopis laevigata*, fitorremediación, cultivo *In vitro*, metales pesados, acumulación simultánea.

1 Introduction

Heavy metals (HM) are present in soil as natural components or as a result of human activity. Toxic levels of some HM appear as a result of environmental pollution due to mining, heavy traffic, smelting, manufacturing, and agricultural wastes (Lagerwerff and Specht, 1970; Buchauer, 1973). Soils, especially those found in or near the metalliferous sites, metal smelters, and sewage treatment plants are highly contaminated with HM, including cadmium (Cd),

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chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), and zinc (Zn) (Boularbah *et al*., 2006). On the other hand, for healthy and vigorous growth, intact plants need to take up from the soil relatively large amounts of some inorganic elements (macronutrients) and small quantities of other elements (micronutrients or trace elements). Cr and Ni are metals that are included in the latter classification so that they have biological functions.

All metals are probably toxic if ingested in sufficient amounts by humans or plants. The toxicity of a metal is greatly influenced by environmental factors as well as the presence of other metals (Moutschen, 1985). Metal ions such as Cd, Cr, Cu, Pb, Zn, and Fe, are commonly detected in natural and industrial effluents and therefore priority is given to regulate these pollutants (Carreño-De León *et al*., 2017). Current clean up technologies for the degradation, removal or immobilization of contaminants involve either bulk removal or *in situ* remediation. In water waste treatment, have been novel technologies as the photocatalysis (Zarazúa-Aguilar *et al*., 2018), or HM removal by use of plant biomass (Corral-Escárcega *et al*., 2017; Carreño-De León *et al*., 2017). However, soil removal requires expensive equipment, disturbs the ecosystem and is not well accepted by the community. While the *in situ* technology depends on the cleaning cost per kg, the selectivity of the treatment agent, and the feasibility of recovering the chemicals used to treat the soil as well as the laden material from the surface and subsurface (Smith and Houthoofd, 1995; Barona *et al*., 2001). Furthermore, soil cannot be used immediately after treatment. In contrast, phytoremediation can be accomplished *in situ*, is relatively inexpensive, environmentally friendly, and the soil can be utilized immediately after treatment application (Ensley, 2000). The phytoremediation implicate the action of various processes that use the plants and the associated microorganisms to rhizosphere, for environmental pollution remediation (Peralta-Pérez and Volke-Sepulveda, 2012).

Although the low availability of metals might represent a disadvantage for phytoremediation, certain strategies such as the use of metal uptake enhancers can alleviate these limitations (van der Lelie *et al*., 2001; Sharma *et al*., 2003). Chelating agents such as ethylenediamine tetraacetic acid (EDTA) have been used in phytoremediation to enhance the extraction of heavy metals by plants from soil (Liphadzi and Kirkaham, 2006). Advantageously, phytoremediation does not generate sludge, and metals accumulated by plants can be recovered by metal extraction processes and incineration. Successful phytoremediation relies mainly on the identification of hyperaccumulator plants. Several studies have been conducted in order to determinate the capability of different plant species for heavy metal uptake. Unfortunately, the studies with plants are selective toward one metal and would not be effective at sites with multiple HM (Wei and Zhou, 2006; Kamnev and van der Lelie, 2000).

In previous works (Buendía-González *et al*., 2010a, b) it was found that *Prosopis laevigata* seedlings cultured on medium supplemented with Cr(VI), Cd(II), Pb(II), or Ni(II), uptake and accumulate large amounts of these HM in the shoots. The aim of this work was to investigate the effectiveness of *Prosopis laevigata* seedlings for the simultaneous phytoremediation of different HM (Cr, Cd, Pb, and Ni), and to assess the effects on the plant growth. In order to eliminate the effect of physicochemical soil factors, the experiment was carried out using an *In vitro* system.

2 Materials and methods

2.1 Plant material

Mature brown pods were collected from adult *Prosopis laevigata* trees growing naturally in the Mexican State of San Luis Potosi, during July 2016. The identification of voucher specimens was confirmed by Botanical taxonomist of the Universidad Nacional Autónoma de México, Campus Iztacala (UNAM-Iztacala). Botanical vouchers from this species were also collected and deposited in the Herbarium, Biology Department, UNAM-Iztacala since 2005. Mature seeds were isolated from the pods and were scarified mechanically. Under laminar flow hood, the seeds were disinfected by immersion in ethanol, followed by immersion in sodium hypochlorite (Buendía-González *et al*., 2010). Seeds were carefully rinsed with sterile deionized water five times and germinated aseptically in culture tubes (25 x 150 mm) containing 15 mL of modified Murashige & Skoog medium (MS), according to Buendía-González *et al*., (2010a) and 2.3 subsection, to ensure the complete HM bioavailability. Filter paper (100 x 15 mm, Whatman 1004917) segments were put inside the culture tubes for the purpose of supporting the seeds. One seed was aseptically placed into each culture tube and a batch of five culture tubes was evaluated for each treatment.

2.2 Tested heavy metals

The heavy metal (HM) sources used in this study were $Pb(NO_3)_2$, $NiCl_2{\cdot}6H_2O$, $K_2Cr_2O_7$ and $CdCl_2{\cdot}2½H_2O$ salts (Baker Analyzed, Phillipsburg, NJ). Stock solutions of each heavy metal salt were prepared at a concentration of 10 g L^{-1}.

2.3 Media preparation and culture conditions

The modified MS culture medium was prepared as follows: (1) NH_4NO_3 was included in all media of treatments without lead. The medium was supplemented with 19.30 mg L^{-1} of this salt to compensate the nitrogen included in the salt, lead source, in the all culture media (64.51 mg N L^{-1}); (2) Fe-EDTA was eliminated, and only the treatment containing the four HM was enriched or not with Fe-EDTA (0.5 μM); (3) 30 g L^{-1} sucrose; and (4) aliquots of the heavy metal salt stock solutions were added in order to achieve HM concentration of 50 mg L^{-1} for each heavy metal individually, combining 2, 3, or 4 HM. All media were adjusted to pH 5.8 with 1.0 N NaOH before autoclaving at 121 ºC for 18 min. All cultures were maintained at 25±2ºC under warm-white fluorescent light at an irradiance of 50 μmol m^{-2} s^{-1} and a 16 h (light) photoperiod.

2.4 Evaluation of plant growth and heavy metal resistance

One seed was inoculated aseptically in each culture tube. The percentage of seed germination was registered after 10 days of culture. Root emergence of the seeds was taken as an index for germination. The survival response was evaluated as the percentage of surviving seedlings from five planted seeds after 50 days of culture. For growth measurement purposes surviving seedlings were harvested after 50 days, rinsed with deionized water three times and then the root and shoot length of the seedlings were measured. Seedling length was measured from the main root apex to the main shoot apex. Furthermore, the seedlings were weighted and then were dried in a convection oven at 60 °C for 72 hours. Their weight was determined and reported on a dry basis (d.b.), and this value was considered the plant biomass. Also, the pH value was determined in the residual culture medium with a potentiometer (Conductronic, model pH120, Puebla, Mexico). Every treatment consisted of a batch of five culture tubes with one seedling. Means derived from three batches were used for statistical analysis (n = 3). The relative water content (RWC) was determined as follows (Henson *et al*., 1981):

$$RWC(\%) = \frac{\text{plant biomass (FW)} - \text{plant biomass (DW)}}{\text{plant biomass (FW)}} \times 100 \quad (1)$$

Growth measurements were used for evaluating the weigh to length ratio (WRL), which are defined as follows (Baker, 1987):

$$WLR(mgcm^{-1}) = \frac{\text{root biomass (DW; mg)}}{\text{root length (cm)}} \quad (2)$$

growth ratio (GR)

$$GR(\%) = \frac{\text{plant biomass with HM (DW)}}{\text{plant biomass without HM (DW)}} \times 100 \quad (3)$$

and the heavy metal tolerance index (TI) resistance

$$TI = \frac{\text{root length with HM (DW)}}{\text{root length without HM (DW)}} \quad (4)$$

All of which are indices of the plant growth and tolerance to metals.

2.5 Analysis of the content of heavy metals in plant biomass

Fifty-day old surviving seedlings were separated into root and aerial parts and were dried as indicated in the previous section. Dried tissue was weighed, powdered and digested with 5 mL of concentrated HNO_3 in a microwave oven (CEM Mars5, CEM Corporation, Mathews, North Carolina). Finally, the final sample volume was adjusted to 10 mL with deionized water and placed in HDPE flasks. The metals concentration was analyzed from the samples using a Varian Spectra AA-220 FS Atomic Absorption Spectrometer (Varian Australia Pty Ltd, Victoria, Australia). The concentration of Cd, Pb, Ni, and Cr were determined by calibration curves obtained using standards solutions of pure metal ions (Baker Analyzed, Phillipsburg, NJ). The standard calibration curves had correlation coefficients (R^2) of 0.9 or higher (1-30 mg L^{-1}). All glassware and apparatuses were washed with 0.1 N HNO_3 before use. The shoot and root heavy metal contents were determined for each seedling. Every treatment consisted of a batch of five seedlings. Means derived from three batches were used for statistical analysis (n = 3). Metal concentration measurements were used for evaluating the bioaccumulation factor (BF) and translocation

factor (TF), which are defined (Zhao *et al*., 2003; Niu *et al*., 2007) as:

$$BF = \frac{[HM]_{shoot}}{[HM]_{medium\ culture}} \quad (5)$$

and

$$TF = \frac{[HM]_{shoot}}{[HM]_{root}} \quad (6)$$

respectively.

2.6 *Statistical analysis*

All the experiments were carried out in a batch of five culture tubes with one seedling with three replicates. All the experimental data obtained in this work were subjected to a variance analysis (ANOVA) using the statistical software NCSS V.5 (Wireframe Graphics, Kaysville, UT). Comparisons of means were performed using Tukey's multiple range tests at the 5% level of probability for all experiments.

3 Results and discussion

3.1 *Seed germination and survival response*

P. laevigata seeds showed germination of 100%, irrespectively of the different HM combinations used in experiments. However, different HM treatments affected differently the survival of the seedlings after 50 days of culture (Table 1). Single and two HM treatments, tended to exhibit survivals of 100%, with the exception of the Cd and CrNi that showed significantly minor survivals (86.66%) with respect to control treatment. In contrast, the combination of 3 or 4 HM produced a significantly reduced survival percentage (ranging from 80 to 93.33 %) in all cases, the exception being treatment PbCrCd (100%). These results demonstrate that *P. laevigata* is a tolerant species, which grow on soil with HM concentrations that are toxic to most other plants (Peer *et al*., 2005).

On the other hand, the culture media containing the heavy metal combinations CrNi and PbCrNi showed significantly higher pH values than the control treatment, devoid of HM (Fig. 1a). In a study conducted by Luo *et al*., (2000), the pH value of rhizosphere of *Thlaspi caerulescens* soil solution increased due to the presence of Zn, indicating that the presence of the HM induced a slight modification of the pH values. The relative water content (RWC) is an important parameter indicative of plant tolerance to HM as it can be altered under HM stress. It is well-known that the embryo water potential needs to overpass a critical threshold for a seed to germinate (Kranner and Colville, 2010). Table 2 presents the percentage RWC for *P. laevigata* tissue seedlings after 50 days of culture in media supplemented with HM separately or combined. Single Ni and Cd significant reduced the total RWC with respect to the control treatment. The total RWC reduction had a major effect on shoots, despite that the water reduction in the roots was non-statistically significant ($p<0.05$). The different combinations of HM tended to significant decrease the total RWC, the exceptions being PbCr, PbCd, and CrCdPbNi. The larger reductions were of about five percentage points, with Cr and Ni involved in this situation. This is not surprising at all since Cr can reduce the hydric potential and the transpiration rate and also can increase the resistance to diffusive transport, impacting negatively on nutrients and water uptake by the plant (Chatterjee and Chatterjee, 2000). It has been also reported that Cd can inhibit seed imbibition, reducing seedling water content in *Dorycnium pentaphylum* (Lefèvre *et al*., 2009). In all cases, when HM combinations produced a reduction in total RWC, they also tended to produce a reduction in the RWC in shoots and roots.

3.2 *Shoot and root length*

The effects of HM combinations in shoot and root length of *P. laevigata* seedlings are shown in Figure 1b. In general terms, data show a reduction in shoot and root length as the number of HM increased. Besides, all the seedlings showed visible stress symptoms reflected as stunted growth, necrotic zones at the leaf margins and reddish brown roots. The effect was more pronounced in the root than in the shoot, maybe caused by limitations in the absorption of nutrients by the presence of HM. These results are congruent with the fact that the root is the organ that first enters in contact with the HM. Figure 1b shows that the significant highest reductions of shoot and root lengths were observed for Ni, Cd, PbNi, CdNi, PbCrNi, PbCdNi, PbCrNiCd and PbCrNiCd-EDTA with respect to control treatment (32-47% and 77-83%, respectively). Similar results were reported for *Apium graveolens* in *In vitro* cultures, showing important reductions in shoot length (about 65%) when exposed to different concentrations of Cr(III) in the range 0.1-1.0 mM (Scoccianti *et al*., 2006).

Table 1. Resistance indicators for *P. laevigata* seedlings after 50 days of culture in MS medium supplemented with different metals.

HM Treatment	Survival response (%)	Tolerance Index	Growth Ratio (%)	Weight-to-length Ratio (mg cm^{-1})
Control	100.00 ± 0.00a	1.00 ± 0.1a	100.00 ± 6.15a	5.41 ± 0.69cd
Pb	100.00 ± 0.00a	0.85 ± 0.3a	114.74 ± 16.56a	7.43 ± 3.36b
Cr	100.00 ± 0.00a	0.58 ± 0.12b	99.47 ± 2.08a	6.94 ± 1.11bc
Ni	100.00 ± 0.00a	0.23 ± 0.0c	78.87 ± 9.21b	5.13 ± 1.08cd
Cd	86.66 ± 11.55b	0.22 ± 0.0c	70.85 ± 5.02bc	4.91 ± 0.12d
PbCr	100.00 ± 0.00a	0.51 ± 0.08b	97.91 ± 5.2 a	7.86 ± 1.29ab
PbNi	100.00 ± 0.00a	0.16 ± 0.02d	80.49 ± 3.9 b	7.39 ± 1.37ab
PbCd	100.00 ± 0.00a	0.22 ± 0.02c	73.07 ± 1.50bc	7.36 ± 0.58b
CrNi	86.66 ± 11.55b	0.19 ± 0.02cd	81.87 ± 7.20b	8.09 ± 1.44a
CrCd	100.00 ± 0.00a	0.24 ± 0.01c	86.57 ± 12.83ab	8.10 ± 0.15a
CdNi	100.00 ± 0.00a	0.19 ± 0.01cd	70.35 ± 12.89bc	7.72 ± 0.61ab
PbCrNi	86.66 ± 11.55b	0.21 ± 0.01c	81.46 ± 3.15b	6.24 ± 0.48c
PbCrCd	100.00 ± 0.00a	0.17 ± 0.03cd	80.71 ± 3.95b	9.76 ± 1.84a
PbCdNi	93.33 ± 11.55ab	0.16 ± 0.04cd	68.67 ± 2.76c	6.84 ± 1.25bc
CrCdNi	80.00 ± 0.00bc	0.19 ± 0.04cd	73.67 ± 5.57bc	6.67 ± 1.26bc
PbCrNiCd	86.66 ± 11.55b	0.19 ± 0.03 cd	68.77 ± 6.25c	5.35 ± 0.74cd
PbCrNiCd-EDTA	80.00 ± 00.00bc	0.20 ± 0.02c	66.99 ± 8.42c	7.62 ± 3.63ab

Data correspond to the average of three repetitions by treatment ± SD. The values with the same letter in columns are not statistically different (Tukey, 0.05).

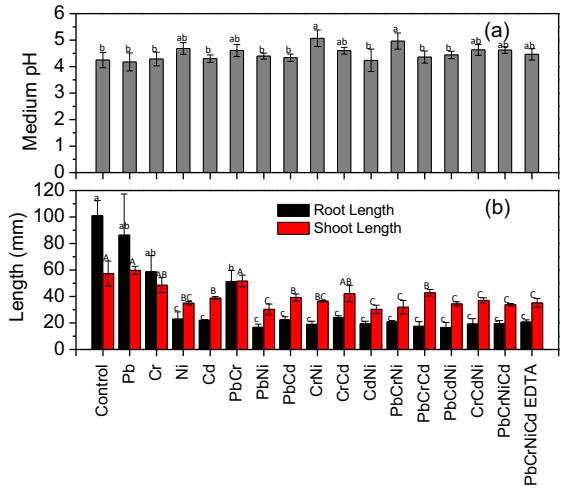

Fig. 1. (a) Medium pH and (b) root and shoot lengths obtained for the different combinations of heavy metals used in the culture medium.

Studies with *Hordeum vulgare* under hydroponic conditions with 0.1 mM of Cr(VI) found shoot reductions as high as 75% (Skeffington *et al*., 1976). It has been suggested that the transport of HM from root to shoot tissue drastically impacts the cellular metabolism, contributing to the reduction of seedling size (Santiago-Cruz *et al*., 2014). Into treatments containing 4 HM, with or without supplement of EDTA, the root and shoot length were non-significantly affected, indicative that *P. laevigata* seedlings were not affected by the chelating effects of EDTA, added for the purpose of enhancing heavy metal uptake.

3.3 Weight-to-length (WTL) ratio, growth ratio (GR) and tolerance index (TI)

WTL and TI are parameters used to characterize the effect of heavy metal stress in root tissue (Baker, 1987). *P. laevigata* seedlings exhibited lower tolerance index (TI) values for Ni, Cr, Cd and all combinations for 2, 3 and 4 HM treated seedlings than for the control and Pb treated seedlings (Table 1). The GR evaluates the effect of HM stress on the whole plant. GR values lower than 100.0 indicate a net decrease in biomass and suggest that the seedlings are stressed, whereas GR values equal to 100.0 indicate no difference relative to control treatment. Also, GR values greater than 100.0 indicate a net increase in biomass and suggest that plants express a growth dilution effect in tolerating HM stress (Audet and Charest, 2007), a process in which the concentration of any compound decreases subsequent to its distribution in the growing biomass (Newman and Unger, 2003). The GR of *P. laevigata* was not affected by Pb, Cr and PbCr treatments. However, the GR decreased as the number of HM increased.

Table 2. Percentage of relative water content for *P. laevigata* tissue seedlings after 50 days of culture in media enriched with different metals.

HM treatment	RWC_{Shoots} (%)	RWC_{Roots} (%)	RWC_{Total} (%)
Control (without HM)	84.02 ± 0.54[a]	85.36 ±0.37[a]	84.35 ± 0.47[a]
Pb	83.65 ± 0.35[a]	85.16 ±0.51[a]	84.02 ± 0.38[a]
Ni	78.81 ± 1.45[c]	84.25 ±0.25[a]	79.23 ± 1.34[c]
Cd	78.82 ± 0.69[c]	86.55 ±0.15[a]	79.57 ± 0.61[c]
Cr	85.24 ± 0.17[a]	84.75 ±0.08[a]	85.16 ± 0.14[a]
PbCr	84.61 ± 0.79[a]	83.83 ±0.33[b]	84.48 ± 0.62[a]
PbNi	82.11 ± 0.72[b]	78.91 ±1.22[b]	81.93 ± 0.71[b]
PbCd	83.69 ± 0.11[a]	83.40 ±0.79[b]	83.66 ± 0.13[a]
CrNi	79.07 ± 0.89[c]	75.77 ±1.98[c]	78.85 ± 0.96[c]
CrCd	79.47 ± 1.37[c]	80.76 ±0.50[b]	79.61 ± 1.17[c]
CdNi	80.95 ± 0.56[c]	81.90 ±0.91[b]	81.04 ± 0.43[b]
PbCrNi	82.59 ± 0.17[b]	85.09 ±0.72[a]	82.79 ± 0.20[b]
PbCrCd	81.10 ± 0.54[b]	80.65 ±0.83[b]	81.06 ± 0.50[b]
PbCdNi	79.27 ± 0.38[c]	75.72 ±0.82[c]	79.06 ± 0.38[c]
CrCdNi	82.33 ± 0.48[b]	81.69 ±0.12[b]	82.28 ± 0.46[b]
CrCdPbNi	83.80 ± 0.64[a]	74.03 ±0.57[c]	83.37 ± 0.62[a]
CrCdPbNi-EDTA	81.74 ± 0.33[b]	84.05 ± 1.96[a]	81.96 ± 0.49[b]

The data correspond to the average of three repetitions by treatment ± SD. The values with the same letter in columns are not statistically different (Tukey, 0.05).

The highest GR reduction occurred for the PbCdNi (31.33%), PbCrNiCd (31.22%), and PbCrNiCd-EDTA (33.00%) treatments. Finally, except for Cd, the WTL ratio increased when the medium contained single or combinations of HM. This in turn indicates an increasing production of roots seedling biomass. This effect is known as hormesis, which occurs when low doses cause an increase in growth, while high doses inhibit growth (Poschenrieder *et al*., 2013). The dose taken up by a plant is largely dependent of the availability of the HM in the external medium, as well on the capacity of the plant tissues to mobilize and absorb the elements through the roots (Poschenrieder *et al*., 2013). Probably due to the bioavailability of HM, the *P. laevigata* seedlings showed growth stimulation at low doses, despite the combination of several metals. Besides, the addition of EDTA led to important increase of the WTL, backing up the idea that bioavailability at low doses enhances the production of root tissue.

3.4 Heavy metal uptake

Figures 2a and 2b presents the results of single heavy metal accumulation in shoots and roots. All values are expressed as mg kg^{-1} of dry tissue for total medium concentrations of 50 mg L^{-1}. The order of HM uptake for shoots was Pb≥Ni>Cd≫Cr. However, this order is not maintained for roots, for which the order was Cr≫Pb≫Ni>Cd. Plants can adopt different strategies to deal with the presence of metals in the medium. The tolerance response of plants to heavy metal stress is given by accumulation and exclusion mechanisms. In the accumulation mechanisms, the plants accumulate the metal in all tissues by triggering highly specialized physiological processes of detoxification (Baker, 1987). In contrast, in the exclusion mechanism the plant restricts the transport of HM to the shoots. Pb, Cd, and Ni were preponderantly accumulated in the shoots, indicating a response by accumulation mechanism. On the other hand, Cr was concentrated mainly in the root, reflecting a response mechanism by exclusion. These results are in line with previous reports showing that *P. laevigata* cultured in media containing 2.0 mM of Cr(VI) accumulated 2364 mg kg^{-1} in shoot and 5035 mg kg^{-1} in root (Buendía-González *et al*., 2010a).

The results in Figures 2c and 2d, show that the response of the *P. laevigata* seedlings depended on heavy metal combination. The uptake results for combinations of two and three HM were puzzling. Figures 2c and 2d, show that the combination of two HM affected strongly the HM distribution in shoots and roots. For instance, the combination PbCr promoted the Pb absorption in roots and the Cr

absorption in shoots. The effect was inverted for the combination CrNi where Cr was preponderantly accumulated in roots. On the other hand the combination CdNi seems to inhibit the absorption of Pb in both shoots and roots. In contrast, the combination of CrNi had a synergistic effect as reflected by an even adsorption of both metals in shoots and roots. Thus, the accumulation and exclusion mechanisms depended on the specific combination of HM. The combinations PbNi, PbCr, PbCd and CdNi showed a dominant absorption in shoots, reflecting an accumulation mechanism. However, the uptake for the combinations CrNi and CrCd exhibited strong absorption in roots, indicating exclusion mechanism. The combination of three HM was even more complex (Fig. 2e and 2f). For instance, the PbCrNi combination increased the uptake of Cr and Ni, but not of Pb. Cd was preponderantly accumulated in roots in the presence of Pb and Cr, indicating mixed defense mechanisms, with exclusion for Pb and Cr and accumulation for Cd. Overall, the results in Figure 2 showed that the uptake ratio and the plant defense mechanisms depend strongly on the heavy metal combinations, finding synergetic and inhibition effects, as well as accumulation and exclusion mechanisms. In fact, Pb and Cd enter the cell through the uptake and transport pathways of Zn and Fe, and Ni only through the Fe pathways. While the chromium, enters the cell through the uptake and transport pathway of Mn. However, Cd, Cr, and Ni share the formation of element-organic complexes with plant thiols (Peer *et al*, 2005).

Figures 3a and 3b present the results for the combined four HM with and without EDTA addition. The accumulation of HM in shoots was not affected by the presence of EDTA. However, EDTA promoted greatly the HM adsorption in roots (3, 22, 39, and 61 times for Cd, Pb, Ni, and Cr, respectively). In a study conducted by Liphadzy and Kirkham (2006), showed similar results, in sunflower plants grown on the composted biosolids with 1.0 g EDTA kg^{-1}, was increased the accumulation of Cd and Ni in the roots and reduced the concentrations of Ni, Cd, and Pb in the leaves and stems of plants. The transport from roots to shoots was not increased by EDTA, caused probably by the establishment of the complex formed by EDTA and the HM.

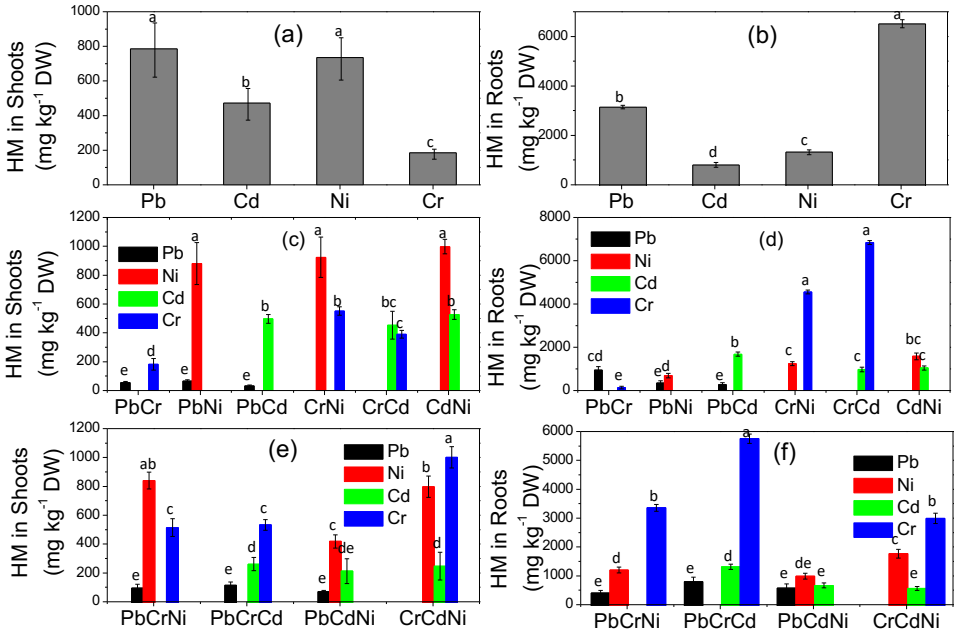

Fig. 2. (a) and (b) Heavy metal contents in shoots and roots for culture medium with a single heavy metal. (c) and (d) Heavy metal contents in shoots and roots for culture medium with double combinations of heavy metals. (e) and (f) Heavy metal contents in shoots and roots for culture medium with a triple combinations of heavy metals.

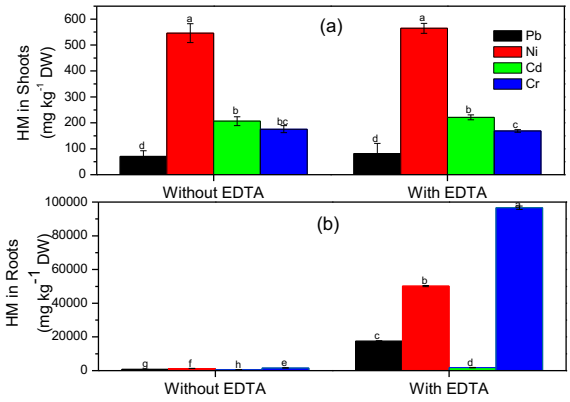

Fig. 3. Effect of EDTA in the heavy metal contents in (a) shoots and (b) roots.

3.5 *Bioaccumulation and translocation factors*

The bioaccumulation factor (BF) is a parameter reflecting the phytoextraction and hyper-accumulation efficiency, providing valuable insights regarding the phytoremediation potential of a given plant species. It determines the relation between the concentration in shoot tissue and the concentration in medium culture. The BF was greater than 1.0 for all the single HM (Fig. 4a) with the following order Pb>Ni>Cd>Cr. Hyperaccumulator plant species showing a BF≫1 can reach HM concentrations in shoot tissue of 10-100 times higher than the concentrations considered as normal (Chaney *et al*. 2000). Excepting Cr, the other three HM exhibited BF≫1, suggesting that *P. laevigata* is a normal hyper-accumulator with respect to these metals. It can be noticed that the BF was of the order of 1.0 for Cr. *In vitro* cultures of other plant species showing high BF values are the following: *Medicago sativa* (BF of 6.9 at 40 mg L^{-1} (~0.7 mM) of Cr(VI); Peralta *et al*., 2001), and *Amaranthus dubius* (BF of 2.6 at 25 ppm (~0.48 mM) and of 4.6 at 100 ppm (~1.92 mM; Mellen, 2008).

The BF for combinations of HM is shown in Figures 4c and 4e. It is interesting to note the synergistic effect exhibited by some cases. For instance, single Ni presented a BF of about 14.0 (Fig. 4a). However, when Ni was combined with Cd and Cr, BF increased to about 20.0. Also, the BF of Cr was greatly increased from about 2.5 to 10.5 and 7.5 when this metal was combined with Ni and Cd, respectively. However, Pb exhibited the opposite behavior as the BF of the single metal was about 15.0 and decreased to about 2.0 when combined with Cr.

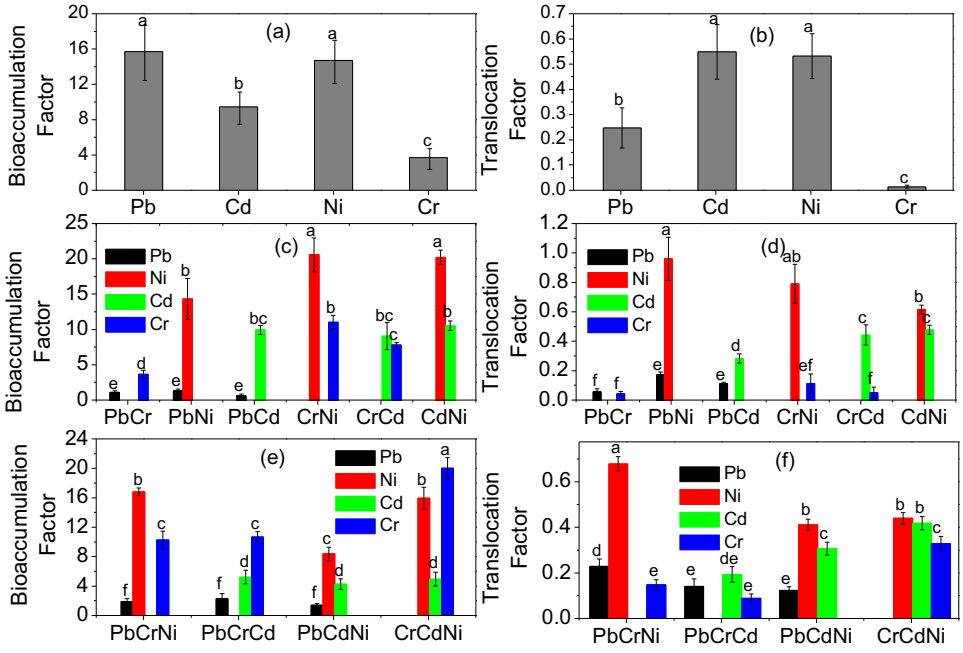

Fig. 4. (a) and (b) Bioaccumulation factor and translocation factor for culture medium with a single heavy metal. (c) and (d) Translocation factor for culture medium with double combinations of heavy metals. (e) and (f) Bioaccumulation factor and translocation factor for culture medium with triple combinations of heavy metals.

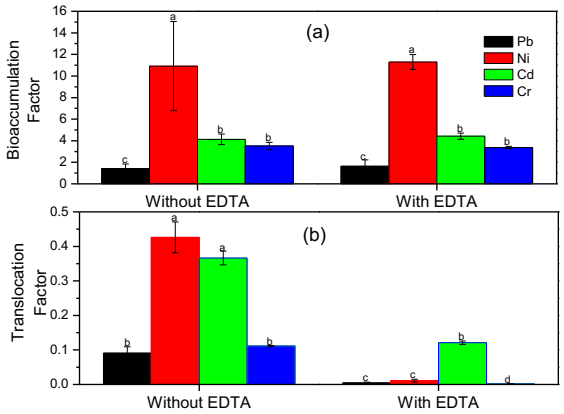

Fig. 5. Effect of the EDTA addition in (a) bioaccumulation factor, and (b) translocation factor.

Triple combinations of HM also displayed interesting effects (Fig. 4e). The most salient effect was presented by Cr as its BF was significantly higher (3-5 times) relative to the value for the single metal case. The BF of Ni was also increased after triple combinations with other HM. The addition of EDTA has a weak effect in the BF (Fig. 5a), indicating that this compound has not important effects in the phytoremediation potential of *P. laevigata*.

Another parameter used for measuring the phytoremediation efficiency is the TF, representing the HM transport from root to shoot. For all cases, single metals and combinations of them, presented TF values smaller than 1.0 (Fig. 4b to 4f). This means that the transport of metals from root to shoot was limited for *P. laevigata* seedlings. *P. laevigata* seedlings showed a TF of 0.52 when the seedlings were treated with 2.0 mM Cr(VI) (Buendía-González *et al*., 2010a). However, Ni retained the value of the TF after combinations with other metals, but Cd exhibited a huge uptake decrease when combined with other HM, mainly with Pb. Finally, the TF was greatly affected by EDTA (Fig. 5b), indicating that this chelating agent does not participate in translocation mechanisms of metals from root to shoots. The Pb showed the largest decrease of the TF relative to the case of single HM, with a decrease as high as ten times.

Conclusions

P. laevigata was able to germinate and grow in different culture media containing combinations of HM (Pb, Cd, Ni, and Cr). However, the survival response was decreased when two or more HM were used in the culture medium. *P. laevigata* exhibited different results with respect to the uptake of combinations of HM. The accumulation of HM, was present in both tissues, shoots and roots, being the predominant accumulation in the root, due to the mechanism of exclusion. *P. laevigata* can be used as a model plant to study the detoxifying mechanisms triggered under the presence of several HM, which in turn contribute to a better understanding of the process of heavy metal hyperaccumulation in plants. In fact, *P. laevigata* seedlings showed high bioaccumulation factors ($\gg$1) for all HM, indicative of their potential to grow and colonize contaminated soils containing different HM, representing an excellent candidate for phytoremediation purposes.

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