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ESTABLISHMENT OF CELL SUSPENSION CULTURES OF *Prosopis laevigata* (HUMB. & BONPL. EX WILLD) M.C. JOHNST TO DETERMINE THE EFFECT OF ZINC ON THE UPTAKE AND ACCUMULATION OF LEAD

ESTABLECIMIENTO DE CULTIVOS DE CÉLULAS EN SUSPENSIÓN DE *Prosopis laevigata* (HUMB. & BONPL. EX WILLD) M.C. JOHNST PARA DETERMINAR EL EFECTO DEL ZINC EN LA ABSORCIÓN Y ACUMULACIÓN DE PLOMO

A. Maldonado-Magaña[1], J. Orozco-Villafuerte[2], L. Buendía-González[3*], M.E. Estrada-Zúñiga[3], A. Bernabé-Antonio[4] and F. Cruz-Sosa[1]

[1]*Departamento de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa. Av. San Rafael Atlixco No. 186, Col. Vicentina C.P. 09340, México D.F., México.*
[2]*Facultad de Química, Universidad Autónoma del Estado de México. Paseo Colón esq. Paseo Tollocan s/n, Col. Residencial Colón, C.P. 50120 Toluca, Estado de México, México.*
[3]*Facultad de Ciencias, Universidad Autónoma del Estado de México. Campus El Cerrillo, Piedras Blancas, Carretera Toluca-Ixtlahuaca km. 15.5, C.P. 50200, Toluca, Estado de México.*
[4]*Centro de Investigaciones Químicas, Universidad Autónoma del Estado de Morelos. Av. Universidad 1001 Chamilpa 62210, Cuernavaca, Morelos, México.*

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Abstract

In vitro studies indicate that *Prosopis laevigata* can be considered a potential hyperaccumulator of lead. Likewise, lead uptake has been related to protein transporters for zinc. In this work presents a protocol for the establishment of cell suspension culture to determine the effect of zinc on the uptake and accumulation of lead. A bioassay with Pb^{2+}/Zn^{2+} (0.0, 0.5, and 1.0 mM) was carried out on cell suspension cultures derived from callus induced in half-strength Murashige and Skoog (MS) medium added with 6.8 μM 2,4-dichlorophenoxyacetic acid (2,4-D) with 4.5 μM kinetin (KIN). Cells showed significant tolerance to growth (GR>60%) at all concentrations and combinations of Pb and Zn (0.0, 0.5 and 1.0 mM). When the Pb with or without Zn were added to the culture medium, the cells showed the highest accumulation efficiency for non-essential (lead) metal over essential (zinc) metal (*BF* values for Pb $\gg$ *BF* values for Zn; 2-33 times). Scanning electron micrographs evidenced the accumulation of Pb in the cells walls. These results provide insights about the tolerance and accumulation mechanisms of Pb occurring in *P. laevigata*.

Keywords: *Prosopis laevigata*, lead, zinc, competitive transport, bioaccumulation, phytoremediation.

Resumen

Estudios de cultivos *in vitro*, indican que *Prosopis laevigata* puede ser considerada como una especie potencialmente hiperacumuladora de plomo. En este trabajo se presenta un protocolo para el establecimiento de un cultivo de células en suspensión de *P. laevigata*, para determinar el efecto del Zn^{2+} sobre la absorción y acumulación de Pb^{2+}. Se realizó un bioensayo con Pb^{2+}/Zn^{2+} (0.0, 0.5, and 1.0 mM) en cultivos de células en suspensión establecidos a partir de callos inducidos en cotiledones en medio Murashige & Skoog (MS) a la mitad de su concentración y suplementado con 2,4-D (6.8 μM) y KIN (4.5 μM). Las células presentaron un crecimiento relativo del 63-98% en todas las concentraciones y combinaciones de Pb y Zn (0.0, 0.5, 1.0 mM). Respecto a la acumulación, cuando el Pb fue adicionado al medio con o sin Zn, las células mostraron mayor eficiencia de acumulación para el metal no esencial (Pb) sobre el metal esencial (Zn) (valores de *BF* para Pb $\gg$ valores de *BF* para zinc; 2-33 veces). Imágenes de las células observadas con microscopía electrónica de barrido evidencian la acumulación del plomo en la pared celular. Estos resultados proporcionan información sobre los mecanismos de tolerancia y acumulación de plomo que se llevan a cabo en *Prosopis laevigata*.

Palabras clave: *Prosopis laevigata*, plomo, zinc, transporte competitivo, bioacumulación, fitorremediación.

[*]*Corresponding author. E-mail*: lety_sax@yahoo.com.mx
Tel. +52-722-296-5556, Fax+52-722-296-5554

1 Introduction

Plants have the ability to absorb soluble mineral from soils, including metals such as iron, copper, manganese, zinc, among others, which play a crucial role in metabolic processes and exert a significant impact on vegetal nutrition, growth and development. The uptake of the essential minerals into roots takes place by two main pathways: (i) Entry through cytoplasm of endodermic cells by selectively using a protein transport system which is localized in the plasma membrane and comprehended by channels, carriers and co-transporters (symplastic pathway), or (ii) across the exterior of the plasma membrane by diffusion (apoplastic pathway) (Peer *et al*., 2006). Besides, mineral uptake involves accumulation of nutrient molecules to higher concentrations than in the surrounding medium, so the primary mechanisms for uptake imply the use of protein transport system. However, in the case of metals, due to the involvement of a nonselective cation uptake mechanism, plants are also able to absorb metals that are non-essential minerals, which can be toxic such as the heavy metal (HM) lead. Competition for transport sites would favor the uptake of this metal at higher concentrations, at the expense of those whose supply is limiting, thereby exacerbating nutrient deficiencies (Reid and Hayes, 2003).

The lead uptake has been associated to transporters implied in zinc and iron entry (Peer *et al*., 2006). Once lead has been absorbed, this can become toxic causing the inhibition of several metabolic activities in plant cells, e.g., the biosynthesis of nitrogenous compounds and of photosynthetic pigments, carbohydrate metabolism, water absorption, inter alia (Sharma *et al*., 1995; Azooz *et al*., 2011). In contrast, there are some species showing few or none phytotoxicity symptoms despite high heavy metal exposure, which are capable to tolerate and grow successfully, as well to accumulate high metal concentrations in shoots (Sarma, 2011). This seems to be the case of *Prosopis laevigata*, a leguminous tree endemic of Mexico capable to grow in mining zones, which has been identified as a potential hyperaccumulator species for chromium, cadmium, nickel, and lead, in *in vitro* experiments (Buendía-González *et al*., 2010a, 2010b). Due to serious contamination problems, studies on the mechanisms of HM transport are being carried out in hope of developing biotechnological applications to remediate HM contaminated soils. Plant tissue culture is considered an important tool for fundamental studies that provide information about the plant-contaminant relationships, help to predict plant responses to environmental contaminants, and improve the design of plants with enhanced characteristics for phytoremediation. Callus, cell suspensions, hairy roots, and shoot multiplication cultures are currently used as model systems for understanding the uptake, localization, metabolism, toxicity, and tolerance of pollutants under aseptic conditions. *in vitro* cultures can be propagated indefinitely, are available all year around, and their use enables a great reduction in the amount of whole plant material required for research (Couselo *et al*., 2012). To our knowledge, there are no studies on the accumulation or tolerance of heavy metals in *Prosopis laevigata* cell suspension cultures.

The aim of this work was establishing *Prosopis laevigata* cell suspension culture to determine the effect of Zn^{2+} on the uptake and accumulation of lead in cell suspension cultures.

2 Materials and methods

2.1 Plant material

Seeds of *P. laevigata* (Hum. and Bonpl. ex Willd M.C. Johnston) were isolated from mature pods which were collected from adult mesquite trees. Seeds were scarified mechanically under laminar flow hood, disinfected by immersing in ethanol, followed by immersion in sodium hypochlorite (Buendía-González *et al*., 2007). Seeds were carefully rinsed five times with sterile distilled water, and germinated aseptically in culture tubes (25 x 150 mm) containing 15 mL of half-strength Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) added with 3% (w/v) sucrose. At this point it must be stated that half-strength MS contains basal amounts of Zn^{2+} (0.015 mM), that must be distinguished from the Zn^{2+} that are further added to MS for bioassay described in section 2.4. Once all the components of medium culture were added, the pH value was adjusted to 5.8 with 1N NaOH or 1N HCl. Phytagel at 0.2% (w/v) was used as a gelling agent. Finally, medium culture was sterilized by autoclaving at 121°C for 18 min. Seedlings (15 days old) were used as source of explants (cotyledon, hypocotyl, and roots). All cultures were maintained at 25±2°C under warm-white fluorescent light at an irradiance of 50 μMol m^{-2} s^{-1} and 16 h (light)/8 h (dark) photoperiod.

2.2 *Callus induction*

Explants (1.5 cm in length) of hypocotyl, cotyledon or roots from *P. laevigata* seedlings were used to callus induction. Explants were transferred to each culture tube (5 per treatment, 2 replicates) containing half-strength MS medium and supplemented with different combinations and concentrations of plant growth regulators (PGRs): 2,4-dichlorophenoxyacetic acid (2,4-D; 0.0 and 6.8 μM) combined with 6-benzyladenine (BA; 0.0, 2.3, 4.5, and 6.8 μM) or with kinetin (KIN; 0.0, 2.3, 4.5, and 6.8 μM). All cultures were added with 3% (w/v) sucrose and solidified with 2% (w/v) phytagel. Treatments were coded as D_xB_y or D_xK_y, where D_x stands for the 2,4-D at concentration x, and B_y or K_y for *BA* or *KIN*, respectively, at concentration y (Table 1). Data of percentages of callus induction were recorded at 30 days of culture. These were determined as a ratio of the number of the explants showing callus with respect to total tested explants in each treatment. Callus induced from cotyledons with 6.8 μM 2,4-D and 4.5 μM KIN ($D_{6.8}K_{4.5}$) was selected for further experiments as it showed high percentage of callus induction, better growth and friability. Callus derived from $D_{6.8}K_{4.5}$ treatment were subcultured continuously to propagate biomass and perform further experiments in cell suspension cultures. A subculture cycle consisted of transferring callus to fresh medium culture (same formulation), allowing growing for 30 days.

2.3 *Establishment of cell suspension cultures and growth kinetics*

Friable callus (3 g FW) of 30 days-old induced in cotyledon explants from $D_{6.8}K_{4.5}$ treatment was transferred to 125 mL Erlenmeyer flasks containing 25 mL of callus induction medium (phytagel-free). All cultures were incubated in an orbital shaker at 110 rpm at 25 ± 2°C under warm-white fluorescent light at an irradiance of 30 μMol m^{-2} s^{-1} and 16 h (light) photoperiod. Cultures were subcultured every 15 days for 3 months in liquid MS culture medium with $D_{6.8}K_{4.5}$ formulation, and supplemented with 5% (v/v) coconut water, and each cycle was passed through a cell dissociation sieve (60 mesh screen; Sigma Chemical Co., USA) in order to get homogeneous cultures. The resulting cultures were transferred (6 % (w/v) of inoculum) into Erlenmeyer flasks of 500 mL containing 100 mL of MS liquid culture medium with $D_{6.8}K_{4.5}$ formulation, and supplemented with 5% (v/v) coconut water and subcultured every 15 days for 6 months. Suspension cell cultures of the latter subcultured (10 days-old) were used to perform growth kinetics and also for heavy metal bioassays. Erlenmeyer flasks of 125 mL containing 25 mL of MS liquid medium culture with $D_{6.8}K_{4.5}$ formulation and 5% (v/v) coconut water were inoculated with 6% (w/v) cell biomass. Harvesting of cells was made during 21 days at intervals of two or three days. At every interval, the cells were separated from liquid medium culture by filtering, and the recovered cells were weighed (fresh weight, FW), dried into an oven at 70°C for 24 h and weighed (dry weight, DW). The specific cell growth rate (μ), defined as the increase in cell mass per unit time, was calculated by plotting the cell growth data in the form of natural logarithm versus time. This yields a straight line over the exponential phase growth. The slope of the linear part of the plot corresponds to specific cell growth rate and is given in 1 per units of time (Trejo-Espino *et al*., 2011)). The time required for biomass to double (doubling time, t_d) was computed from the μ experimental data. All experiments were done in duplicate with three replicates each.

2.4 *Bioassay of Pb^{2+}/Zn^{2+}*

In order to evaluate the effect of zinc in the accumulation of Pb, the cell suspension cultures were grown in liquid MS medium with $D_{6.8}K_{4.5}$ formulation, and supplemented with 5% (v/v) coconut water. Cultures were supplied with Pb (0.0, 0.5 and 1.0 mM) combined with the essential ion Zn^{2+} (0.0, 0.5 and 1.0 mM). These treatments were coded as the Pb_xZn_y, where Pb_x stands for the lead at concentration x and Zny for essential ion (Zn^{2+}) at concentration y. The $Pb(NO_3)_2$ and $ZnSO_4 \cdot 7H_2O$ (Baker Analyzed, Phillipsburg NJ) salts were used as the source of the corresponding metal, and thus the appropriate stock solution (20 mg mL^{-1}) was prepared with deionized water. The respective aliquots of metal stock solutions were added to achieve the desired concentrations. Every experiment was done in duplicate with three replicates. Cultures were incubated for 10 days and were simultaneously harvested. Cell biomass was filtered, washed (three times with deionized water to remove extracellular adsorbed metals) and then was dried at 70°C for 24 h. The *DW* measurements were used to determine the growth ratio (*GR*) defined as (Baker, 1987):

$$GR = \frac{\text{Plant biomass with Pb and/or Zn (DW)}}{\text{Plant biomass without Pb (control; DW)}} \times 100 \quad (1)$$

Moreover, the harvested biomass (*DW*) was used to determine the metal content.

2.5 Analysis of Pb and Zn content in biomass

Dry biomass was weighed (100 mg), powdered and digested with concentrated HNO_3 (5 mL) and deionized water (4 mL) in a microwave (MARS-Xpress, CEM corporation, Mathews, North Carolina) and the final sample volume was adjusted to 10 mL with deionized water, filtered (0.45 μM, GN-6, Metricell) and placed in high density polyethylene (HDPE) flasks. The metal concentration was analyzed from digested samples using a Varian Spectra AA-220 FS Atomic Absorption Spectrometer (Varian, Australia). The concentration of metals (Pb and Zn) was 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 0.99 or better. All glassware and apparatus were washed with 0.1 N HNO_3 before their use. Metal concentration measurements were used for evaluating the bioconcentration factor (*BF*); which estimates the efficiency of a plant in taking up heavy metals from medium culture and is defined as the ratio of metal concentration in plant tissues ([Pb or Zn]$_{biomass}$) to *HM* concentration in medium ([Pb or Zn]$_{\text{medium culture}}$) (Audet and Charest, 2007):

$$BF = \frac{[\text{Pb or Zn}]_{\text{biomass}}}{[\text{Pb or Zn}]_{\text{medium culture}}} \quad (2)$$

2.6 Surface cellular wall analysis by scanning electron microscopy (SEM-EDS)

Biomass samples from the control or Pb-treated cell suspension cultures were analyzed using a *SEM* (JEOL JSM-5900 LV, Oxford, Japan), equipped with energy-dispersive X-rays spectroscopy (EDS 7274, England). Samples of cell suspension cultures (1 mL) were removed from flasks before filtering procedure and were centrifuged at 1000 rpm for 5 min. The supernatant was discarded and cells were fixed by adding a solution of glutaraldehyde 2% (v/v) for 24 h at 4°C. Samples were carefully rinsed with a buffer solution (phosphate 2 mM, pH 7.2) to remove the fixer, followed by immersion in 1% (w/v) osmium tetraoxide solution for 2 h, dehydrated through a graded ethanol series (30-100%, v/v) sequentially for 20 min at each step. All dehydrated samples were desiccated at critic point with carbon dioxide (CO_2) (Samdri-795, USA). The samples were covered with a layer of carbon and gold (Bozzola and Russell, 1999) with a Denton Vacuum Desk III equipment (Denton, USA). Lastly, the samples were observed, analyzed and photographed in SEM-EDS (2000x).

2.7 Statistical analysis

The results were subjected to variance analysis and Tukey's multiple range test ($P \leq 0.05$) with NCSS version five statistical software (Wireframe Graphics, Kaysville, UT) in order to determine significant differences.

3 Results and discussion

3.1 Callus induction

All the immature *P. laevigata* explants tested showed the callus development after 10 days of culture. The phenotypic characteristics of callus corresponded to green or beige color and friable or compact morphology. The percentages of callus induction from *P. laevigata* were significantly affected by the type of explant and PGRs ($P \leq 0.05$) (Table 1). The cotyledon and the auxin 2,4-D were the prime factors promoting the callus response. Also, the combination of 2,4-D with cytokinin (BA or KIN) increased the percentages of callus induction compared to the treatment consisting of only 2,4-D. The highest percentage (100%) of callus induction occurred in cotyledon explants treated with 6.8 μM 2,4-D combined with 4.5 μM of BA or 4.5 μM KIN ($D_{6.8}B_{4.5}$ or $D_{6.8}K_{4.5}$, respectively) (Table 1). However, the cotyledons from the treatment containing 6.8 μM 2,4-D with 4.5 μM KIN produced a beige callus ($D_{6.8}K_{4.5}$), and showed better growth and friability. Therefore, the cotyledon cultures from $D_{6.8}K_{4.5}$ were selected for propagating the cellular line and to establish the cell suspension cultures. In contrast, in a study conducted by Trejo-Espino *et al*. (2011) reported low *P. laevigata* callus induction ($\leq$ 18.7%) from cotyledons when treated with 5.0 μM 2,4-D without/with *KIN* or *BA* (5.0 μM). Nevertheless, these same authors achieved high callus induction (100%) from hypocotyls treated with 5.0 μM 2,4,5-T plus 5.0 μM KIN or 5.0 μM BA. The differences found between this work and that by Trejo-Espino *et al*. (2011) can probably be attributed to genetic factors and the age of explants, as in the former

Table 1 Callus induction percentage in *Prosopis laevigata* explants treated with different Plant Growth Regulators (PGRs) after 30 days of culture.

Treatments	Callus induction in explants (%)		
	Cotyledon	Hypocotyl	Root
D_0 B_0K_0 (Control)	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
$D_0B_{2.3}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
$D_0B_{4.5}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
$D_0B_{6.8}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
$D_0K_{2.3}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
$D_0K_{4.5}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
$D_0K_{6.8}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
$D_{6.8}$ B_0K_0	80.63 ± 0.0^{c}	40.16 ± 9.7^{bc}	66.67 ± 14.8^{d}
$D_{6.8}B_{2.3}$	93.33 ± 11.5^{de}	32.48 ± 1.5^{b}	45.67 ± 3.1^{c}
$D_{6.8}B_{4.5}$	100.00 ± 0.0^{e}	45.24 ± 4.1^{c}	28.00 ± 4.0^{b}
$D_{6.8}B_{6.8}$	81.67 ± 3.5^{c}	32.59 ± 12.2^{b}	43.33 ± 5.8^{c}
$D_{6.8}$ $K_{2.3}$	91.53 ± 7.5^{d}	46.29 ± 3.2^{c}	43.33 ± 5.8^{c}
$D_{6.8}$ $K_{4.5}$	100.00 ± 0.0^{e}	50.00 ± 0.0^{c}	46.67 ± 11.5^{c}
$D_{6.8}$ $K_{6.8}$	70.33 ± 2.5^{b}	42.43 ± 2.3^{c}	49.44 ± 22.4^{c}

The data correspond to the average of five repetitions by treatment ± SD. The values with the same letter in columns are not statistically different (Tukey's range test cotyledon $p = 1.89e^{-26}$; hypocotyls $p = 7.23e^{-16}$; root $p = 4.26e^{-14}$). Critical Value = 5.1766

study the germination of *P. laevigata* seeds took place between 3 and 5 days and in the latter study between 5 and 7 days. Burbulis and Blinstrubiené (2011) reported that a specific combination of PGR was necessary for callus induction of *Linum usitatissimum* according genotype. Likewise, the age of different *Amaranthus* species explant tissues produced different *in vitro* responses (Bovelli *et al*., 2001). Other *Prosopis* species such as *P. juliflora* and *P. tamarugo* required the combination of auxins with cytokinins to develop callus in hypocotyl explants (Nandwani and Ramawat, 1991; Nandwani and Ramawat, 1992).

3.2 Growth kinetics of cell suspension cultures

P. laevigata cell suspension culture was established and the growth curve is shown in Fig. 1. The lag phase lasted 5 days, followed by an exponential phase, which ended after 21 days of culture, and the maximum accumulated biomass (MAB) was produced (15.61 g DW L^{-1}). The duplication time (t_d) was 14.1 days, and specific growth rate (μ) was 0.049 $days^{-1}$. However, studies conducted by Trejo-Espino *et al*. (2011) in a *Prosopis leavigata* cell suspension culture reported a lower MAB (11.9 g *DW* L^{-1}).

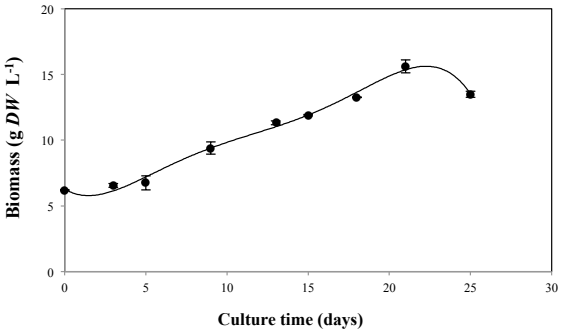

Fig. 1. *Prosopis laevigata* cell suspension cultures growth kinetics. Vertical bars denote SD. $y = -3 \times 10^{-5}x^5 + 0.0016x^4 - 0.0346x^3 + 0.3198x^2 - 0.7345x + 6.2745$; $R^2 = 0.9927$.

Instead the t_d (6.6 days) and μ (0.104 $days^{-1}$) were higher than those found in this study. Tissue culture and plant cells are more likely possess good phytoremediation potential if they exhibit high growth rate parameters (Doran, 2009). Izquierdo *et al*. (2004) pointed out that to avoid incurring in the erroneous measurements of the heavy metal net effect on the metabolism of cells, sampling should be performed before reaching the end of exponential stage of growth since by this time, substrate depletion may commence or toxic products accumulated.

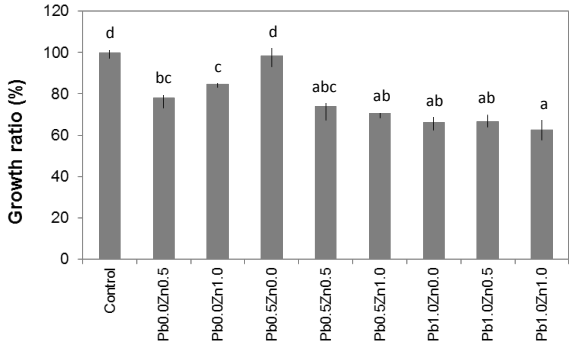

Fig. 2. Growth ratio (GR) of *Prosopis laevigata* cell suspension cultures treated with different concentrations of Pb^{2+} and Zn^{2+}. The data correspond to the average of three repetitions by treatment ± SD. Vertical bars denote SD. The values with the same letter in columns are not statistically different (Tukey's range test; $p = 7.12 \times 10^{-9}$). Critical Value = 4.9552.

3.3 Tolerance to lead and competitive transport between Pb^{2+} and Zn^{2+} in cell suspension cultures

The cell suspension cultures exposed to $Pb_{0.0}Zn_{0.5}$ (78%), $Pb_{0.0}Zn_{1.0}$ (85%), $Pb_{0.5}Zn_{0.5}$ (74%), $Pb_{0.5}Zn_{1.0}$(71%), $Pb_{1.0}Zn_{0.0}$(66%), $Pb_{1.0}Zn_{0.5}$ (67%), and $Pb_{1.0}Zn_{1.0}$(63%) showed significant lower growth ratio (GR) than the control (100%) and $Pb_{0.5}Zn_{0.0}$ (98%) treatments (Fig. 2). Similar results were reported for *P. laevigata* seedlings, where the exposition to 1.5 (84.59%) and 3.0 mM (77.72%) Pb^{2+} induced a GR reduction (Buendía-González *et al*., 2010b). Hu *et al*. (2012) reported that *Chenopodium album* seedlings showed decreased growth (FW) when exposed to 150 mg L^{-1} Pb^{2+} (~0.7 mM); Muschitz *et al*. (2009) reported that the growth of seedlings from *Solanum lycopersicum* was decreased when the concentration of Zn was higher to 0.5 mM, whereas the growth of cells from *Arabidopsis thaliana* was decreased by adding over 30 μM of Zinc (Klein *et al*. 2008). Also, Azooz *et al*. (2011) reported that *Hibiscus esculentus* seedlings showed decreased growth when exposed to Pb^{2+} and/or Zn^{2+}. Growth inhibition and reduction of biomass production are considered as general responses of higher plants to heavy metal toxicity. Inhibition of both cell elongation and division by heavy metals could explain, in part, the decline in biomass production (Arduini *et al*., 1994; Ouariti *et al*., 1997). However, in the treatment with $Pb_{0.5}Zn_{0.0}$, there were no differences among GR and the control (Fig. 2). This behavior suggests the operation of a tolerance mechanism against adverse effects of lead at concentration of 0.5 mM. The effect might be attributed to a phenomenon known as hormesis, an adaptive response characterized by increasing and reducing responses at low and high concentrations, respectively, of a given pollutant (Calabrese *et al*. 2007). Thus, based on the above considerations, it is possible that a lead concentration of 0.5 mM could induce low levels of stress by activating the cellular and molecular mechanisms enhancing the ability of this species to with stand more severe stresses.

P. laevigata cell suspension cultures treated with both Pb^{2+} (0.5 and 1.0 mM) and Zn^{2+} (0.5 and 1.0 mM) showed that lead accumulation increased as the concentration of both metals increased (Table 2). Moreover, Zn^{2+} absorption vary among the different treatments. In cultures treated only with Pb^{2+} (0.5 and 1.0 mM), the concentration of Zn in biomass was decreased significantly compared with to the control. Whereas, in cultures with $Pb_{0.5}Zn_{0.5}$, the concentration of Zn in biomass decreased significantly with respect to $Pb_{0.0}Zn_{0.5}$ treatment. However, the Pb does not affect the absorption of Zn when the cells were treated with $Pb_{1.0}Zn_{0.5}$ or $Pb_{1.0}Zn_{1.0}$. This is because the concentration of Zn was increasing as the concentration of Pb in the medium was also increased (Table 2). The accumulation in *P. laevigata* cell suspension was greater for Pb (7-101 times) than Zn, when the culture media containing Pb with or without Zn.

The bioconcentration factor (*BF*) has been used as a measure of the metal accumulation efficiency. In accordance to Audet and Charest (2007), *BF* values higher than 1 are indicative of potential hyperaccumulator species. The *BF* values were greater for Zn than Pb for the *P. laevigata* cell suspension cultures, when the medium was supplemented only with Zn^{2+} or in the control treatment. However, *BF* values for Zn drastically decreased as the combined Pb^{2+} and Zn^{2+} concentration increased ($Pb_{0.5}Zn_{0.0}$, $Pb_{0.5}Zn_{0.5}$, $Pb_{0.5}Zn_{1.0}$, $Pb_{1.0}Zn_{0.0}$, $Pb_{1.0}Zn_{0.5}$ and $Pb_{1.0}Zn_{1.0}$) with respect to control, $Pb_{0.0}Zn_{0.5}$ and $Pb_{0.0}Zn_{1.0}$ treatments (Table 2). With respect to *BF* values for Pb, the showed that *BF* value increased as the concentration of both metals increased, except to $Pb_{1.0}Zn_{1.0}$ treatment where *BF* was significantly lower than $Pb_{1.0}Zn_{0.5}$ treatment, which suggests that the Zn (1.0mM) decreases Pb accumulation in cell suspension.

Table 2. Accumulation and bioconcentration factors (BF) for Pb and Zn in cell suspension cultures of *Prosopis laevigata*, after 10 days of culture.

Treatment	Pb		Zn	
	Accumulation (mg kg^{-1})	BF	Accumulation (mg kg^{-1})	BF
Control	00.00 ± 00.00^{a}	00.00 ± 00.00^{a}	368.44 ± 68.17^{b}	153.13 ± 28.33^{c}
$Pb_{0.0}Zn_{0.5}$	00.00 ± 00.00^{a}	00.00 ± 00.00^{a}	641.74 ± 43.07^{c}	266.72 ± 17.90^{d}
$Pb_{0.0}Zn_{1.0}$	00.00 ± 00.00^{a}	00.00 ± 00.00^{a}	1184.33 ± 68.73^{d}	492.23 ± 28.57^{e}
$Pb_{0.5}Zn_{0.0}$	1481.87 ± 48.15^{b}	14.30 ± 0.46^{b}	190.92 ± 53.48^{a}	5.44 ± 1.52^{a}
$Pb_{0.5}Zn_{0.5}$	5788.03 ± 40.97^{c}	55.87 ± 0.40^{c}	384.69 ± 57.84^{b}	10.96 ± 1.65^{ab}
$Pb_{0.5}Zn_{1.0}$	13399.44 ± 48.84^{d}	129.34 ± 0.47^{g}	1834.82 ± 27.49^{f}	52.28 ± 0.78^{b}
$Pb_{1.0}Zn_{0.0}$	14086.05 ± 54.33^{e}	67.98 ± 0.26^{d}	138.46 ± 35.91^{a}	2.04 ± 0.53^{a}
$Pb_{1.0}Zn_{0.5}$	25990.94 ± 49.65^{g}	125.44 ± 0.24^{f}	1007.62 ± 45.88^{d}	14.86 ± 0.68^{ab}
$Pb_{1.0}Zn_{1.0}$	18286.21 ± 63.37^{f}	88.25 ± 0.31^{e}	1383.03 ± 45.65^{e}	20.40 ± 0.67^{ab}

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's range test; Pb $p = 6.04e^{-42}$; Pb FB $p = 6.16e^{-40}$; Zn $p = 1.20e^{-18}$; Zn FB $p = 1.43e^{-18}$). Critical Value = 4.9552

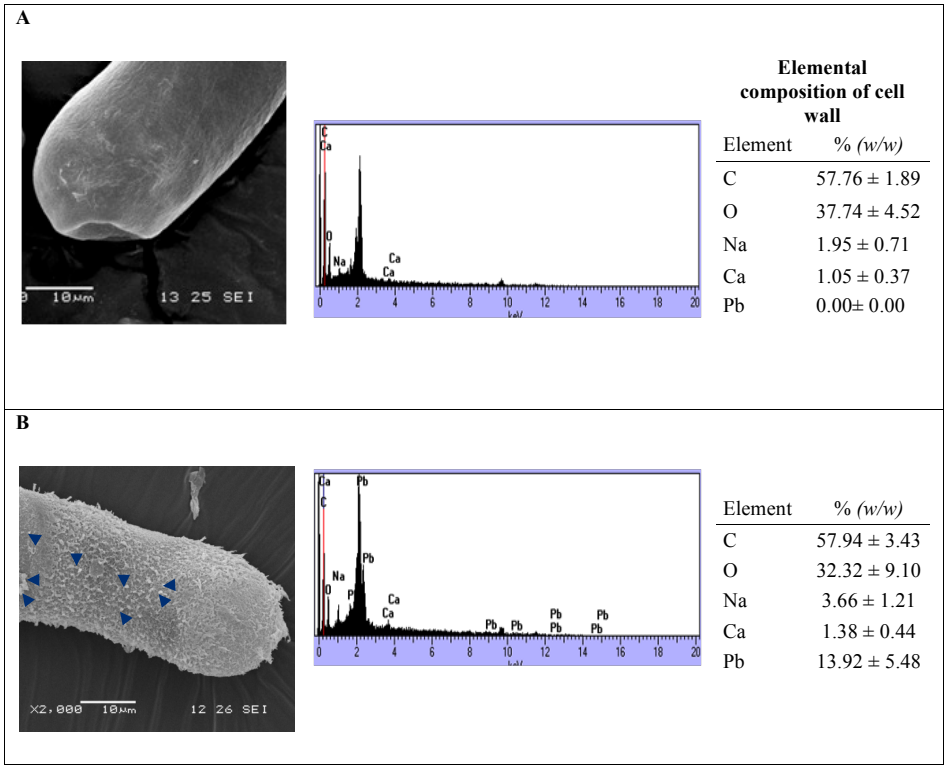

Fig. 3. Micrographs (SEM 2,000x magnification) of *Prosopis laevigata* cell suspension cultures grown in: (A) without heavy metals and (B) with Pb^{2+} 1.0 mM. The arrows indicate the Pb deposition.

Our results show that the Pb was accumulated more efficiently than Zn. The *BF* values were greater for Pb (2-33 times) than Zn for the *P. laevigata* cell suspension cultures. These results imply that competitive transport of both metals occurs through transporter channels, with saturation occurring at higher metals concentrations. It has been reported that Zn supply increased Pb uptake in *Phaseolus vulgaris* plants (Geebelen *et al*., 2007), but reduced Pb uptake in *Brasica rapa, Lactuca sativa* (He *et al*., 2004) and

in *Elsholtzia argyi* plants (Islam *et al*., 2011). In the first case, Pb increase caused a decrease of Zn uptake (Geebelen *et al*., 2007). A pre-treatment with Zn in *Dunalliela tertiolecta* cells resulted in a significant improvement to tolerance capability for Pb (Tsuji *et al*., 2002).

3.4 Surface cellular wall analysis by SEM-EDS

SEM micrographs of cells suspension culture of *P. laevigata* treated with 0.0 and 1.0 mM Pb^{2+}, displayed elongated morphology cells (Fig. 3A-B). The SEM-EDS analysis showed significant differences in the elemental composition of the cellular wall between the control and the 1.0 mM Pb^{2+} treatments (Fig. 3A-B). While in the control treatment Pb was not detected, 13.92% Pb was found in the Pb^{2+} treatment, those were observed as such deposits or aggregates (Fig. 3B, arrows). Furthermore, the surface morphology of the cells treated with 1.0 mM Pb^{2+} showed substantial differences from that of the control treatment. While control treatment exhibited a smooth surface (Fig. 3A), the 1.0 mM Pb^{2+} treatment was characterized by a multitude hairy-like protuberances or bumps (Fig. 3B). Plant cells can resort to several defense mechanisms in response to HM stress including exclusion, immobilization, chelation and compartmentation of metal ions. Also, the plant cells can prevent HM excess entry to cytosol and force them into a limited area e.g. cell wall (Liu and Kottke, 2003). Significant amounts of Pb have been found in the cell walls of many plant species, and this has been attributed to Pb detoxification mechanism. It has been suggested that Pb is adsorbed firstly in cell wall by its union to carboxyl groups, followed by cell uptake, where endoplasmic reticulum vesicles are formed, which can participate in the repair of plasma membrane or be secreted through plasmalemma and fused with the cell wall (Jiang and Liu, 2010). It has been suggested that Pb deposition in cell walls was the main mechanism for tolerance and detoxification to this *HM* in two species of *Lespedeza* (Zheng *et al*., 2012).

Conclusions

Cell suspension cultures of *P. laevigata* showed significant tolerance to growth at different Pb^{2+} concentrations. The cells showed a growth ratio for heavy metal greater than 63%, and also a capacity to uptake Pb. Additionally, the cells showed the highest accumulation efficiency for non-essential metal over essential metal. Scanning electron micrographs evidenced the accumulation of Pb in the cells walls. These results provide insights about the tolerance and accumulation mechanisms of lead occurring in *P. laevigata*.

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Notation

2,4-D	2,4-dichlorophenoxyacetic acid
BA	6-benzyladenine
BF	bioconcentration factor
DW	dry weight; mg
FW	fresh weight; mg
GR	growth ratio; %
HDPE	high densitypolyethylene
HM	heavy metal
KIN	kinetin
MAB	maximum accumulated biomass (g DW L^{-1})
MS	murashige and Skoog medium
PGRs	plant growth regulators
SEM-EDS	scanning electron microscopy equipped with energy-dispersive X-rays spectroscopy
t_d	duplication time; days
μ	growth specific rate; $days^{-1}$

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