
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

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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

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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 Pb²⁺/Zn²⁺ (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 ≫ 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 Zn²⁺ sobre la absorción y acumulación de Pb²⁺. Se realizó un bioensayo con Pb²⁺/Zn²⁺ (0.0, 0.5, y 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 ≫ 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, fitoremediación.
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 able 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 phyto remediation. 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 D6.8K0.5, D6.8K2.3, D6.8K4.5, and D6.8K6.8, where D6.8 stands for the 2,4-D at concentration 6.8 μM, K stands for the BA 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 (D6.8K4.5) was selected for further experiments as it showed high percentage of callus induction, better growth and friability. Callus derived from D6.8K4.5 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 D6.8K4.5 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 D6.8K4.5 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 D6.8K4.5 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 D6.8K4.5 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. 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 Pb²⁺/Zn²⁺

In order to evaluate the effect of zinc in the accumulation of Pb, the cell suspension cultures were grown in liquid MS medium with D6.8K4.5 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 Zn²⁺ (0.0, 0.5 and 1.0 mM). These treatments were coded as the Pb_z0.5y, where Pb₂ stands for the lead at concentration x and Zn for the essential ion (Zn²⁺) at concentration y. The Pb(NO₃)₂ and ZnSO₄·7H₂O (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):

\[
GR = \frac{\text{Plant biomass with Pb and/or Zn}}{\text{Plant biomass without Pb (control; DW)}} \times 100
\]
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 HNO₃ (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, Metrical) 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 (r²) of
0.99 or better. All glassware and apparatus were
washed with 0.1 N HNO₃ before their use. Metal
collection 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
collection in plant tissues ([Pb or Zn]biomass) to
HM concentration in medium ([Pb or Zn]medium culture)
(Audet and Charest, 2007):

\[ BF = \frac{[\text{Pb or Zn}]_{\text{biomass}}}{[\text{Pb or Zn}]_{\text{medium culture}}} \] 

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
tetroxide 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 critical point with carbon dioxide (CO₂)
(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 ≤ 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 ≤ 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 (<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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Callus induction in explants (%)</th>
<th>Cotyledon</th>
<th>Hypocotyl</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₀ B₀ K₀ (Control)</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
</tr>
<tr>
<td>D₀ B₂ K₃</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
</tr>
<tr>
<td>D₀ B₄ K₅</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
</tr>
<tr>
<td>D₀ B₆ K₈</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
</tr>
<tr>
<td>D₀ K₂ K₆</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
</tr>
<tr>
<td>D₀ K₄ K₈</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
</tr>
<tr>
<td>D₀ K₆ K₈</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
<td>0.0±0.0^a</td>
</tr>
<tr>
<td>D₆ K₀ B₀ K₀</td>
<td>80.63±0.0^c</td>
<td>40.16±9.7^b,c</td>
<td>66.67±14.8^d</td>
<td></td>
</tr>
<tr>
<td>D₆ B₂ K₃</td>
<td>93.3±11.5^de</td>
<td>32.48±1.5^b</td>
<td>45.67±3.1^c</td>
<td></td>
</tr>
<tr>
<td>D₆ B₄ K₅</td>
<td>100.0±0.0^e</td>
<td>45.24±4.1^c</td>
<td>28.0±4.0^b</td>
<td></td>
</tr>
<tr>
<td>D₆ B₆ K₈</td>
<td>81.67±3.5^c</td>
<td>32.59±12.2^b</td>
<td>43.33±5.8^c</td>
<td></td>
</tr>
<tr>
<td>D₆ K₂ K₆</td>
<td>91.53±7.5^d</td>
<td>46.29±3.2^c</td>
<td>43.33±5.8^c</td>
<td></td>
</tr>
<tr>
<td>D₆ K₄ K₈</td>
<td>100.0±0.0^e</td>
<td>50.00±0.0^e</td>
<td>46.67±11.5^c</td>
<td></td>
</tr>
<tr>
<td>D₆ K₆ K₈</td>
<td>70.3±2.5^b</td>
<td>42.43±2.3^c</td>
<td>49.44±22.4^c</td>
<td></td>
</tr>
</tbody>
</table>

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-15; 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 (9.61 g DW L⁻¹). The duplication time (tₜ) 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).

Instead the tₜ (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.
3.3 Tolerance to lead and competitive transport between Pb²⁺ and Zn²⁺ in cell suspension cultures

The cell suspension cultures exposed to Pb₀.₅Zn₀.₅ (78%), Pb₀.₁Zn₀.₁ (85%), Pb₀.₅Zn₀.₅ (74%), Pb₀.₅Zn₁.₀ (71%), Pb₁.₀Zn₀.₀ (66%), Pb₁.₀Zn₀.₅ (67%), and Pb₁.₀Zn₁.₀ (63%) showed significant lower growth ratio (GR) than the control (100%) and Pb₀.₅Zn₀.₀ (98%) treatments (Fig. 2). Similar results were reported for *Prosopis laevigata* seedlings, where the exposition to 1.5 (84.59%) and 3.0 mM (77.72%) Pb²⁺ 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 Pb²⁺ (~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 Pb²⁺ and/or Zn²⁺. 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 Pb₀.₅Zn₀.₀, 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 withstand more severe stresses.

*P. laevigata* cell suspension cultures treated with both Pb²⁺ (0.5 and 1.0 mM) and Zn²⁺ (0.5 and 1.0 mM) showed that lead accumulation increased as the concentration of both metals increased (Table 2). Moreover, Zn²⁺ absorption vary among the different treatments. In cultures treated only with Pb²⁺ (0.5 and 1.0 mM), the concentration of Zn in biomass was decreased significantly compared with to the control. Whereas, in cultures with Pb₀.₅Zn₀.₅, the concentration of Zn in biomass decreased significantly with respect to Pb₀.₀Zn₀.₅ treatment. However, the Pb does not affect the absorption of Zn when the cells were treated with Pb₁.₀Zn₀.₅ or Pb₁.₀Zn₁.₀. 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 Zn²⁺ or in the control treatment. However, BF values for Zn drastically decreased as the combined Pb²⁺ and Zn²⁺ concentration increased (Pb₀.₅Zn₀.₀, Pb₀.₅Zn₀.₅, Pb₀.₅Zn₁.₀, Pb₁.₀Zn₀.₀, Pb₁.₀Zn₀.₅ and Pb₁.₀Zn₁.₀) with respect to control, Pb₀.₀Zn₀.₅ and Pb₀.₀Zn₁.₀ 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 Pb₁.₀Zn₁.₀ treatment where BF was significantly lower than Pb₁.₀Zn₀.₅ 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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pb Accumulation (mg kg(^{-1}))</th>
<th>Pb BF</th>
<th>Zn Accumulation (mg kg(^{-1}))</th>
<th>Zn BF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>368.44 ± 68.17</td>
<td>153.13 ± 28.33</td>
</tr>
<tr>
<td>Pb(<em>{0.0})Zn(</em>{0.5})</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>641.74 ± 43.07</td>
<td>266.72 ± 17.90</td>
</tr>
<tr>
<td>Pb(<em>{0.0})Zn(</em>{1.0})</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>1184.33 ± 68.73</td>
<td>492.23 ± 28.57</td>
</tr>
<tr>
<td>Pb(<em>{0.5})Zn(</em>{0.0})</td>
<td>1481.87 ± 48.15</td>
<td>14.30 ± 0.46</td>
<td>190.92 ± 53.48</td>
<td>5.44 ± 1.52</td>
</tr>
<tr>
<td>Pb(<em>{0.5})Zn(</em>{0.5})</td>
<td>5788.03 ± 40.97</td>
<td>55.87 ± 0.40</td>
<td>384.69 ± 57.84</td>
<td>10.96 ± 1.65</td>
</tr>
<tr>
<td>Pb(<em>{1.0})Zn(</em>{0.0})</td>
<td>13999.44 ± 48.84</td>
<td>129.34 ± 0.47</td>
<td>1834.82 ± 27.49</td>
<td>52.28 ± 0.78</td>
</tr>
<tr>
<td>Pb(<em>{1.0})Zn(</em>{0.5})</td>
<td>14086.05 ± 54.33</td>
<td>67.98 ± 0.26</td>
<td>138.46 ± 35.91</td>
<td>2.04 ± 0.53</td>
</tr>
<tr>
<td>Pb(<em>{1.6})Zn(</em>{0.5})</td>
<td>25990.94 ± 49.65</td>
<td>125.44 ± 0.24</td>
<td>1007.62 ± 45.88</td>
<td>14.86 ± 0.68</td>
</tr>
<tr>
<td>Pb(<em>{1.6})Zn(</em>{1.0})</td>
<td>18286.21 ± 63.37</td>
<td>88.25 ± 0.31</td>
<td>1383.03 ± 45.65</td>
<td>20.40 ± 0.67</td>
</tr>
</tbody>
</table>

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 \(p = 6.64 \times 10^{-42}\); Pb FE \(p = 6.16 \times 10^{-40}\); Zn \(p = 1.20 \times 10^{-18}\); Zn FE \(p = 1.43 \times 10^{-18}\)). 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 *Dunaliella 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 density polyethylene
- **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
- $\mu$: growth specific rate; days$^{-1}$

### References


