Cell Viability and Leakage of Electrolytes in *Avicennia germinans* Exposed to Heavy Metals

Daniel Gonzalez-Mendoza[a,d,*], Adriana Quiroz-Moreno[b], Rosa Escobedo Gracia Medrano[c], Onecimo Grimaldo-Juarez[a], and Omar Zapata-Perez[d]

[a] Instituto de Ciencias Agrícolas de la Universidad Autónoma de Baja California (ICA-UABC), Carretera a Delta s/n C.P. 21705, Ejido Nuevo León, Baja California, Mexico. E-mail: daniasaf@gmail.com
[b] Unidad de Biotecnología, CICY, Merida, Yucatan, Mexico
[c] Biología Molecular, CICY, Merida, Yucatan, Mexico
[d] Departamento de Recursos del Mar, Cinvestav Unidad Merida, Merida, Yucatan, Mexico

* Author for correspondence and reprint requests

The effect of heavy metal stress on the cell viability and leakage of electrolytes of *Avicennia germinans* leaf discs was investigated by the tissue tolerance test. Foliar discs were incubated with different Cd^{2+} or Cu^{2+} concentrations for 24 h; thereafter, the cell membrane stability of the tissue was assayed by the cell viability Evans blue and leakage electrolytes methods. The results indicated that electrolyte leakage of the leaf discs increased 24 h after exposure to heavy metal stress, as shown by a reduction of the cell viability by 30% in discs exposed to higher doses of Cd^{2+} (0.546 M) and Cu^{2+} (0.7 M), respectively. Additionally, the histological analysis of the leaf discs exposed to heavy metal stress revealed that at higher Cd^{2+} and/or Cu^{2+} concentrations an increase in the intercellular spaces and destruction of mesophyll cells was observed 24 h after exposure. In summary, the biochemical and structural changes observed in foliar tissues of *A. germinans* suggest that higher cadmium and copper concentrations may result in structural changes and altered physiological characters in leaves.

Key words: Avicennia germinans, Cadmium, Copper, Cell Viability

Introduction

Metal toxicity has great impact and relevance in coastal environments. In contrast with organic pollutants, heavy metals can not be biologically or chemically degraded, and thus may accumulate locally or be transported over long distances. Cadmium (Cd^{2+}) and copper (Cu^{2+}) ions often occur in high concentrations, up to 200 and 900 mg/kg, in mangrove forests due to their prevalence in sediments (Nascimento *et al.*, 2006; Chen *et al.*, 2007). Along with other mangroves, the different species of *Avicennia* are of great ecological importance; they are regarded as tolerant to heavy metals (Peng *et al.*, 1997). *Avicennia* species have different adaptations for coping with hypersaline environments, which might affect the uptake, distribution, loading and excretion of heavy metals within the plant. *A. germinans* plants treated with Cd^{2+} have an exclusion strategy with the retention of metals in their roots, as well as some degree of ion transfer to leaves, as evidenced by a positive correlation of bioaccumulation between roots and leaves (Gonzalez-Mendoza *et al.*, 2007a). In addition, when there is a greater bioaccumulation of metals in the roots, metals can move up to the leaves where they can be accumulated and cause a disruption of the photosynthetic activity (Gonzalez-Mendoza *et al.*, 2007b). However, the effect of Cd^{2+} and/or Cu^{2+} on the cellular stability and structural changes in foliar tissues (*e.g.* leaves) of *A. germinans* is not yet well understood. The aim of our present study was to evaluate the cadmium and copper toxicity with respect to the cellular stability in foliar tissues of *A. germinans*, one of the dominant mangrove species found in the Gulf of Mexico and Yucatan Peninsula coastline.

Material and Methods

Field collection and seed germination

Black mangle seeds were collected from Chabihau Bay (21° 20′ 38″ N and 89° 05′ 08″ W), Yucatan, Mexico, and transported to the laboratory

for further examination. The disinfection process was achieved with 1% NaOCl (Clorox) for 5 min, followed by a rinse with deionized sterile water. The seeds were then peeled with a sterile scalpel and the pericarp was discarded. The naked seeds were further disinfected with 0.1% NaOCl and finally rinsed with deionized sterile water six times altogether. All steps were carried out under sterile conditions. After disinfection, 150 seeds were germinated in sand (grain size 3–6 mm) in the greenhouse under the following conditions: 28–35 °C during the day and 24–26 °C during the night; 60% relative air humidity; 12-h light/dark photoperiods. Seedlings (12 cm of height) with well developed roots were transplanted to single pots (500 ml) containing a commercial soil mixture for horticulture mixed with quartz sand and peat moss (50% soil, 20% sand, and 30% peat moss). Plants were irrigated daily with water and, every other week, fertilized with ¼ strength Hoagland's solution.

Preparation of leaf slices and heavy metal exposure

Leaf discs of *Avicennia germinans* fresh seedlings were cut into 5-mm pieces with cork borers. Sets of five leaf discs were randomly allocated and transferred to Petri dishes ($n = 4$) containing 10 ml prepared with 0.054, 0.136, 0.267 and 0.546 M of cadmium chloride ($CdCl_2$) and 0.06, 0.160, 0.320 and 0.700 M of copper sulfate ($CuSO_4 \cdot 5H_2O$), respectively, prepared with bidistilled water (bdw). Control leaf discs of plants were transferred to plastic Petri dishes containing 10 ml bdw. The incubation of the Petri dishes with leaf slices was performed at 23 °C and 60% relative air humidity for 24 h.

Membrane permeability (solute leakage)

Electrolyte leakage was determined as described by Lin *et al*. (1985) in control and heavy metal-treated leaf discs. Petri dishes with leaf discs were incubated for 24 h, and then the leakage of electrolytes into bdw was determined with a conductivity meter (Jenway 4010, Jenway Ltd., Dunmow, Essex, UK)

Determination of cell viability

In order to determine changes in the cell viability during heavy metal stress of *A. germin-* ans leaves, we used the Evans blue assay (Baker and Mock, 1994). This cell viability assay is based on the uptake of Evans blue by nonviable cells. Briefly, non-treated and heavy metal-treated leaf discs were stained with 0.25% (v/v) Evans blue aqueous solution for 15 min, and then they were extensively washed with distilled water for 30 min to remove excess and unbound dye. The dye bound to dead cells was solubilized in 50% (v/v) ethanol with 1% (w/v) SDS at 60 °C for 30 min and quantified by measuring the absorbance at 600 nm. An internal control of heat-killed cells from leaf discs was used to estimate the percentage of relative viability (Escobedo and Miranda, 2003). The experiment was repeated twice with four replicates for each treatment.

Histology and light microscopy

The histological procedures were according to Buffard-Morel *et al*. (1992) with slight modifications. Samples of leaf discs treated with heavy metals and nontreated discs (control) were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.2) for 24 h under reduced pressure. After samples were dehydrated with ethanol solutions of increasing content [30, 50, 70, 80, 90, 95% (v/v)] and absolute ethanol for 1 h each, the samples were impregnated with JB-4R resin (Polyscience, Niles, IL, USA). Sections of 3 μm were prepared from the resin-impregnated tissues with a microtome (HM 325, MICROM), equipped with knife steel blades. Thin sections mounted in slices were double stained with periodic acid-Schiff (PAS) reagent and naphthol blue-black. The PAS reagent stains starch reserves and cell walls in pink, while naphthol blue-black specifically stains soluble or reserve proteins dark blue (Fisher, 1968).

In the present study, the anatomical changes of leaf discs were studied evaluating changes in the morphology of the upper epidermis (UE), palisade parenchyma (PP), spongy parenchyma (SP), lower epidermis (LE), and intercellular spaces (IS), using images captured with a digital camera (AxioCam MRm, Carl Zeiss) coupled to a compound microscope (Axioplan, Carl Zeiss). The images were processed with the image software AxioVision (Version 3.1, Carl Zeiss).

The data were analyzed by one-way analyses of variance (ANOVAs), and the means were separated using Tukey's test (Statistical Package version 5.5, Statsoft, USA). Significant differences

were accepted, if $p < 0.05$ and data was expressed as mean ± SEM.

Results and Discussion

Membrane permeability and leaf disc viability

The extent of membrane damage was estimated by electrolyte leakage from leaf tissues. The values of electrical conductivity in leaf discs, an indicator of ion leakage, increased with higher doses of Cd^{2+} (0.267 and 0.546 M) and Cu^{2+} (0.320 and 0.700 M) after 24 h of exposure (Table I). Clearly, the results suggest that Cd^{2+} and Cu^{2+} treatments might have caused the disruption of the membrane integrity of *A. germinans* leaf tissues at concentration of 0.267 and 0.546 M or higher.

Changes in the cell membrane permeability of leaf discs due to Cd^{2+} or Cu^{2+} treatments were observed (Table I). The cell membrane is impermeable to macromolecules such as Evans blue, but, when it undergoes an injury or loses its selective permeability, macromolecules can penetrate it. Thus, the amount of Evans blue entering and binding dead cells reflects the degree of loss of cell membrane integrity and is an estimate of cell death. As shown in Table I, the cell viability of leaf discs was reduced to 50% and 30% in response to higher doses of Cd^{2+} (0.267 and 0.546 M) as compared to lower Cd^{2+} concentration after a 24-h exposure.

Comparable results were observed in leaf discs treated with 0.320 and 0.700 M Cu^{2+}; the cell viability was reduced to 40% and 30% 24 h after exposure (Table I).

Table I. Effect of heavy metal stress on cell viability and electrolyte leakage in *A. germinans*.

Heavy metal	Cell viability (%)	Electrolyte leakage (%)
Cd		
0.054	75 ± 1.56^a	70 ± 4.55^a
0.136	60 ± 2.08^b	70 ± 3.87^a
0.267	50 ± 1.78^c	66 ± 2.97^c
0.546	30 ± 2.64^d	30 ± 3.51^d
Cu		
0.06	80 ± 2.64^a	70 ± 4.99^a
0.160	70 ± 2.79^b	70 ± 3.34^a
0.320	40 ± 3.65^c	55 ± 2.45^c
0.700	30 ± 1.89^d	30 ± 4.13^d

Means with different letter are significantly different from one another ($p < 0.05$, Tukey's test).

These results suggest that heavy metal stress induces membrane damage in leaf discs of *A. germinans*, that might be attributable to the accumulation of Cd^{2+} and Cu^{2+}, and, perhaps, generation of reactive oxygen species (ROS). An increase in ROS can induce a cyclic cascade of reactions causing a distortion of the lipid bilayer, of the membrane proteins and disruption of the membrane by the Fenton reaction that might be further enhanced by these metals. Cd^{2+} and Cu^{2+} ions can interact with S and N groups present in cell proteins and cause an alteration of the ionic channels of the membrane, which promotes a higher flow of ions of the cell into the leaf discs. Similar results were reported by Xiong and Wang (2005) and Tamas *et al.* (2006) who showed that electrolyte leakage and cell viability (evaluated by the Evans blue staining method) were increased by the generation of ROS in *Brassica pekinensis* and *Hordeum vulgare*, respectively, exposed to heavy metals.

Histological changes

The main alteration observed in the leaf disc tissue was related to membrane disruptions initially observed after 24 h of exposure to Cd^{2+}. When leaf discs were treated with 0.267 and 0.546 M Cd^{2+} for 24 h, the integrity of cells was more irregular and showed notable structural changes. Similar phenomena have been described by Vitória *et al.* (2003) for *Raphanus sativus* where the structural changes observed in seedlings treated with Cd^{2+} were mainly caused by a Cd-induced decrease in water uptake. On the other hand, not much alteration of cell arrangement in size of the intercellular spaces, mesophyll structure and palisade parenchymas was found in leaf discs treated with 0.060 and 0.320 Cu^{2+} 24 h after exposure with respect to the control (data no show). Similar phenomena have been described by Panou-Filotheou *et al.* (2001) for *Origanum vulgare* where the intercellular spaces of untreated leaves were more numerous than of heavy metal-treated leaves.

In summary, our results showed that mangrove leaf discs exposed to Cu^{2+} or Cd^{2+} initiate a variety of subcellular responses, *i.e.*, metabolic reactions, which can cause damage at the cellular level, lead by their phytotoxic effects. However, cadmium was more toxic than copper causing membrane damages and structural changes in foliar tissues of *A. germinans*. Future work on *Avicennia ger-*

minans should focus on evaluation of the ROS production, identification of antioxidant enzymes and gene regulation in order to achieve a better understanding of the regulation of damage at the cellular level in mangrove species exposed to heavy metal stressors.

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