

Nickel hyperaccumulation “in vitro” by *Leucocroton havanensis* (*Euphorbiaceae*)

Hiperacumulación “in vitro” de Ni en *Leucocroton havanensis* (*Euphorbiaceae*)

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RESUMEN

Leucocroton havanensis es un arbusto perenne que hiperacumula níquel. Esta especie es endémica de los suelos serpentina, en las áreas protegidas “Lomas de Galindo” y “La Coca”, en el occidente de Cuba. En este trabajo se estudiaron diferentes tratamientos para la desinfección, y la germinación de semillas de *L. havanensis*, así como la hiperacumulación de níquel en condiciones de cultivo “in vitro”. Los tratamientos para la desinfección de las semillas fueron: NaClO (1% y 2%) por 5, 10 y 15 minutos, y HgCl₂ (0,01% y 0,05%) por 3, 5 y 10 minutos. Todos los tratamientos contenían Tween 20 al 1%. La germinación tuvo lugar sobre medio basal MS. La determinación cualitativa del níquel acumulado por las plantas fue realizada mediante el método de la dimetilgloxima, y su cuantificación por Espectrometría de Emisión (Plasma-Óptico) Acoplada Inductivamente (ICP-OES). El mejor tratamiento para la desinfección de las semillas es el que emplea HgCl₂ (0,05 %) con Tween 20 (1 %) por 10 minutos. El porcentaje de germinación de la semilla fue del 98,5 %. *L. havanensis*, en condiciones de cultivo “in vitro”, es capaz de absorber el NiSO₄ del medio e hiperacumularlo (5000,03 mg·g⁻¹) en todos los órganos de la planta. El cultivo “in vitro” de esta especie puede utilizarse como sistema modelo para el estudio de los mecanismos moleculares de hiperacumulación de este metal en el género *Leucocroton*.

Palabras clave: *Leucocroton havanensis*; hiperacumulación de Ni, cultivo “in vitro”

ABSTRACT

Leucocroton havanensis, a perennial shrub endemic to ultramafic (serpentine) soils of “Lomas de Galindo” and “La Coca” in western Cuba, is a known nickel hyperaccumulator. The present work examined different treatments for the disinfection and germination of seeds from *L. havanensis*, examining nickel hyperaccumulation under “in vitro” culture conditions. The following seed disinfection treatments were assayed: NaClO (1% and 2%) for 5, 10 and 15 minutes, and HgCl₂ (0.01% and 0.05%) for 3, 5 and 10 minutes, combined with 1% Tween-20 in all cases. MS basal medium was used for germination, employing the dimethylglyoxime method for qualitative nickel determinations and Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) to quantify Ni levels. Treatment with 0.05% HgCl₂ – 1% Tween-20 for 10 minutes was shown to be optimal, producing germination rates of 98.5%. Under “in vitro” culture conditions, *L. havanensis* absorbed nickel from the culture medium, accumulating it to levels of 5000.03 mg·g⁻¹ in all organs of the plant. The “in vitro” culture system described here for *L. havanensis* can be used as a model system to study the molecular mechanisms of nickel hyperaccumulation in the genus *Leucocroton*.

Keywords: *Leucocroton havanensis*; Ni hyperaccumulation, “in vitro” culture

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INTRODUCTION

Leucocroton havanensis Borhidi (*Euphorbiaceae*) is a shrub commonly known by the name of “Cuaba Amarilla”, geographically restricted to xeromorphic scrublands of thorny shrubs on serpentine soils (*cuabales*) of *La Coca*, province of Havana, and *Lomas de Galindo*, province of Mayabeque, Cuba. The genus *Leucocroton* is endemic to Cuba and is composed of 26 separate taxa, all of which are known to be Ni hyperaccumulators. Most of them contain over 1% of Ni (dried weight) in their leaves (Reeves & al. 1996, Jestrow & al. 2010).

The first Ni hyperaccumulator plant was discovered in Italy by Minguzzi & Vergnano (1948), who quantified the concentration of this metal in ashes from the leaves of *Alyssum bertolonii*. A species is classified as a Ni hyper-

accumulator if its aerial organs contain more than 1000 µg·g⁻¹ (dried weight) of this metal (Brooks & al. 1977, Reeves 1992). *Leucocroton havanensis* hyperaccumulates up to 19,160 µg·g⁻¹ (dried weight) of Ni in its leaves (Reeves & al. 1996); a phenomenon interpreted as a response to the high concentrations of this metal characterizing ultramafic rock-derived soils such as peridotites, dunites and serpentinites, collectively known as serpentines. These chemically complex soils exhibit high concentrations of Mg, Fe, Cr, Co and Ni, and are typically poor in elements playing an essential role in plant development, such as Ca, K and N (Reeves & al. 1999).

Serpentines are hostile to vegetation developing in nearby soils, being therefore characterized by a unique flora with a high number of endemic species, which has caught the attention of scientists studying speciation and the conservation of relict flora (Borhidi 1988). In Cuba, serpentine soils are distributed throughout the island, and host approximately 30% of its endemic flora despite the fact that they cover only 7% of the country surface area

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(Reeves & *al.* 1996). Hyperaccumulator plants have potential applications in phytomining and the phytoremediation of soils contaminated with heavy metals (Brooks 1998, Lasat 2002, Mc Grath & *al.* 2002, Chaney & *al.* 2005, Banuelos 2006).

A number of studies have been performed on the molecular mechanisms underlying the phenomenon of heavy metal hyperaccumulation in plants. The results have depended on different factors, including the specific metal under examination, hyperaccumulator species and its origin and habitat, among others (Rawser 1999, Cobbett 2000). Although “*in vitro*” culture techniques are often used to examine molecular mechanisms that are difficult to study under natural conditions (Che & *al.* 2002, Che & *al.* 2006, Su & *al.* 2009) and are therefore well suited for studying endemic Cuban Ni hyperaccumulators, such studies have not been performed so far. In the present work we use “*in vitro*” culture to demonstrate that *Leucocroton havanensis* hyperaccumulates nickel under these conditions, which is a necessary first step for later studies on the molecular mechanisms of Ni hyperaccumulation in the *Leucocroton* genus.

MATERIALS AND METHODS

Plant material

Leucocroton havanensis fruits were collected during July 2011 in serpentine soils at *Lomas de Galindo* (23°03'55.0" N, 81°42'57.0" W) in the province of Mayabeque, Cuba.

Culture media

MS (for 1L): Murashige & Skoog (1962) salts with vitamins 4.4g; sucrose 20g and micro agar 8g. pH was adjusted to 6.6-6.8 with 1M KOH before autoclaving at 103.5 kPa and 121°C for 20 minutes.

MS-Ni (for 1L): MS medium with NiSO₄ (50; 100; 250; 500 and 1000 mg).

Reagents

All chemicals were purchased from Duchefa Biochemie (Netherlands), Fluka (Switzerland), Carlo Erba (Italy), Oxoid (U.K.), Mallinckrodt Baker (USA), Shelton Scientific (USA), Sigma – Aldrich (USA) and Sigma (USA).

Disinfection of seeds and germination of *Leucocroton havanensis*

Leucocroton havanensis seeds were washed for 10 minutes in 5% Tween-20 and then rinsed thoroughly with sterile distilled water. Surface sterilization was performed with disinfecting solutions of sodium hypochlorite (1% and 2% NaClO) and mercuric chloride (0.01% and 0.05% HgCl₂) containing 1% Tween-20 as tensoactive agent. Incubation times of 5, 10 and 15 minutes were assayed for the sodium hypochlorite solutions; in the case of mercuric chloride solutions, the assayed incubation times were 3, 5 and 10 minutes

instead. Once treated, the seeds were rinsed 4 times with sterile distilled water and transferred onto MS or MS-Ni culture media. Seeds were distributed at a density of 10 seeds per flask (Sigma-Aldrich, Inc.), each containing 40 mL of solid culture medium, and incubated at 23 ± 2 °C under a light-darkness photoperiod of 16-8 h respectively with a light intensity of 50 μmol×m⁻²×s⁻¹. The plants were passaged into fresh medium every 6 weeks.

Semi-quantitative detection of Ni using the dimethylglyoxime method

A piece of filter paper was wetted with 1% dimethylglyoxime (in ethanol) and left to dry at room temperature. Six week old plants were pressed against the filter paper, monitoring the resulting changes in color.

Determination of Ni concentration by Inductively Coupled Plasma-Optical Emission Spectroscopy

Five grams of plant material (6 week old plants) were weighed and grounded to a fine powder in a mortar containing liquid nitrogen. The resulting sample was transferred to a 50 mL FALCON tube and dehydrated by incubation for 24 hours in a 120°C oven. Later 205 mg of the sample were then weighed into a Teflon tube, to which 4 mL of 65% HNO₃ P.A. and 2 mL of 48% HF 48% P.A. were added. The tubes were placed into the press and introduced into the digester (Advanced Microwave Labstation Ethos Touch Control Milestone S.r.l. Italy).

The following program was used:

T _{initial} = 3°C	Steps	t (minutes)	Power (Watts)
T _{maximal} = 1300°C	1	1	250
T _{final} = 3°C	2	5	250
	3	4	400
	4	4	600
	5	5	250

T: temperature

After diluting the digested sample to a final volume of 50 mL with Milli-Q water, the samples were analyzed in a Perkin-Elmer Optima™ 7000 DV ICP-OES inductively coupled plasma optical emission spectrometer.

RESULTS

Disinfection of seeds and germination from *Leucocroton havanensis*

Treatment with 1% NaOCl for 5 min. failed to completely eliminate fungal and bacterial contamination in the treated seeds. No bacterial and/or fungal contamination was observed in seeds undergoing the remaining sodium hypochlorite-based treatment regimes (1% NaOCl for 10 or 15 min., or 2% NaOCl for 5, 10 or 15 min.) after 4 weeks of culture. Under these conditions, however, seed germination was also suppressed, and therefore NaOCl was discarded as seed disinfection treatment.

When using 0.01% HgCl_2 , there was detectable bacterial and fungal growth for 100% of treated seeds after one week, regardless of treatment time. The same result was obtained when using 0.05% HgCl_2 for 3 minutes. However, when this treatment was extended to 5 minutes, there was detectable bacterial growth in 45% of the treated seeds, and both fungal and bacterial growth in 20% of the seeds. There was germination only in uncontaminated seeds. Further extending of this treatment to a total incubation time of 10 minutes completely eliminated fungal and bacterial contamination without affecting germination.

Four weeks after germination the seedlings exhibited two round and green cotyledonary leaves, a stem approximately 5 cm long and a white, thick and long main root; two to four mature leaves were already present by the sixth week. Although germination percentage was high for this treatment (98.5%), this parameter decreased

with storage time at 10°C, and no seeds germinated after 4 months of storage at this temperature (data not shown).

Semi-quantitative detection of Ni by the dimethylglyoxime method

There were no apparent morphological differences between six week old *Leucocroton havanensis* plants obtained by “in vitro” culture on either MS or MS-Ni (0–1000 mg/L) culture media. When the dimethylglyoxime method was applied, these plants left marks on the filter paper with colors ranging from light pink to dark red. The intensity was directly proportional to Ni concentration in the culture medium, and the color was most intense for the plants grown in 500 $\text{mg} \times \text{L}^{-1}$ of NiSO_4 . The seeds incubated on MS medium containing 1000 mg/L of NiSO_4 did not germinate (data not shown).

When the plants were grown directly on MS medium without Ni supplementation (Figure 1A and 1B) only the cotyledonary leaves left a light pink imprint on the paper (Figure 1B). The remaining parts of the plant (true leaves and stem) only left a light green imprint, attributable to the presence of chlorophyll in these organs.

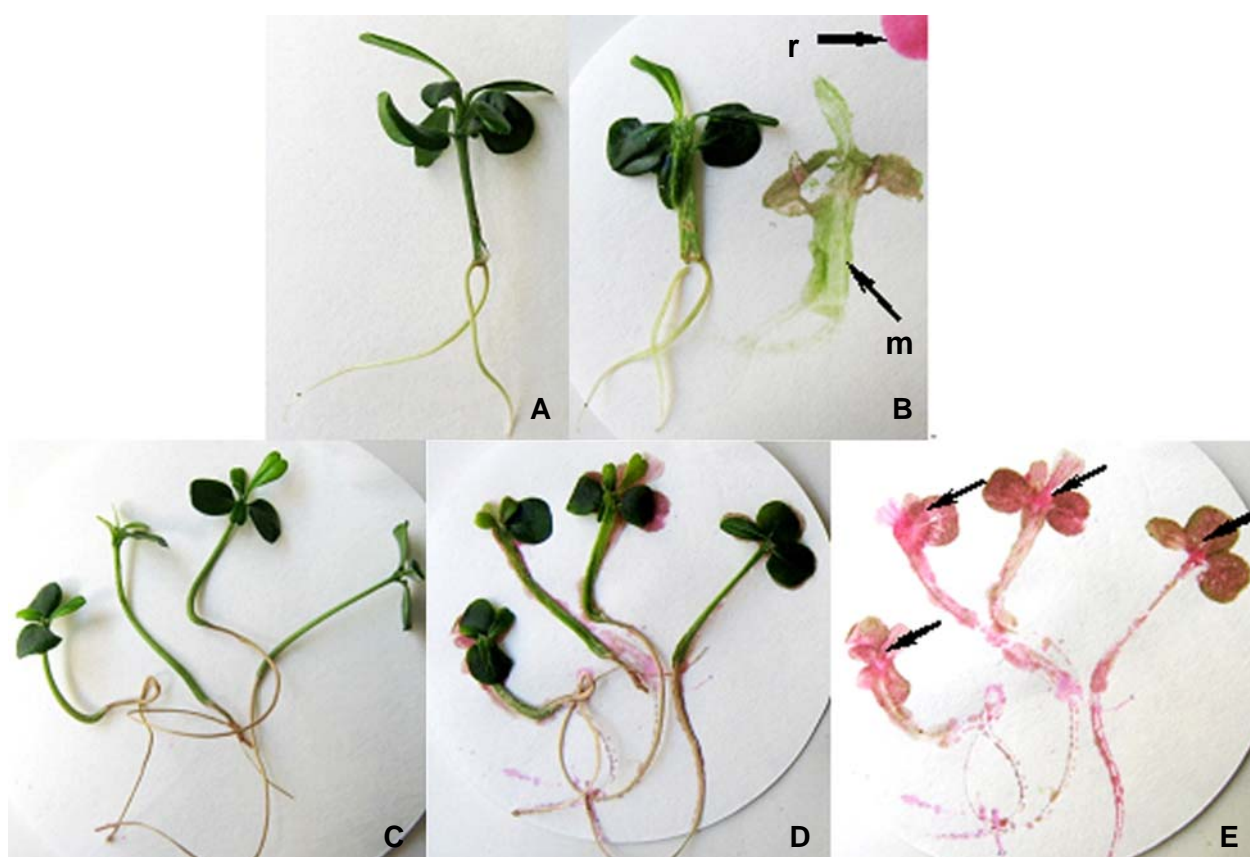


Fig. 1: Qualitative Ni detection for *in vitro*-grown seedlings of *Leucocroton havanensis*, using the dimethylglyoxime method. **A)** Plant grown for 6 weeks in MS medium. **B)** The same plant (A) pressed against a piece of filter paper impregnated with detection reagent, together with the imprint it leaves on the filter (**m**); the right top corner of the picture contains, for comparison purposes, the signal left by the reaction of a 250 $\text{mg} \times \text{mL}^{-1}$ NiSO_4 solution (**r**). **C)** Plants grown for 6 weeks on MS-Ni medium (500 $\text{mg} \times \text{mL}^{-1}$ NiSO_4). **D)** The same plants (C) pressed against a piece of filter paper impregnated with detection reagent. **E)** Dark red imprint left by the plants shown in (C). The black arrows point to the most vividly colored areas

When the same procedure was performed (Figure 1C, 1D and 1E) with plants grown on NiSO₄-supplemented culture media, all organs left a vivid red imprint on the filter paper (Figure 1E). This result indicates that *Leucocroton havanensis*, when cultured “in vitro”, is able to transport NiSO₄ from the culture medium, accumulating it in every organ of the plant. In Figure 1E the most intensely colored areas correspond to the apical bud.

Determination of Ni concentration by Inductively Coupled Plasma Optical Emission Spectroscopy

Determinations of Ni concentration for “in vitro”-cultured plants were performed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). The concentration of this mineral in plants grown without NiSO₄ supplementation was 58.45±1.47 µg×g⁻¹, and nickel concentrated predominantly in their cotyledonary leaves (Figure 1B). By comparison, Ni concentration in plants grown in culture medium containing 500 mg×L⁻¹ of NiSO₄ was 5000.03±296.79 µg×g⁻¹, indicating that the plants actively absorb nickel from the medium and, therefore, the species *Leucocroton havanensis* hyperaccumulates Ni when cultured “in vitro”.

DISCUSSION

The present work reports that *Leucocroton havanensis*, an endemic species from serpentine soils of Cuba, is able to hyperaccumulate nickel during “in vitro” culture; a finding that will facilitate closer examination of the molecular basis of this phenomenon. Examining molecular mechanisms that are difficult to study in natural habitats is, precisely, one of the most frequent applications of “in vitro” culture (Che & al. 2002, Che & al. 2006, Su & al. 2006).

NaClO has found wide application as a disinfecting agent for surface sterilization of starting plant material during “in vitro” culture (Yucesan & al. 2007, Steinmacher & al. 2007, García & al. 2008). In our case, however, the disinfection of *Leucocroton havanensis* seeds with this agent inhibited their germination permanently; a symptom suggesting the presence of embryo damage (Ramírez-Villalobos & al. 2002). Disinfection with HgCl₂ was, however, very efficient, and the seeds germinated after 7 to 10 days of culture on MS medium. Similar results were reported by Daquinta & al. (2003) while studying “in vitro” culture techniques to propagate forest and bamboo species such as *Gradua angustifolia* and *Dendrocalamus strictus*. The high rates of seed germination observed during the present work suggest that embryo viability is optimal and there is no dormancy, in contrast with previous reports such as that of *Stackhousia tryonii*, an Australian Ni-hyperaccumulator grass exhibiting long dormancy periods that are only broken by forest fires in its natural habitat (Bhatia & al. 2005), requiring the use of axillary buds as starting material for propagation “in vitro” (Bhatia & al. 2002).

As mentioned above, the present work demonstrated that *Leucocroton havanensis*, when cultured “in vitro”, is able to absorb NiSO₄ from the culture medium and accumulate it throughout the plant (Figure 1C, 1D and 1E). The levels of Ni accumulation of “in vitro”-grown plants, according to ICP-OES determinations, are comparable to those found in samples taken from its natural habitat (Reeves & al. 1996).

De Souza & al. (1999) and Whiting & al. (2001) reported that soil microflora plays an important role in the incorporation of heavy metals by hyperaccumulator species. However, when *Leucocroton havanensis* was cultured under aseptic conditions, it still exhibited a Ni-hyperaccumulator phenotype. This finding coincides with a previous report for *Alyssum markgrafii*, which hyperaccumulated nickel in calluses and shoots at 2.65 µg×g⁻¹ and 2.37 µg×g⁻¹ respectively when cultured in MS medium supplemented with this metal (Vinterhalter & Vinterhalter 2005). The results, therefore, indicate that the soil microflora is not indispensable for nickel hyperaccumulation in these species. In addition, the fact that calluses are able to hyperaccumulate nickel demonstrates that the process does not require fully formed organs such as roots, being instead an intrinsic property of cells and tissues from particular species.

The presence of hyperaccumulated Ni in the cotyledonary leaves of control plants grown in the absence of this metal (Figure 1B) demonstrates that nickel, in this case, was not obtained from the culture medium, but was already present in the cotyledons, generated during seed formation. In other words, this result indicates that *Leucocroton havanensis* also accumulates Ni in the embryo; specifically in the cotyledons.

Nickel hyperaccumulation during growth “in vitro” was first reported for *Hybanthus floribundus* (Violaceae family), an endemic species from serpentine soils of southern Australia (Bidwell & al. 2001). Micropropagated shoots of this species maintained the nickel hyperaccumulator phenotype despite the absence of roots, and exhibited, similar to our results, dose-dependent hyperaccumulation.

Previous studies in *Leucocroton flavicans* (a species closely related to *L. havanensis*) have shown nickel hyperaccumulation to be most intense at the stem, close to the apical bud and to young leaves (Berzaín & al. 2007). A similar phenomenon was observed on “in vitro”-cultured *L. havanensis* seedlings, confirming the importance of this mineral for growth in these species (Borhidi 1988). Surprisingly, there were no statistically significant size differences between plants grown in the presence or absence of nickel, both here with *L. havanensis* (data not shown) and in a previous report for *Hybanthus floribundus* (Bidwell & al. 2001).

Heavy metal hyperaccumulator plants have obvious application in phytoremediation and phytomining (Robinson & al. 1999). *Leucocroton havanensis*, a shrub from serpentine soils whose leaves contain close to 2% Ni (dry weight), can potentially be used for this purpose (Reeves & al. 1999).

The results of the present study clearly indicate that this species also exhibits a Ni-hyperaccumulator phenotype during growth "in vitro". This finding can be leveraged for further studies of the molecular mechanism underlying this phenomenon in the *Leucocroton* genus.

CONCLUSIONS

We recommend a 10-minute incubation in a solution of 0.05% HgCl₂ containing 1% Tween-20 for disinfecting seeds of *Leucocroton havanensis* prior to their propagation "in vitro", for which they constitute an excellent starting material. The best results to the semi-quantitative Ni detection by the dimethylglyoxine method was the most intense red color for the plants grow in 500 mg×L⁻¹ of NiSO₄. The nickel accumulated in cotyledonary leaves of control plants does not originate from the culture medium, but from the cotyledons, organs that develop during seed formation. Under "in vitro" culture conditions, *L. havanensis* absorbs nickel from the culture medium and hyperaccumulates it in all organs of the plant. Ni hyperaccumulation does not depend on pre-existing soil microflora or the presence of organized structures, constituting instead an intrinsic property of cells and tissues of certain species. "In vitro" cultures of *L. havanensis* can be used as starting material for studying the molecular mechanisms underlying the phenomenon of nickel hyperaccumulation in the *Leucocroton* genus.

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