



UNIVERSIDAD AUTÓNOMA CHAPINGO

DEPARTAMENTO DE FITOTECNIA

UNIVERSITÉ DE LORRAINE

ECOLE DOCTORALE SIRENA



**PLANT METAL HIPERACCUMULATION IN MEXICO:
AGROMINING PERSPECTIVES**

**PLANTAS HIPERACUMULADORAS DE METALES EN MEXICO:
PERSPECTIVAS PARA LA AGROMINERÍA**

**THESIS IN JOINT SUPERVISION
WITH INTERNATIONAL MENTION**

Submitted as a partial requirement to obtain the double degree of:

**DOCTOR EN CIENCIAS EN AGRICULTURA MULTIFUNCIONAL
PARA EL DESARROLLO SOSTENIBLE
(UACH, México)**

**DOCTOR IN AGRONOMIC SCIENCES
(UL, France)**

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APROBADA



Noviembre 2021

Chapingo, Estado de México

Thesis approval document by the advisory committee

Plant Metal hyperaccumulation in Mexico: agromining perspectives

Thesis carried out by **Dulce Montserrat Navarrete Gutiérrez** under the supervision of the committee advisor indicated, approved by the same, and accepted as a partial requirement to obtain the grade of:

DOCTOR OF SCIENCE IN MULTIFUNCTIONAL AGRICULTURE FOR SUSTAINABLE DEVELOPMENT

DIRECTOR MEXICO:



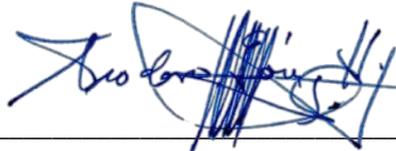
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To my parents and grandparents

A mis papas y abuelitos

Acknowledgements

A personal and professional adventure that I never imagined and that I will never forget. I am very proud of this thesis, a small contribution to science and the fruit of the effort and dedication not only of me but of many people. All these people contributed in different ways and were indispensable to the culmination of this research. These words that I write are not enough to express my sincere gratitude.

I would like first to thank my thesis director in France, Guillaume Echevarria, for stimulating my scientific curiosity and inspiring me with his energy and passion for discovery and learning. Thank you for always trusting me, for the adventures during field explorations, for your friendship, and for all the advice and support during these 4 years of thesis. I also express my gratitude to my thesis co-director in France, Marie-Noëlle Pons, for her valuable feedback and assistance, mainly at the end of the PhD.

I would like to acknowledge Dr. Jesús Cuevas, my thesis director in Mexico, for his unconditional support, his valuable teachings and for always reminding me the relevance of the cultural aspect in research.

To my following-up committee members in France (Joe Pollard and Laetitia Minguez) and in Mexico (Dr. Teodoro Gómez) for their disposal and their precious guidance during my thesis. Your feedback, always constructive, helped me to keep the thread of the research and to question my work from different perspectives.

My sincere gratitude to Christina Siebe Grabacht and Clístenes W. Araújo do Nascimento, for the careful revision of this manuscript and for your participation in my thesis defense, it was a really honor for me.

I express my sincere thanks to my collaborators in France, Michel Cathelineau; in USA, Joe Pollard, Grace McCartha and Halley Disinger; in Australia and the rest of the world, Alain Baker, Antony van der Ent and Philip Nkrumah. Thank you for your guidance during field explorations, for your help in the analyses, for sharing all your expertise, and for your substantial contribution to the articles. It was a real pleasure and an honor to have collaborated with you.

I want to specially thank the “Consejo Nacional de Ciencia y Tecnología” in Mexico (CONACYT), for sponsored my studies of master and doctorate in France. I also thank the “Agence Nationale de la Recherche” (ANR) through the program "Investissements d'avenir" ANR-10-LABX-21 -RESSOURCES21, and the “Centre National de la Recherche Scientifique” (CNRS) through the X-LIFE Research Program for their financial support for this research project.

I am deeply grateful to all members of the “Laboratoire Sols et Environnement (LSE)”, specially to Christophe Swartz, the head of the laboratory, for accepting me into the research unit and for all your support during all these years. I want to specially thank the technical team: to Lucas Charrois and Romain Goudon, for your patience, for always helping me with the analyses in the laboratory and for answering all my questions; to Adeline Bouchard, for managing so well the quality in the laboratory, for your good mood and your availability; to Alain Rakoto, for all your precious and effective help in computer science and for your sympathy; to Stéphane Colin, for your always good attitude and your great assistance; and finally to Jean-Claude, for your help in grinding the roots of my plants and for the jokes that I rarely understood hahaha.

My sincere gratitude to Laetitia Despouy, for handling the administrative part of the LSE laboratory so well, I really appreciate all your support, especially for my displacements to Mexico.

Thanks to all the professor-researchers and researchers in the LSE laboratory, to Stéphanie Ouvrard, Catherine Sirguy, Apolline Auclerc, Thibault Sterckeman, Geoffroy Séré, Pierre Leglize, Noelle Enjelvin, Frédéric Darboux, Sophie Leguedois and Gaylord Machinet (Microhumus). Thanks for the short or long discussions we had, I am glad to have shared good moments with you. I regret that my insecurity did not let me to interact a little bit more. I want to particularly thank Jean-Louis Morel, for being an excellent co-supervisor during my master internship and to encourage me to continue my doctorate in the LSE.

Many thanks to all my labmates in the LSE (doctorate students and new doctors), it was a real pleasure to have met all of you and to have shared both, good and bad moments. Thanks for the moral, scientific, and linguistic support you gave me. Thanks to you, I can almost pronounce "mon œil" perfectly XD . I had a lot of fun during the coffee breaks, lunch, parties, cinemas, Saint-Nicolas race, and the doctoral weekends in the Vosges. Thanks to the old doctorate students (now doctors), Ramez, Ali, Marie, Robin, Quentin, for their warm welcome to the laboratory in 2015. I

wish to also thank the postdocs we had in the lab, to Clement for the always interesting conversations and to Tino, for your cheer and support. Anne, thanks for your particular sense of humor; Clementine, thanks for your constant kindness and caring, and also for cooking such delicious desserts; Severine, thank you for always being so nice with me, for organizing my “cagnotte” and for my wonderful thesis album; Victor, thanks for your help in R, for the discussions, for correcting my refined French XD , and for the knitting classes. Gwendal, thank you for livening up the atmosphere when you arrived at the lab and for your concern for others; Alexis, thank you for being my office colleague for a few months, for the discussions, for being quiet that I could write in peace (not as in the TD4 hahaha), for your kindness and your passion for research; Gabin, thanks for being my second officemate, although it was complicated due to the pandemic, thank you for sharing with me the difficult stage of the thesis writing and for the encouragement; Serge, thank you for the discussions, I send you much encouragement and strength to finish your last year; Xueqian or “Shuetie” for me hahaha, thank you for your good mood, for the laughs and the outings to the "cafe de langues".

Romane, you arrived at the lab a year after me and I knew from the first moment that you would become one of my best friends. We share the same thesis director and even almost the same thesis topic but in such different places haha. I liked it when people asked us, "What is your thesis topic?" and you answered : the same as Dulce but in Malaysia haha. Thank you for having been my great support, both scientifically and emotionally, I will always be grateful for your consolation when I was sad or down, especially at the end of my thesis. Petit Julien, thank you very much for the trust you had from the beginning, friendships like yours nourish the soul. I am proud of what you have accomplished and what you will continue to accomplish. You and Romane are my best thesis gifts. Thank you very much for being the way you are, and even though I don't know what will happen with our lives in the next years, I am sure we will continue the friendship.

I will never forgive the interns in the LSE, Alice, Antonin, Vincent, Marina, Zeinab, Dani, etc... you were simply exceptional. Specially Dani, thank you very much for taking care of my baby plants in the phytotron during my fieldwork in Mexico.

I want to express my sincere gratitude to Christine Fivet and Estelle Barranco, for their incredible work managing everything related to administration of doctorate students at the doctoral school, bravo. Many thanks to Savrina Divoux, for your assistance for the co-mentorship agreement.

Mi sincera gratitud al anterior y actual director del "Departamento de Fitotecnia" de la Universidad Autónoma Chapingo (UACH), Dr. Juan Martínez y Dr. Oscar Mascorro, por aceptarme en esta honorable universidad y por ayudarme a afrontar la complejidad de hacer un doctorado en doble titulación y en dos países. Estoy profundamente agradecida al Dr. Raúl Nieto por todo el apoyo recibido y por haberme ayudado a establecer los acuerdos con la UACH. Un reconocimiento especial a Angeles Pérez, Ana Candelas y Rogelio Deheza por su asistencia en trámites administrativos y por toda su amabilidad. Muchas gracias a Ma. De los Ángeles Cruz por su ayuda en el establecimiento de mi convenio de co-tutela. Gracias al Dr. Joel Nieto por todo el apoyo.

Muchas gracias a mis colegas de doctorado en agricultura multifuncional de la UACH, de las distintas generaciones: Indu y Reylbeck de la primera, Diana y Daniel de la segunda, Lulu, Lau, Vane, Asuka, Luis, Gera, Paty y Hugo de la tercera. Imagino como debo haberlos confundido con mi situación nada estable, entre idas y vueltas Mexico - Francia. Agradezco mucho su apoyo, y disfruté mucho haber compartido con ustedes clases y reuniones. Los admiro mucho y estoy muy orgullosa de todos ustedes. Un agradecimiento especial también a todas las personas que me ayudaron, me dieron palabras de ánimo o solo una risa contagiosa en mi paso por la UACH, por ejemplo Cami, Rubí, Prisco, Tomás y Laura.

Quiero agradecer enormemente a mis invaluable colaboradores de la Universidad Tecnológica de la Selva (Lacandona) en Chiapas, al Biólogo Jorge Arturo Ramírez, por haber facilitado el trabajo de investigación en campo, por su energía y su siempre buena disposición. A los estudiantes de la carrera de "Manejo y Conservación de Recursos Naturales": Nancy, Simón, Moisés, Francisco, Sebastián y Saúl, por haber decidido hacer sus prácticas conmigo, por su curiosidad y ganas de aprender, y por haber trabajado de forma excelente en sus proyectos de investigación. Los datos obtenidos contribuyeron a la realización de esta tesis.

Muchas gracias a los que facilitaron las exploraciones en Chiapas, a Carlos Chambor y Saúl Miguel, gracias a ustedes y sus conocimientos. Gracias a la familia Top Ché por su amabilidad y gran apoyo. Mi sincera gratitud a Rosamond Coates, por todas las facilidades durante nuestra

estancia en la Estación de Biología Los Tuxtlas, sin su ayuda no habiéramos podido completar con éxito la misión. Mis reconocimientos a Santiago Sinaca, quedamos muy sorprendidos con tu habilidad para reconocer las especies de plantas en campo, muchas gracias por todo.

Quiero expresar mi gratitud al personal del herbario MEXU, especialmente a Blanca Verónica Juárez, por su amabilidad y gran apoyo en la determinación de las especies de plantas que colectamos en campo. Muchas gracias a Vanessa Invernon, responsable del herbario P del MNHN de Paris, por su asistencia y gentileza. A Lourdes Rodas y a María Álvarez, de los herbarios USCG y UVAL en Guatemala, por permitirnos hacer análisis de los ejemplares de herbario.

Muchísimas gracias a todos mis amigos, que no se hartaron de mis quejas sobre la tesis y de lo difícil que se me hacía siempre, pero sobre todo de sacarme un poco de mi estrés constante y hacerme reír en momentos duros y lo siento por a veces no estar disponible. Mi Ilse, gracias por estar siempre pendiente de mí, perdóname por no haber estado en momentos importantes de tu vida en estos últimos años, sabes que te quiero muchísimo a pesar de la distancia. Araviux, tqm, gracias por esas pláticas que me llenan de alegría cada que vuelvo a Puebla, me recargaban baterías. Tiki, gracias por tu amistad, me dio ánimos para seguir. Indu, gracias por tu apoyo incondicional, por las pláticas de 3 horas por teléfono jajajajaa para ponernos al día, me siento muy afortunada de tenerte como amiga, ánimo con esa tesis. Lulú y Lau, muchas gracias por todo su cariño y apoyo, son fuente de inspiración para mí.

A mi grupo de latinos en Francia, muchas gracias por todo el apoyo, los quiero mucho: Manu, Clau, Andrés, Nata, Pau, Cony, Leyla, Sergio, Audrey y Lore. En especial a Manu y Claudia, mis escapadas a Paris con ustedes me daban alivio y me motivaban a continuar. Andrés y Natalia, gracias por las tantas discusiones (filosóficas o no jajaja) y por levantarme el ánimo. Paula, mil gracias por estar cerca (aunque fuera con llamadas) en cada momento, me encanta ver como nuestra amistad ha ido creciendo poco a poco. A los amigos que hice durante estos años y que ahora están repartidos por el mundo, muchas gracias: Valentina, Cami, Cata, Meli, Moi, Damien, Anne-Claire, Aramis, Magali, Aliz.

Mis adorada Carmencita, tqm, muchísimas gracias por tu linda amistad (de hace 4 años omg! jajaja) y por animarme cuando lo necesité. Mariana, te agradezco como no tienes idea el haberme invitado a hacer coloc contigo, me permitió conocerte mucho más y fortalecer la amistad; tu

ejemplo me motivó siempre a terminar la tesis a pesar de las dificultades, ahora tienes un lugar muy especial en mi corazón, te quiero mucho. Jordan, merci beaucoup pour les mots d'encouragement, d'aimer Mariana et d'être un si bon propriétaire, je te promets de mieux prendre soin de la maison hahaha.

Merci beaucoup les filles !!, Estibaly, Claudia, Estelle, Noemí, Caro, Shirin. Merci pour les petit déjeuner devant Saint Epvre, les rires au barami, les pique-niques dans le parc de la pépinière, les concerts de Nancy Jazz, les soirées, les barbecues, les danses, enfin, tous les moments incroyables que j'ai passés avec vous. Un grand merci à mon ex-coloc Pauline, de m'avoir supporté à la fin de la thèse et avoir été si gentil avec moi.

Merci beaucoup Damien, mon coeur, tu es arrivé à la fin de ma thèse et je pense que c'était le meilleur moment. Merci de me soutenir autant et de ne pas fuir avec mes crises d'angoisse et mes sautes d'humeur hahaha. Je suis très heureuse avec toi mi osito.

Finalmente, termino con la parte más importante, mi familia. Definitivamente de no ser por ustedes, no hubiera llegado hasta aquí. Mami, gracias por haber trabajado tanto para darme una buena educación, por motivarme a estudiar idiomas, gracias por haber confiado en mí cuando te dije que quería cambiar de profesión e irme a estudiar fuera de México, tal vez ya presentías que sería por más de un año (ahora mas de 5 años!!) y aun así me animaste, gracias por ser mi mejor amiga y estar siempre cerquita de mí, te amo hasta el infinito. Papá, gracias por todo tu esfuerzo por darme una buena educación, por animarme siempre, por todos los consejos sabios que siempre me has dado por ser el mejor papá que una hija pueda pedir jeje. Esta tesis también es suya, mamá, papá, les voy a entregar su título de doctores. Papá, mil gracias por colaborar conmigo, por acompañarte a todas las exploraciones en campo, por emocionarte conmigo con los descubrimientos, por hacerte cargo de gran parte de los experimentos en Chiapas, por estar pendiente de los estudiantes de la Selva; mami, muchas gracias por ayudarme en el herbario de la UNAM a hacer los análisis, por ayudarme a ordenar mis muestras, por acompañarme a veces a explorar y por emocionarte, por escuchar mis historias de la tesis y nunca cansarte. Pandota Carlos, muchas gracias por ser mi hermano, estoy muy orgullosa de ti, te quiero muchísimo y disfruto como no te imaginas los momentos que pasamos en familia. Anita, gracias por las pláticas cuando coincidíamos en Puebla, los mensajes de ánimo que siempre me mandabas, las fotos y videos de

bombachin, que me hacían ver como crecía poco a poco mi hermoso ahijadito jejeje, hubo momentos que solo ver la risa de bombachín me animaba a seguir escribiendo jejeje. Pandota Paco, muchas gracias por estar confinado conmigo tres meses en Nancy jeje, me ayudaste mucho a poner en orden mis ideas y sobre todo me diste mucho ánimo en ese momento especialmente difícil, te admiro y te quiero muchísimo. Tía Lupita (pipis), te quiero mucho, muchas gracias por darme siempre ánimos para seguir adelante y por cuidar a mi abuelita. Gracias a mis tíos, Felipe, Chucho y Victor, que me daban ánimos desde el cielo, los recuerdo con mucho cariño. Gracias a mis primos, Richi, Chuchin, Felipito, me reconfortaba mucho estar con ustedes en familia, los quiero. Gracias, tío Raúl por tu apoyo y tus bromas.

Gracias inmensas a mis abuelitos, que se me fueron durante estos años de tesis. Doy gracias a la vida de haber tenido estos abuelitos tan increíbles, y por haber tenido la oportunidad de disfrutarlos y quererlos tanto. Mi Nichito, tu sola presencia me daba tanta paz, extraño tanto tus historias, gracias por enseñarme a actuar con valores. Mi Quechita, nunca entendí lo apegada que estaba a ti hasta que te fuiste en cuerpo, agradezco tanto las largas pláticas que tuvimos, las risas, las películas en blanco y negro que vimos juntas, los churros con chocolate que nos tomamos, los masajitos de espalda que nos dábamos, los abrazos que me llenaban de alegría el alma. Nichito, Quechita los llevo siempre en mi corazón, esta tesis es para ustedes también, su amor siempre me recordó lo realmente importante en la vida.

Gracias a la Dios y a la Virgen, por tantas bendiciones.

Plant metal hyperaccumulation in Mexico: agromining perspectives

Agromining technology involves the recovery of strategic metals from metalliferous soils through the cultivation of metal(loid) hyperaccumulator plants. The impetus of this research was to evaluate the potential of Mexican plant resources for the future development of agromining. The main objectives were then to identify and to study some metal hyperaccumulator plant species in Mexico, and to assess the agronomy of one promising “metal crop” for agromining. Field explorations in three nickel-rich ultramafic regions of central and southern Mexico were undertaken. Despite the availability of soil and climatic conditions, no nickel (Ni) hyperaccumulation was found in any of these regions. A second strategy based on plant phylogeny as a prediction tool for metal hyperaccumulation was followed. In total, ten plant metal hyperaccumulator species were identified during this research (Rubiaceae and Violaceae) in Ni-enriched soils influenced by volcanic activity in Southeastern Mexico; most of them were priorly unknown. The studies revealed two of the strongest hypernickelophores reported so far (>4%wt Ni) and two new ones Ni hyperaccumulator genera (*Orthion* and *Mayanaea*). Special focus was given to the hypernickelophore tree *Blepharidium guatemalense*. The phloem on leaves, roots, stems and petioles of this plant are the richest in Ni suggesting an unusual re-distribution mechanism via the phloem. Different agronomic practices were tested for this plant. Synthetic fertilization strongly increased nickel uptake without any change in plant growth or biomass, whereas organic fertilization enhanced plant shoot biomass with a negligible effect on foliar Ni concentrations. A 5-year-old stand which was subsequently harvested twice per year produced the maximum Ni yield tree⁻¹ yr⁻¹, with an estimated total nickel yield of 142 kg ha⁻¹ yr⁻¹. *Blepharidium guatemalense* is a prime candidate for Ni agromining on the account of its valuable traits: extremely efficient Ni uptake, high biomass production, fast growth rate, and easy to reproduce.

Keywords: metal hyperaccumulation, metalliferous soils, Mexico, agromining, nickel, biogeochemistry.

Plantas hiperacumuladoras de metales en México:

Perspectivas para la agrominería

La agrominería se ocupa de la recuperación de metales estratégicos de los suelos metalíferos mediante el cultivo de plantas hiperacumuladoras de metales (y metaloides). El motor de esta investigación fue evaluar el potencial de los recursos vegetales mexicanos para el desarrollo de la agrominería. Los objetivos principales fueron identificar y estudiar algunas especies vegetales hiperacumuladoras de metales en México, y evaluar el potencial agrícola de una de estas especies con características prometedoras para la agrominería. Primero realizamos exploraciones en tres regiones ultramáficas enriquecidas en níquel (Ni) del centro y sur de México. A pesar de la disponibilidad de níquel en el suelo y de las condiciones climáticas, no se encontró hiperacumulación de Ni en estas regiones. Se siguió una segunda estrategia basada en la filogenia de las plantas como herramienta para predecir la hiperacumulación de metales. En total, en esta investigación se identificaron diez especies hiperacumuladoras de metales (Rubiaceae y Violaceae) en suelos enriquecidos en Ni influenciados por la actividad volcánica, en el sureste de México; la mayoría de ellas no fueron identificadas como hiperacumuladoras. Nuestros estudios revelaron dos de los hiperníquelóforos más potentes detectados hasta ahora (>4% de Ni en peso) y dos nuevos géneros hiperacumuladores de níquel (*Orthion* y *Mayanaea*). En la segunda parte de la investigación está dirigida al estudio de la hiperacumuladora *Blepharidium guatemalense*. El floema de las hojas, raíces, tallos y pecíolos de esta planta es muy rico en Ni, lo que sugiere un mecanismo de redistribución del Ni a través del floema. Se probaron diferentes prácticas agronómicas para esta planta. La fertilización inorgánica aumentó en gran medida la absorción de Ni sin alterar el crecimiento o la biomasa de la planta, mientras que la fertilización orgánica aumentó la biomasa de la planta con un efecto insignificante sobre las concentraciones de Ni en las partes aéreas. Una parcela con un cultivo de 5 años, con una cosecha de dos veces al año, produce el máximo rendimiento de Ni de 142 kg ha⁻¹ año⁻¹. *Blepharidium guatemalense* es un candidato ideal para la agrominería de Ni debido a sus valiosas características: absorción de Ni extremadamente eficiente, alta producción de biomasa, rápida tasa de crecimiento y facilidad de reproducción.

Palabras clave: hiperacumulación de metales, suelos metalíferos, México, agrominería, níquel, biogeoquímica.

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General Introduction

In recent decades, global demand for strategic metals has strongly increased and so the volume of wastes produced by the mining industry. Some metals, including nickel (Ni) and cobalt (Co) are now considered strategic or even critical raw materials. Thus, the creation of strategies to recycle metals under a context of “circular economy” has gained much importance in the last years. One of these strategies is agromining, that allows the recovery of metals from secondary sources from which the extraction by conventional metallurgical methods are not economically attractive. Agromining is a type of phytomining involving the recovery of strategic metals from metalliferous soils through the cultivation of metal(loid) hyperaccumulator plants which can accumulate extraordinarily high concentrations of metals in their aerial tissues. Agromining falls into the “phytoremediation” techniques since it slowly reduces metal toxicity in soils with the possibility to obtain metals from the pyro/hydrometallurgical processing of the harvested plant biomass. Although the concept of “metal farming” is relatively recent, the technical and economic viability of this technique for certain metals (e.g. nickel) has been demonstrated and its environmental effects (positive and negative) are constantly being assessed. Long-term experiments evidenced maximum nickel yields of 112 kg Ni ha⁻¹ in Albania and of 400 kg Ni ha⁻¹ in the USA. However, most investigations have been carried out under a Mediterranean climate (e.g. Albania) and have focused only on a few hyperaccumulator species. Indeed, not all hyperaccumulator species are suitable candidates for agromining but only those who possess specific traits such as very high biomass production rate and metal uptake. The search of new species of metal “hyperaccumulator” plants and the assessment of their suitability for metal agromining are key steps for the expansion of the agromining technology well beyond its current limits (including geographic).

Even though the number of hyperaccumulator plant species identified globally is constantly increasing, some regions in the world remain understudied, and therefore their agromining suitability potential. In the tropics, some hotspots of hyperaccumulators biodiversity such as New Caledonia and Cuba possess potential candidates for agromining. Nevertheless, at the global scale, only the agronomic performance of two nickel “metal crops” has been tested for nickel agromining at a pilot-scale in Sabah (Malaysia); these test crops showed very promising results in terms of nickel yields.

Mexico is among the 12 megadiverse countries that together hold between 60 and 70% of the total biodiversity of the planet (genetic, species and ecosystem level). Moreover, Mexico is the fourth largest floristic richness country in the world with around 23 000 vascular plant species and of which about 40% is endemic. The factors that most contribute to the great Mexican biodiversity are: i) the topography, ii) the large variety of climates, and iii) the complex geological history, resulting in a mosaic of environmental and micro-environmental conditions that creates a wide variety of habitats. Indeed, some climatic variations (e.g. atmospheric humidity, intensity of irradiation, temperature, etc.) result from changes in altitude as well as from the alignment of the mountain ranges and the shape of the country's coastline. Biogeographically, Mexico is the transition zone between the Neotropical and the Nearctic region which favored a rich mix of flora from the moment they made contact (~ 6 million years ago). There are six ecological regions identified in Mexico: i) tropical warm-humid, ii) tropical warm-sub-humid, iii) temperate humid, iv) temperate sub-humid, v) arid and semi-arid, and vi) sea-land transition. The arid and semi-arid regions occupy more than half of the country's territory followed by the sub-humid temperate regions (20%) and the warm-sub-humid tropical zones (18%). Furthermore, more than 20 mafic and ultramafic complexes, which are typically primary habitats for nickel hyperaccumulator species, are distributed in Mexico from the northwest in Baja California to the southeast in Chiapas. Despite its apparent potential in terms of plant diversity and nickeliferous soils, no research has ever been carried out in the field of metal hyperaccumulation or agromining/phytomining in this country nor in the Mesoamerican Region at large. Under this context, the following scientific questions guided the research work:

- 1) Is there nickel (or other metal) hyperaccumulation occurring in the flora of some selected ultramafic complexes existing in Mexico? What could be the characteristics that seem to determine the occurrence of nickel hyperaccumulation in the flora of ultramafic regions? In the case of the discovery of metal hyperaccumulation in non-ultramafic substrates: what are the possible contributors to the metal-enrichment in those soils?.
- 2) Knowing the phylogeny of metal hyperaccumulation in a given plant family, is it possible to predict and to discover new hyperaccumulator plant species? In the case of the successful discovery of metal hyperaccumulator species, many specific questions could be addressed:

What is their biochemistry? What is the geographic distribution of metal hyperaccumulation in those species? Are they restricted to metalliferous soils? How is the distribution of the metal(s) within their tissues? Do they accumulate more than one metal? Do they share similar characteristics with other hyperaccumulator species reported worldwide?

- 3) In case of the discovery of several metal hyperaccumulator species in Mexico: Are there some species that possess desirable traits to be good candidates for “metal cropping” ? How do those traits could be responsibly exploited for agromining ? What is the appropriate agronomic management for the optimization of the metal yield?

Therefore, to fully evaluate the potential of Mexican natural resources for the development of agromining, the main objectives of this research were:

- 1) The identification of metal hyperaccumulator plant species in targeted metalliferous regions of Mexico;
- 2) The study of the discovered metal hyperaccumulator plant species (ecology, taxonomic and geographic distribution, phylogeny, biochemistry, metal localization in plant tissues) in Mexico;
- 3) The selection of one promising “metal crop” among potential hyperaccumulators discovered and the assessment of its agronomic potential for nickel agromining in Mexico.

To address these objectives, this thesis is structured in four chapters, the first chapter corresponds to the state of the art which shows the most recent stage of the research in the fields covered by this work emphasizing on the hyperaccumulation phenomenon as a tolerance mechanism of plants to metal toxicity as well as the practical application of these plants for agromining. The second chapter includes the results of the geobotanical exploration in three different ultramafic regions in central and southern Mexico. The third chapter exhibits the results of the phylogenetic prediction and search of metal hyperaccumulation in other metalliferous regions of Mexico. Finally, the fourth chapter reveals the potential of one selected hyperaccumulator species for nickel agromining in southern Mexico. As the selected format of this thesis is by compilation of published or submitted works, the materials and methods are described in each article.

The project X-TrEM aims to study the ionomics and transcriptomics of hyperaccumulator plant species around the world. It belongs to X-LIFE program of the Centre National de la Recherche Scientifique (CNRS) and has financed part of this research work.

Chapter I

State of the art
From metalliferous soils to Ni agromining

The objective of this chapter is to present the state of the art that served as the basis for this research. It is composed by three main sections: the first one widely describes metalliferous soils, the second one explains the strategies of plants against metal toxicity including metal hyperaccumulation, the ecology and their distribution of such hyperaccumulating plants all over the world and the existing methods for the search of these unusual plants. This section also includes the review of the last knowledge of the molecular mechanisms involved in plant metal hyperaccumulation. Finally, the technology of agromining (phytomining) is explained in detail as a sustainable approach to benefit from the extraordinary accumulation ability of hyperaccumulator plants while valorizing degraded metal enriched soils.

A soil is an organic/inorganic layer on the earth's surface which is in constant evolution and in dynamic equilibrium with the atmosphere and biosphere as well as with the geological sediments (Sumner, 2000). Soil, which is formed by slow and permanent processes of weathering and pedogenesis, is also considered an open system with specific physical, chemical and biological properties that result from the physical and chemical equilibrium between its different phases (solid, liquid and gaseous) (Kabata-Pendias and Pendias, 2001). The soil solid phase is made up of both mineral compounds and organic matter. The earth's crust (lithosphere) provides the chemical elements in the mineral part, while the atmosphere provides CO₂ and water, and sometimes N, which are the main components of organic matter (Sumner, 2000).

Chemical elements in soils are commonly categorized in major (O, Si, Al, Fe, Ca, Na, K, Mg, Ti, and P) and minor (i.e. *trace*) (B, Cl, Cu, Mo, Se, Co, Zn, Cr, Ni, Cd, etc.) elements. Major elements are present in sufficient concentrations (> 1%) in the lithosphere that they can form discrete phases able to influence soil properties, whereas minor elements are generally found at very low concentrations (< 0.1 %) so they do not usually affect soil properties (Sumner, 2000). Some trace elements can occur in soils in their metallic form (metal ions) or complexed in more-or-less stable compounds such as oxides, silicates, sulfates and carbonates (Sumner, 2000).

1. Metalliferous soils

The content of trace elements in soils is very variable and it depends on many factors including the concentration of trace elements in the parent rock and the process involved in soil formation. The metalliferous soils, defined as soils enriched in at least one metal ion from the class B and borderline group (Nieboer and Richardson, 1980; Preite et al., 2018), can be thousand-fold higher in some trace elements (e.g. Zn, Cu, Ni, Cr, Cd) resulting from lithogenic, pedogenic or anthropogenic inputs (Kabata-Pendias and Pendias, 2001; Reeves, 2006). Even though it is impossible to get exact representative concentrations of elements in a soil type, “normal concentrations” of trace elements in soils have been estimated and could serve to detect abnormal concentrations (Kabata-Pendias and Pendias, 2001; Sumner, 2000).

Lithogenic origin refers when trace elements are directly inherited from the lithosphere (parent rock) (Kabata-Pendias and Pendias, 2001). The concentrations of trace elements in rocks depend

on many factors such as their mineralogic properties and their capacity of adsorption. Clays and schists, for example, have a great capacity to adsorb metallic ions and are therefore generally more concentrated in trace elements (Rose et al., 1979). Pedogenic inputs concern trace elements of lithogenic origin that have undergone changes in their concentration and distribution in soil layers during soil weathering and pedogenesis (Kabata-Pendias and Pendias, 2001). Anthropogenic activities are also important sources of trace elements in soils such as mine exploitation, agriculture, metallurgy and other types of industry (electronic, chemical, etc.) (Alloway, 1995).

Metalliferous soils can be classified according to their mineral composition as: i) calamine, ii) seleniferous, iii) copper/cobalt-rich soils or iv) ultramafic (serpentine) (Wójcik et al., 2017).

Calamine soils are naturally rich in Zn, Pb and Cd because they derive from galena (PbS), zinc blende (ZnS) and other calamine deposits; they can be found in some European and South Asian countries such as Belgium, France, China and Iran. Seleniferous soils, derived from Se-rich rocks such as Cretaceous shales, are found in Australia, southern Asia, Ireland and USA. Soils rich in Cu and Co develop from argillites and dolomites containing Cu /Co sulphides and occur mainly in Central Africa, in the DR Congo and Zambia (Wójcik et al., 2017).

Ultramafic soils are some of the most important sources of nickel (Ni) and Co to terrestrial ecosystems (Estrade et al., 2015). They derive from ultramafic (ultrabasic) rocks which are defined as rocks containing more than 90% mafic minerals (rich in Mg and Fe) and less than 45% silica (Le Bas and Streckeisen, 1991). Ultramafic outcrops are mainly localized in ophiolite belts along tectonic plates margins (Vaughan and Scarrow, 2003; Echevarria, 2018). Ultramafic rocks are classified based on their modal proportions of olivine, orthopyroxene and clinopyroxene minerals (Le Bas and Streckeisen, 1991). Dunite, harzburgite, lherzolite and pyroxenite are the most frequently ultramafic rocks found in outcrops (Echevarria, 2018), and can experience some metamorphism (O'Hanley, 1996). For example, serpentine 1:1 clay mineral (*i.e.* chrysolite, lizardite, antigorite) could be formed as a result of the “serpentinization” of olivine and pyroxene (hydration under temperature and pressure changes) (Coleman and Jove, 1992; McCollom et al., 2016). Soils derived from serpentines are commonly poor in essential pedogenetic elements (Al, Ca, K, and P) and rich in some trace elements such as Ni, Co, Cr, Fe, Mg and Cd (Proctor and Woodell, 1975; Martin and Coughtrey, 1982). Whereas total Ni content in soils globally varies

between 2 and 750 mg kg⁻¹, ultramafic soils can easily reach 3,600 mg kg⁻¹ Ni (Sparks, 2003) and they are usually low Ca:Mg ratio, limited organic matter and low water holding capacity (Brooks, 1987).

Metals in soils are present in different geochemical forms: water-soluble, exchangeable, carbonate associated, Fe/Mn oxide-associated or organic associated. In general, plants take up metals from the water-soluble and exchangeable fractions. The “phytoavailability” of an element can be defined as its capacity to be transferred from any compartment of the soil to the plant’s root system (Barber, 1995). Many factors can affect metal mobility and availability in soils. Trace metals behave like bivalent cations (e.g. Ni²⁺, Zn²⁺, Cd²⁺) in soil phases and their mobility is controlled by dynamic equilibria between solid and liquid phases. Metal phytoavailability in soils depends mainly on the capacity of retention of soil constituents, the composition of the soil solution and on several soil properties (e.g. pH, redox potential) (Kabata-Pendias and Pendias, 2001). In general, at low pH, H⁺ ions displace metal cations from the cation exchange complex (CEC) of soil resulting in the release of metals into the soil solution (McBride, 1994).

2. Plant response to metal toxicity

There are seventeen essential elements for plant growth, strictly needed to complete their life cycle. They are commonly divided into major (macronutrients) and minor (micronutrients) elements depending on plant nutrition needs. Macronutrients (C, H, O, N, P, K, Ca, Mg and S) are the major constituents of the organic compounds (nucleic acids, proteins, etc.) that build the structure of a plant, so they are required in large quantities. Conversely, micronutrients (Cl, Fe, Mn, B, Zn, Cu, Ni and Mo) are needed in very small quantities acting mainly as cofactors for enzyme activity (Campbell et al., 2012). Mineral nutrition of plants depends partly on the genetic heritage of the plant itself and on many developmental and environmental factors (White and Brown, 2010).

Some metals are essential micronutrients. Plants roots absorb metal cations from the soil solution though passive or active transport (Campbell et al., 2012). The passive transport occurs along a concentration gradient through diffusion across cell membranes, whereas the active transport requires metabolic energy because it takes place against a concentration gradient (Campbell et al.,

2012). The mechanism of absorption depends on the given element; for example, Ni is preferably absorbed passively, while Zn is preferably absorbed actively (Kabata-Pendias and Pendias, 2001).

However, some metals can become toxic for most plants when they are phytoavailable at very high concentrations such as those occurring in some metalliferous soils (e.g. ultramafic soils). Plants can be “sensitive” (negative response to stress up to plant death) or “tolerant” (positive response to stress and survival) to this metal toxicity (Levitt 1980). Sensitive plants may exhibit signs of metal toxicity: for example in the case of Co and Ni toxicity, there may be inhibition of plant growth, decrease in shoot biomass, and chlorosis/necrosis of leaves. These symptoms are the consequence of oxidative stress, inhibition of photosynthesis, and Fe deficiency (competition for nutrient uptake) (Vergnano and Hunter, 1953; Lešková et al., 2017; van der Ent et al., 2018). Tolerance to soil metal toxicity implies an adaptation which results in a change in the physiological response of the plant. Indeed, tolerant plants have developed different strategies to protect their metabolisms from metal toxicity (DalCorso et al., 2019). Thus, plants can exclude, indicate or (hyper)accumulate metallic elements in their aerial tissues depending on their level of metal tolerance (Baker, 1981; McGrath et al., 2000; van der Ent et al., 2013; Wójcik et al., 2017). Metal exclusion is the most common strategy performed by tolerant plants. Some excluders impede the entry of metals in roots while others allow a minimal entry of metals in roots where they are retained and detoxified (Wójcik et al., 2017). Excluders can restrict metal transport to the shoots over a wide range of phytoavailable metal contents in soils up to a critical level (Baker, 1981). Bioindicator plants tolerate metal entry and translocation to the shoots over a wider range than “sensitive” non-tolerant plants up to a certain phytotoxicity limit. In bioindicator plants, shoot concentrations usually reflect metal concentrations in soils (Baker, 1981; van der Ent et al., 2013). Accumulator plants can uptake, translocate, detoxify, and concentrate metals in different plant tissues but preferably in their aerial shoots even from soils with low metal concentrations. Finally, hyperaccumulator plants are very strong accumulators, able to withstand higher phytoavailable metal concentrations in soils than excluders and bioindicators (Baker, 1981; van der Ent et al., 2013).

Ultramafic soils are examples of stressful environments, so their unusual geochemical composition has prompted the evolution of some of the above-mentioned tolerant strategies in plants. Therefore,

ultramafic flora worldwide can be easily differentiated from the flora of neighboring areas because of their high rates of endemic tolerant plant species (Brooks, 1987; Bergmeier et al., 2009). The majority of Ni hyperaccumulators are ultramafic-endemic species and many have very restricted distribution (R. D. Reeves et al., 2018b).

2.1. Metal hyperaccumulation

Metal hyperaccumulator plants are physiologically able to accumulate specific metals or metalloids in their leaves (and other plant tissues) to concentrations that could be thousands of times greater than the typical values found in most plants. The foliar metal (or metalloid) concentration thresholds for “hyperaccumulation” has been established at 2-3 orders of magnitude higher than in plants growing on normal soils and at least one order of magnitude greater than in plants from metalliferous soils (van der Ent et al., 2013). The values determined for a “reference plant” (Markert, 1992), as well as reported critical toxicity levels of metals in plants (Krämer, 2010), can serve as a basis for comparing and identifying any abnormal metal concentration (Table 1). Other characteristics are also common in hyperaccumulators such as high bioconcentration (>1) and translocation factors (>1).

Element	Concentration in “standard plant” (mg kg⁻¹)^a	Critical toxicity concentration (mg kg⁻¹)^b	Threshold for hyperaccumulation (mg kg⁻¹)^c
Cd	0.05	6–10	100
Se	0.02	3–100	100
Tl	0.05	20	100
Cu	10	20–30	300
Co	0.2	0.4–several	300
Cr	1.5	-	300
Ni	1.5	10–50	1000
As	0.1	<2–80	1000
Pb	1	0.6–28	1000
Zn	50	100–300	3000
Mn	200	200–3500	10 000

Table 1. Reference concentrations in “normal” and hyperaccumulator plants. Data from ^a(Markert, 1992b), ^b(Krämer, 2010), and ^c (van der Ent et al., 2013).

2.2. Purpose of hyperaccumulation

Hyperaccumulator plants have developed many physiological adaptations representing an important expense in terms of energy. The fact that this trait has persisted during evolution suggests that it should confer some advantages for the survival of these species.

Many hypotheses have been then proposed to explain the ecological role of metal hyperaccumulation: i) metal tolerance, ii) drought resistance, iii) allelopathic response, iv) consequence of an efficient nutrient uptake, and v) (elemental) defense against herbivores or pathogens (Boyd and Martens, 1992; Boyd, 2007; Pollard et al., 2014).

Among all those possible explanations, the “elemental defense” has obtained the most supporting evidence (Boyd, 2004, 2007). According to this hypothesis, elevated metal concentrations in plant tissues can negatively affect some of their natural herbivores and/or pathogens (but not all of them). Defensive effects of metal hyperaccumulators in herbivores can significantly decrease their performance (e.g. lesser survival, slower growth, preference for low element tissues). However, certain herbivores have been reported to consume metal hyperaccumulator plants without being harmed, in some cases co-evolution can result in the selection adapted highly-tolerant herbivores such as the beetle *Chrysolina pardalina* (Chrysomelidae) which only feeds on the Ni-hyperaccumulator *Berkheya coddii* from South Africa without accumulating Ni in its body and essential tissues (Mesjasz-Przybyłowicz and Przybyłowicz, 2001; Przybyłowicz et al., 2003). Also, there is evidence that Ni hyperaccumulation in *Streptanthus polygaloides* reduced susceptibility to bacterial and fungal pathogens (Boyd et al., 1994). Studies verifying this defense hypothesis exists for only certain elements (i.e. Ni, Zn, Cd, As) whereas the others remain to be tested (Boyd, 2004, 2007).

2.3. Taxonomic distribution (evolution, phylogeny)

By 2018, there were 721 metal (and metalloid) hyperaccumulator plants reported worldwide, of which 532 were Ni hyperaccumulators. Following in number of species, there were hyperaccumulators of Cu (53), Co (42), Mn (42), Se (41), Zn (20), Pb (8), Cd (7), As (5), Tl (2) and other few of rare elements (R. Reeves et al., 2018). These numbers are constantly increasing. For example, recent discoveries in Sabah (Malaysia) have added many new hyperaccumulator

plant species to the global database (at least 18 Mn and 7 Co hyperaccumulators) (Gei et al., 2020b; van der Ent et al., 2019b).

Hyperaccumulator plants are taxonomically scattered in more than 50 families and 130 genera, almost exclusively in angiosperms (Krämer, 2010; R. Reeves et al., 2018). However, Ni hyperaccumulation has been also found in ferns (*Adiantum* genus), bryophytes and liverworts suggesting that this trait could have appeared early in the evolution of terrestrial plants (Brooks et al., 1990; Boyd et al., 2009; Jaffré et al., 2013). The hyperaccumulation character is of polyphyletic origin, occurring in a wide range of unrelated families (Macnair, 2003; Verbruggen et al., 2009). Indeed, this trait has appeared many times during evolution of plants although it is displayed more often in certain lineages. Most hyperaccumulators are within the Eudicots and around one third of the Ni hyperaccumulators belong to the COM clade (Celestrales, Oxidiales and Malpighiales) which form part of the Rosids (Jaffré et al., 2013). A recent massive study of the flora of New Caledonia confirms the predominance of Ni hyperaccumulation in the Oxidiales and Malpighiales (Gei et al., 2020a). The families with the highest number of Ni hyperaccumulator species are the Brassicaceae, Euphorbiaceae, Phyllanthaceae, Salicaceae and Violaceae (Krämer, 2010; R. Reeves et al., 2018; Bouman et al., 2018). In some Ni hyperaccumulator genera (*i.e.* *Buxus*, *Leococroton*, *Phyllanthus*), the large number of Ni hyperaccumulating endemics suggest an active and continuous evolving speciation. On the contrary, some phylogenetically isolated hyperaccumulators such as *Phyllomelia coronata* (Rubiaceae), may be “palaeo-endemics”, *i.e.*, survivals of formerly widespread species adapted to metalliferous soils having no or few closely related species (R. D. Reeves et al., 2018b). In the Mediterranean area, Ni hyperaccumulation is mainly found in the *Odontarrhena*, *Bornmuellera*, and *Thlaspi* genera (Brassicaceae).

2.4. Physiology of metal hyperaccumulator plants

The hyperaccumulation phenomenon is rare since most plants respond with the exclusion strategy when dealing with soil metal toxicity (Krämer, 2010). The complexity of metal hyperaccumulation is linked to the fact that plants must maintain normal cellular functions despite high concentrations of metals in their aerial shoots, which is potentially harmful to sensitive photosynthetic structures and processes (Boyd and Martens, 1992).

The molecular mechanisms involved in metal hyperaccumulation result essentially from the mechanisms involved in plant metal homeostasis. Several molecular studies using hyperaccumulator and non-hyperaccumulator related species (*e.g. Arabidopsis halleri* and *A. thaliana*) have demonstrated that some genes involved in metal hyperaccumulation are not species-specific or novel, but rather genes involved in metal homeostasis which are differentially expressed compared to related non-hyperaccumulator species as a result of gene duplication and/or changes in promoter activity (Krämer et al., 2007; Verbruggen et al., 2009; Merlot et al., 2018). Certain mechanisms involved in metal hyperaccumulation may be specific to a plant family, genus or species whereas cases of convergent evolution among distant species within the same family have also resulted in convergent mechanisms (Merlot et al., 2018).

Metal exclusion and metal hyperaccumulation are two opposed strategies used by plants when facing soil metal toxicity. Whereas most metal excluders avoid metal accumulation in their photosynthetically active aerial tissues (*e.g.* limitation of metal absorption by roots, metal storage in root cell walls and vacuoles, etc.), metal hyperaccumulators need to ensure a metal translocation to the aerial tissues where metals will be finally stored. Thus, metal hyperaccumulation (and associated hypertolerance) involves complex alterations to several processes of plant metal homeostasis (Krämer, 2010; Merlot et al., 2018):

- a) An enhanced metal mobility (within root symplasm) and uptake in roots metal uptake
- b) An efficient radial metal transport and a reduction of metal sequestration in root vacuoles.
- c) An increase of root to shoot translocation of metal, with efficient xylem loading.
- d) An enhanced ability for metal storage and detoxification in shoot tissues.
- e) An efficient mechanism for xylem unloading and metal distribution in shoots together with a higher vacuolar storage capacity.

2.4.1. Mechanisms of Ni hyperaccumulation

Although several studies are still needed to fully understand the molecular basis of these processes, enormous progress has been made. Most of the research has been dedicated to *Arabidopsis halleri* and *Noccaea caerulea* (Zn/Cd hyperaccumulators) because they are relatively recent diverged

species from the non-hyperaccumulator *A. thaliana*. Indeed, a complete genome sequence as well as many genomic tools are available for the latter (Krämer et al., 2007). Studies revealed that *A. halleri* and *N. caerulescens* had constitutively elevated expression of genes involved in the uptake, chelation and xylem loading of Zn/Cd in comparison to related non-hyperaccumulators (Weber et al., 2004; van der Pas and Ingle, 2019).

The lack of genomic resources for Ni hyperaccumulator species has resulted in a reduced number of studies in the mechanisms involved in this phenomenon. However, recent RNA-seq technology has improved the understanding of the molecular basis of Ni hyperaccumulation (van der Pas and Ingle, 2019). Nickel is an essential micronutrient for plants being associated as the metallic co-factor of the enzyme urease, which contains a bi-nickel center. Therefore, Ni hyperaccumulation mechanisms may have derived from the mechanisms evolved for the regulation of Ni homeostasis (Merlot et al., 2018). A proposed model of Ni transport is described below (**Figure 1**):

- 1.- An efficient uptake of Ni²⁺ cations by roots is mediated by divalent metal transporters (e.g. ZIP, NRAMP) located in the plasma membrane of root epidermal cells.
- 2.- Ni is transported through the cortex and the endodermis by a combination of Ni export and import transporter activities. In this step, Ni is chelated by ligands (carboxylic acids such as citrate or malate, or amino acids such as histidine) to reduce its reactivity in the cytoplasm. The formation of these organic complexes may impede vacuolar sequestration of Ni in roots to favor radial transport.
- 3.- In the pericycle, Ni is loaded in the xylem as free cation or as Ni-ligand complex may be by YSK and MATE transporters and transported to the shoots.
- 4.- The mechanisms involved in xylem unloading and transport to the epidermal cell are not well known
- 5.- Ni is transported and stored mainly in the vacuoles of leaf epidermis in most species by IREG/FPN transporters.
- 6.- In the vacuoles, Ni is complexed by carboxylic acids, with Ni-citrate or Ni-malate.

7.- In many species, Ni is redistributed through the phloem, in particular to reproductive parts (flowers and fruits). Ni in the phloem is generally complexed with the same ligands (Ni-citrate or Ni-malate) but the transport mechanisms are unknown (Deng et al., 2016)

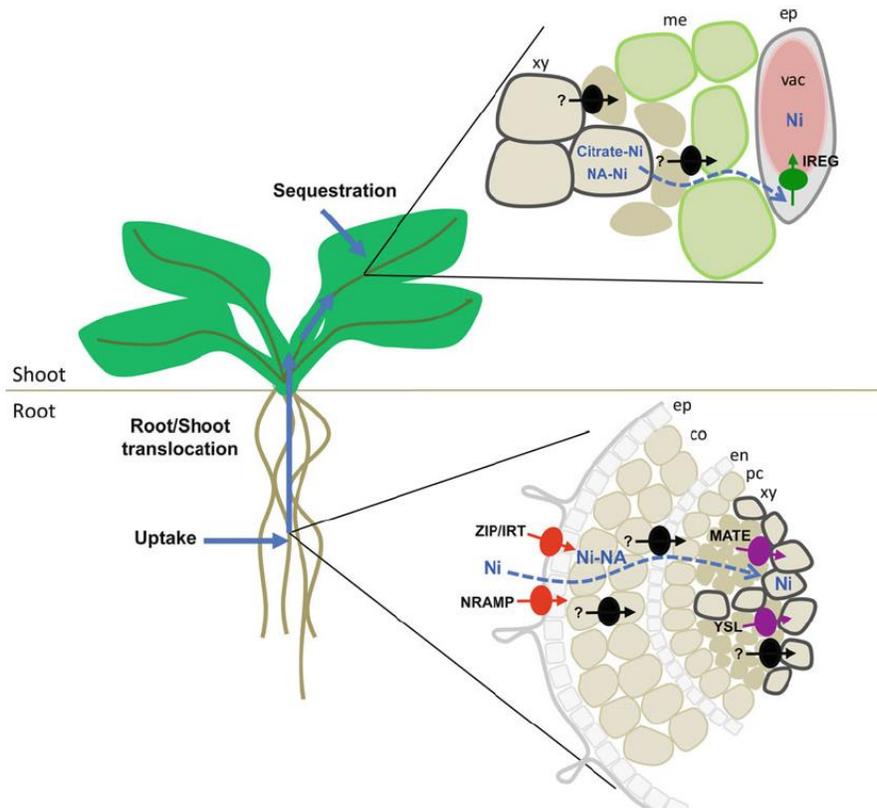


Figure 1. Proposed mechanisms of Ni transport in hyperaccumulators (Merlot et al., 2018)

2.4.1.1. Simultaneous hyperaccumulation of metals

Some plant species reported to accumulate only one metal in its natural habitat were also able to simultaneously tolerate and accumulate several metals when exposed to sufficient available metal contents in ex-situ experiments suggesting the low specificity of some of the molecular mechanisms involved in metal transport and chelation (Merlot et al., 2018).

There is evidence of simultaneous hyperaccumulation in some plants when growing in their natural environment. *Noccaea caerulescens* has been reported to accumulate Ni, Cd and Zn, with a wide genetic variability within its different populations (Assunção et al., 2003; Gonneau et al., 2014).

In Sabah (Malaysia), Ni and Co co-hyperaccumulation has been reported in *Ashtonia excelsa* (Borneo), *Walsuria pinnata*, *Rinorea cf bengalensis*, *R. cf javanica* (Violaceae) and *Glochidion cf. sericeum* (Phyllanthaceae) (van der Ent et al., 2018, 2019b). Metal tolerance and accumulation mechanisms may differ among and within species. However, this phenomenon is not very well understood due to a scarcity of research (besides for *N. caerulea*). Recent studies on the hyperaccumulator *G. cf sericeum* showed that Ni tolerance did not confer Co tolerance suggesting that the species possess different tolerance mechanisms for each metal (van der Ent et al., 2018). Simultaneous hyperaccumulation of Co and Ni is facilitated by their similar chemical properties such as their oxidation state (2+) and their close atomic weights (58.69 and 58.93 respectively) (Albanese et al., 2015; Rue et al., 2020). Nevertheless, Ni and Co compete for the same root transporters and intercellular ligands decreasing the overall uptake of both elements (Keeling et al., 2003; Rue et al., 2019). In the South African Ni hyperaccumulator *B. coddii*, Co hyperaccumulation is favored at high and equal soluble concentrations in soils of both metals (Ni:Co ratio of 100:100), confirming the antagonism between these two elements (Rue et al., 2020). It was shown that the hyperaccumulating Violaceae *Rinorea cf. bengalensis* can accumulate high concentrations of Co at the same time as Ni, but is intrinsically more tolerant to nickel (Paul et al., 2020b). Furthermore, hyperaccumulation does not rely on a similar sequestration mechanism for both metals, which could explain the lesser tolerance for Co. Nickel appears to be essential for the plant to tolerate high Co concentrations.

2.4.2. Metal distribution within plant tissues

The distribution of metals in the different tissues of the hyperaccumulator plants depend on the physiology of each species and on the metal considered (Merlot et al., 2018). The majority of hyperaccumulator plants have been reported to store metals in the epidermis of leaves, specifically in the vacuoles, preventing metals to negatively affect photosynthesis (Küpper et al., 1999, 2001; Lombi et al., 2002; Cosio et al., 2005; Merlot et al., 2018). Indeed, vacuoles are preferred as main storage organelles since they only contain enzymes (e.g. phosphatases) not targeted of metal toxicity (Leitenmaier and Küpper, 2013). Nevertheless, metal accumulation can occur also in the mesophyll cells which are the main sites of photosynthesis. There is evidence of Zn and Cd storage in the vacuoles of mesophyll cells in *A. helleri* and *Sedum alfredii* where Zn form ligands with malate (Küpper et al., 2000; Sarret et al., 2002). In the hyperaccumulator *Noccaea tymphaea*, Ni

and Zn display contrasting localizations: whereas Ni is present in the vacuoles of epidermal cells, Zn is mainly in the mesophyll cells (van der Ent et al., 2019c). In *Berkheya coddii*, Ni is present in the epidermis, but the highest concentrations are found in the mesophyll cells (Mesjasz-Przybyłowicz et al., 2001; Groeber et al., 2015). Recently, with non-destructive microXRF techniques on frozen-hydrated specimens of *Rinorea cf. bengalensis*, it was shown that some plants store large Ni concentrations in the apoplastic space of the leaf epidermis, *i.e.* cell walls (van der Ent et al., 2020).

Some hyperaccumulator plants can concentrate metals not only in leaves but also in other tissues. Extremely high Ni concentrations have been reported in flowers and seeds of some hyperaccumulators. In seeds of *N. tymphaea*, Ni was found associated with carboxylic acids (mainly Ni-malate) and stored in the vacuoles of the cotyledon and hypocotyl epidermal cells (van der Ent et al., 2019c). In *B. coddii*, Ca and Ni are co-accumulated in seeds and actively translocated to the mesophyll of emerging leaves (Groeber et al., 2015). The trunk of *Phyllanthus cf. securinegoides* and *Rinorea bengalensis* reported concentrations exceeding 1000 $\mu\text{g g}^{-1}$ Ni (van der Ent and Mulligan, 2015). In *N. tymphaea*, moderate Ni concentrations were found in roots (epidermis, phloem bundles and pericycle), in stems (collenchyma and phloem bundles) (van der Ent et al., 2019c). There is evidence of Ni-enriched phloem in different tissues (stems, roots and/or leaves) of many other Ni hyperaccumulator species such as *Senecio coronatus*, *Alyssum murale*, *Noccaea caerulescens* and *Stackhousia tryonii*. (Mesjasz-Przybyłowicz et al., 1997; Bhatia et al., 2004; McNear, et al., 2005; Deng et al., 2016). The tropical Malaysian Ni hyperaccumulator trees *R. bengalensis* and *Phyllanthus balgooyi* are known to have extremely high Ni concentrations in their phloem tissues (17.7 wt% and 6.5 wt% Ni respectively) (van der Ent et al., 2017a). However, the most astonishing report of Ni-enrichment in a plant tissue is the 26 wt% Ni in the latex of the New Caledonian tree *Pycnandra acuminata* (Jaffre et al., 1976). High concentration of Ni in the phloem may be a common feature in Ni hyperaccumulator plants (van der Ent et al., 2017a)

2.5. Ecology of hyperaccumulator plants

Habitats of metal hyperaccumulator plants can be classified by the origin of the metal supply in primary, secondary and tertiary (Baker et al., 2010; Wójcik et al., 2017; R. D. Reeves et al., 2018b).

In primary habitats, the elevated metal contents in soils are due to the weathering of metal ore outcroppings or naturally enriched metalliferous rocks (e.g. ultramafics) (Baker et al., 2010; R. D. Reeves et al., 2018b). Other phenomena such as volcanic eruptions may also contribute to the metal input in primary habitats (Kabata-Pendias and Mukherjee, 2007; Wójcik et al., 2017). Secondary habitats resulted from ore mining and processing, spoil and slag heaps or other derived soil alterations from anthropogenic origin. Tertiary habitats result from the atmospheric deposition of dust and particles derived from smelting operations or by alluvial deposition of metal-rich substrates in river floodplains or riverbanks (Baker et al., 2010; R. D. Reeves et al., 2018b; Wójcik et al., 2017).

In temperate climates, ultramafic soils usually host a monospecific community of a Ni hyperaccumulator whereas in tropical climates, ultramafic soils present a high density of woody species with hyperaccumulators and non-hyperaccumulators growing together (R. D. Reeves et al., 2018b). Research undertaken in Cuba revealed that the oldest and undisturbed ultramafic regions in the eastern part of the country hosted the highest number of Ni hyperaccumulator plant species (Reeves et al., 1999, 1996).

Most metal hyperaccumulator plant species are obligate endemic to metalliferous soils (primary, secondary, or tertiary), although a minority can occur in both, metalliferous and non-metalliferous soils, displaying metal hyperaccumulation only when growing in the latter. This phenomenon was defined as facultative hyperaccumulation (Pollard et al., 2014). The reason for the lack of hyperaccumulation in these species may be due to specific soil conditions (e.g. differences in metal availability, pH, etc.) or to genetic/physiological mechanisms differences among populations. However, some studies have evidenced that the trait of hyperaccumulation is present in all the populations of the facultative hyperaccumulators species (Boyd and Martens 1998; Pollard et al. 2014). Some examples of these rare species are the tropical *Psychotria grandis* and *Rinorea bengalensis* (Brooks and Wither, 1977; Campbell et al., 2013; Pollard et al., 2014). The current evolutionary models proposed to explain the adaptive role of hyperaccumulation did not fit well to this facultative hyperaccumulation. Possible explanations seem to depend on the distribution of the species in relation to metalliferous and normal (i.e. non-metalliferous) soils. In facultative hyperaccumulators commonly occurring on metalliferous soils and occasionally on normal soils,

the genetic traits related to hyperaccumulation may have evolved as a selective advantage in these metal-enriched soils and then phylogenetically preserved (or transferred) to non-metalliferous populations. In the contrary, facultative species mainly growing in normal soils and rarely in metalliferous soils were probably predisposed to hyperaccumulation (by incremental advantage or inadvertent uptake), or they may developed the ability to hyperaccumulate in metalliferous populations and it was spread throughout the species (Pollard et al., 2014).

2.6. Global distribution of Ni hyperaccumulator plants

The prevalence of the hyperaccumulation of Ni coincides with the large number of Ni-enriched ultramafic (serpentine) outcrops existing worldwide (Krämer, 2010). In general, Ni hyperaccumulators are geographically grouped in two main regions (R. D. Reeves et al., 2018b): i) the Mediterranean Basin, extending from Portugal to Italy and the Balkans to Turkey (Brooks et al., 1979; Reeves and Adigüzel, 2008), and ii) humid tropical and subtropical ultramafic regions, with three important hotspots which are Cuba, New Caledonia, and Southeast Asia (Galey et al., 2017a; Reeves, 2003). By 2017, the countries with the greatest number of hyperaccumulators were Cuba (128), followed by New Caledonia (65), Turkey (59), and Brazil (30) (R. Reeves et al., 2018).

While some regions have been extensively studied, many others remain unexplored, opening opportunities for the search for new metal hyperaccumulating taxa in those regions.

In the Neotropics, Cuba and Brazil are well known hotspots of hyperaccumulator biodiversity with at least 128 and 30 plant species correspondingly (Reeves et al., 1999; Reeves et al., 2007a; Reeves et al., 2018). Nickel hyperaccumulation was confirmed in the ultramafic regions of Cuba including 16 species of *Buxus* (Buxaceae), 24 species of *Phyllanthus* (Phyllanthaceae) and 28 species of *Leucocroton* (Euphorbiaceae) (Reeves et al., 1999, 1996) whereas in Brazil it is well distributed within 17 different genera such as *Lippia* (Verbenaceae), *Justicia* (Acanthaceae), *Pfaffia* (Amaranthaceae), *Cnidoscolus* (Euphorbiaceae), and *Turnera* (Passifloraceae) (R. D. Reeves et al., 2007). Dominican Republic has some Ni hyperaccumulator species in common with Cuba, and has two more endemic species: *Senecio plumbeus* (Ginocchio and Baker, 2004) and *Phyllanthus nummularioides* Muell. Arg (Reeves, 2003; Reeves et al., 1999). No abnormal metal accumulation was detected in the ultramafic regions of Santa Elena peninsula in Costa Rica (Reeves et al., 2007). In Mexico, no cases of metal hyperaccumulation had been reported at the beginning of this

research, despite the existence of many ultramafic outcrops in that country (Delgado-Argote, 1988; Delgado-Argote et al., 1992; Ortiz-Hernández et al., 2006).

2.7. Discovery of hyperaccumulator plants

The search for metal hyperaccumulator plant species in the field has been long performed using a spot test consisting on a paper impregnated with dimethylglyoxime (DMG) followed by Atomic Absorption Spectrometry (AAS) and Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) of the digested plant leaf samples to confirm the hyperaccumulation status (Gei et al., 2018). Nevertheless, the DMG-test can only be used for Ni hyperaccumulators. The discovery of new metal hyperaccumulator plants has been also performed through the sampling of herbarium specimens and their consequent elemental analysis by ICP-AES or AAS (Brooks et al., 1977a; Reeves, 2003). However, this method has also some disadvantages: i) AAS analysis is limited to a single element at a time, ii) the preparation of the samples is generally time consuming, and iii) the cost of analyzing a large number of samples can be very high. Other methods have been developed such as the use of portable X-ray fluorescence (XRF) spectrometer for the systematic screening of herbarium specimens. This XRF instruments can measure the concentrations of different elements in 30s (to 2 min) with detection limits of $\sim 100 \text{ mg g}^{-1}$ for most transition elements (van der Ent et al., 2019a). The advantages of this method are : i) it is non-destructive, ii) it allows to scan a vast number of samples in a short period of time, iii) it is cost-effective for a great number of samples. For example, a recent massive systematic screening of more than 11 000 herbarium specimens in New Caledonia has allowed to substantially increase their number of metal hyperaccumulator species (34 more for Ni, 63 for Mn, 6 for Co, and 4 for Zn) (Gei et al., 2020a). This technology allows to study the evolution, the phylogeny and the geographic distribution of plants. However, XRF instruments are usually designed for rock and soil elemental measuring, but not for dried plant leaves. For this, a proper calibration of the XRF data must be performed through the calculation of “correction factors”. Parallel foliar samples are analyzed by both XRF and any destructive analytical method (e.g. ICP-AES) to determine the needed correction factors (Gei et al., 2018; van der Ent et al., 2019a).

3. Phytomining- Agromining

Besides the efforts to search for new metal hyperaccumulator plants and to understand the mechanisms involved in this trait, there has been also an enormous advance in the study of practical applications of these unusual plants. Indeed, metal hyperaccumulator plants can be used as indicators of metals in soils for geochemical prospecting (Baker and Brooks, 1988; Nkoane et al., 2005), or for phytoremediation of polluted soils (Baker et al., 1994). Phytoremediation includes phytostabilization (metals are transformed into non phyto/bioavailable forms), phytoextraction (using plants to remove metals from the soil), and phytomining (growing plants to recover metals from soils as an alternative agriculture). The concept of phytomining was first introduced by Chaney in studies related to the land treatment of hazardous wastes (Chaney et al., 1981; Chaney, 1983). A bit later, this concept of growing crops of hyperaccumulator plants to remove metals from soils was also exposed by Baker et al., (1988). The term “agromining” was proposed by Morel (2015) to include the entire soil-plant-ore system.

Agromining can be defined as a complete chain of agro-metallurgical processes that aims to recover strategic metals from low-grade ore bodies, natural metal-enriched soils (*e.g.* ultramafics) or anthropogenic metal-enriched soils (*e.g.* industrial sludge) by means of the cultivation of selected hyperaccumulator plant species (Echevarria et al., 2015; Morel et al., 2015; van der Ent et al., 2015). This technology requires the harvesting of the biomass, the incineration of the dry biomass and the processing of the ashes to provide marketable products (*e.g.* Ni metal, Ni salts, Ni-based catalysts) (Nkrumah et al., 2018a). Agromining of high-valuable elements such as Ni, Co or Au are considered economically viable (Chaney et al., 2018a).

The main process of agromining are: i) identification of potential sites, ii) search for local hyperaccumulator plant species, iii) selection of the most promising hyperaccumulators (or “metal-crops”), iv) development of agronomic practices to optimize metal yields, and v) study of possible metal recovery methods (Nkrumah et al., 2016; Chaney et al., 2018a).

3.1. Identification of potential sites

The cropping of metal hyperaccumulator plants can be carried out in large metalliferous surface areas. In the specific case of Ni, it could be in large natural Ni-rich soils (ultramafic areas) or other

anthropogenic Ni-rich soils (mine wastes). For example in Albania, large ultramafic surface areas (~11% of the country) are suitable for agromining implementation since they are low-productive for traditional food crops (Bani et al., 2014, 2015a). The success of economic agromining partly

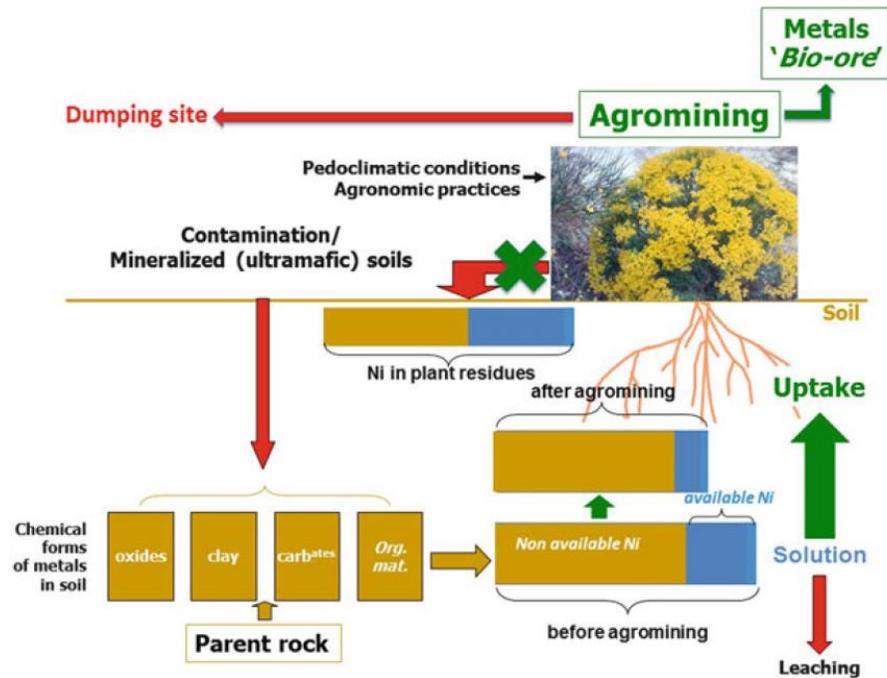


Figure 2. Ni cycle in hyperaccumulator-soil system (Morel, 2012; Nkrumah et al., 2018b)

depends on soil properties and especially on the high Ni phytoavailability in soils (Massoura et al., 2004; Chaney et al., 2018a). It has been demonstrated that both hyperaccumulator and normal plants absorb metals from the same available (labile) pool (Echevarria et al., 1998; Shallari et al., 2001). A proposed Ni cycle in an agromining system is shown in **Figure 2**.

Physical soil properties are also important parameters to consider when selecting potential sites such as good soil drainage (to facilitate root penetration), and good soil water-holding capacity (to enhance biomass production) (Angle et al., 2003; Chaney et al., 2007; Nkrumah et al., 2016, 2018a).

3.2. Selection of the most promising hyperaccumulators

Potential “metal crops” for Ni agromining should have certain characteristics (Nkrumah et al., 2018b) such as high shoot Ni concentrations, high biomass production, fast-growing, easy to propagate and preferably native species. Hyperaccumulators reporting Ni concentrations exceeding 1% wt Ni (*hypernickelophores*) in their shoots are ideal candidates. Tropical Ni hyperaccumulator species seem to have the greatest potential for Ni agromining because they are usually woody perennial species that rapidly regenerate their biomass after harvesting (Nkrumah et al., 2020). Hyperaccumulators possess inherent large natural variation for metal accumulation. Therefore, breeding techniques could be used to improve cultivars and metal yields (Li et al., 2003).

3.3. Development of agronomic practices to optimize metal yields

The study of the agronomy of each “metal crop” is important for the development of profitable agromining systems. Optimal agronomic practices, based on laboratory and field experiments, have significantly increased overall Ni yields of some metal hyperaccumulators. The improvement of the agronomy of *Odontarrhena muralis sensu lato* (syn. *Alyssum murale s.l.*) resulted in a maximum yield of 112 kg Ni ha⁻¹ in Albania (Bani et al., 2015b) and of 400 kg Ni ha⁻¹ in USA (Yin-M Li et al., 2003; Chaney et al., 2007). Pilot-scale agromining trials in western, central and southern Europe have been set-up to improve plant yields of selected “metal crops” (e.g. *Bornmuellera emarginata*) while enhancing soil quality (Kidd et al., 2018). Among the agronomic practices usually tested for “metal crops” are pH adjustment, inorganic fertilization, bacterial inoculation, plant growth regulators, organic matter amendments, herbicide needs, soil tillage and plant density.

Although most of the agronomic studies have been performed in temperate regions, research on tropical Ni agromining has recently started and progress rapidly. In South-East Asia (Sabah, Malaysia), two promising species: *Phyllanthus rufuschaney* and *Rinorea cf bengalensis*, were tested in pots and in the field to evaluate their response to inorganic fertilization, pH adjustment and organic amendments addition, etc (Nkrumah et al., 2019b, 2019c, 2019d). A maximum Ni yield of 250 kg Ni ha⁻¹ has been estimated for large-scale agromining using *P. rufuschaney* (Nkrumah et al., 2019a). The reduction of soil pH in ultramafic substrates of Sabah (Ferrasols,

Leptosols and Cambisols) did not increase Ni extractability because it is more dependent on the Ni-bearing phases and mineralogy (Nkrumah et al., 2019c). As a result of the fertilization test, *P. rufuschaney* significantly increased growth and biomass production in response to N and P additions but significantly decreased under K amendments (Nkrumah et al., 2019a). *Rinorea cf bengalensis* had positive effect on growth and biomass in response to N, P and K additions (Nkrumah et al., 2019a). Shoot Ni concentrations were not significantly reduced except for N addition which caused a significant decrease in Ni uptake by *P. rufuschaney* (Nkrumah et al., 2019a). In the contrary, Ca and S amendments did not significantly affect the growth of both species but rather increased their Ni uptake (Nkrumah et al., 2019b). Organic amendments significantly reduced the shoot Ni concentrations in both species (Nkrumah et al., 2019b).

In the Neotropics, species from the genera *Buxus* and *Leucocroton* in Cuba have been identified to have potential as “metal crops” (>2% wt Ni in leaves) (Reeves et al., 1996; Nkrumah et al., 2016). Nevertheless, agromining in those regions remains unstudied.

3.4. Metal recovery

The transformation of the biomass of “metal crops” into valuable products is also an important stage in the agromining chain. Once the biomass is harvested, it must be dried and incinerated to remove organic matter and to concentrate the metals in the resulting ashes. For example, ashes of Ni hyperaccumulator plants can contain up to 20 wt % of Ni (Simonnot et al., 2016). These ashes are considered “bio-ores” that can be hydro-metallurgically processed to obtain a variety of Ni compounds such as ferronickel metals, Ni metals, Ni salts, Ni oxides or Ni-based catalysts (Simonnot et al., 2018). These materials possess convenient mechanical and physical properties (e.g. corrosion resistance, durability) for their use in many industrial sectors such as construction, aeronautics, chemical and petrochemical, electronics, and battery production. Hydrometallurgical recovery of metals is an aqueous chemical process performed in a relatively low-temperatures and includes different stages such as leaching, solution-phase upgrading, purification and precipitation. A successful example of this metal recovery is the patented synthesis of ammonium nickel sulfate hexahydrate (ANSH) salt from the biomass of *O. muralis s.l.* (Barbaroux et al., 2012). The different steps of the process have been improved allowing to obtain ANSH salts with a purity of

>99.1 % (Zhang et al., 2016). These salts are commonly used for surface treatment in the chemical and paint industry.

Conclusion

Metalliferous soils are toxic environments for most plant species but may favor the emergence of metal hyperaccumulation in a few of them as an adaptative response to these extreme conditions. The physiology of the metal hyperaccumulation in plants as well as its ecological role are not yet fully understood but a substantial scientific progress has been done to contribute to the better understanding of these unusual species. Potential applications of metal hyperaccumulator plants are also investigated, such as their use for agromining (phytomining). The recovery of nickel through agromining technology has been demonstrated to be economically viable and is currently in a transition stage from a pilot-scale to a large-scale operation. Most of the research in the optimization of nickel agromining has focused on a few nickel hyperaccumulator species and on Mediterranean climate. Some pioneering short-scale studies have been carried out in recent years in tropical climates with many promising results. This agromining technology may be extended to other parts of the world, mainly in those countries with a visible potential in terms of plant diversity and metalliferous extents. Mexico is one of the countries with the highest number of reported vascular plants in the world and possess large extents of metalliferous (e.g. ultramafic) soils suggesting a high potential for the identification of new “metal crops” as well as for the implementation of agromining. If nickel (or other metal) hyperaccumulation can be detected in some plant species in Mexico, their agronomic suitability for agromining can be therefore investigated.

Chapter II

Metal hyperaccumulator plants research in Mexico:

Geobotanical exploration in Ultramafic regions

In order to successfully achieve the first objective of the thesis, which was the identification of metal hyperaccumulator plant species in Mexico, we followed a first strategy of research consisting in the exploration of the regions with geologic anomalies (*i.e.* ultramafic soils), which are commonly primary habitats for nickel and cobalt hyperaccumulator plants. The three areas selected displayed some differences in climatic conditions and they were explored for soil conditions and plant absorption of metals of interest. Although no metal hyperaccumulation was detected during the geobotanical explorations carried out in three different ultramafic complexes of Mexico, this chapter aimed to provide a general characterization of the soil and the flora of these unexplored regions in central and southern Mexico.

This work has been exposed in a poster for the 9th International Conference on Serpentine Ecology in Albania (2017) and subsequently published in the peer-reviewed journal *Ecological Research* (2018) 31, 641-649 : <https://doi.org/10.1007/s11284-018-1574-4>.

Is metal hyperaccumulation occurring in ultramafic vegetation of central and southern Mexico?

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Abstract

In Mexico, ultramafic complexes are present in different regions from the northwest (Baja California Norte) to the southeast (Chiapas). In this paper, we present the results of the exploration of three ultramafic (serpentine) habitats in central and southern Mexico: Cuicatlán-Concepción Pápalo (Oaxaca), Tehuitzingo-Tecomatlán (Puebla), and San Juan de Otates (Guanajuato). Previous geology studies showed that these complexes are mainly made up of serpentinized peridotites. Soil analyses demonstrated typical ultramafic characteristics such as high content of Mg in relation to Ca, and high concentrations of Fe, Cr, Co, and Ni. Soil samples from Oaxaca and Puebla had similar Ni contents around 2300 mg kg⁻¹, while samples of Guanajuato showed the lowest Ni levels with an average of 200 mg kg⁻¹ as well as for other metals such as Co, Cr, Mn, and Zn. During this study, 83 plant specimens were collected, of which 52 were identified at genus level and 40 at species level. The collected plants belong to 19 different families such as Anacardiaceae, Fabaceae, Acanthaceae, Asteraceae, Sterculiaceae, and Verbenaceae which are also widely present in other ultramafic areas in Iran, Brazil, Sri Lanka, and Costa Rica. Only two Mexican endemic species are included in the collection. Ni hyperaccumulators were not detected at any of the studied sites. Therefore, hyperaccumulation, as a tolerance mechanism of the flora in response to ultramafic geochemical stress, does not seem to be developed in Central Mexico, as observed in the close Costa Rican site of Santa Elena.

Keywords: *Ultramafic Flora; Serpentine; Nickel; Hyperaccumulator; Geochemical anomaly.*

1. Introduction

Ultramafic soils (*e.g.* serpentine soils) are distributed all around the world and cover > 3 % of Earth's surface (Guillot and Hattori, 2013). They are derived from ultramafic rocks and are usually characterized by high Mg concentrations, deficient levels of macronutrients (N, P, K), low concentrations of Ca, and high contents of metallic trace elements (MTE) such as Cu, Cr, Co, Mn and Ni (Chaney et al., 2008; Boyd and Jaffré, 2009; Kabata-Pendias and Pendias, 2001). Because of their very high Mg/Ca quotient and the presence of heavy metals, these soils are edaphically stressful for the survival and growth of most plants. These conditions generally result in infertile soils with stunted, slow growing vegetation normally hosting endemic plant species (Anacker, 2014; Galey et al., 2017b). Ultramafic species have evolved morphological and physiological adaptations allowing them to tolerate those stressful environments (Kazakou et al., 2008; Anacker, 2014). Within this ultramafic flora, there is a group of plants termed 'nickel hyperaccumulators' that have developed specific metal sequestering and transporting mechanisms enabling them to accumulate extraordinary high concentrations of nickel (Ni) in their aboveground biomass (> 1000 $\mu\text{g g}^{-1}$) (Baker and Brooks, 1989; van der Ent et al., 2013, 2017a). To date, approximately 450 plant species have been reported as Ni hyperaccumulators in many places throughout the world (van der Ent et al., 2017a). Most of these plants are endemic to nickel-rich soils derived from ultramafic rocks (Brooks, 1987; Baker and Brooks, 1989), however, some hyperaccumulator species called "facultative" can be found in both metalliferous and nonmetalliferous soils such as *Psychotria grandis* in Puerto Rico (Reeves, 2003) and Central America (Campbell et al., 2013). Many hyperaccumulator species have been identified in the tropical ultramafic soils of the Neotropics. Cuba hosts the largest number of Ni hyperaccumulators reported from any one country with more than 130 species from different genera such as *Buxus* (Buxaceae), *Leucocroton* (Euphorbiaceae), *Phyllanthus* (Phyllanthaceae), etc. (Reeves et al., 1996). Also, the ultramafic flora of Goiás State in Brazil includes more than 30 Ni hyperaccumulators species (R. D. Reeves et al., 2007). Outside Cuba and Brazil, very few Ni hyperaccumulator plants have been reported in the Neotropics, *e.g.* *Phyllanthus nummularioides* in the Dominican Republic (Reeves et al., 1996) and *Psychotria costivenia* in Central America and the Caribbean (Campbell et al., 2013),

this may be due to the scarcity of scientific studies of native vegetation on natural mineralized or metal-contaminated areas (Ginocchio and Baker, 2004).

Mafic and ultramafic (M-UM) complexes are present in different regions in Mexico, from the northwest (Baja California Norte) to the southeast (Chiapas). The geological characteristics and the metallogenic potential of the 20 main M-UM complexes in Mexico have been treated in more detail by Ortiz-Hernández et al., (2006). Mexico is among the five countries with the largest number of vascular plants, hosting around 25,000 known species of which approximately 40 % are endemic (Conabio, 1998; Sarukhán et al., 2009). However, there are no specific studies of the ultramafic flora and their associated soils from these areas. This study does not attempt to be exhaustive but rather provides a first approach to the exploration of Mexican ultramafic (serpentine) habitats. Three M-UM complexes were selected as study sites from different States of Mexico: Oaxaca, Puebla, and Guanajuato, that could be representative of climatic conditions of central and southern Mexico. Ultramafic soils in these areas developed upon ultramafic rocks (mainly serpentized harzburgites and peridotites) from the Paleozoic and Cretaceous age (Ortiz-Hernández et al., 2006); this implies that these surfaces may have had a very long continuous period of exposure and availability for plant colonization, suggesting the existence of serpentine endemic and Ni hyperaccumulator species. The objectives of this research were to document the edaphic properties of soils and the Ni hyperaccumulator status of a previously underreported UM areas in Mexico.

2. Materials and methods

2.1. Sample sites

In this study, three ultramafic-mafic complexes were chosen for investigation because of their location and surface extent: (1) Cuicatlán-Concepción Pápalo (state of Oaxaca), (2) Tehuitzingo-Tecomatlán (state of Puebla), and (3) San Juan de Otates (state of Guanajuato) (see **Figure 3**). They are all situated within mountain ranges, resulting an undulating, rugged, and stony relief on mountain slopes. Climate ranges from temperate to tropical, with a marked dry season. Those serpentine soils are derived from weathering of ultramafic rocks (mainly serpentized harzburgites and peridotites) (Ortiz-Hernández et al., 2006). Nevertheless, they all differ in

geology, climate, and vegetation. The main characteristics of the three sampled sites are presented below.

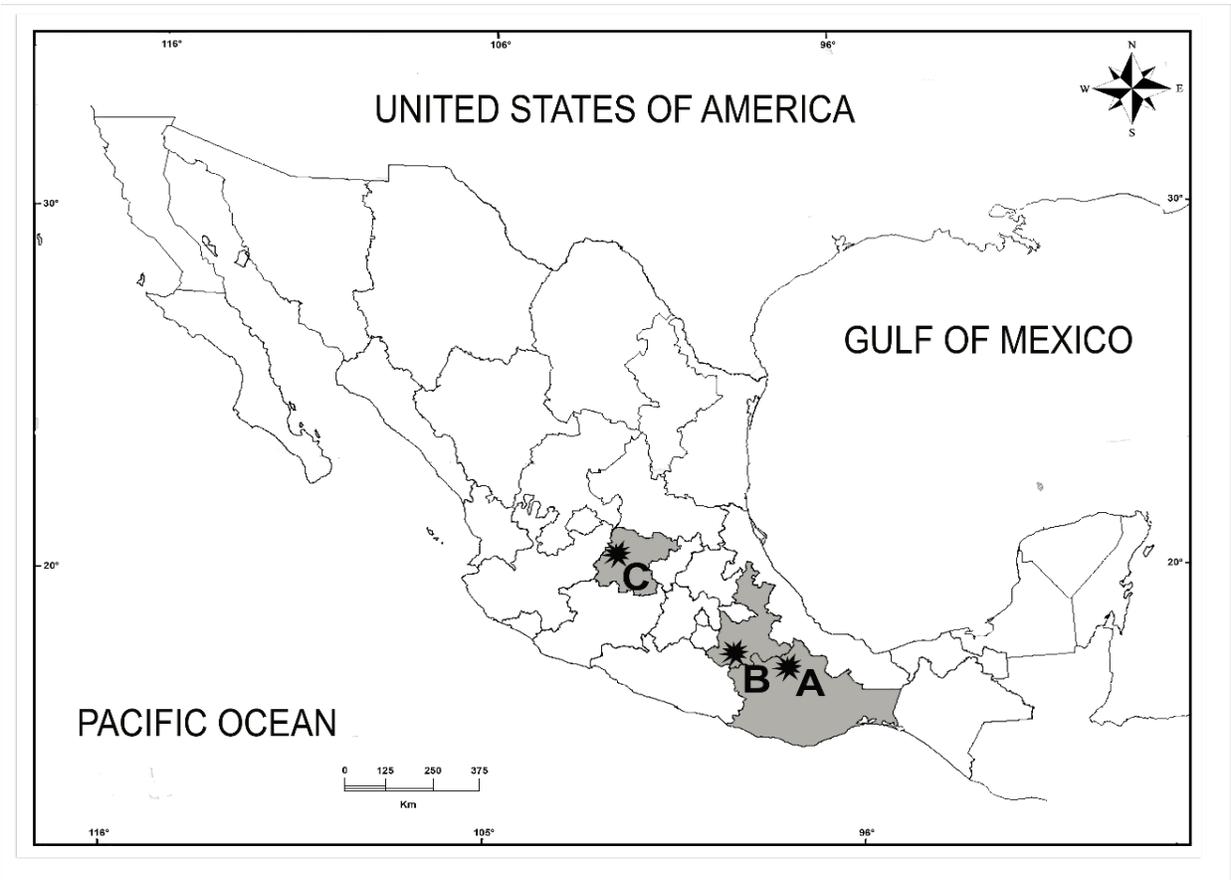


Figure 3. Ultramafic study sites location: A = Cuicatlán-Concepción Pápalo, B = Tehuiztingo-Tecomatlán, and C = San Juan de Otates (modified from Ortiz-Hernández et al., (2006))

(Ortiz-Hernández et al., 2006)

2.1.1. Cuicatlán- Concepción Pápalo

This ultramafic-mafic complex, situated in the state of Oaxaca in southern Mexico, belongs to the Cuicateco tectonostratigraphic terrain, the Juarez mountain range and the physiographic region of Sierra Madre del Sur. It is constituted by unmetamorphosed sedimentary rocks of Cenozoic and Mesozoic age (130 Ma) rocks and includes a large body of serpentinites in the form of concordant mantles 100–300 m thick. These serpentine bodies are constituted mainly by lizardite, antigorite, chlorite, talc schist, primary oxides, and secondary magnetite; their protoliths correspond to harzburgites, wherlites, and dunites (Ortiz-Hernández et al., 2006). Asbestos fibers, such as

chrysolite, are also present within the serpentinites (Delgado-Argote et al., 1992; Delgado-Argote, 1988).

The predominant vegetation is a deciduous tropical mountain forest, *Quercus*, and *Quercus-Pinus* forest (Solis-Rojas, 2006). There is a low diversity of plant species, mainly related to the intensive disturbance by local communities. The climate is classified as temperate sub-humid with rain in summer (Cw2 (w) (i) g); rainfall is typically about 916.7 mm yr⁻¹ and strongly seasonal: there is normally less than 35 mm from November to April. The mean annual temperature is 16.3 °C. The survey was carried out in December 2016.

Collection sites are summarized by the following data and are indicated in Fig. 1:

Site A: M-UM complex Cuicatlán-Concepción Pápalo

SL-S1: San Lorenzo Pápalo; 17°53'3'' N, 96°52'34'' W; altitude 1864 m.

SF-S1: Cuicatlán - San Francisco Pueblo Nuevo; 17°51'50' N, 96°52'24'' W; altitude 1655 m.

SF-S2: San Francisco Pueblo Nuevo; 17°51'46'' N, 96°52'25'' W; altitude 1777 m.

2.1.2. Tehuizingo-Tecomatlán

Located in Puebla State, also in the southern part of the country, this UM complex belongs to the Mixteco tectonostratigraphic terrain, the Sierras del Sur mountain range and the physiographic region of Sierra Madre del Sur. As part of this complex, there is an elongated basic-ultrabasic sequence from the Paleozoic of 7 km long and 500 m wide orientated N-S formed by serpentine schist, associated with spilitic dikes and metagabros. Its protolith is composed by harzburgites (Ortiz-Hernández et al., 2006). Ultramafic rocks are strongly serpentized and are mainly composed of antigorite-lizardite, talc schist and chlorite schist (Carballido-Sánchez and Delgado-Argote, 1989; Ortiz-Hernández et al., 2006). The sampled area supports low elevation deciduous forest vegetation mixed with Cactaceae. Climate is tropical sub-humid with rains in summer (Aw'0 (w) (i) g). The mean annual precipitation is 530 mm yr⁻¹ with less than 15 mm during the dry season (November–April) and the mean annual temperature is 23.7 °C. Sampling was carried out at the end of December 2016.

Collection sites are summarized by the following data and are indicated in Fig. 1:

Site B: M-UM complex Tehuiztingo-Tecomatlán

Tec-S1: Tecolutla, on top of the mountain; 18°17'51" N, 98°19'37" W; altitude 1200 m.

Tec-S2: Tecolutla, downslope; 18°17'51" N, 98°19'37" W; altitude 1194 m.

2.1.3. San Juan de Otates

This 12-km long M-UM complex from the Lower Cretaceous (113 ± 6.8 Ma) is situated in the center of the country, in the State of Guanajuato. It belongs to the Tepehuano tectonostratigraphic terrain, the Sierras del Guanajuato mountain range and the physiographic region of Mesa del Centro. Within this volcanic complex, in the village of San Juan de Otates, there is a geologic formation of pyroxenites constituted mainly by pyroxenes, hornblende, olivine and calcic plagioclase with different degrees of serpentinization (Ortiz-Hernández et al., 2006). Soils derived from these rocks are underdeveloped and have poor plant diversity. Predominant vegetation in the region is oak forest and grassland. The region has a temperate climate with tendency to be warm ((A)C (w) (e) g); the mean annual temperature is 18.5 °C. The mean annual rainfall is 559 mm yr⁻¹ with a marked increase in summer reaching 144 mm in July while in the dry season precipitation does not exceed 20 mm monthly (November-April). The survey was in March 2016.

Collection sites are summarized by the following data and are indicated in Fig. 1:

Site C: M-UM complex San Juan de Otates

SA-S1: Sierra de Guanajuato en San Juan de Otates, 21°10'27.05" N, 101°31'57.11" W; altitude 1950 m.

SA-S2: Sierra de Guanajuato en San Juan de Otates, 21°10'19.45" N, 101°32'41.28" W; altitude 1930 m.

SA-S3: Sierra de Guanajuato en San Juan de Otates, 21° 8'56.25" N, 101°32'39.95" W; altitude 1910 m.

2.2. Collection

Soil samples as well as herbarium and analytical plant specimens were collected from the three ultramafic sites mentioned above. The collection comprised 8 soil samples and 83 plant specimens

of which 50, 17, and 16 were from Oaxaca, Puebla, and Guanajuato respectively. At each study site, three random soil samples over a 2-m² area were taken from the 0–30 cm depth after discarding fresh organic matter particles, then mixed thoroughly in a clean bucket to form a representative composite sample. Soil samples were put in plastic bags and labeled.

Plant specimens were collected in duplicate, numbered, put in a press for drying and prepared as voucher specimens for subsequent identification in the MEXU herbarium at the Universidad Nacional Autónoma de México (UNAM), Mexico City, Mexico.

2.3. Analytical procedures

Soil samples were air-dried at room temperature for 15 days, sieved (2 mm) and sent to UNAM for chemical analysis. Electric conductivity and pH were determined at a soil: solution ratio (SSR) of 1:2.5, stirring for 18 hours and taking the measurements with a Beckman 34 pH Meter and a LaMotte conductivity meter. Exchangeable cations were determined in an ammonium acetate extract at pH 7; Ca²⁺ and Mg²⁺ were determined by atomic absorption and K⁺ and Na⁺ by inductively coupled plasma atomic emission spectroscopy (ICP-OES). Available P content was determined applying the Olsen method and using a Genesys 20 spectrophotometer with a calibration curve between 0 and 12.5 mg l⁻¹ of P. For total C and N contents, a Flash 2000 Thermo elemental analyzer was used with He as carrier gas, and methionine as a calibration compound. Available Ni, Cu, Fe, Mn and Zn contents (extractable DTPA) were determined after extraction (Lindsay and Norvell, 1978; Echevarria et al., 2006) by ICP-OES using a Perkin Elmer Optima 8300 equipment. The digestion of samples was carried out by microwave-assisted digestion (following the method EPA 3051A) and using 9 mL of concentrated nitric acid (JT Baker Instra-Analysed 69 %) and 3mL of concentrated hydrochloric acid (JT Baker Instra-Analysed 38 %) for the extraction.

For expeditions in Oaxaca and Puebla, most of the plants were first screened in the field for Ni accumulation by a semi-quantitative test, which consisted of pressing the leaves against filter paper impregnated with the Ni-specific colorimetric-reagent dimethylglyoxime (1 % solution in ethanol) and then leaves were scanned by a portable X-ray fluorescence spectrometer in “soil” mode (Thermo Scientific GOLDD+ XL3T-980). In the case of Guanajuato, only the filter paper test was used for field Ni accumulation screening. Color reaction (red-magenta) appears when foliar Ni is

> 500–700 $\mu\text{g g}^{-1}$, which is below the threshold defining for Ni hyperaccumulation, *i.e.* 1000 $\mu\text{g g}^{-1}$ (van der Ent et al., 2013).

Small portions of leaf (approximately 3 g) from 25 specimens collected within the three sites were set aside and sent to the laboratory of the Geology Faculty at UNAM University for analysis. The plant material was first washed with tap water for several minutes to discard organic matter particles, then immersed in hydrochloric acid, rinsed with distilled water and finally put into a preheated oven at 60–65 °C until the plant samples were completely dried; 100 mg of the plant tissue of each sample was then weighed and transferred to digestion glass tubes of 100 ml ensuring that the sample had minimal contact with the tube walls and the majority of the sample was settled to the bottom. Subsequently, 3 mL of nitric acid (JT-Baker 69 %) and 3 ml of cold hydrogen peroxide (JT-Baker 30 %) were slowly added dropwise. Samples were then digested by microwave-assisted digestion using an Anton paar Multiwave 3000 microwave; completing a heating cycle in which a power of 1400 W was reached in 10 minutes and maintained for another 15 minutes. Once the heating period was elapsed, samples were left to cool for 30 minutes. The digests were filtered and diluted with deionized water to a volume of 25 ml. Resulting solutions were analyzed by ICP-OES for Ni, Co, Zn, Fe, Mn, Ca, and Mg using a Perkin Elmer Optima 8300 equipment and a Cetac 5000+ nebulizer for ultrasonic nebulization.

3. Results and discussion

3.1. Soils

All soil samples showed ultramafic chemical characteristics (**Tables 2 and 3**), such as high content of Mg in relation to Ca, resulting in very low total and exchangeable Ca/Mg quotient (below 0.37 and 2.2 respectably). Available P was quite low ranging from 1.1 to 5.4 $\mu\text{g g}^{-1}$. They also contain high levels of Mg, Fe, Cr, Co, and Ni, that are typical for ultramafic (*i.e.* serpentine) soils (Whittaker, 1954). Soil samples from Oaxaca and Puebla had similar Ni total concentrations around 2300 $\mu\text{g g}^{-1}$, while Guanajuato soils showed the lowest Ni levels (average of 200 $\mu\text{g g}^{-1}$) as well as other metals such as Co, Cr, Mn and Zn which supports what is reported by (Hernández-Silva et al., 2000; Ortiz-Hernández et al., 2006) for this locality. The pH ranged from neutral to alkaline (6.6–8.3), with the highest amount of available Ni in soils with the lowest pH (Oaxaca).

Cation exchangeable capacity values were in general high and were slightly dominated by Mg^{2+} in most of the cases followed by Ca^{2+} , K^+ and Na^+ . Nevertheless, exchangeable Mg^{2+} was not always higher than Ca^{2+} . An interesting fact is that the concentration of K^+ in all soil samples was extremely high.

Soil sample	pH	EC (dS m ⁻¹)	Total Org C (%)	Org. Matter (%)	Total N (%)	P Olsen (µg g ⁻¹)	CEC (cmol kg ⁻¹)	Exchangeable cations (cmol kg ⁻¹)				Exchan geable Ca/Mg	Total Ca/Mg
								Ca ²⁺	Mg ²⁺	Na ⁺	K ⁺		
SL-S1	6.61	0.08	2.92	5.03	0.18	2.08	20.6	3.05	13.49	< LOD	4.13	0.2	0.01
SF-S1	6.38	0.10	3.90	6.73	0.22	1.07	22.42	4.20	15.22	< LOD	3.01	0.3	0.01
SF-S2	8.06	0.17	2.24	3.85	0.14	5.42	43.68	21.56	10.16	0.77	11.21	2.1	0.37
Tec-S1	8.07	0.09	0.93	1.60	0.04	2.18	7.89	4.34	3.28	< LOD	< LOD	1.3	0.05
Tec-S2	7.54	0.16	2.54	4.38	0.25	4.46	26.59	11.28	5.05	10.43	26.59	2.2	0.02
SA-S1	6.91	0.086	1.16	2.21	0.09	1.73	41.00	13.62	18.32	0.55	7.55	0.8	0.27
SA-S2	8.31	0.139	0.29	1.12	0.01	1.34	43.16	21.68	15.16	0.88	4.83	1.4	0.12
SA-S3	7.07	0.126	1.24	2.36	0.11	2.05	29.29	10.09	13.50	< LOD	4.09	0.6	0.29

Table 2. Soil analyses of ultramafic soils in Mexico (concentrations in % or µg g⁻¹ where indicated). SL = San Lorenzo Pápalo, Oaxaca; SF = San Francisco Pueblo Nuevo, Oaxaca; Tec = Tecolutla, Puebla; SA = San Juan de Otates, Guanajuato; LOD = Limit of detection; CEC= Cation-exchangeable capacity.

Soil sample	Total major elements							Total trace elements					
	Si	K	Na	Ca	Mg	Al	Fe	Co	Cr	Mn	Cu	Zn	Ni
	mg kg ⁻¹			g kg ⁻¹				mg kg ⁻¹					
SL-S1	1790	476	101	1.1	99	6.1	50.5	108	1510	1080	20	70	2740
SF-S1	1010	703	151	1.4	115	11.7	81.3	158	2000	1330	11	71	2480
SF-S2	53	4620	200	17.5	47	21.8	35.9	49	394	725	55	166	442
Tec-S1	148	285	123	9.4	201	4.4	71.7	128	1220	878	4	66	2180
Tec-S2	88	856	135	3.4	151	6.0	59.3	109	1020	1050	23	58	2030
SA-S1	106	828	142	3.8	14	17.5	36.5	37	285	612	30	48	136
SA-S2	416	744	116	4.5	37	13.4	29.3	45	142	649	101	38	192
SA-S3	201	616	161	9.2	32	17.1	47.0	60	325	736	29	53	274

Table 3. Total major and trace elements of serpentine soils in Mexico. SL = San Lorenzo Pápalo, Oaxaca; SF = San Francisco Pueblo Nuevo, Oaxaca; Tec = Tecolutla, Puebla; SA = San Juan de Otates, Guanajuato.

Table 4 shows the availability of several elements (estimated by the DTPA extraction) , in which we observe that Ni values are very high in two sites from Oaxaca reaching $159 \mu\text{g g}^{-1}$ while soils from Puebla and Guanajuato have medium ($4.1\text{--}32.6 \mu\text{g g}^{-1}$) and low values ($0.5\text{--}3.7 \mu\text{g g}^{-1}$), respectively. Only the sites from Oaxaca and Puebla may present stressful Ni conditions for the flora.

Soil sample	Extractable elements DTPA ($\mu\text{g g}^{-1}$)				
	Cu	Fe	Mn	Zn	Ni
SL-S1	0.54	23.1	17.2	2.21	159
SF-S1	0.67	41.8	26.7	2.11	145
SF-S2	0.67	3.40	3.20	5.66	1.00
Tec-S1	0.67	6.00	7.40	0.71	4.00
Tec-S2	1.35	10.5	28.6	1.04	32.0
SA-S1	0.67	9.00	7.50	0.47	2.00
SA-S2	1.53	4.60	7.50	0.71	1.00
SA-S3	0.95	11.6	7.20	0.54	3.00

Table 4. Extractable elements with DTPA from serpentine soils in Mexico. SL = San Lorenzo Pápalo, Oaxaca; SF = San Francisco Pueblo Nuevo, Oaxaca; Tec = Tecolutla, Puebla; SA= San Juan de Otates, Guanajuato.

3.2. Plants

3.2.1. Flora

From a collection of 83 plant specimens, 31 could not be identified due to lack of fertile specimens with features (inflorescences, fruits, etc.). The remaining 52 specimens were identified at genus level and 40 of them at species level. The list of identified genera and species is given in Table 4. The genera identified are from 19 different families, the most represented families are: Compositae (Asteraceae) and Leguminosae (Fabaceae) which is a common feature of the Mexican flora (Villaseñor, 2004). Two Mexican endemic species were included in the collection: *Plumbago pulchella* (Plumbaginaceae) which is distributed in 20 States of Mexico and *Quercus konzattii* (Fagaceae), which can be found in some states in the northern part of the country such as Durango

and Zacatecas, as well as in Oaxaca State in the south. Nevertheless, they cannot be considered as serpentine endemics as they are also found in non-ultramafic soils. Specimens from genera *Bidens*, *Gomphrena*, *Penstemon*, *Pherotrichis*, *Pseudognaphalium*, *Simsia*, *Verbesina*, and *Zaluzania* were also reported. Plants collected included several from genera *Calea*(☒), *Crotalaria*(±), *Lippia*(+☒), *Mimosa*(☒), *Ruellia*(+), *Simsia*(☒), *Stevia*(+), *Stachys*(±), and *Waltheria*(±☒) that are also present in other ultramafic areas in Iran(*), Brazil(+), Sri Lanka(±), and Costa Rica(☒) (*Ghaderian and Baker, 2007; +, ☒Reeves et al., 2007 a, 2007 b; ±Rajakaruna and Bohm, 2002).

Family	Genus and species	Study site	n
Acanthaceae	<i>Ruellia inundata</i> Kunth.	C	1
Amaranthaceae	<i>Gomphrena</i> sp.	C	1
Anacardiaceae	<i>Schinus molle</i> L.	S	1
Asclepiadaceae	<i>Asclepias linaria</i> Cav.	S	1
Asclepiadaceae	<i>Pherotrichis</i> sp.	C	1
Buddlejaceae	<i>Buddleja cordata</i> Kunth.	S	1
Compositae	<i>Baccharis mexicana</i> Cuatrec.	C	1
Compositae	<i>Baccharis multiflora</i> Kunth.	C	1
Compositae	<i>Baccharis salicifolia</i> (Ruiz & Pav.) Pers.	S	1
Compositae	<i>Barkleyanthus salicifolius</i> (Kunth) H. Rob. & Brettell.	S	1
Compositae	<i>Bidens</i> sp.	C	1
Compositae	<i>Calea</i> sp.	C	2
Compositae	<i>Critoniopsis uniflora</i> (Sch. Bip.) H. Rob.	C	1
Compositae	<i>Piqueria trinervia</i> Cav.	C	2
Compositae	<i>Pseudognaphalium</i> sp.	C	1
Compositae	<i>Pseudognaphalium purpurascens</i> (DC.) Anderb.	C	1
Compositae	<i>Simsia</i> sp.	C	1
Compositae	<i>Stevia</i> sp.	C	1
Compositae	<i>Stevia lucida</i> Lag.	C	1
Compositae	<i>Tagetes erecta</i> L.	C	1
Compositae	<i>Verbesina</i> sp.	S	2
Compositae	<i>Zaluzania</i> sp.	S	1
Convolvulaceae	<i>Ipomoea murucoides</i> Roem & Schult.	S	1
Convolvulaceae	<i>Porana nutans</i> (Moc. & Sessé) O'Donell.	T	2
Fagaceae	<i>Quercus acutifolia</i> Neé.	C	2
Fagaceae	<i>Quercus conzattii</i> Trel.*	C	1
Gramineae	<i>Melinis rosea</i> (Nees) Hack.	C	1
Hydrophyllaceae	<i>Wigandia urens</i> (Ruz & Pav.) Kunth.	S	1
Lamiaceae	<i>Stachys pilosissima</i> M. Martens & Galeotti.	C	1
Leguminosae	<i>Acacia farnesiana</i> (L.) Willd.	C, S	2

Leguminosae	<i>Acacia schaffneri</i> (S. Watson) F.J. Herm.	S	1
Leguminosae	<i>Crotalaria sagittalis</i> L.	C	1
Leguminosae	<i>Havardia acatlensis</i> (Benth.) Britton & Rose.	T	1
Leguminosae	<i>Mimosa albida</i> Humb. & Bonpl. ex Willd. var. <i>strigosa</i> (Willd.) B.L. Rob.	C	1
Leguminosae	<i>Senna wislizenii</i> (A. Gray) Irwin & Barneby var. <i>pringlei</i> (Rose) Irwin & Barneby.	T	1
Leguminosae	<i>Prosopis laevigata</i> (Humb. et Bonpl. ex Willd.) M. C. Johnst.	S	1
Plumbaginaceae	<i>Plumbago pulchella</i> Boiss.*	C	1
Polemoniaceae	<i>Loeselia pumila</i> (M.Martens & Galeotti) Walp.	C	1
Rubiaceae	<i>Bouvardia ternifolia</i> (Cav.) Schtdl.	C	2
Scrophulariaceae	<i>Penstemon</i> sp.	C	1
Sterculiaceae	<i>Waltheria indica</i> L.	C	2
Solanaceae	<i>Nicotiana glauca</i> Graham.	S	2
Verbenaceae	<i>Lippia alba</i> (Mill.) N.E. Br. ex Britton & P. Wilson.	S	1

Table 5. Plant species collected from ultramafic soils in Mexico. C = Cuicatlán- Concepción Pápalo in Oaxaca, T = Tehuiztingo-Tecomatlán in Puebla, S = San Juan de Otates in Guanajuato, n = number of specimens collected,* = Mexican endemic species.

3.2.2. Plant geochemistry

The results from colorimetric-reagent dimethylglyoxime test, X-Ray Fluorescence spectrometry ($9.0\text{--}609\ \mu\text{g g}^{-1}$) and ICP-OES ($0.8\text{--}43.1\ \mu\text{g g}^{-1}$) did not reveal any Ni hyperaccumulator in all sampled sites; this is based on recent recommended ranges for hyperaccumulation of $> 1000\ \mu\text{g g}^{-1}$ (van der Ent 2013a). This may be related to the low available Ni content in soils of Puebla ($4.0\text{--}32\ \mu\text{g g}^{-1}$) and Guanajuato ($0.5\text{--}3.7\ \mu\text{g g}^{-1}$). However, in the case of Oaxaca, Ni availability is very high ($146\text{--}160\ \mu\text{g g}^{-1}$). Thus, the lack of hyperaccumulation may be related to other causes, such as the particular geomorphologic position of the serpentine bands, which are a few hundreds of meters wide in steep slope areas experiencing continuous soil erosion. Such a feature could have impeded the evolution of the Ni-hyperaccumulation trait in the ultramafic flora because the surface of the outcrop is not significant enough in the landscape. Another possible cause is human impact on the vegetation (deforestation, asbestos mining, etc.). However, a much more detailed study of soils related to plant physiology is needed to confirm the causes. **Table 5** lists the elemental concentrations in leaves of 24 species collected within the three explored sites. The ranges are typical of plants in ultramafic soils. The maximum Ni concentration of $43.1\ \mu\text{g g}^{-1}$ was found in a specimen of *Wigandia urens*, collected in San Juan de Otates (Guanajuato), which also showed slightly positive result to the test with dimethylglyoxime. The highest Co concentration was also observed in *W. urens* ($7.4\ \mu\text{g g}^{-1}$) while in most other plants it was $< 1.2\ \mu\text{g g}^{-1}$. For Zn, *Nicotiana*

glauca (37.9 $\mu\text{g g}^{-1}$) had the highest concentration of all plants. The highest concentration of Mn was found in *Quercus acutifolia* (234 $\mu\text{g g}^{-1}$), more than 47 x higher than the lowest Mn concentration reported in the other plants analyzed. Values above 1000 $\mu\text{g g}^{-1}$ of Fe concentrations were found in *Buddleja cordata* (1788 $\mu\text{g g}^{-1}$), *W. urens* (7831 $\mu\text{g g}^{-1}$) and *Lippia alba* (1089 $\mu\text{g g}^{-1}$), while the lowest value was found in *Quercus acutifolia* (67 $\mu\text{g g}^{-1}$). The highest Ca and Mg concentrations were found in *N. glauca* (44,136 $\mu\text{g g}^{-1}$ and 11,478 $\mu\text{g g}^{-1}$ respectively), while the lowest Ca and Mg concentrations were reported for a species of *Calea sp.* The Ca/Mg quotients ranged from 0.73 to 5.7, this may be a consequence of the low total and extractable Ca/Mg quotient values.

Family	Species	Co	Ni	Zn	Fe	Mn	Ca	Mg	Ca:Mg
Anacardiaceae	<i>Schinus molle</i> L.	0.1	3.4	22.0	141	51	32 123	5637	5.70
Asclepiadaceae	<i>Asclepias linaria</i> Cav.	0.1	0.8	19.7	169	136	17 475	6536	2.67
Buddlejaceae	<i>Buddleja cordata</i> Kunth	1.2	10.0	13.6	1788	22	6889	4481	1.54
Compositae	<i>Baccharis salicifolia</i> (Ruiz & Pav.) Pers.	0.2	1.9	33.9	142	22	6619	1909	3.47
Compositae	<i>Baccharis mexicana</i> Cuatrec.	0.4	26.1	18.6	154	18	2717	3711	0.73
Compositae	<i>Barkleyanthus salicifolius</i> (Kunth) H. Rob. & Brettell	0.6	7.2	17.7	373	20	14 796	5041	2.93
Compositae	<i>Calea sp.</i>	0.2	8.3	7.3	111	5	809	677	1.20
Compositae	<i>Calea sp.</i>	0.4	33.9	8.8	346	10	19 54	2324	0.84
Compositae	<i>Critoniopsis uniflora</i> (Sch. Bip.) H. Rob.	0.1	9.5	14.9	106	49	6127	3809	1.61
Compositae	<i>Stevia lucida</i> Lag.	0.2	2.3	13.2	363	17	2279	845	2.70
Compositae	<i>Verbesina sp.</i>	0.3	2.6	7.6	771	7	1693	904	1.87
Compositae	<i>Verbesina sp.</i>	0.3	2.4	10.3	705	7	1427	754	1.89
Convolvulaceae	<i>Ipomoea murucoides</i> Roem & Schult.	0.4	7.3	22.3	215	25	4552	2989	1.52
Fagaceae	<i>Quercus acutifolia</i> Neé	0.1	29.2	9.7	67	234	5194	3304	1.57
Fagaceae	<i>Quercus conzattii</i> Trel	0.2	25.6	9.5	191	108	3332	3075	1.08
Hydrophyllaceae	<i>Wigandia urens</i> (Ruz & Pav.) Kunth	7.4	43.1	17.9	7831	74	13 375	8008	1.67
Leguminosae	<i>Acacia farnesiana</i> (L.) Willd.	0.8	2.8	9.5	285	20	11 996	1301	9.22
Leguminosae	<i>Acacia schaffneri</i> (S. Watson) F.J. Herm	0.1	4.6	13.6	220	14	2484	1056	2.35
Leguminosae	<i>Mimosa albida</i> Humb. & Bonpl. ex Willd. var. <i>strigosa</i> (Willd.) B.L. Rob	0.6	13.9	5.4	397	13	2338	1568	1.49
Leguminosae	<i>Prosopis laevigata</i> (Humb. et Bonpl. ex Willd.) M. C. Johnst.	0.2	5.9	33.0	167	14	5045	1884	2.68
Convolvulaceae	<i>Porana nutans</i> (Moc. & Sessé) O'Donell	0.1	5.9	19.8	78	63	22 472	7027	3.20
Solanaceae	<i>Nicotiana glauca</i> Graham	0.5	8.6	19.1	503	18	12 031	2595	4.64
Solanaceae	<i>Nicotiana glauca</i> Graham	0.4	14.1	37.9	177	83	44 136	11 478	3.85
Verbenaceae	<i>Lippia alba</i> (Mill.) N.E. Br. ex Britton & P. Wilson	0.5	3.7	14.2	1089	17	8334	1942	4.29

Table 6 Elemental concentrations in the leaf dry matter of selected plants in Mexican serpentine soils ($\mu\text{g g}^{-1}$).

Baccharis and *Bidens* species have been found as tolerant to zinc (Zn) and arsenic (As) (respectively) in some South America countries, such as Ecuador and Peru, (Ginocchio and Baker, 2004) or in Niquelândia (Brazil) on ultramafic soils (unpublished data). *Acacia farnesiana* was found in both Oaxaca and Guanajuato ultramafic soils and its suitability for lead (Pb) phytostabilization has been demonstrated by Maldonado-Magaña et al., (2011) and Ruiz-Olivares et al., (2016). Barazani et al., (2004) has studied the potential of *N. glauca* for heavy metal accumulation. *Waltheria indica* was reported to be a copper (Cu) hyperaccumulator in Sri Lanka (Rajakaruna and Bohm, 2002) and a nickel (Ni) hyperaccumulator in Venezuela (Barretto and Casale, 2002) whilst in Queensland and Costa Rica it did not show any nickel or copper accumulation (Reeves et al., 2007b). *Schinus molle* L. has been reported as a Cd tolerant species (Pereira et al., 2015). Buendía-González et al., (2010) demonstrated Pb and Ni accumulation as well as Cr and Cd hyperaccumulation in *Prosopis laevigata* through in-vitro culture. However, this study showed that *P. laevigata* did not accumulate any of those metals in natural conditions (Guanajuato). Some other species of *Prosopis* have been reported as metal indicators in Argentina (Ginocchio and Baker, 2004). *Tagetes erecta* has been studied for Zn, Cu, Pb, and Cd phytoremediation purposes (Sinha et al., 2010).

This study results found normal levels of Ni in genera that have been reported with species containing high Ni in other parts of the world (e.g. *Stevia*, *Ruellia* and *Lippia* species in Brazil, *Waltheria* species in Venezuela, *Stachys* in Italy).

4. Conclusion

Soil samples from all sites (Oaxaca, Puebla, and Guanajuato) showed ultramafic chemical characteristics such as high levels of Mg, Fe, Cr, Co, Ni, and low Ca/Mg quotients. DTPA-extractable Ni concentrations were very high in two sites from Oaxaca reaching $159 \mu\text{g g}^{-1}$ whilst soils from Puebla and Guanajuato reported medium ($4.1\text{--}32.6 \mu\text{g g}^{-1}$) and low values ($0.5\text{--}3.7 \mu\text{g g}^{-1}$) respectively. A total of 40 plant specimens were fully determined at species level (from 19 different families) and 12 more at genus level. The most common families were Asteraceae and Fabaceae. Two endemic species were among the native plants on ultramafic sites: *P. pulchella*

(Plumbaginaceae) and *Q. conzattii* (Fagaceae). No Ni-hyperaccumulator was discovered in this survey, even from genera that have been reported elsewhere as Ni hyperaccumulators (*e.g.* *Stevia*, *Ruellia* and *Lippia* species in Brazil). Nickel levels in the plants collected (leaf dry matter) ranged from 0.8 to 43.1 $\mu\text{g g}^{-1}$. The absence of Ni hyperaccumulation in the study sites of Puebla and Guanajuato may be related to the low available Ni content in soils, whereas in the study site of Oaxaca may be due to the particular topographic position of the serpentines in mountain ranges forming narrow strips of a few hundred meters wide on steep slopes continuously exposed to soil erosion. Such a non-continuous exposure to pedogenesis over time probably impeded the establishment of Ni-hyperaccumulators. The lack of Ni-hyperaccumulators in ultramafic areas was also observed in the nearby Santa Elena Peninsula in Costa Rica (Reeves et al., 2007b), where serpentine soils are also much eroded and inserted in a hilly landscape. Nevertheless, this study only covers three ultramafic sites out of the 20 main ultramafic complexes that exist in Mexico. It does not pretend to be exhaustive but rather provides a first approach to Mexican ultramafic habitats. Further studies are needed on these three sites and because Ni hyperaccumulation is reported in Mexico from the analysis of herbarium specimens, the geobotanical exploration of the other ultramafic habitats in Mexico would provide useful information, since they have remained uninvestigated. This raises the possibility of finding new endemic and Ni hyperaccumulator species, especially on isolated areas such as Margarita and Magdalena Islands in northwest Mexico (Baja California).

Chapter III

Metal hyperaccumulator plants research in Mexico:

Geobotanical exploration in other metalliferous regions in
Mexico

Although interesting results were obtained from the explorations in the ultramafic regions of Central and Southern Mexico (Navarrete Gutiérrez et al., 2018), no abnormal metal accumulation was detected in the flora of those regions. Shortly after, McCartha et al., (2019) reported Ni hyperaccumulation in herbarium specimens of several *Psychotria* (Rubiaceae) species collected from south-eastern Mexico through Central America to northwestern South America. This was the first successful prediction of metal hyperaccumulation based on molecular phylogeny and facilitated by a systematic XRF screening in the *Missouri Botanical Garden's Herbarium* (MO). The new Ni hyperaccumulator species were *Psychotria costivenia* Griseb., *Psychotria lorenciana* C.M. Taylor and *Psychotria papantlensis* (Oerst.) Hemsl. The distribution range of these species included areas that were apparently non-ultramafic. Under this context, a new scientific question arose:

- Knowing the phylogeny of metal hyperaccumulation in a given plant family, is it possible to predict and to confirm hyperaccumulation in some plant species despite their geographical dispersion ?

Subsequently, we undertook field surveys in specific locations in Mexico based on the geographic coordinates of the *Psychotria* herbarium specimens exhibiting Ni hyperaccumulation. In parallel, several screenings were performed in different herbaria including the MEXU (Mexico City, Mexico), P (Paris, France), and MO (Saint Louis, U.S.A.) to complete field explorations. Three regions belonging to different states were selected for field surveys : i) Los Tuxtlas (Veracruz), ii) Ocosingo (Chiapas), and iii) Tacotalpa (Tabasco). Our field surveys revealed total soil Ni concentrations ranging from 80–500 mg kg⁻¹ in Los Tuxtlas, 30–1600 mg kg⁻¹ in Ocosingo, and 200–500 mg kg⁻¹ in Tacotalpa. These Ni contents are clearly lower than those typically found in ultramafic soils (average of 2000 mg kg⁻¹ Ni, Mielke, (1979)), but they are notably elevated compared to typical non-ultramafic soils and high enough to host plant metal hyperaccumulation. Until now, there is no specific and complete research about the origin of the Ni in those sites. However, all these regions have been strongly influenced by intense regional volcanic activity, which may partly explain the Ni content in soils.

The Reserve of “Los Tuxtlas” Biology Station, located in the eastern of Veracruz, is within the “Sierra de Los Tuxtlas” volcanic mountain range where the most important active volcanos are: San Martín Tuxtla, Santa Marta and San Martín Pajapan. Soils in this region derived from the weathering of volcanic ashes of basaltic and basaltic-andesite origin (Díaz Gonzalez, 2010; Flores-Delgadillo et al., 1999). High Ni (and other metal) contents can be commonly found in soils formed from basic and volcanic rocks, and in some organic-rich soils (Kabata-Pendias and Pendias, 2001). In basalts, Ni has been reported in a range of 28 to 589 mg kg⁻¹ (Farmer, 2014), whereas in andesites it varies from 3 to 28 mg kg⁻¹ (Komatina, 2004). The main minerals conforming the volcanic ashes in “Los Tuxtlas” Biology Station are olivine, pyroxene and amphibole (Aguilera-Gómez, 1988; González Soriano et al., 1997; Flores-Delgadillo et al., 1999). During this research, soil samples were analyzed for mineralogical characterization reporting the presence of secondary clay minerals (kaolinite) and Fe oxides (goethite, magnetite, hematite) confirming in part the results from previous investigations (Flores-Delgadillo et al., 1999).

The soils from the sites of Ocosingo and Tacotalpa are mainly derived from sedimentary calcareous soils (limestone) but are moderate Ni-enriched probably due to volcanic ash deposits from the nearest volcano “El Chichón”. There is evidence of crystals of plagioclase, amphibole, clinopyroxene and Fe-Ti oxides found in pumice samples from “El Chichon volcano”. Micro-XRF analysis of one piece of rock collected in the studied site of Ocosingo (Chiapas) was performed during this study to give a first insight on the petrology of the region; the results revealed a detrital sedimentary rock formed by ferromagnesian clays, Fe-Ti oxides, quartz and plagioclases. Ni was associated mainly with Mn oxides in that rock. Mineralogical analysis was also performed in several soil samples taken in the same site: kaolinite, albite, dolomite, clinocllore, goethite, quartz were the main minerals present in the soils. Another potential source of Ni in these soils may be some lateritic material transported by water from near ultramafic regions. Hernández-Quiroz *et al.* (2012) evidenced that lateritic nodular fragments, probably transported from preexisting soils that were possibly derived from ultramafic rocks situated in the margins of the Caribbean plate, contributed to the Ni content in soils near a petrochemical facility in Tabasco.

During this second step on the search of hyperaccumulation in non-ultramafic regions in Mexico, field and herbarium surveys were also based on the phylogeny of nickel hyperaccumulation of

some plant families known to hyperaccumulate elsewhere (*i.e.* Rubiaceae and Violaceae) and that were present in the selected study sites.

Species	Family	Geographic distribution of hyperaccumulation	Hyperaccumulated Element	Shoot concentration (%)
<i>Blepharidium guatemalense</i> Standl.	Rubiaceae	MX, GT	Ni	0.05 – 4.3
			Co	<0.01 – 0.2
<i>Psychotria costivenia</i> Griseb.	Rubiaceae	MX, GT BZ, CR, CU	Ni	<0.02 – 1.8
<i>Psychotria lorenciana</i> C.M. Taylor.	Rubiaceae	MX, GT	Ni	0.04 – 2.1
<i>Psychotria papantlensis</i> (Oerst.) Hemsl.	Rubiaceae	MX, GT, BZ	Ni	<0.02 – 1.3
<i>Mayanaea caudata</i> (Lundell) Lundell	Violaceae	GT	Ni	0.5
<i>Orthion guatemalense</i> Lundell	Violaceae	GT	Ni	0.03 – 0.5
<i>Orthion malpighiifolium</i> (Standl.) Standl. & Steyerm.	Violaceae	MX	Ni	<0.02 – 0.6
<i>Orthion montanum</i> Lundell.	Violaceae	MX	Ni	0.2 – 0.6
			Co	0.03 – 0.04
<i>Orthion oblanceolatum</i> Lundell	Violaceae	MX	Ni	<0.02 – 0.1
			Co	0.02 – 0.08
<i>Orthion veracruzense</i> Lundell	Violaceae	MX	Ni	<0.02 – 0.4
			Co	0.01 0.07
<i>Orthion subsessile</i> (Standl.) Steyerm. & Standl.	Violaceae	MX, GT, BZ	Ni	0.05 – 4.2
			Co	0.01 0.3

Table 7. Metal hyperaccumulator plant species identified during this research. Key for countries: BZ– Belize, GT – Guatemala, MX – Mexico, CU- Cuba, CR – Costa Rica.

In total, 10 metal hyperaccumulator species were identified in Mexico under the context of this research (**Table 7**) including the confirmation of Ni hyperaccumulation in the field on the three *Psychotria* species only reported from herbarium XRF screening.

However, this chapter focused on the study of the hyperaccumulator species we considered the most outstanding. The first article titled “Nickel hyperaccumulation in *Orthion* and *Mayanaea* (Violaceae) from Mesoamerica” is in preparation for submission to the journal “*Phytochemistry*”. It describes the discovery of the new Ni hyperaccumulator genus *Orthion* and its relative species *Mayanaea caudata* (Violaceae). The second article “*Blepharidium guatemalense*, an obligate nickel hyperaccumulator plant from non-ultramafic soils in Mexico” has been accepted for publication (minor revisions) to the journal “*Chemoecology*” in July 2020 and it reveals the astonishing traits of one of the strongest hypernickelophore reported so far: *Blepharidium guatemalense* (Rubiaceae).

The main objectives of this chapter were : i) to describe the discovery of some new metal hyperaccumulator plant species in localized Ni-enriched (non-ultramafic) soils in Mexico, ii) to provide valuable information about some of the discovered metal hyperaccumulator plant species (ecology, taxonomic and geographic distribution, phylogeny, biochemistry, metal localization in plant tissues) in Mexico.

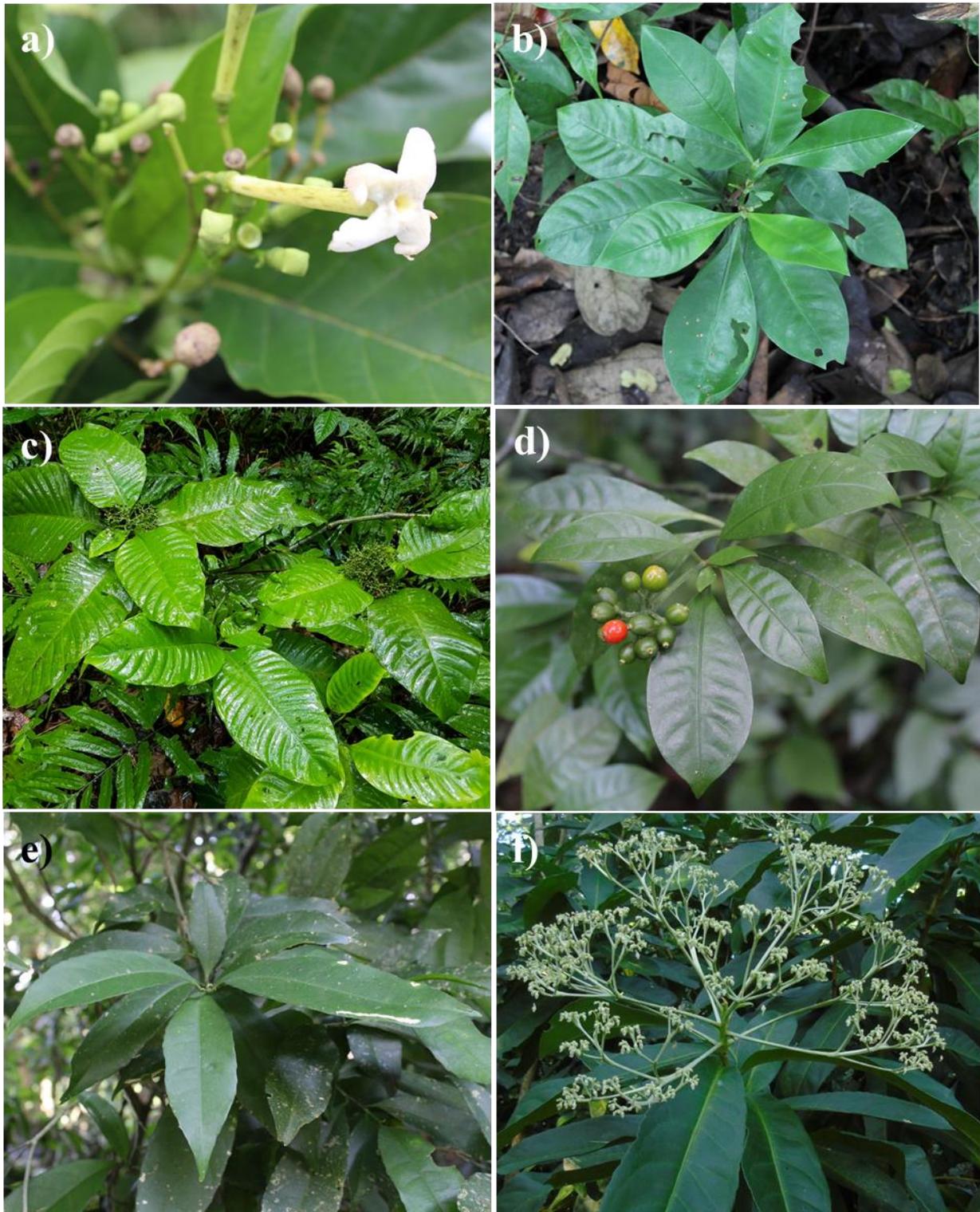


Figure 4. Photos of metal hyperaccumulator plants found in the evergreen forest of southeastern Mexico: a) *Blepharidium guatemalense*, b) *Psychotria costivenia*, c) *Psychotria lorenciana*, and f) *Orthion subsessile* (photo S. Álvarez) in Ocosingo (Chiapas); d) *Psychotria papantlensis* and e) *Orthion veracruzense* in San Andres Tuxtla (Veracruz).

Chapter III a. Nickel hyperaccumulation in *Orthion* and *Mayanaea* (Violaceae) from Mesoamerica

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Abstract

Background and Aims

Metal hyperaccumulation plants have been mostly documented from New Caledonia, the Mediterranean basin, and Southeastern Asia, while many other regions in the world remain understudied. This work is one of the pioneers in the search for new hyperaccumulator plant species in Mesoamerica. The impetus of this study was to determine whether Ni hyperaccumulation occurred in *Orthion* spp., originally placed within the *Hybanthus* genus, and in its close related genus *Mayanaea*.

Methods

This study carried out a large handheld x-ray fluorescence (XRF) spectroscopy screening in major herbarium collections as well as field surveys to measure metal hyperaccumulation in the genera *Orthion* and *Mayanaea*. Field samples were analysed with their associated rhizosphere soils.

Key results

Herbarium specimens from eastern Mexico down to south Nicaragua were screened. The Ni concentration in leaves were as follows: *Orthion guatemalense* (380-5100 $\mu\text{g g}^{-1}$, n=6), *O. malpighiifolium* (24-5620 $\mu\text{g g}^{-1}$, n=7), *O. montanum* (1630-5850 $\mu\text{g g}^{-1}$, n=6), *O. ob lanceolatum* (60-

1440 $\mu\text{g g}^{-1}$, n=6), *O. subsessile* (450-18 700 $\mu\text{g g}^{-1}$, n=77), and *O. veracruzense* (22-3660 $\mu\text{g g}^{-1}$, n=89). In the field, *Orthion veracruzense* reported mean Ni concentrations of 2520 $\mu\text{g g}^{-1}$ and *O. subsessile* of 14 600 $\mu\text{g g}^{-1}$. *Orthion* species are also frequent facultative cobalt hyperaccumulators.

Conclusions

The present study revealed for the first time Ni hyperaccumulation in the Violaceae family in the American continent. Ni hyperaccumulation was discovered in all *Orthion* species and in the closely related *Mayanaea* genus with at least one obligate Ni hyperaccumulator: *O. subsessile*. *Orthion* and *Mayanaea* are phylogenetically related to the strong Ni hyperaccumulators of the *Hybanthus* genus from Australia and New Caledonia (same clade). *Orthion subsessile* has suitable traits to become a potential Ni agromining crop.

Keywords: *Hyperaccumulation, Ultramafic soils, Hybanthus, Orthion, Mayanaea caudata, phylogeny, Nickel, Cobalt, Agromining, Neotropics, Mexico, Central America.*

1. Introduction

Metal hyperaccumulation by plants is defined as the ability to accumulate one or several metallic (or metalloid) elements in foliar tissues at concentrations that are two to three orders of magnitude higher than in plants growing on non-metalliferous soils (van der Ent et al., 2013). In total, there have been 721 hyperaccumulator plant species reported, of which 532 are nickel (Ni) hyperaccumulators (R. D. Reeves et al., 2018b). This global inventory is constantly growing as a result of ongoing research, with many recent discoveries aided by a novel method employing X-ray fluorescence (XRF) measurements of large numbers of herbarium specimens (van der Ent et al., 2019a). The hyperaccumulation phenomenon can help to better understand the mechanisms underlying regulation of essential and non-essential trace elements in plants (Pollard et al 2002). Hyperaccumulator plants also have practical applications in phyto-extraction and phytomining/agromining (Brooks et al., 1999; Nkrumah et al., 2016; Chaney et al., 2018b). Hyperaccumulator plants mainly occur on geological outcrops of metalliferous substrates (*e.g.* ultramafic soils), but they can also occur on soils with elevated metal concentrations resulting from

anthropogenic activities such as mining or smelting (Baker et al., 2010; R. D. Reeves et al., 2018b).

Major centres of hyperaccumulator biodiversity include New Caledonia (Boyd and Jaffré, 2009; Jaffré et al., 2013; Gei et al., 2020b), the Mediterranean basin (Reeves and Adigüzel, 2008; R. D. Reeves et al., 2018b), and the Malaysian state of Sabah in northern Borneo (Bouman et al., 2018; van der Ent et al., 2017a, 2019b), while many other regions in the world remain under-studied. The first reported centres of hyperaccumulator biodiversity in the Western Hemisphere were in Cuba and Brazil (Reeves et al., 1996, 1999; R. D. Reeves et al., 2007), with hyperaccumulating taxa concentrated mainly in the families Euphorbiaceae, Phyllanthaceae, Asteraceae, Buxaceae and Rubiaceae. In addition, a few cases of Ni hyperaccumulation have been reported from the western United States (Reeves et al., 1983), Puerto Rico and the Dominican Republic (Reeves, 2003; Ginocchio and Baker, 2004; Campbell et al., 2013). Until recently there were no known examples of hyperaccumulation in Central America, but herbarium surveys using XRF have identified four species of Ni hyperaccumulators in the genus *Psychotria* (Rubiaceae) (McCarthy et al., 2019) with ranges extending from southern Mexico (states of Veracruz, Tabasco, Campeche and Chiapas) through Central America to northwestern South America.

In 2017 we visited several localities in Mexico, selected on the basis of herbarium specimens of *Psychotria* spp. with high Ni concentrations. During surveys of associated species in these communities, we discovered Ni hyperaccumulation in *Orthion veracruzense* Lundell (Violaceae) growing in tropical forests in the state of Veracruz. Initial observations of high Ni concentrations in leaves were made with a semi-quantitative colorimetric test using dimethylglyoxime (DMG) suggesting possible hyperaccumulation by this species. Following on, an exhaustive elemental analysis of herbarium specimens of all the species of *Orthion* was undertaken in different herbaria. Because of many positive responses, a second field exploration was conducted in 2018, in Veracruz and Chiapas. This paper presents the results from herbarium and field surveys to determine the taxonomic distribution of hyperaccumulation in *Orthion* and the related genus *Mayanaea*, along with detailed characterization of plant-soil relationships and the localization of Ni in plant tissues.

2. Materials and methods

2.1. Species studied

The genus *Orthion* (Violaceae) as currently circumscribed comprises six species native to Mexico, Guatemala and Belize. The first to be described were published as *Hybanthus subsessilis* and *H. malpighiifolius* by Standley (1935), but separated into the new genus *Orthion* by Standley and Steyermark (1940) on the basis of morphological differences including longer-pedunculate inflorescences and distinctive leaves. Four other species were later added to the genus: i) *O. guatemalense* Lundell, ii) *O. montanum* Lundell, iii) *O. oblanceolatum* Lundell and, iv) *O. veracruzense* Lundell (Gleason et al., 1984; Lundell, 1984). A seventh species was originally published as *O. caudatum* Lundell, but subsequently segregated as *Mayanaea caudata* (Lundell) Lundell, based on differences in the inflorescences and flowers (Lundell, 1984).

Previous research and available herbaria databases show that *Orthion* spp. are mainly distributed in the evergreen tropical forests of southeastern Mexico and Central America, surprisingly matching with the distribution ranges of some of the previously mentioned hyperaccumulating *Psychotria* species (Guevara et al., 1994; Ibarra-Manríquez and Colín, 1996a; Ibarra-Manríquez et al., 1997).

Intriguingly, the literature includes one reference to Ni accumulation in an *Orthion* specimen from Mexico identified as *Hybanthus malpighiifolius*, with a Ni concentration of 638 $\mu\text{g g}^{-1}$ dry weight (Brooks et al., 1977a). This is an unusually high value but falls short of the 1000 $\mu\text{g g}^{-1}$ threshold for hyperaccumulation. Apart from this one report, no other studies have examined Ni in this genus.

2.2. Handheld XRF survey on herbarium collections

X-ray Fluorescence (XRF) measurements were performed in five herbaria: (i) the National Herbarium of Mexico (MEXU) in Mexico City, Mexico; (ii) the National Museum of Natural History's Herbarium (P) in Paris, France; (iii) the Missouri Botanical Garden's Herbarium (MO) in Saint Louis, U.S.A.; (iv) the Universidad San Carlos de Guatemala Herbarium (USCG); and (v)

the Universidad Del Valle Herbarium (UVAL) in Guatemala City, Guatemala. All specimens of *Orthion* and *Mayanaea* in these collections were analyzed using XRF as described below. A handheld X-Ray fluorescence spectrometer (Thermo Scientific Niton XL3T-980 GOLDD+) was used. Herbarium specimens were scanned for 30 s in ‘Soils’ mode with a titanium plate (99.995 % purity) underneath the herbarium sheet. During the survey, specimens are subjected to a beam of high-energy X-rays generated by a miniature X-ray tube [Ag anode (6–50 kV, 0–200 μ A max)] (main excitation source). The resulting spectrum of excited fluorescent X-rays is picked up by a Silicon Drift Detector (SDD). Therefore, a range of different elements can be detected and quantified simultaneously with detection limits of about 140 μ g g⁻¹ for most transition elements such as Ni, cobalt (Co), manganese (Mn), zinc (Zn).

2.3. Calibration of the XRF data

A total of 256 leaf samples of different Ni hyperaccumulator plant species were collected from different plant species in the field. Leaf squares of 1 cm² in size of each sample were cut and analyzed for 30 s in ‘soils’ mode with three replicated readings per sample. After XRF analysis, leaf samples were digested and analyzed with Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES, Thermo Fisher CAP 6300 Duo). Paired ICP and XRF data were then analyzed by logarithmic linear regression. It was not necessary to discard any data point because the minimum XRF value was about 500 μ g g⁻¹, greatly higher than the limit of detection (LOD < 190) estimated by (Gei et al., 2020b; van der Ent et al., 2019b). Residuals (observed vs. predicted values) were used to detect and remove outliers (± 3 SD of the residual). A secondary linear regression was then derived and transformed to power regression generating the following calibration equation: $y = 1.1216x^{0.953}$ (x = XRF measurement, y = ICP predicted value), R^2 of 0.87. Nickel XRF measurements were finally corrected by using the resulting equation.

2.4. Study and collection sites

2.4.1. “Los Tuxtlas” Biology station

The first survey was conducted in lot no. 67 of the Reserve of “Los Tuxtlas” Biology Station which belongs to the Universidad Nacional Autónoma de Mexico. The Reserve is situated in the state of Veracruz (eastern Mexico), in the foothills of the San Martin volcano which is part of the Los Tuxtlas mountain range. Since the Oligocene, this region has experienced strong volcanic activity

which has influenced soil pedogenesis. The last explosions of the San Martín volcano (1664 and 1793) caused soil rejuvenation in the lower part of the Reserve (Flores-Delgadillo et al., 1999). Soils have been identified as “eutrophic brown” derived from the fast weathering of volcanic ashes of basaltic and basaltic-andesite origin (Díaz Gonzalez, 2010; Flores-Delgadillo et al., 1999). Fulvic Andosols were found in the upper part of the Reserve whereas Alfisols were found in the lower part. The main minerals composing the volcanic ashes are olivine, pyroxene and amphibole (Aguilera-Gómez, 1988; González Soriano et al., 1997; Flores-Delgadillo et al., 1999). The predominant vegetation is evergreen rain forest and altitudes range from 150 to 530 m. a.s.l (Lot-Helgueras, 1976). Weather is warm humid (Af(m)w’(i’)g) with rains during the whole year and humid winds from the Gulf of Mexico (annual rainfall between 3000 and 4900 mm), and an average annual temperature of 24°C (García, 2004; Guevara et al., 2004).

2.4.2. Ocosingo, Chiapas

The surveyed sites were in the surroundings of the communities of Lacanhá Chansayab, Nuevo Guerrero, San Javier and Cojolita, at altitudes ranging from 100 to 300 m.a.s.l. The area is partly covered by the “Lacandona” tropical evergreen rainforest, but also by secondary forests or fallow fields locally known as “acahuales”, paddocks and grassland. The region is dominated by rolling hills made up mainly of limestone sedimentary rocks from the Mesozoic with overlapped layers of marine origin from the lower Tertiary, whereas marls and clays occur on the foot of the slopes and in alluvial areas (Müllerried, 1957; Levy Tacher et al., 2006). Soils are predominantly calcareous, including Rendzinas, Gleysols and Litosols (INIFAP-CONABIO, 1995). Loamy Rendzina is reported in secondary forests and Calcaric Phaeozem in primary forests (Mendoza-Vega and Messing, 2005). Climate is humid warm with abundant rains in summer (Am (i’)gw”) (García, 2004; Köppen, 1936), and a slight decrease in rainfall from February to April. The average annual temperature is 25°C with low thermal oscillation and average annual precipitation of ~2500 mm (García, 2004).

2.5. Plant and soil sampling

In Veracruz, the exploration extended over a radius of two kilometres from the facilities of “Los Tuxtlas” Biology Station. In the case of Chiapas, sampling was over a radius of no more than 35 km from the locality of Lacanhá Chansayab. Different footpaths were traversed to survey the

existing flora. Filter papers impregnated with dimethylglyoxime (DMG) were used as a rapid test (it gives a pink reaction when contacted with Ni). If Ni hyperaccumulation was suspected, three plant leaves of the individual and a rhizosphere soil sample (15 cm depth) were taken. Other plant tissues such as bark, roots and flowers were also collected when available. In total, 11 sites were sampled (five from Veracruz and six from Chiapas). For identification purposes, duplicate plant samples were collected and deposited as vouchers at MEXU.

2.6. Bulk analysis of plant tissues

Plant tissues samples were dried at 40°C for 5 days in a drying oven, ground and digested using 1 mL HNO₃ (65%) and 2 mL H₂O₂ (30%) and then left to stand overnight. The next day, samples were placed in a heating block (*DigiPREP*) for 2h at 95°C, left to cool to 40°C, filtered at 0.45µm, and adjusted in volume to 10 mL for minor elements quantification. A dilution to 1:10 with ultrapure water was executed for the quantification of major elements. Finally, digests were also analyzed by ICP-AES.

2.7. Bulk analysis of rhizosphere soil samples

Soil samples were air-dried for one week, placed into identified plastic bags and then transported to the laboratory for chemical analysis. Soil pH was measured with in a 1:5 soil: water (5 mL soil with 25 mL ultrapure water) suspension after 1 h shaking at 16 rpm and 1 h standing. Cation exchange capacity (CEC) of soils was determined by means of cobalt-hexamine trichloride extraction by adding 50 mL of solution (4.458 g of Co(NH₃)₆Cl₃ with 700 mL of distilled water); after 1 h shaking, 10 mL of each sample were filtered at 0.45 µm and measured by spectrophotometry (BioRad, SmartSpec Plus). Available P was determined according to the Olsen method. For total major and minor elemental quantification, soil samples were dried at 70°C for 3 days in a drying oven and sieved (< 2 mm). Subsequently, 0.5 g (± 0.001 g) of each sample, finely ground (< 250 µm), were digested by adding 6 mL of 37% HCl and 2 mL of 70% HNO₃ per sample, left to stand overnight, placed in a heating block (*DigiPREP MS*) for 3h at 100°C and then left to cool to a minimum temperature of 40°C; solutions were adjusted in volume to 45 mL, filtered at 0.45 µm and then readjusted to 50 mL with ultrapure water. A final dilution of 1:20 was necessary for major elements. The resulting soil extracts were analyzed by ICP-AES for the quantification of major and minor elements. Trace elements availability in soil samples was

determined by using diethylenetriaminepentaacetic acid (DTPA-extractant) according to the method of Lindsay and Norvell, (1978), and the soil extracts were also analyzed by ICP-AES.

2.8. Micro X-Ray fluorescence microscopy

High spatial resolution (~20 µm) distribution maps of Ni, Ca and potassium (K) were obtained using a micro-XRF (Bruker M4 Tornado). The device has a Rh X-ray tube (50 kV and up to 50 W) as the main excitation source coupled to polycapillary optics. A large and flat sample of a dry leaf of *Orthion subsessile* collected from its natural habitat in Chiapas was subjected to an X-ray beam with a diameter of 20–30 µm inside a chamber under controlled 20 mbar vacuum. X-rays are then detected by two 30 mm² Xflash® Silicon Drift detectors (energy resolution of <135 eV FWHM at Mn K α). The leaf was then powdered and analysed for major and minor elements by ICP-AES.

2.9. Statistical analyses

Soils and plants data were analyzed using Microsoft Excel 2010 (XL STAT) for descriptive statistics (*e.g.* histograms and boxplots). Key symbols for boxplots: open boxes are inter-quartile ranges, bold crosses are \pm mean, whiskers are \pm standard deviation and bold circles are outliers. Maps were generated using ArcGIS version 10.3.

3. Results

3.1. Herbarium XRF survey

A total of 191 herbarium specimens were analysed by XRF, corresponding to the six species of *Orthion*: *O. guatemalense* (n = 6), *O. malpighiifolium* (n = 7), *O. montanum* (n = 6), *O. oblanceolatum* (n = 6), *O. subsessile* (n = 77), *O. veracruzense* (n = 89) and *Mayanaea caudata* (n=1). **Figure 5** shows specimens of *Orthion* spp. and *Mayanaea* analysed in the different herbaria.

Nickel concentrations and the percentage of specimens exceeding the Ni hyperaccumulation threshold in each species of *Orthion* are shown in **Table 8**.



Figure 5. Herbarium voucher specimens of a) *Orthion guatemalense* with 2700 $\mu\text{g g}^{-1}$ Ni collected in Petén (Guatemala), b) *O. malpighiifolium* with 3800 $\mu\text{g g}^{-1}$ Ni collected in Chiapas (Mexico), c) *O. montanum* with 2500 $\mu\text{g g}^{-1}$ Ni collected in Chiapas, d) *O. sessile* with 4400 $\mu\text{g g}^{-1}$ Ni collected in Chiapas, e) *O. veracruzense* with 2300 $\mu\text{g g}^{-1}$ collected in Veracruz (Mexico), and f) *Mayanaea caudata* with 5390 $\mu\text{g g}^{-1}$ Ni collected in Izabal (Guatemala). Specimen numbers in order of appearance: 513651, 1261364, 1257801, 11511696, from Herbario Nacional de México (MEXU), Departamento de Botánica, Instituto de Biología (IBUNAM). The last specimen number is P00426145 from the National Museum of Natural History's Herbarium (P) in Paris, France.

Plant species	Number of specimens	Number and (%) of Ni hyper-accumulators	Ni concentration ($\mu\text{g g}^{-1}$)	Number and (%) of Co hyper-accumulators	Co concentration ($\mu\text{g g}^{-1}$)
<i>O. guatemalense</i>	6	4 (67%)	380 – 5100 (1940 \pm 1780)	0 (0%)	<140 – 277
<i>O. malpighiifolium</i>	7	2 (29%)	24 – 5620 (3160 \pm 2860)	0 (0%)	<140
<i>O. montanum</i>	6	6 (100%)	1630 – 5850 (2550 \pm 1650)	2 (40%)	323 – 439
<i>O. oblanceolatum</i>	6	2 (33%)	60 – 1440 (680 \pm 600)	1 (17%)	156 – 774
<i>O. subsessile</i>	77	71 (92%)	450 – 18 700 (5360 \pm 3910)	26 (36%)	144 – 2660 (516 \pm 459)
<i>O. veracruzense</i>	89	11 (12%)	22 – 3660 (520 \pm 650)	9 (10%)	141 – 709 (259 \pm 119)

Table 8. General information of the discovered hyperaccumulator species in the genus *Orthion* based on data from X-ray fluorescence spectroscopy screening in P, MO and MEXU herbaria. Nickel and cobalt concentrations are presented in ranges and means between parentheses. Cobalt concentrations below the limit of detection ($140 \mu\text{g g}^{-1}$) were not included.

All the species of the genus could be considered Ni hyperaccumulators since at least one specimen of each exceeded $1000 \mu\text{g g}^{-1}$ Ni. The highest concentration was found in one specimen of *O. subsessile* ($18\,700 \mu\text{g g}^{-1}$) collected in the department of Izabal in Guatemala. This species displays an apparently log-normal frequency distribution **Fig. 6a**, and 75 % of the specimens had Ni concentrations within a range from 2100 to $7800 \mu\text{g g}^{-1}$ **Fig. 6b**. About 92% of the specimens of *O. subsessile* exceeded the hyperaccumulator threshold (mean of $5360 \mu\text{g g}^{-1}$) and 9% reached Ni concentrations above $10\,000 \mu\text{g g}^{-1}$. Conversely, only 12% of the specimens of *O. veracruzense* had Ni concentrations above $1000 \mu\text{g g}^{-1}$. The specimen with the highest value of $3340 \mu\text{g g}^{-1}$ Ni was collected in the municipality of Ocosingo in Chiapas, Mexico. The frequency distribution of this species was multimodal (**Fig. 6c**). Although the mean Ni concentration in *O. veracruzense* was $451 \mu\text{g g}^{-1}$, 50% of the specimens had values below $236 \mu\text{g g}^{-1}$ Ni (**Fig. 6d**). Only few specimens of the other four species of *Orthion* ($n < 7$) were available for XRF screening. All of the specimens of *O. montanum* had concentrations above the hyperaccumulation threshold with a mean of $2550 \pm 1650 \mu\text{g g}^{-1}$. Although *O. guatemalense* and *O. malpighiifolium* had mean values above $2000 \mu\text{g g}^{-1}$, only 67% and 29 % (respectively) of the specimens exceeded $1000 \mu\text{g g}^{-1}$. *Orthion oblanceolatum* showed the weakest Ni accumulation with a mean of 680 ± 600 and only two specimens had concentrations above the hyperaccumulation threshold. The only specimen

available for measurement of *Mayanaea caudata* had 5390 $\mu\text{g g}^{-1}$ Ni and it was collected in the department of Izabal (El Estor) in Guatemala **Fig. 5f**.

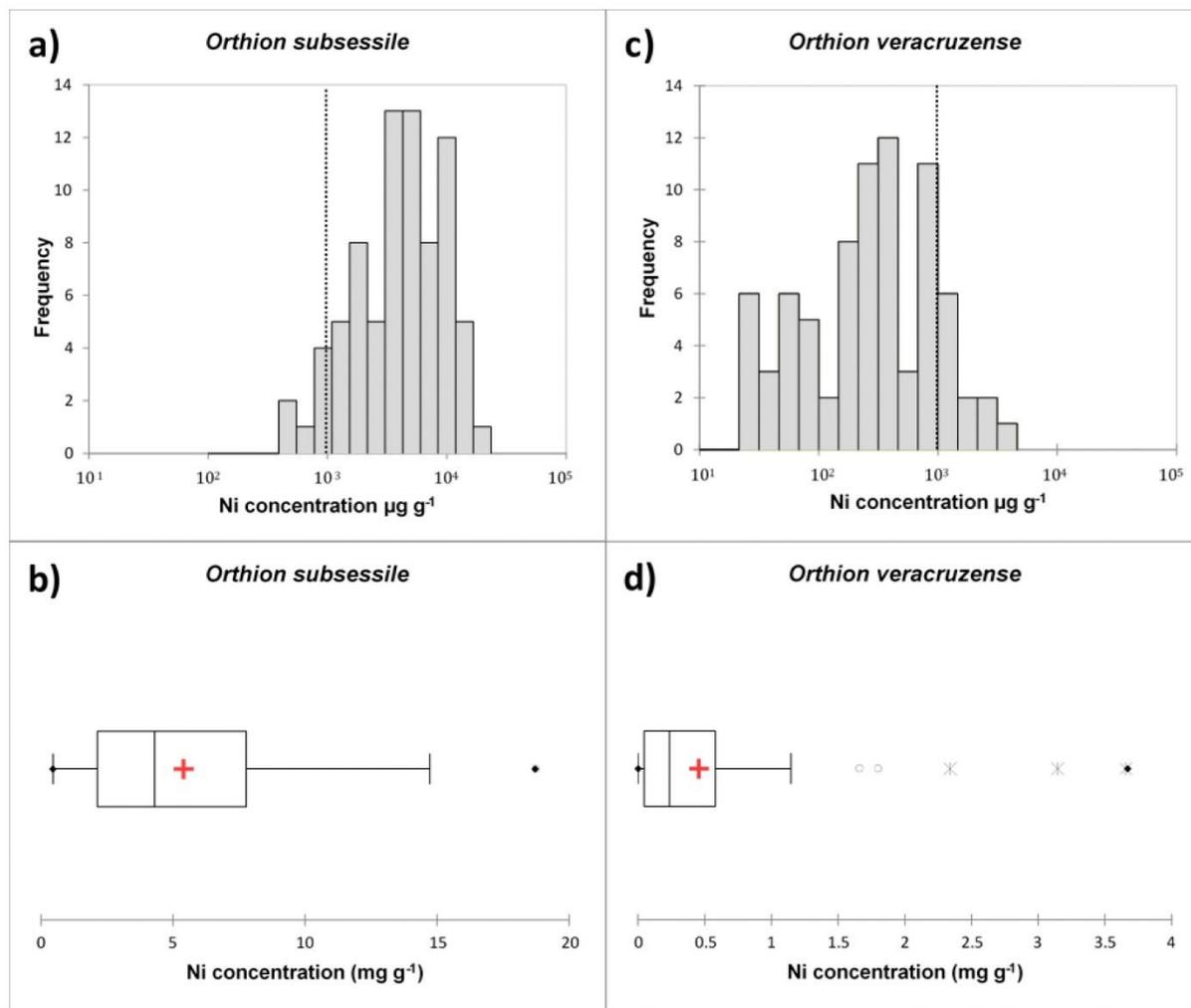


Figure 6. Histograms representing frequency distribution of foliar Ni concentrations (logarithmic scale) in **a)** *O. subsessile* and **c)** *O. veracruzense* based on XRF measurements in herbaria. Nickel concentrations in herbarium specimens of **b)** *O. subsessile* and **d)** *O. veracruzense* presented as boxplots.

Cobalt hyperaccumulation ($>300 \mu\text{g g}^{-1}$) was detected in one specimen of *O. oblancoelatum*, two specimens of *O. montanum*, 9 specimens of *O. veracruzense* and 26 specimens of *O. subsessile* (**Table 8**). The maximum concentration of $2660 \mu\text{g g}^{-1}$ Co was found in one specimen of *O. subsessile* collected in a 20-year fallow field in Chiapas (Mexico) which was simultaneously hyperaccumulating Ni reaching $2870 \mu\text{g g}^{-1}$. A total of 29 voucher specimens also displayed simultaneous hyperaccumulation of Ni and Co: *O. oblancoelatum* (1), *O. veracruzense* (1), *O.*

montanum (2) and *O. subsessile* (25). The frequency distribution of Co concentration among the specimens of *O. subsessile* was not clearly normal and although the mean was about 500 $\mu\text{g g}^{-1}$ Co, half of the specimens had Co concentrations below 360 $\mu\text{g g}^{-1}$ Co (**Fig. 7a, b**). *Orthion veracruzense* had a poorly defined bimodal distribution with a mean of 271 $\mu\text{g g}^{-1}$ Co and a maximum Co concentration of 709 $\mu\text{g g}^{-1}$ (**Fig. 7c, d**).

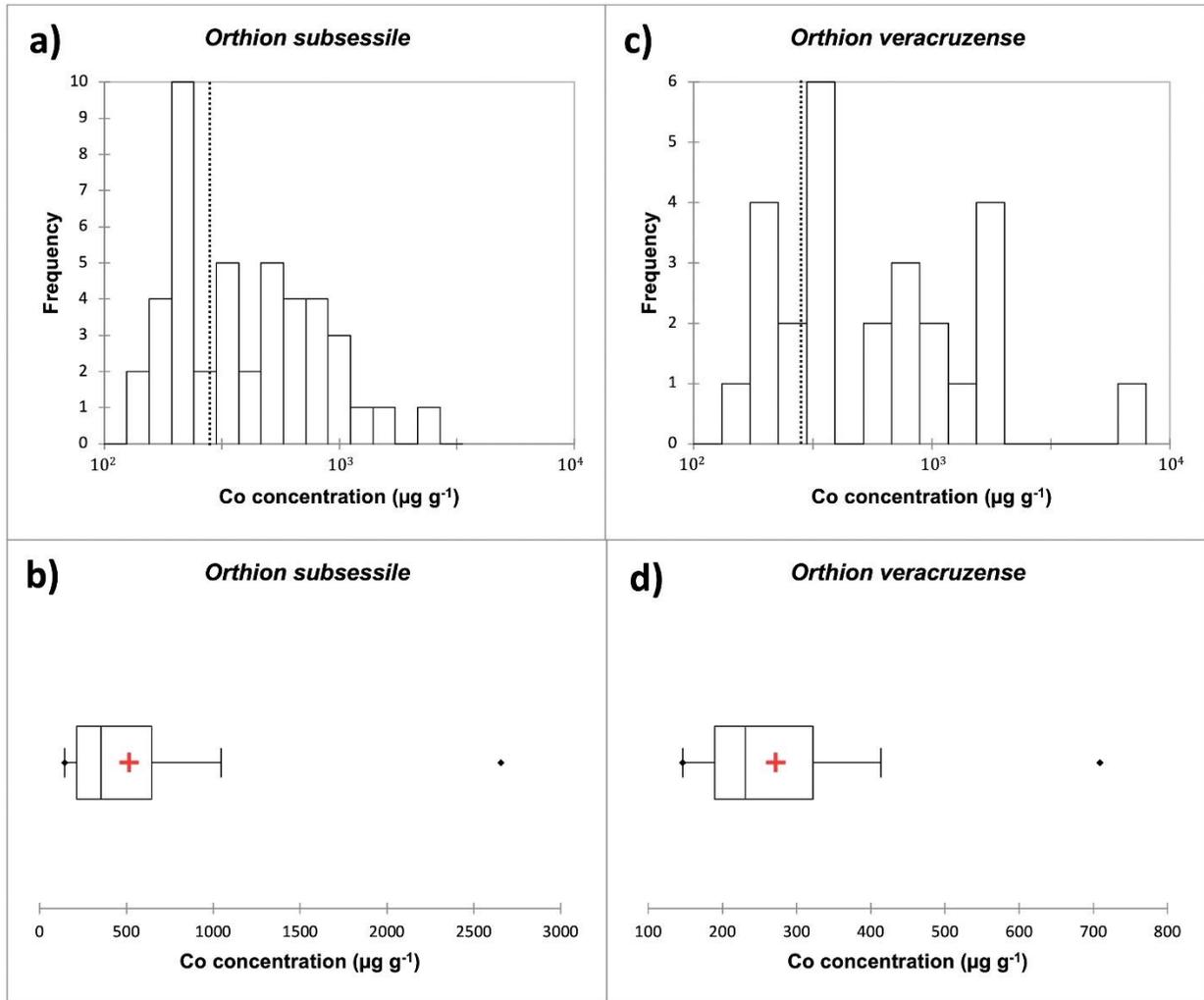


Figure 7. Histograms representing frequency distribution of foliar Co concentrations (logarithmic scale) in a) *O. subsessile* and c) *O. veracruzense* based on XRF measurements in herbaria. Cobalt concentrations in herbarium specimens of b) *O. subsessile* and d) *O. veracruzense* presented as boxplots.

Specimens with available latitude and longitude coordinates were georeferenced in a map **Figure 8a**. The geographic distribution of the specimens included Mexico, Guatemala, Belize and

Nicaragua but most of the specimens were from Mexico (~85%). Three of the species are Mexican endemics: *Orthion montanum*, *O. oblanceolatum* and *O. veracruzense*. Within Mexico, Ni hyperaccumulation was found in the states of Veracruz, Tabasco, Campeche and Chiapas and in almost all the *Orthion* spp. except for *O. guatemalense* **Fig. 8b**. In Guatemala, Ni hyperaccumulation was found in specimens of *O. guatemalense* and *O. subsessile*, most of them collected within the ophiolitic ultramafic belt (Polochic-Motagua faults) in the department of Izabal. In Belize, only three specimens of *O. subsessile* with Ni hyperaccumulation were found in the department of Toledo. Substrates where Ni hyperaccumulators occur are many and varied, ranging from ultramafic soils in Guatemala, to volcanic (basalt-andesite) soils in Veracruz (Mexico) to calcareous soils in Chiapas, Tabasco and Campeche (Mexico).

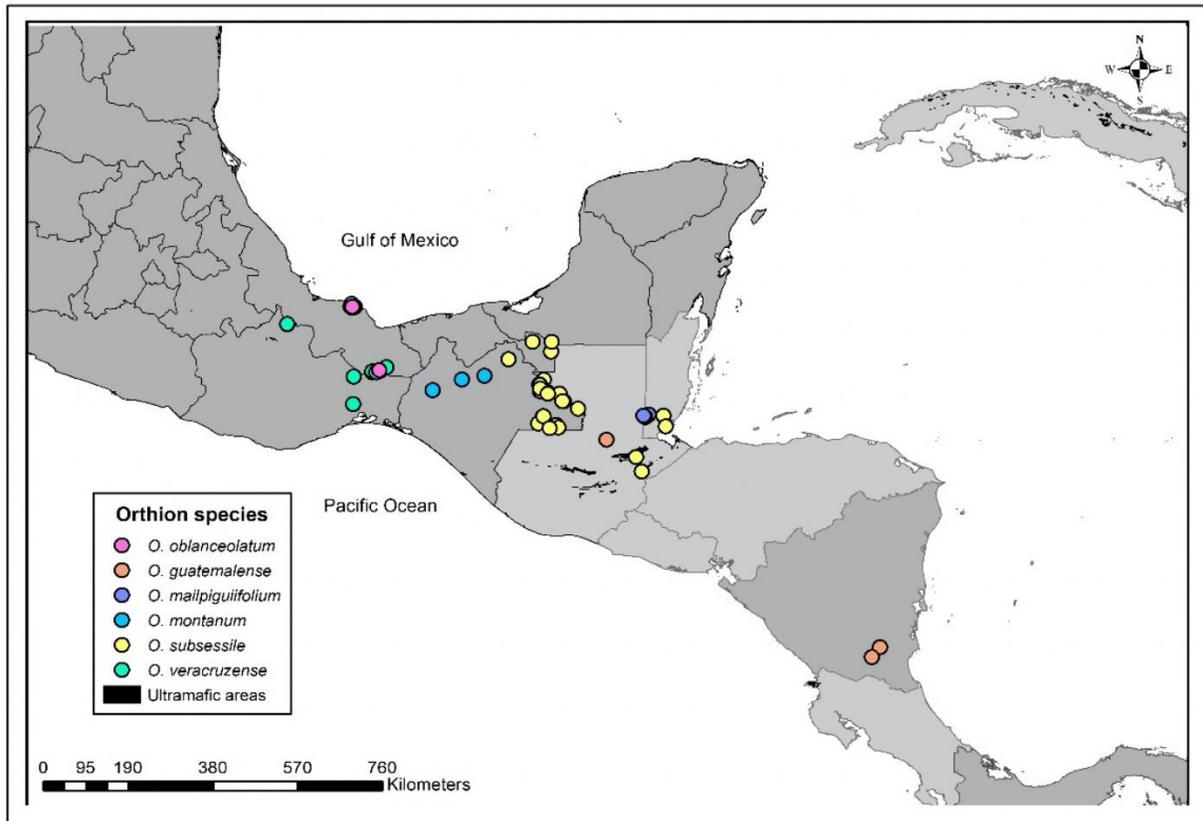


Figure 8a Map showing the geographic distribution of the *Orthion* species based on available geographic coordinates of herbarium voucher specimens from the three herbaria surveyed (MEXU, MO and P).

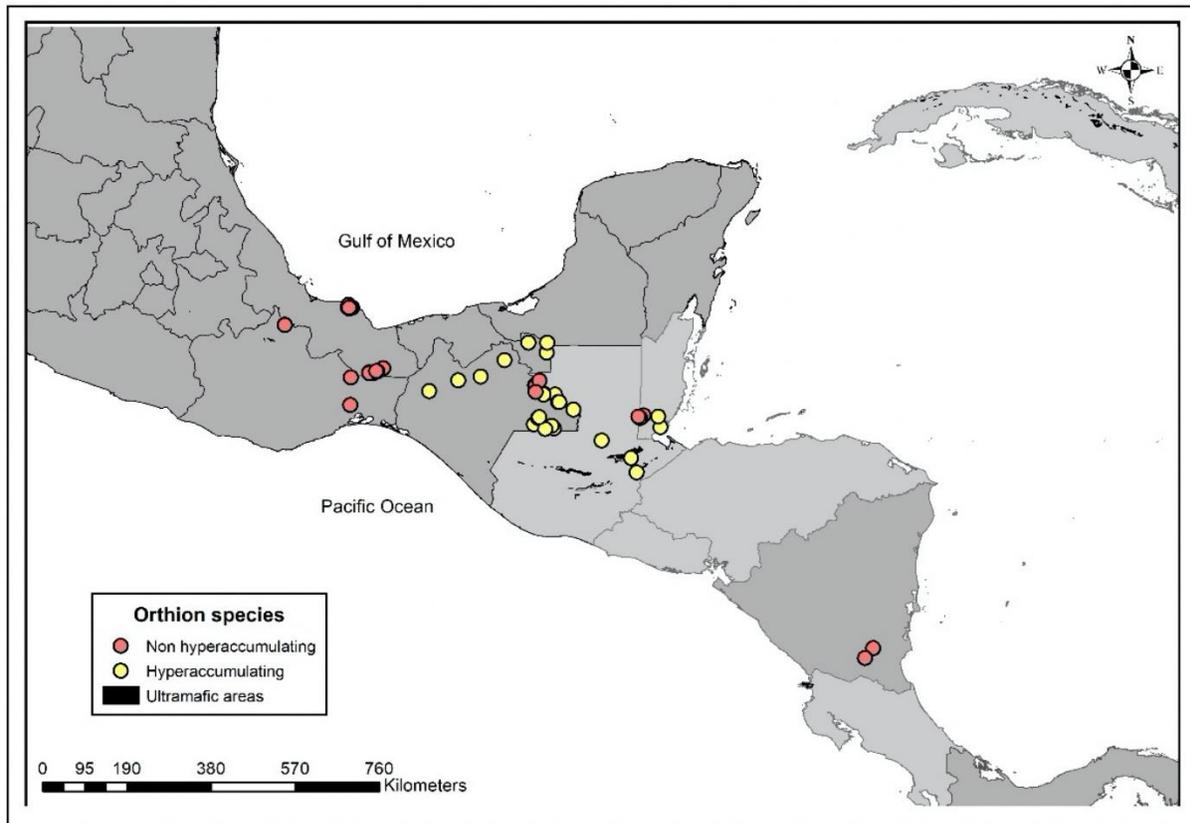


Figure 8b. Map showing the hyperaccumulation occurrence of *Orthion* species, based on available geographic coordinates of herbarium voucher specimens from the three herbaria surveyed (MEXU, MO and P).

3.2. Field studies of *O. veracruzense* at “Los Tuxtlas” Veracruz

Nickel hyperaccumulation was first detected in *O. veracruzense* by means of the DMG test and then confirmed by ICP-OES analysis. This species was observed growing in the lowland forest (150–185 m.a.s.l.) coexisting with *Dendropanax arboreus* (L.) Decne. & Planch., *Nectandra ambigens* (S.F.Blake) C.K.Allen, *Siparuna andina* (Tul.) A. DC., *Costus scaber* Ruiz & Pav., *Brosimum alicastrum* Sw., *Bursera simaruba* (L.) Sarg., among others (Ibarra-Manríquez and Colín, 1995, 1996b, 1996a). *Orthion veracruzense* may occur together with the Ni hyperaccumulator *Psychotria papantlensis* (Oerst.) Hemsl. Although most of the observed individuals were shrubs, the tallest tree was 12-m tall. Elemental concentrations of foliar tissues

and rhizosphere soil samples of *O. Veracruzense* are presented in **Table 9**. Rhizosphere soils presented a pH close to neutral in a range between 5.9 to 6.8. Total organic carbon (TOC) was high with a mean of 5.3% (organic matter ~9.1%) which is a common property in soils derived from volcanic ashes under humid warm weather. Soils had a medium concentration of total nitrogen with a mean of 0.6%. Mean exchangeable cations were high, mainly for calcium (Ca^{++}) (18.5 mEq/100 g) and magnesium (Mg^{++}) (7.4 mEq/100 g), with CEC reaching a maximum of 36 mEq/100 g (mean of 27 mEq/100 g). Regarding total elements, rhizosphere soils had normal total concentrations of the major elements calcium (Ca), sodium (Na), and phosphorus (P), but were very low in K. A relatively low exchangeable Ca:Mg ratio (2.5) was also observed although this was not reflected in plant leaves. Soils were very rich in iron (Fe) ($>110 \mu\text{g g}^{-1}$) and aluminium (Al) ($>80 \mu\text{g g}^{-1}$). Concentrations of trace elements (*e.g.* Cu, Co, Zn) were relatively low. Available (*i.e.* DTPA-extractable) Zn, Co and Cu concentrations in soils were also very low ($< 3.6 \mu\text{g g}^{-1}$). Total and DTPA-available Ni were not very high with means of $189 \mu\text{g g}^{-1}$ and $2.3 \mu\text{g g}^{-1}$, respectively. Foliar samples of *O. Veracruzense* reported a minimum Ni concentration of $815 \mu\text{g g}^{-1}$ and a maximum of $4290 \mu\text{g g}^{-1}$ (mean of $2520 \mu\text{g g}^{-1}$). Plant leaves were particularly high in K, Ca, magnesium (Mg), and sulphur (S) while concentrations of minor elements were unremarkable. Cobalt concentration in the leaves was slightly high reaching a maximum of $123 \mu\text{g g}^{-1}$ Co.

Elements	Plant leaves	Associated soil	
		Total	DTPA-extractable
K	15 800 ± 6150	717 ± 65	18 ± 14
Ca	19 300 ± 3810	5740 ± 2110	-
Mg	4370 ± 872	10 900 ± 211	139 ± 42
P	2200 ± 882	1320 ± 254	26 ± 11*
S	8780 ± 1870	587 ± 106	-
Al	<90	84 100 ± 4460	4.44 ± 3.36
Fe	<154	114 000 ± 12 000	56 ± 33
Mn	347 ± 185	2180 ± 269	22 ± 10
Cu	2.25 ± 1.29	145 ± 10	2.56 ± 1.17
Co	52 ± 43	70 ± 11	0.57 ± 0.36
Ni	2520 ± 1490	189 ± 17	2.13 ± 1.19
Zn	101 ± 43	163 ± 17	3.62 ± 1.93

Table 9. Element concentrations for leaves and rhizosphere soil samples of *O. Veracruzense* collected in the native habitat at the Biology Station “Los Tuxtlas” (Veracruz, Mexico). Concentrations are given in $\mu\text{g g}^{-1}$ and the number of samples is five. Other mean soil parameters: pH is 6.28, CEC is 27 meq/100 g. and C org is 5.3 % (~9.1% M.O.), *phosphorus was obtained by Olsen P extraction.

3.3. Field studies of *O. subsessile* in Ocosingo, Chiapas

Driven by the geographic coordinates of the XRF screened herbarium specimens, an individual of *Orthion subsessile* was identified growing in the backyard of a house in the small locality of Nuevo Guerrero (Ocosingo, Chiapas). Subsequently, the species was also found in primary and secondary forests coexisting with other reported Ni hyperaccumulators: *Blepharidium guatemalense*, *Psychotria costivenia* and *P. lorenciana*. Elemental concentrations of leaves and rhizosphere soil samples of *O. subsessile* are presented in **Table 10**. Rhizosphere soils had a neutral pH (mean of 6.6) ranging between 5.8 to 7.4. Total Nitrogen and TOC were very high with a mean of 0.5 wt% (organic matter ~ 8.6 wt%). Cation Exchange Capacity was high with a mean of 36 mEq/100 g. Exchangeable Base cations were very high for Ca²⁺ (26.1 mEq/100 g) and Mg²⁺ (8.9 mEq/100 g) and relatively high for K⁺ (0.26 mEq/100 g). Considering total concentrations, soils are rich in Fe, Mg and Mn which is normal for clay soils. The ratio of exchangeable Ca:Mg (2.9) was also low in these soils. Trace elements were not remarkable except for total and available Ni concentrations (means of 698 ± 376 and 62 ± 74 respectively). Available Co, Zn and Cu were very low (<2.5 µg g⁻¹) whereas major element concentrations in plant leaves were particularly high in K, Ca, Mg, and S. Foliar tissues had slightly high Co and Zn concentrations whereas the maximum value was for Ni reaching a maximum concentration of 47 200 µg g⁻¹ (mean of 14 600 ± 16 800 µg g⁻¹). Elevated Ni concentrations also occurred in other tissues of *O. subsessile* such as flowers (13 900 µg g⁻¹, *n* = 1), bark (mean of 11 300 µg g⁻¹, *n* = 5) and roots (mean of 2560 µg g⁻¹, *n* = 3).

Elements	Plant leaves	Associated soil	
		Total	DTPA-extractable
K	16 530 ± 2280	2250 ± 1270	12 ± 11
Ca	10 600 ± 4020	6690 ± 2710	-
Mg	2940 ± 850	12 400 ± 3930	292 ± 111
P	1170 ± 280	222 ± 49	8.8 ± 1.7*
S	4800 ± 2540	504 ± 71	-
Al	<195	29 800 ± 5880	<3.86
Fe	<50	45 700 ± 7470	46 ± 38
Mn	104 ± 47	1300 ± 314	13 ± 10
Cu	8.9 ± 3.6	30 ± 3.3	2.4 ± 1.3
Co	26 ± 19	58 ± 21	<0.29
Ni	14 600 ± 16 800	698 ± 376	62 ± 74
Zn	138 ± 112	54 ± 5.6	1.1 ± 0.7

Table 10. Element concentrations for leaves and rhizosphere soil samples of *Orthion subsessile* collected in the evergreen rainforests of south-eastern Chiapas, Mexico. Concentrations are given in µg g⁻¹ and the number of samples is six. Other mean soil parameters: pH is 6.63, CEC is 36 meq/100 g. and C org is 5.0 % (~8.6% M.O.), *phosphorus was obtained by Olsen P extraction.

3.4. Elemental distribution within *O. subsessile* tissues

Elemental maps obtained from Micro-XRF revealed that in *O. subsessile*, Ni was distributed throughout the leaf with a higher signal in the leaf midrib (**Figure 9**). Calcium was mainly concentrated in the secondary veins whereas K was mainly localized in the leaf midrib and nearby areas.

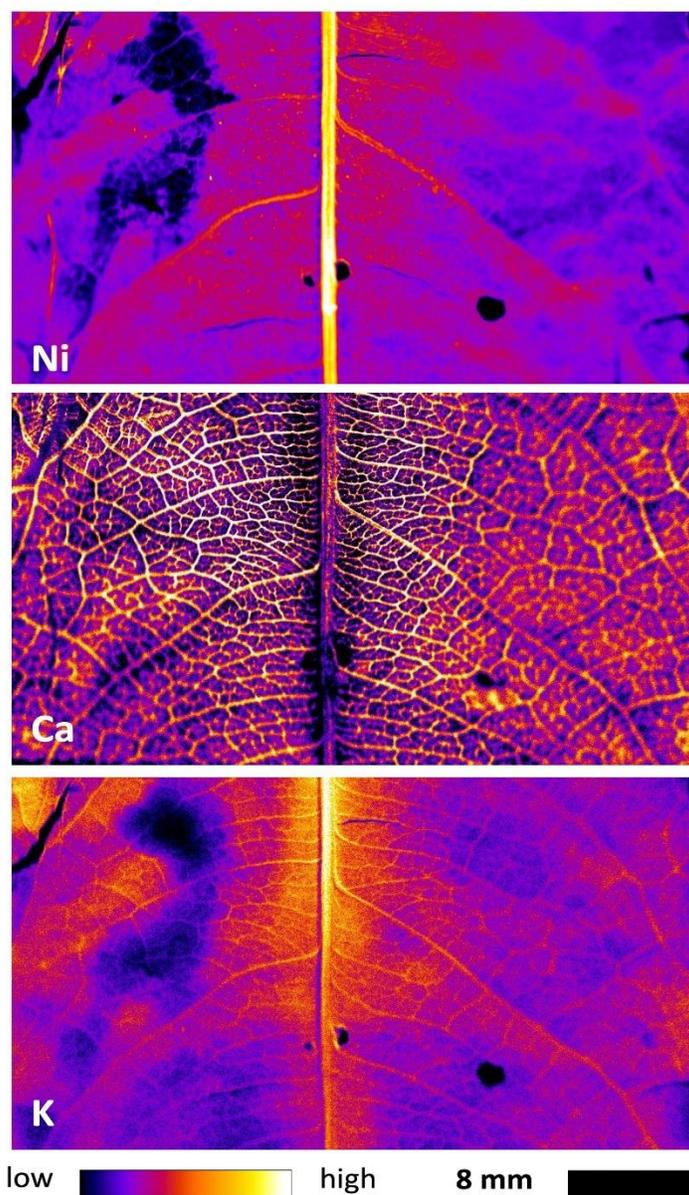


Figure 9. Maps showing elemental distribution in a mature leaf collected in the field (Chiapas) of *Orthion subsessile* generated using micro-XRF (20 μm resolution). Mean concentrations in the leaf: 42 200 $\mu\text{g g}^{-1}$ Ni, 12 500 $\mu\text{g g}^{-1}$ Ca and 9800 $\mu\text{g g}^{-1}$ K.

4. Discussion

The present study is the first report of Ni hyperaccumulation in the family Violaceae in the Western Hemisphere, as Ni hyperaccumulation in the Violaceae had been only reported in three genera (*Rinorea*, *Hybanthus* and *Agatea*) distributed in New Caledonia and Southern Asia (Brooks et al., 1974; Jaffré, 1980; Fernando et al., 2014; R. Reeves et al., 2018). In total, seven new Ni hyperaccumulator species were discovered through field exploration and herbarium survey. From the seven species, six belonged to the genus *Orthion* and the other one to the monotypic genus *Mayanaea*. *Orthion veracruzense* was the first Ni hyperaccumulator species to be discovered during the field exploration in “Los Tuxtlas”, Veracruz (Mexico). Although this species is not amongst the strongest hyperaccumulators, it was able to accumulate up to 4300 $\mu\text{g g}^{-1}$ Ni from soils with very low DTPA-available Ni concentrations ($<2.0 \mu\text{g g}^{-1}$). The presence of Ni in the soils from “Los Tuxtlas” Reserve is due to the fact that they originated from volcanic ash (basaltic and basaltic andesite) rich in mafic minerals such as pyroxene, olivine and amphibole (Jacob-Albarrán and Aguilera-Gómez, 1988; Flores-Delgadillo et al., 1999). Soils derived from basaltic volcanic ashes are also present in soils around the Popocatepetl and Parícutin volcanos (Central Mexico), suggesting that these areas should also be investigated for new hyperaccumulator plants (Flores-Delgadillo et al., 1999). Other chemical properties of rhizosphere soils such as high OM and CEC (mainly Ca^{2+} and Mg^{2+}) as well as the almost neutral pH were in accordance with previous research conducted at these sites (Flores-Delgadillo et al., 1999).

The presence of Ni hyperaccumulation in the six species of the genus *Orthion* was confirmed through XRF herbaria surveys. More than 90% of the specimens of *O. subsessile* exceeded the Ni hyperaccumulator threshold (1000 $\mu\text{g g}^{-1}$) suggesting an obligate pattern of hyperaccumulation. The last means that the geographic distribution of the species is restricted to metalliferous soils (e.g. Ni-rich) and this is known to happen in around 85–90% of all hyperaccumulator species (Reeves and Adigüzel, 2008; Pollard et al., 2014). The opposite case is that of *O. veracruzense* with only 12% of the specimens with Ni concentrations above the hyperaccumulation threshold. This pattern is known as facultative hyperaccumulation and describes a behaviour in which the species can grow in both non-metalliferous and metalliferous soils only displaying hyperaccumulation in the last (Pollard et al., 2014). Previous research demonstrated that the trait

of hyperaccumulation is present in all the populations of the facultative hyperaccumulators species (Boyd and Martens, 1998; Pollard et al., 2014). Based on the latter, the lack of Ni hyperaccumulation in most of the specimens of *O. veracruzense* may be due to environmental factors such as differences in Ni availability in soils. Unfortunately, there is no information available on the metal concentrations on soils where the herbarium specimens were collected. Nonetheless, foliar samples of *O. veracruzense* collected during the field survey in Veracruz showed a high variability in their Ni concentrations (range of 820–4300 $\mu\text{g g}^{-1}$), apparently not related to the DTPA-available Ni concentrations on rhizosphere soils (range of 1.05–3.80 $\mu\text{g g}^{-1}$).

The other four *Orthion* species all had at least one herbarium specimen that was a Ni hyperaccumulator. However, no further conclusions can be drawn due to the small number of available voucher specimens (<7) of each of the species and due to the possibility of erroneous identifications. For example, one scanned specimen identified as *O. oblanceolatum* may in fact be a novel taxon, based on morphological differences detected by one of the authors of the present study and verified by an expert from the MEXU herbarium. Other potential cases of misidentification could be two specimens of *O. malphighiifolium* collected in Chiapas (Mexico), which are unlikely because the species is only known to occur in the rainforest of Petén (Guatemala) and Toledo (Belize) (Lundell, 1984b).

Cobalt hyperaccumulation was found in almost 20% of all the specimens. Cobalt hyperaccumulation is a rare phenomenon with most of the plant species reported to occur in the Democratic Republic of Congo (Lange et al., 2017). More than a third of the herbarium specimens of *Orthion subsessile* had Co hyperaccumulation with a mean of 516 $\mu\text{g g}^{-1}$. Its maximum concentration of 2660 $\mu\text{g g}^{-1}$ Co (Chiapas, Mexico) was among the strongest reported so far, only surpassed by *Haumaniastrum robertii* (Robyns) P.A.Duvign. & Plancke (Lamiaceae) with 10 220 $\mu\text{g g}^{-1}$ Co (Central Africa) and *Walsura pinnata* Hassk (Meliaceae) with 4580 $\mu\text{g g}^{-1}$ Co (Southeast Asia) (Brooks, 1977; van der Ent et al., 2019b). An interesting discovery is that almost all the specimens that are Co hyperaccumulators simultaneously hyperaccumulate Ni. Cases of co-hyperaccumulation of Ni and Co have been previously reported in other species, such as *Rinorea bengalensis* (Wall.) Kuntze., and *Glochidion cf. sericeum* (Blume) Zoll. & Moritzi (Brooks et al., 1977b; van der Ent et al., 2018). A recent study revealed simultaneous Ni-Co hyperaccumulation

in the genera *Baccaurea*, *Walsura*, and *Ashtonia* from Sabah (Malaysia) through an herbarium XRF survey (van der Ent et al., 2019b). Foliar samples of *O. veracruzense* and *O. subsessile* collected in the field had elevated maximum Co concentrations (123 and 54 $\mu\text{g g}^{-1}$ Co respectively) compared with concentrations commonly found in ‘normal’ plants (0.2 $\mu\text{g g}^{-1}$ Co) (Markert, 1992b). However, these values did not exceed the hyperaccumulation criteria of 300 $\mu\text{g g}^{-1}$ Co (van der Ent et al., 2013), and this may be due to the low Co availability in soils (<0.6 $\mu\text{g g}^{-1}$) or to a high available Ni: available Co ratio which favors Ni uptake (Lange et al., 2017; Paul et al., 2020b; Rue et al., 2020). Also, soil rhizosphere samples collected in the field in both sites, Veracruz and Chiapas, had almost neutral pH whilst there is evidence that most plants accumulate Co at low pH (Kukier et al., 2004a).

An unexpected discovery from the herbarium XRF survey was the Ni hyperaccumulator species *Mayanaea caudata*. The species was first described as *Orthion caudatum* by Lundell in 1968 and then excluded from the genus and described as *M. caudata* (Lundell, 1968, 1984b). Both genera, *Orthion* and *Mayanaea* belong to the Violaceae family which in turn is part of the COM clade (Celestrales, Oxalidales and Malpighiales). Indeed, a third of the reported Ni hyperaccumulators worldwide belongs to that clade (Jaffré et al., 2013). Other interesting phylogenetic relations within the Violaceae family can be observed. The most recent study about the phylogeny of the Violaceae was inferred from plastid DNA sequences and distinguishes four main clades (apart from the *Fusispermum* and *Rinorea crenata* groups and the *Rinorea s.s.* clade). In that study, *Orthion* was resolved as a sister genus of *Mayanaea* and they both were placed in the clade 3 which also includes *Leonia*, *Gleospermum*, *Amphirrhox*, *Hybanthus concolor*, the *Hybanthus havanensis* group and the *Hybanthus caledonicus* group (Wahlert et al., 2014). Nickel hyperaccumulation has long been reported in three species belonging to the *Hybanthus caledonicus* Group in New Caledonia and Australia (**Figure 10**) (Brooks et al., 1974; Paul et al., 2020a). Moderate hyperaccumulation of Ni (n = 12, mean of 134 $\mu\text{g g}^{-1}$) was found in voucher specimens of *Hybanthus yucatanensis* collected in Mexico (Brooks et al., 1977a). The hyperaccumulation trait has appeared many times throughout evolution (Jaffré et al., 2013). Thus, the Ni hyperaccumulation trait could have appeared in a common ancestor of *Orthion*, *Mayanaea*, *Hybanthus havanensis* Group, *Hybanthus concolor* and the *Hybanthus caledonicus* Group. However, no Ni hyperaccumulation in *H. havanensis* nor in *H. concolor* has been reported so far.

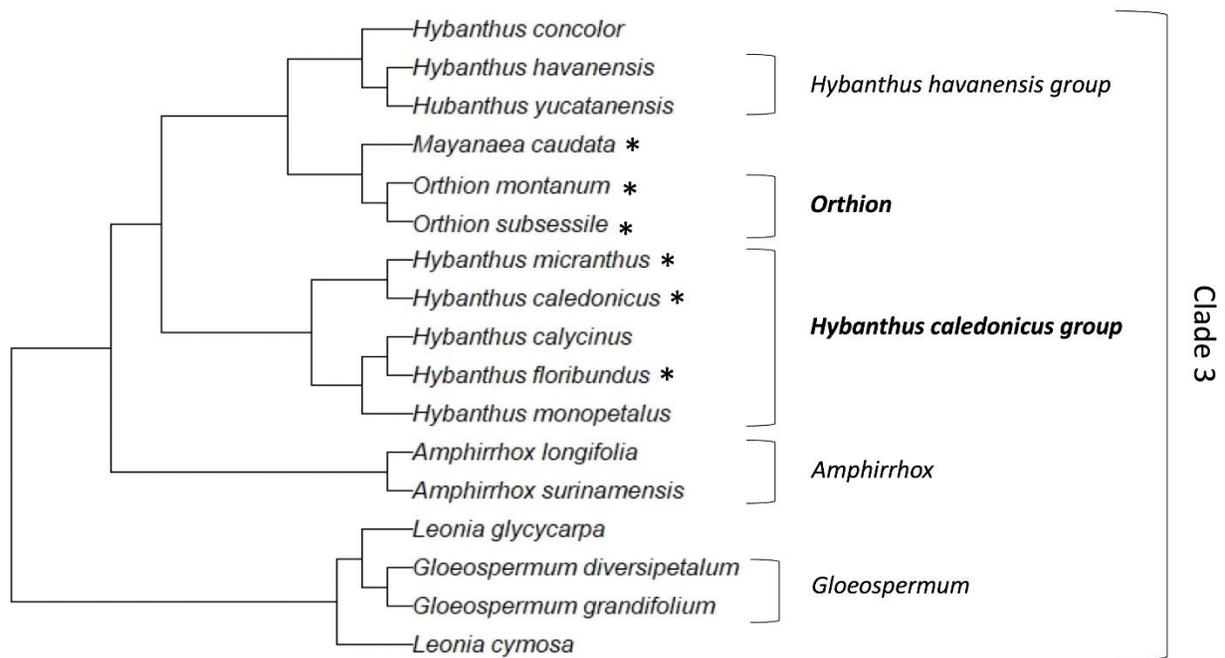


Figure 10. Part of Violaceae family cladogram showing clade 3, adapted from (Wahlert et al. 2014). Ni hyperaccumulator species are indicated by an asterisk (*).

Results from field exploration in Chiapas demonstrated that *O. subsessile* was able to hyperaccumulate up to 4.2 wt% Ni in leaves. Indeed, the species is among the strongest accumulators globally named “hypernickelophores” (>1 wt% Ni) (Jaffré and Schmid, 1974) close to the highest reported concentration record in leaves of 7.6 wt% Ni in *Berkheya coddii* Roessler (Asteraceae) (Mesjasz-Przybyłowicz et al., 2004). Extremely enriched Ni in the bark and flowers of *O. subsessile*, as well as the Ni-enriched leaf midrib (micro-XRF map) suggest a distribution of Ni through the phloem. The reproductive organs and young leaves are the main sinks of phloem translocation (Deng et al., 2018). Nickel-rich phloem has recently been reported in *H. austrocaledonicus* with Ni concentrations exceeding 15wt.% (Paul et al., 2020a). It was also observed in the South-African species *Senecio coronatus* (Thumb) Harv. (Asteraceae), as well as in some tropical hyperaccumulator species from Southeast Asia such as *Actephila alabakeri* Welzen & Ent. (Phyllanthaceae) and *Phyllanthus balgooyi* Petra Hoffm. & A.J. Baker belonging also to the COM clade (Mesjasz-Przybyłowicz et al., 1997; van der Ent et al., 2017a). This feature

was also recently discovered in the phloem tissues of the neotropical Ni hyperaccumulator *Blepharidium guatemalense* Standl. (Rubiaceae) (Navarrete Gutiérrez et al., submitted).

From field samples, the ability of *O. veracruzense* to accumulate Ni, as indicated by its bioconcentration factor of 13.3 (BCF = the ratio of the metal concentration in leaves to the total Ni concentration in soils), also demonstrating its potential for agromining (Nkrumah et al., 2016). Moreover, this species can reach up to 12–15m high according to our field observations in Veracruz and the information found on the herbarium specimen labels. *Orthion oblanceolatum* has been reported to be an indicator species of middle-aged and late secondary vegetation communities in abandoned agriculture fields around “Los Tuxtlas” Reserve (Purata, 1986), but its growth form is irregular (Ibarra-Manríquez et al., 1997). In 2014, all the specimens of *O. oblanceolatum* from the MEXU herbarium were reidentified to *O. veracruzense*, so research conducted before 2014 may then refer to the latter. *Orthion subsessile* could be also a potential candidate for Ni agromining since its BCF was of 21. Furthermore, this species occurs also in coffee plantations in the north of Chiapas (Soto-Pinto et al., 2001) and it grows up to 15 m high (herbarium label information), but not much is known about its growth-rate or possible means of propagation except that it regrows fast when coppiced in pastures (personal field observations). Four of the six *Orthion* species were not found in the field surveys conducted in this study. Unfortunately, there is little research on the taxonomy and ecology of these species (*i.e.* habitat description, distribution, frequency, growth of rate, *etc.*). The scarcity of available specimens in the different herbaria may reflect the rarity of some of these species. For example, *M. caudata* is known only from the tropical forest in the Department of Izabal in Guatemala and has not been collected for decades (Ballard et al., 2014; Lundell, 1984b). Thus, the eventual use of *Orthion* and/or *Mayanaea* species for Ni agromining in degraded lands of the neotropics must be carefully assessed (Nkrumah et al., 2020).

The present study revealed for the first time Ni hyperaccumulation in the Violaceae family in the American continent. Field explorations together with herbarium surveys led to the discovery of seven new Ni hyperaccumulator species. The present study is an example of the immense value of the material and information contained in the different herbaria around the world. Although herbaria have long been used for taxonomic studies, they have now become one of the most

important resources in the search for new metal hyperaccumulator plant species (Brooks et al., 1977a; van der Ent et al., 2019a). New technologies, such as the hand-held XRF spectrometer, have facilitated the exploitation of these herbaria resources. (Gei et al., 2018).

Chapter III b. *Blepharidium guatemalense*, an obligate nickel hyperaccumulator plant from non-ultramafic soils in Mexico

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Abstract

Aims

Nickel hyperaccumulation in *Blepharidium guatemalense* Standl. (Rubiaceae) was found in the tropical forests of south-eastern Mexico. This study aimed to document the geographic extent of nickel hyperaccumulation in this species, to understand its process of hyperaccumulation and to explore nickel distribution within the tissues of this plant.

Methods

Rhizosphere soils and plant tissues were collected in Mexico and analyzed for physical-chemical parameters. Non-destructive elemental screening of herbarium specimens was performed with a hand-held X-ray fluorescence spectrometer. Elemental distribution maps of nickel and other elements in plant tissues were obtained by X-ray fluorescence spectroscopy and microscopy.

Results

Blepharidium guatemalense is distributed throughout Chiapas, Tabasco, and Campeche, reaching the maximum nickel concentration in leaves (4.3 wt%) followed by roots and seeds (2.0 wt%) and bark (1.8 wt%). Simultaneous hyperaccumulation of cobalt and nickel was found in 15% of the herbarium specimens. Phloem has the highest nickel-enriched tissue from all parts of the plant (from roots to leaves). A high total nickel (mean of 610 $\mu\text{g g}^{-1}$) was found in rhizosphere soils even though no evidence of ophiolite emplacement in that area has been reported.

Conclusions

Blepharidium guatemalense has uncommon re-distribution mechanisms via phloem. It represents the first hypernickelophore (>1 wt% Ni) to be reported as growing in soils that are neither ultramafic nor enriched by anthropogenic pollutants.

Keywords: *hypernickelophore; XRF scanning; Rubiaceae; biogeochemistry; ionomics; agromining.*

1. Introduction

Hyperaccumulators are plants that can actively take up and accumulate certain metals or metalloids to extremely high concentrations in their above-ground tissues (especially leaves) when growing in their natural habitats (Baker et al., 1992; van der Ent et al., 2013). To date, 731 hyperaccumulator species are reported, of which 532 are nickel (Ni) hyperaccumulators (Reeves et al., 2018; van der Pas and Ingle, 2019). The nominal threshold values in plant dry matter depend on the element, *e.g.* 100 $\mu\text{g g}^{-1}$ for cadmium (Cd), 300 $\mu\text{g g}^{-1}$ for cobalt (Co), 1000 $\mu\text{g g}^{-1}$ for Ni, 3000 for zinc (Zn) and 10 000 for manganese (Mn) (van der Ent et al., 2013). Among these plants, there is a singular group of about 50 species termed ‘hypernickelophores’, which can accumulate Ni to concentrations exceeding 1 wt% (10 000 $\mu\text{g g}^{-1}$) in their shoots (Boyd and Jaffré, 2009; Jaffré and Schmid, 1974). Hyperaccumulator plants that only grow on metalliferous soils are classified as ‘obligate hyperaccumulators’, meanwhile those that are reported to grow (without exhibiting metal hyperaccumulation) in non-metalliferous soils are ‘facultative hyperaccumulators’ (Pollard et al., 2014). The primary habitats of these hyperaccumulator plants comprise soils developed from the weathering of surficial ore deposits or naturally enriched metalliferous rocks. Most Ni hyperaccumulators grow on soils issued from the weathering of ultramafic rocks, which are the most significant reservoirs of Ni and Co for terrestrial ecosystems (Estrade et al. 2015; Reeves et al. 2018). Since the discovery of the first Ni hyperaccumulator *Odontarrhena* (syn. *Alyssum*) *bertolonii* (Desv.) Jord. & Fourn. (Minguzzi and Vergnano 1948), series of studies of the ultramafic flora provided numerous data on the distribution and number of hyperaccumulator taxa worldwide. Most Ni hyperaccumulator plants occur in: i) ultramafic regions around the Mediterranean Basin, especially in Turkey and the Balkans (Brooks et al., 1979;

Reeves and Adigüzel, 2008), and ii) humid tropical and subtropical ultramafic areas, with three important hotspots which are Cuba, New Caledonia, and Southeast Asia (Galey et al., 2017a; Reeves, 2003). According to the Global Hyperaccumulator Database (Reeves et al. 2017), the eight most diverse families in terms of Ni hyperaccumulator species are the Phyllanthaceae (130 spp.), Brassicaceae (104 spp.), Asteraceae (68 spp.), Cunoniaceae (55 spp.), Euphorbiaceae (45 spp.), Salicaceae (39 spp.), Fabaceae (29 spp.) and Rubiaceae (23 spp.).

Mexico is the country with the fourth-largest floristic richness globally, with a record of 23 314 vascular plant species (Villaseñor, 2016). These species belong to different families including those also present in the most diverse Ni hyperaccumulator families, such as the Asteraceae (3057 spp.), Fabaceae (1903 spp.), Euphorbiaceae (714 spp.), Rubiaceae (707 spp.), and Brassicaceae (210 spp.) (Villaseñor, 2016). Such diversity suggested the possibility of searching for native metal hyperaccumulator plants in Mexico. New techniques for the discovery of hyperaccumulators, such as the non-destructive elemental screening of herbarium specimens through the use of a portable X-ray fluorescence (XRF) instrument, have provided the opportunity to use herbarium specimens to discover new hyperaccumulators (van der Ent et al., 2019a) and new occurrences of mineralized soils.

After several recent attempts to identify Ni hyperaccumulators on ultramafic soils in central and southern Mexico through field surveys, Navarrete Gutiérrez et al. (2018) concluded that Ni hyperaccumulation did not occur in this part of the world. However, a recent XRF screening of herbarium specimens at Missouri Botanical Garden (MO) in Saint Louis, Missouri, USA, revealed for the first time Ni hyperaccumulation in Mexico occurring in several woody species of the genus *Psychotria* (Rubiaceae), namely *Psychotria costivenia* Griseb., *Psychotria lorenciana* C.M. Taylor and *Psychotria papantlensis* (Oerst.) Hemsl., collected in the states of Chiapas, Tabasco and Veracruz (McCartha et al., 2019). These findings led us to undertake new field surveys in these regions and to discover that *Blepharidium guatemalense* Standl. (Rubiaceae) occurs in some of the same communities with the above-mentioned *Psychotria* species, and that also hyperaccumulates Ni (**Figure 11**). *Blepharidium* is a monospecific genus which was first described in 1918 by Paul Carpenter Standley based upon a type collected in the Department of Alta Verapaz in Guatemala by Henry Pittier in 1905 (Standley, 1918). Years later, the species *Blepharidium mexicanum* was

reported as a separate species based on taxonomic features such as smaller flowers and sparse pubescence over the lower leaf surface (Standley, 1940). Despite morphological differences, *B. mexicanum* is now considered a synonym of *B. guatemalense* (WCSP, 2019). The latter is distributed mainly in primary tropical perennial and sub-perennial rainforest of southern Mexico and Guatemala at altitudes ranging from 200 to 500 meters above sea level (m.a.s.l.). ‘Popiste’ or ‘Sayaxché’ (as it is called in the local languages), is a hardwood tree up to 70 m in height (Ricked et al., 2013) with a diameter up to 70 cm, and a straight trunk and rounded crown.

Long and acute stipules occur on younger leaves. The inflorescences are in axillary panicles 10–25 cm long with white flowers and fruits are erect capsules 2–3.5 cm long with abundant brown, flattened and winged seeds 1–1.5 cm long (Pennington and Sarukhán, 1968). *Blepharidium guatemalense* is commonly used for rafters, beams and supports of lowland houses due to the strength of its wood whereas its leaves and bark are reputed poisonous (Standley and Williams, 1975). The species also grows in secondary forests and on agro-pastoral areas (Ochoa-Gaona et al., 2007; Villanueva López et al., 2015). In the secondary forest, however, *B. guatemalense* appears to be a pioneer species that colonizes land after many cycles of ‘slash - and - burn’ which is the ancient cultivation agriculture system still used by local people. In contrast to primary forests, *B. guatemalense* trees are usually between 5–8 m high when growing in secondary forest and in open pasture areas, and they seldom exceed 20 m in these environments. The species is on the IUCN Red List, mainly because of the lack of data (Nelson, 1988).

According to existing geological maps, the distribution range of *B. guatemalense* in Guatemala coincides with ultramafic substrates whereas in Mexico its distribution range is derived mainly from limestone, including alluvial and colluvial deposits of the same materials (Ortega-Gutiérrez, 1992; SGM, 2017). The surprising finding that Ni hyperaccumulation occurs commonly on non-ultramafic soils in south-eastern Mexico was the impetus for this study.



Figure 11. Detail of *Blepharidium guatemalense* plants in the tropical forests of southeast Mexico: a) seeds; b) fruit capsules; c) inflorescences; d) mature leaf of approximately 45 cm long and 18 cm wide; e) detail of bark cutting showing a positive reaction to the field spot test based on DMG; f) 25-m high individual.

The outer bark is blue-grey and scaly whereas the inner bark is cream and granular with a total thickness from 5 to 10 cm. Leaves are decussate, oblanceolate 10–30 cm long by 5–12 cm wide.

The main purposes of this study were: i) to show the geographic extent of the Ni hyperaccumulation phenomenon in *B. guatemalense*, ii) to understand this phenomenon with regard to its rhizosphere soil properties, and iii) to present a first investigation of the Ni distribution within the plant tissues.

2. Materials and methods

Hyperaccumulation of nickel by *Blepharidium guatemalense* was discovered coincidentally in December 2017 when sampling in the field several species of *Psychotria* in the lowland forests of Chiapas. These *Psychotria* species had been priorly reported to hyperaccumulate nickel, hence the interest for Ni hyperaccumulators in this region (McCartha et al., 2019). Therefore, a complete herbarium survey was carried out to determine the extent of Ni hyperaccumulation by this species, followed by a field survey of the interesting regions pointed out by the herbarium study.

2.1. Survey of nickel hyperaccumulation by *B. guatemalense* in herbarium specimens

Three collections were surveyed to get a sufficient distribution of the species in Mexico, these were : MEXU (Mexico City, Mexico), P (Paris, France) and MO (Saint Louis, U.S.A.). Elemental concentrations in mounted herbarium specimens of *B. guatemalense* were measured at the three different herbaria with the same procedure and the same material. The scanning was performed by using a hand-held X-Ray Fluorescence Spectrometer (Thermo Scientific GOLDD+ XL3T-980). The analyzer uses a miniature X-ray tube [Ag anode (6–50 kV, 0–200 μ A max)] as the main excitation source and a Silicon Drift Detector (SDD) with 185 eV, 60 000 cps, 4 μ s shaping time. It can detect a range of different elements simultaneously within 30–60 s with a detection limit of 140 μ g g⁻¹ for most transition elements such as Ni, Cd, Cr, Zn, *etc.* The leaves of dried herbarium specimens were scanned for 30 s in ‘soils’ mode and by placing a titanium plate (99.999% purity) underneath the herbarium sheet. During the scanning, specimens are subjected to a beam of high-energy X-rays generated from the X-ray tube; the resulting spectrum of excited fluorescent X-rays serves to detect the elements and to quantify their concentrations.

To obtain better XRF measurement accuracy, calibration and correction of the data were performed. Hence, a total of 256 leaf samples of different Ni (and some concomitantly Zn)

hyperaccumulator plant species were collected from different plant species including *B. guatemalense* in the field because it was not possible to achieve destructive sampling of herbarium specimens. Leaf squares of 1 cm² in size of each sample were cut and analyzed for 30 s in ‘soils’ mode with three replicated readings per sample. After XRF measurements, leaf samples were digested and analyzed with Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) for major and minor elements following the methodology mentioned below in this section.

Paired ICP and XRF data were analyzed by logarithmic linear regression. No noise was detected because the minimum XRF value was about 500 µg g⁻¹, greatly higher than the limit of detection (LOD < 190) estimated by (Gei et al., 2020b; van der Ent et al., 2019b). Residuals (observed vs. predicted values) were used to detect and remove outliers (± 3 SD of the residual). A secondary linear regression was then derived and transformed to power regression generating the following calibration equation with an R² of 0.87:

$$y = 1.1216x^{0.953}$$

where:

Where: x= XRF measurement, y= ICP predicted value

Nickel XRF measurements were finally corrected by using the resulting equation. A calibration equation for Zn ($y = 1.0041x^{0.9316}$ and R² of 0.93) was also derived by following the same procedure described above. For Co and Mn XRF measurements, data were corrected by using the calibration equations generated by (Gei et al., 2020b): for Co $y = 0.6277x^{1.1706}$ (r² = 0.84) and for Mn $y = 1.148x^{0.937}$ (r² = 0.73).

2.2. Study and collection sites

2.2.1. Ocosingo, Chiapas

In total, 13 sites from the municipality of Ocosingo in Chiapas were sampled. The sites were located in the surroundings of the communities of Lacanhá Chanyasab, San Javier and Cojolita, at altitudes ranging from 100 to 300 m.a.s.l. The area is mainly covered by the “Lacandona” tropical evergreen rainforest, a region of approximately 1,800,000 ha which is part of the main hydrological basin that generates the Usumacinta and Lacanhá rivers. The area is dominated by rolling hills

made up mainly of limestone sedimentary rocks (lutite, limonite) from the upper Cretaceous (Mesozoic) with overlapped layers of marine origin from the lower Tertiary (Cenozoic); marls and clays could be found on the foot of the slopes and in alluvial areas (Müllerried, 1957; Levy Tacher et al., 2006; SGM, 2006). Main soil types are Rendzinas, Gleysols and Litosols (INIFAP-CONABIO, 1995). Loamy Rendzina has been reported in secondary forests and Calcaric Phaeozem in primary forests (Mendoza-Vega and Messing, 2005). According to the nearest meteorological station in Bonampak, the climate is humid warm with abundant rains in summer (Am (i') gw") (García, 2004; Köppen, 1936), and a slight precipitation decrease from February to April. The average annual temperature is approximately 25 °C with low thermal oscillation and total annual precipitation of around 2500 mm (García, 2004). The predominant vegetation is evergreen rainforest alternated with secondary vegetation, rain-fed agriculture, and grassland. The first survey took place in December 2017. Promising results from herbarium XRF screenings of *B. guatemalense* leaves from all over south-eastern Mexico prompted a second survey in November 2018 to study some unexplored sites during the first survey in Chiapas.

2.2.2. Tacotalpa, Tabasco

The second survey in November 2018 included the exploration of one site in Tapijulapa, which is a small village belonging to the municipality of Tacotalpa in the southern state of Tabasco. Several of the highest elevations of the state are here, highlighting among them the hills of El Madrigal (which is the second highest elevation in Tabasco, at 900 m.a.s.l. The climate is humid warm with abundant rainfall all year round and an average annual temperature of 26 °C with low thermal oscillation (Af (m) (i) gw"). The precipitation regime is characterized by a total rainfall of 3554 mm (Díaz P. et al., 2006; García, 2004). Previous predominant vegetation was evergreen rainforest, but this has gradually changed to corn crops, coffee plantations and livestock. Predominant soils are Rendzinas, Humic Acrisols and Eutric Gleysols (CONABIO, 2001; INEGI, 2005), mainly composed of sedimentary rocks (lutite, limonite, limestone and sandstone). This site was also selected for exploration based on previous data from XRF herbarium screening.

2.2.3. Balancán, Tabasco

Balancán is a municipality located in the eastern part of Tabasco. Climate is humid with summer rains in most of the territory and an average annual temperature of 27 °C with low thermal oscillation (Am (f) (i) gw”) (Díaz P. et al., 2006; García, 2004; Köppen, 1936). Altitude varies from 10 to 50 m.a.s.l. and lands are generally flat with small hills. Vertisol is the main type of soil, derived from sedimentary rocks (sandstone, alluvial deposits) (CONABIO, 2001; SGM, 2017). Even though many herbarium specimens of *B. guatemalense* presenting high Ni concentrations in their leaves were collected in the municipality of Balancán, no plant could be found in this municipality during the 2018 survey despite many efforts. Main cause could be the drastic woodland reduction from 49 % to 14% that Tabasco suffered over the last 60 years (Palma-López and Triano, 2007). Large surfaces of evergreen rainforest have been converted to grasslands for livestock.

2.3.Plant and soil sampling

Soils and plant samples were taken from a total of 14 distinct sites (13 sites from Chiapas and one from Tabasco) where *B. guatemalense* was found. Most of the studied areas were previously identified from herbarium collections, but some additional areas uncovered by herbarium collections were also surveyed. Three samples of *B. guatemalense* leaves from a single tree and its associated rhizosphere soil were taken in the 14 sites for chemical analysis. In specific locations, various specimens were collected to account for the local variability of Ni concentrations among individual trees. Leaf samples were selected randomly within a plant, and the rhizosphere soil samples were collected near the roots of each individual at a depth of 15 cm. The different types of habitats where samples were collected corresponded to : i) primary evergreen forests, ii) relatively young secondary forests (or fallow fields), and iii) agro-pastoral areas. Other plant tissue samples of *B. guatemalense*, such as fruits (n=9), flowers (n=16), bark (n=25), and roots (n=11) were also collected when available. In addition, one leaf and two seeds of *B. guatemalense* were collected separately, placed into paper bags, and dried in envelopes placed in silica-gel containing bags for Ni distribution analysis within the plant tissues. Some plant specimens were collected in duplicate, numbered, transferred to a press for drying, and prepared as voucher specimens for identification in the MEXU herbarium at Universidad Nacional Autónoma de México (UNAM).

2.4.Bulk analysis of plant tissues

Plant tissue samples of *B. guatemalense* were dried at 40 °C for 5 days in a drying oven and subsequently packed for transport to the laboratory for chemical analysis. Dried samples were first ground and digested using 1 mL HNO₃ (65%) and 2 mL H₂O₂ (30%) and left to stand overnight. The next day, samples were placed in a heating block (*DigiPREP*) for 2h at 95°C, left to cool to 40 °C, filtered at 0.45µm, and adjusted in volume to 10 mL for minor elements quantification. A 1:10 dilution was carried out with ultrapure water for the quantification of major elements. Finally, digests were analyzed using ICP-AES (Thermo Fisher CAP 6300 Duo). Two blanks and two reference samples of the known hyperaccumulator *Noccaea caeruleascens* (J. Presl & C. Presl) F.K. Mey, were used for each analytical series. The same instrument was used in all subsequent ICP-AES analyses.

2.5. Bulk analysis of rhizosphere soil samples

Rhizosphere soil samples of *B. guatemalense* collected in the field were air-dried during one week, placed into identified plastic bags and then transported to the laboratory for chemical analysis. Soil pH was measured with in a 1:5 soil: water (5 mg soil with 25 mL ultrapure water) suspension after 1 h shaking at 16 rpm and 1h standing. Cation exchange capacity (CEC) of soils was determined using cobalt-hexamine trichloride extraction by adding 50 mL of solution (4.458 g of Co(NH₃)₆Cl₃ with 700 mL of distilled water). After 1 h shaking, 10 ml of each sample were filtered at 0.45 µm and measured by spectrophotometry (BioRad, SmartSpec Plus). Available phosphorus (P) was determined according to the Olsen method. For total major and minor elemental quantification, soil samples were dried at 70°C for 3 days in a drying oven and sieved (< 2 mm). Subsequently, 0.5 g (± 0.001 g) of each sample, finely ground (< 250 µm), were digested by adding 6 mL of 37% HCl and 2 mL of 70% HNO₃ per sample, left to stand overnight, placed in a heating block (*DigiPREP MS*) for 3h at 100°C, and then left to cool to a minimum temperature of 40°C. Solutions were adjusted in volume to 45 mL, filtered at 0.45 µm, and then readjusted to 50 mL with ultrapure water. The final dilution of 1:20 was necessary for major elements. The resulting soil digests/extracts were analyzed by ICP-AES for the quantification of major and minor elements. Trace elements availability in soil samples was determined by using diethylenetriaminepentaacetic acid (DTPA-extractant) according to the method of Lindsay and Norvell, (1978), and then analysis by ICP-AES of the soil extracts.

2.6. Micro X-Ray fluorescence elemental mapping

High spatial resolution (20 μm) distribution maps for Ni and other elements were obtained using a Micro-XRF (M4 Tornado, Bruker). The device has a Rh X-ray tube (50 kV and 200 μA) as a main excitation source with a Be side window and polycapillary optics. A large and flat sample is subjected to an X-ray beam with a diameter of 20–30 μm inside a chamber under controlled 20 mbar vacuum. X-rays are then detected by two 30 mm^2 Xflash® Silicon Drift detectors (energy resolution of <135 eV at 250 000 cps). A leaf of *B. guatemalense* was collected from its natural habitat in Chiapas and dried at 40 °C for 5 days in a drying oven. Afterwards, a flat section of the leaf (13.0 x 1.9 cm) was cut and analyzed with micro-XRF in the GeoRessources laboratory (France).

2.7. X-ray fluorescence microscopy (XFM)

X-ray fluorescence microscopy analysis of different plant tissues of *B. guatemalense* was undertaken at PETRA III (Deutsches Elektronen-Synchrotron; DESY) a 6 GeV synchrotron. The microprobe beamline P06 is equipped with a cryogenically cooled double-crystal monochromator with Si (111) crystals and a K/B mirror focusses a beam of 10^{11} photon/s down to 300 nm size in the energy range 5–21 keV (Schroer et al., 2010). The XRF was collected using a silicon drift detector (Hitachi Vortex EM-90, 80 mm^2 , energy-resolution 120 eV) operated in 90-degree geometry). Incident energy of 12 keV was used in order to excite the element of interest with the highest atomic number (*i.e.*, Zn).

Different plant tissues (root, stem, petiole, and leaf) of a three-month-old individual of *B. guatemalense*, which was grown in pot experiments under controlled conditions in a Ni-contaminated soil, were analyzed. The samples were hand-cut with a stainless-steel razor blade ('dry knife'), mounted between two sheets of 4 μm 'Ultralene' thin film in a tight sandwich to limit evaporation, and analyzed within 10 minutes after excision. X-ray micro-fluorescence was performed at high speed to keep the scan time to a minimum. The fresh and freeze-dried samples were mounted between two sheets of 'Ultralene' thin film (4 μm) stretched over a perspex frame magnetically attached to the *x-y* motion stage at atmospheric temperature (~20 °C). The XRF data

were fitted and processed using PyMCA software (Solé et al., 2007), and exported into ImageJ as greyscale 16-bit TIFF files and displayed using ImageJ's 'Fire' lookup table.

2.8. Statistical analyses

Soils and plants data were summarized by using Microsoft Excel 2010 (XL STAT ECOLOGY version 2019.1.1) for descriptive statistics, Pearson Correlation Coefficient (r) and Principal Components Analysis (PCA). Nickel concentrations are presented as boxplots and in tables. Key symbols: open boxes are inter-quartile ranges, bold crosses are \pm mean, whiskers are \pm standard deviation and bold circles are outliers. Maps were generated using ArcGIS 10.3.

3. Results

3.1. Elemental concentrations from the herbarium XRF screening in relation to geographical distribution

A total of 182 specimens of *B. guatemalense* historically collected in the current territory of Mexico were scanned by using X-ray fluorescence spectroscopy (XRF); 132 from the MEXU herbarium, 47 from MO and 3 from P. The results revealed the 'obligate' character of this species since only 5% of the specimens had Ni concentrations lower than $1000 \mu\text{g g}^{-1}$ (but yet all the values were $> 586 \mu\text{g g}^{-1}$, a value higher than the threshold considered to define 'hemi-accumulators') and about 3% of the specimens exceeded the hypernickelophore threshold ($10\,000 \mu\text{g g}^{-1}$). The mean Ni concentration in all 182 specimens was $4022 \mu\text{g g}^{-1}$ and the maximum Ni concentration of $18\,786 \mu\text{g g}^{-1}$ was recorded in one specimen collected from Ocozocuahtla, Chiapas. The histogram shows an approximately log-normal distribution of Ni concentration in the species (**Figure 12**).

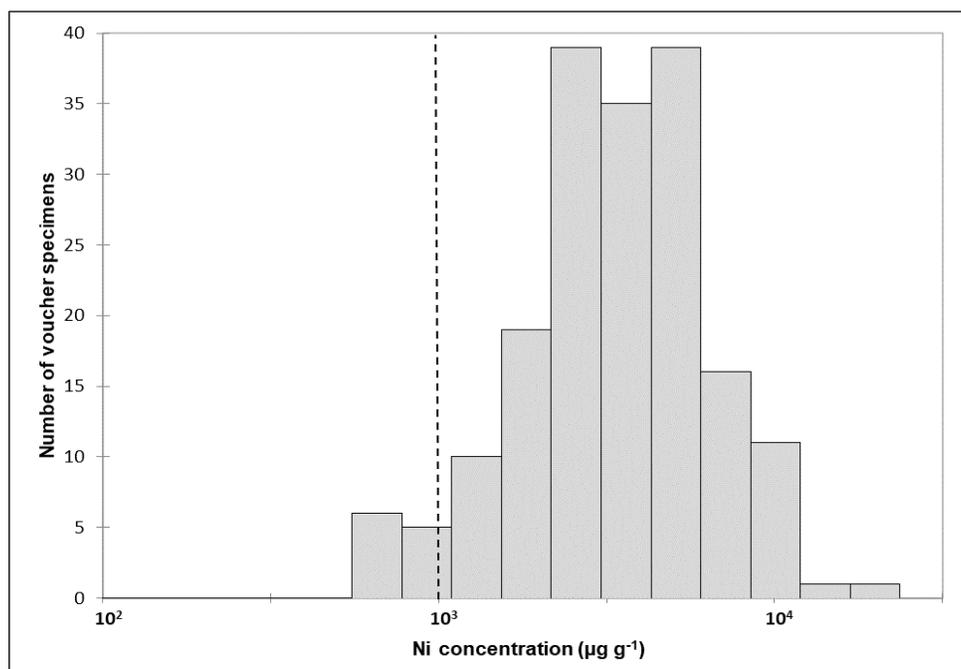


Figure 12. Histogram indicating the frequency distribution of Ni concentration ($\mu\text{g g}^{-1}$) in herbarium specimens of *Blepharidium guatemalense* ($n=182$) analyzed by X-ray fluorescence spectroscopy in MEXU, P and MO herbariums. The abscissa is plotted on a logarithmic scale.

Specimens with available geographical coordinates were georeferenced in a geological map (**Figure 13**) which confirms that the plant grows across different geological basements but never on ultramafic bedrocks. Ultramafic rock outcrops in Chiapas are found in the southern city of Motozintla, outside the apparent range of distribution of the plant and in a different climatic zone. Occurrences of the species were found on sedimentary formations that include limestone, sandstone, lutite, limonite, marls, clays and alluvial formations derived from the previous.

Cobalt hyperaccumulation ($>300 \mu\text{g g}^{-1}$) was found in 27 specimens (*i.e.* 15 %), from which one was collected in Campeche, 11 in Tabasco and 15 in Chiapas with the highest Co concentration of $1994 \mu\text{g g}^{-1}$ being found in a specimen collected in Tacotalpa, Tabasco. The mean concentration of Co was $446 \mu\text{g g}^{-1}$ for the 83 specimens that showed values above detection limit ($140 \mu\text{g g}^{-1}$). The mean Mn concentration for the 121 specimens with values above the detection limit was $260 \mu\text{g g}^{-1}$ with a maximum value of $1220 \mu\text{g g}^{-1}$. Although they were sometimes high, the concentrations never reached the hyperaccumulation threshold for this metal (*i.e.* $10000 \mu\text{g g}^{-1}$). In the case of Zn, the maximum concentration was $476 \mu\text{g g}^{-1}$, always well below the

hyperaccumulation threshold of $3000 \mu\text{g g}^{-1}$. In general, values found for herbarium specimens were lower than in field collected specimens.

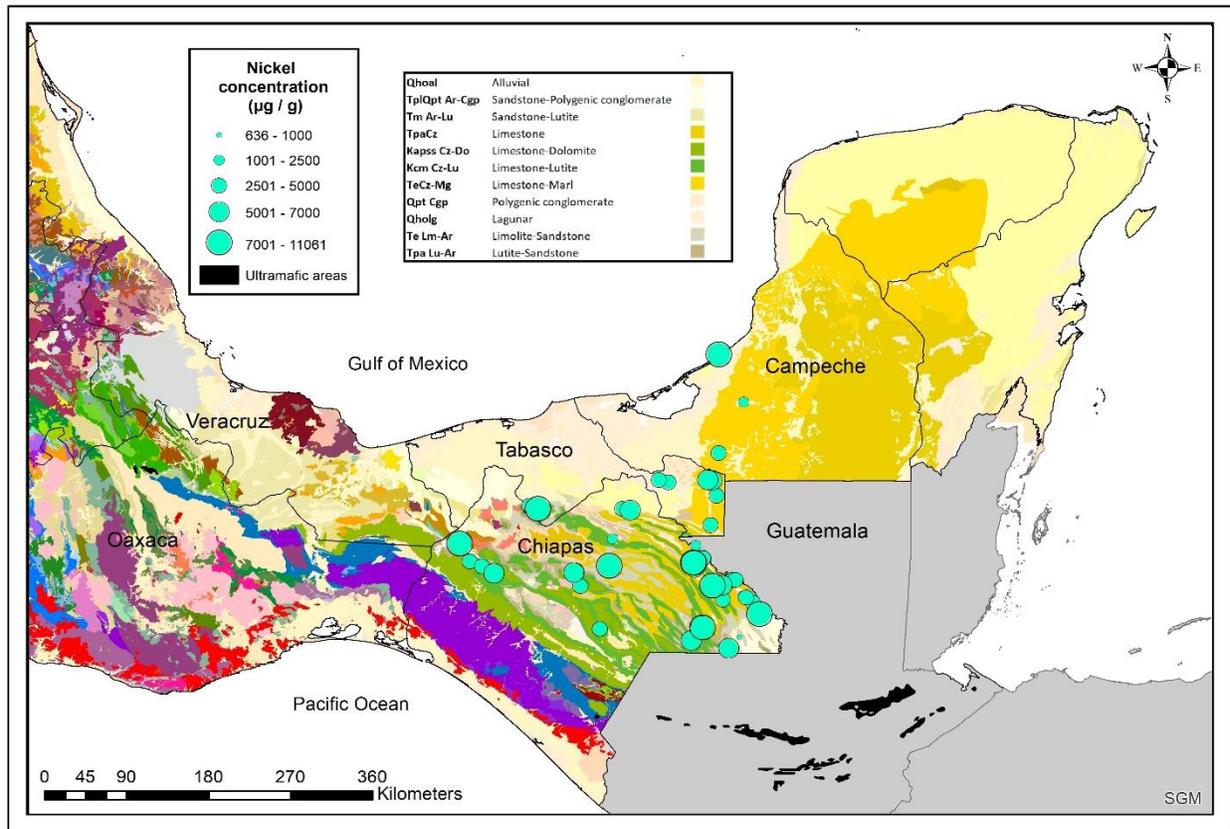


Figure 13. Map showing the geographic distribution of *B. guatemalense* herbarium specimens analyzed with XRF spectroscopy in the herbariums MEXU, P and MGB (number of voucher specimens with available geographic coordinates = 83). Ultramafic complexes are shown in black: Motozintla (Chiapas) and Polochic-Motahua (Guatemala). Geologic map sources: “Surface Geology of the Caribbean Region” of the USGC (United States Geological Survey) and Sistema Geológico Mexicano (SGM 2006, 2017).

3.2. Elemental concentrations in plant tissues of *B. guatemalense* collected in the field

The bulk elemental concentrations in the different tissues of *B. guatemalense* are reported for leaves, bark, flowers, and fruits (**Table 11**). The results were compared with the concentrations of a “reference plant” proposed by Markert (1992). Some major element concentrations were in the expected ranges for most in the parts of the plant, such as for P, sulphur (S), and magnesium (Mg). Mean potassium (K) concentrations in leaves, flowers and fruits were slightly low (mean < 1.8 wt%), whereas in the roots K concentration barely reached a mean of 0.7 wt%. The highest concentration of K was found in the bark with a maximum concentration exceeding 5.4 wt%. Calcium had typical concentrations in almost all parts of the plant except in the inner bark (with phloem) with a maximum concentration of 3.4 wt%. Copper (Cu) concentrations in the different plant parts were within the normal range (mean < 12 $\mu\text{g g}^{-1}$). Roots were particularly enriched in aluminium (Al) (mean 3910 $\mu\text{g g}^{-1}$) and iron (Fe) (mean 4200 $\mu\text{g g}^{-1}$) compared to other parts of the plant but this could be due to some minor soil contamination, despite thorough rinsing of roots after collection.

Plant part	n	Elemental concentration ($\mu\text{g g}^{-1}$)					
		Mn	Fe	Co	Cu	Ni	Zn
Leaf	78	22–587 (249)	< 50–561 (64)	7.4–149 (39)	< 0.6–22 (8.5)	2700–42 900 (13 500)	< 2.2–304 (96)
Flower	16	48–338 (112)	56–241 (83)	14–40 (24)	6.0–14 (11)	4420–9690 (5930)	44–80 (59)
Seed	5	217–297 (253)	< 50	27.4–44.2 (27.4)	< 0.6	16 700–19 800 (18 400)	< 2.2
Fruit	9	34–95 (61)	< 50–482 (133)	11–30 (17)	6.1–9.2 (7.8)	1260–4160 (2650)	17–56 (33)
Bark	25	36–764 (265)	< 50–333 (122)	1.9–116 (29)	< 0.6–10 (6.2)	1124–18 000 (4360)	20–215 (72)
Root	11	112–792 (447)	1460–6890 (4200)	11–120 (39)	6.9–23 (12)	1780–20 200 (12 300)	32–133 (99)

Plant part	n	Elemental concentration ($\mu\text{g g}^{-1}$)					
		P	K	Ca	S	Al	Mg
Leaf	78	682–2150 (1210)	5700–28 900 (14 500)	2990–14 200 (7200)	1730–7060 (3070)	< 163–227 (85)	877–4700 (2260)
Flower	16	1790–2970 (2480)	13 500–24 200 (17 100)	2700–8850 (4835)	1680–2660 (2370)	< 163	1650–5490 (2440)
Seed	5	7730–10 300 (9050)	6420–10 600 (8630)	3110–3460 (3340)	2040–2340 (2210)	< 163	2610–3850 (3130)
Fruit	9	1490–2850 (2070)	13 400–26 400 (18 100)	1330–2690 (2090)	947–2050 (1340)	< 163–443 (122)	1190–2540 (1760)
Bark	25	178–1680 (606)	10 700–54 600 (25 100)	3970–33 900 (16,800)	566–3910 (1660)	< 163–2960 (277)	274–2370 (743)
Root	11	232–532 (308)	5800–10 900 (7703)	4470–10 800 (7350)	719–4180 (2080)	1500–8380 (3910)	1170–2860 (1990)

Table 11. Elemental concentrations in plant tissues of *B. guatemalense* from individuals collected in their native habitat at Lacandonian rainforest (Chiapas, Mexico) and Tacotalpa (Tabasco, México). The elemental concentrations are shown in ranges and means in $\mu\text{g g}^{-1}$ (n= number of samples).

Nickel hyperaccumulation > 1 wt% characterize all the different tissues of *B. guatemalense* (**Figure 14**). Leaves showed the highest concentrations reaching 4.3 wt% Ni with a mean value of 1.4 wt% which is beyond the threshold that defines hypernickelophores. The lowest concentrations of Ni in the leaves were of $2700 \mu\text{g g}^{-1}$. The other parts of the plant also had quite elevated concentrations, especially seeds with a mean Ni concentration of 1.8 wt%. Roots show a mean Ni concentration of 1.2 wt% (ranging from 0.2 to 2 wt%) and bark inner tissues (largely phloem) showed the same trend as roots (*i.e.* from 0.1 to 1.8 wt% Ni). The lowest values of Ni concentrations were observed in fruits with a maximum concentration of $4160 \mu\text{g g}^{-1}$. Maximum Co accumulation values did not reach the hyperaccumulation threshold ($> 300 \mu\text{g g}^{-1}$) but exceeded $100 \mu\text{g g}^{-1}$ in leaves, bark, and roots which correspond to the parts with maximum Ni accumulation values. Manganese was not accumulated in any of the plant parts and ranged from $22\text{--}587 \mu\text{g g}^{-1}$, and similarly Zn with a maximum concentration of $304 \mu\text{g g}^{-1}$ reported in leaves.

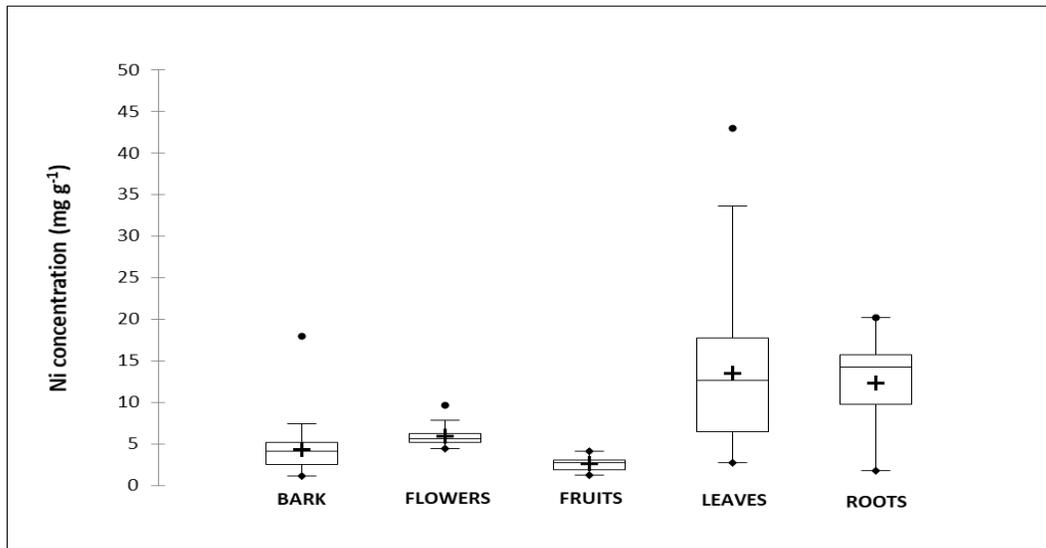


Figure 14. Nickel concentrations in plant tissues of *B. guatemalense* presented as boxplots. Key symbols: open boxes are interquartile ranges, bold crosses are \pm mean, whiskers are \pm standard deviation and bold circles are outliers.

3.3. Soil chemistry in the rhizosphere of *B. guatemalense*

Primary habitats of this species correspond mainly to evergreen tropical forests, but it is most often found in secondary forest and in pasture lands where it is quite abundant. Rhizosphere soil samples from *B. guatemalense* presented different physical properties according to the localities they were collected. Soils from the primary forest were sandy-clay-loam (12% sand, 56% silt, 32% clay), with an olive colour and a granular structure. In the secondary forest, soils were silty-clay (48% silt and 52% clay), exhibiting a gray colour and angular blocks structure, whereas in the pasture lands they were silty-clay (3% sand, 43% loam and 54% clay), with an olive color and a granular structure.

The general chemical properties of rhizosphere soil samples were not consistent with an ultramafic origin. The results reveal a mean pH value of 6.53 which is slightly acid with some variation between habitats. Mean pH values for the primary evergreen forest, secondary forest and pasture lands were 6.36, 6.58 and 6.93 respectively. The maximum pH in the primary forest was 6.72, whereas in secondary forest it was 7.36. Soils revealed a high CEC (mean $40 \text{ cmol}^+ \text{ kg}^{-1}$) as expected for tropical clay soils; the mean total carbon (C) and nitrogen (N) were 75 g kg^{-1} and 6.16 g kg^{-1} , respectively. Mean exchangeable Ca:Mg ratio was around 4:1. **Table 2** shows the total and

available concentrations of some major and minor elements. High total concentrations were recorded for some metals such as Fe (mean 45 300 $\mu\text{g g}^{-1}$) and Mn (mean 1380 $\mu\text{g g}^{-1}$), but concentrations of Co (mean 50 $\mu\text{g g}^{-1}$) and Zn (mean 65 $\mu\text{g g}^{-1}$) were within the normal range. Total Ni concentration was surprisingly high for non-ultramafic soils (with a mean value of 610 $\mu\text{g g}^{-1}$). Mean DTPA-extractable Ni concentration was also very high (and similar to ultramafic soils) with a mean value of 50 $\mu\text{g g}^{-1}$, and the maximum value almost reached 200 $\mu\text{g g}^{-1}$. Total major nutrient elements were relatively high in the case of Ca (7360 $\mu\text{g g}^{-1}$) and K (3370 $\mu\text{g g}^{-1}$). The available P concentrations were moderate (9.4 $\mu\text{g g}^{-1}$).

Elements	Elemental concentrations ($\mu\text{g g}^{-1}$)	
	Total	DTPA-extractable
Mg	5200–15 800 (10 500)	158–362 (247)
Ca	3550–14 900 (7360)	-
P	129–483 (287)	4.5–16 (9.4) *
K	1200–5560 (3370)	3.4–40 (18)
Al	20 900–67 700 (40 000)	< 0.8–9.6 (2.4)
Fe	36 500 –58 500 (45 300)	15–113 (54)
Mn	773–2660 (1380)	4.5–49 (25)
Cr	180–635 (310)	< 0.09
Co	28–101 (50)	< 0.08–1.8 (0.5)
Ni	240–1650 (610)	4.90–198 (50)
Zn	50–86 (65)	0.40–6.60 (2.9)

Table 12. Total and available elements of the rhizosphere soil collected in the native habitat of *B. guatemalense* at Lacandonian rainforest (Chiapas, Mexico). Concentrations are given in ranges and means in $\mu\text{g g}^{-1}$ and the number of samples is 19.

3.4. Statistical correlations between plant ionomics and rhizosphere soil composition

A Principal Components Analysis (PCA) was performed to reveal the existing relations between the different elemental concentrations in plant leaves and their associated rhizosphere soils collected in the sampling sites. The first three principal components together explained 60.4 % of the total variance, with 30.5 % of the variance on the first axis, 18.2 % on the second, and 11.7 % on the third. Projections of factor scores and loadings for the first and second components are presented in **Figure 15**. There was no apparent relation among soil and plant variables, except for a positive association between Ni concentration in leaves and the available Ni content in soils ($r = 0.510$). Three Pearson correlation coefficients were obtained separately: i) plant elemental concentrations, ii) total elemental contents in soils and iii) DTPA-extractable concentrations in

soils. Within plant leaves, Ni was highly positively related with Zn ($r = 0.970$). Other strong positive correlations could be observed, such as Ca-Mg ($r = 0.807$). In soils, CEC is highly positive associated with the N ($r = 0.766$) and C ($r = 0.775$) content in soils. Pearson correlation matrix with all the significant correlations is shown in **Annex 1**.

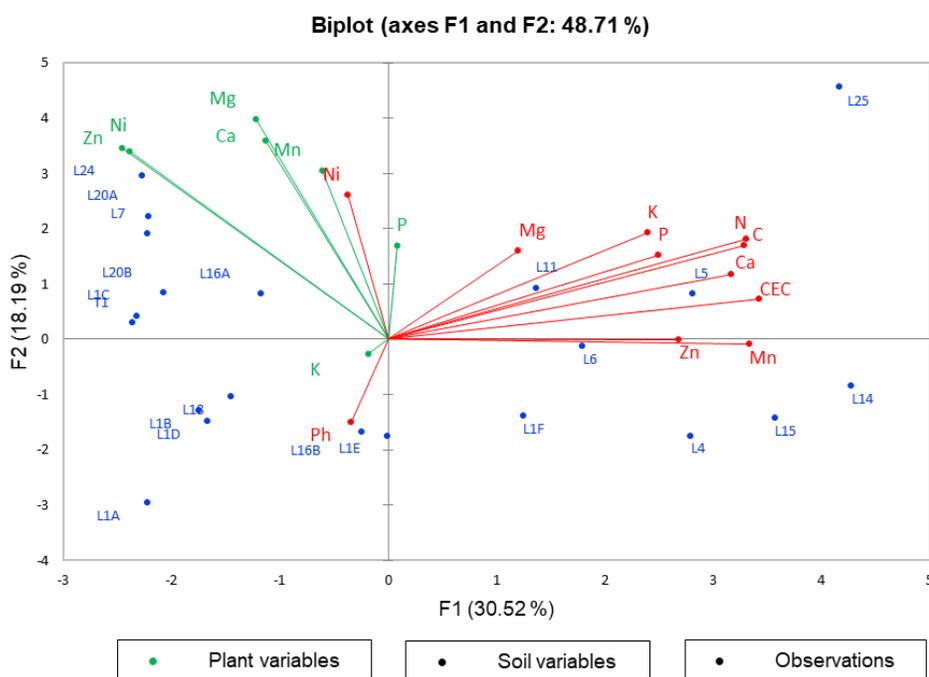


Figure 15 Biplot of the Principal Components Analysis (PCA) based on foliar elemental concentrations of *B. guatemalense* and the properties of its associated rhizosphere soils, $n=21$.

3.5.Desktop fluorescence microscopy on plant tissues

The micro-XRF maps show the spatial distribution of Ni and other elements in one leaf and two seeds of *B. guatemalense*. Leaf elemental maps revealed a much higher signal of Ni in the midrib and veins with a lower signal in the epidermis (**Figure 6**). Calcium was present all over the leaf whereas K, Fe, Zn and Mn were mainly present in the midrib and secondary veins. Cobalt, Mg and S were detectably higher in the leaf blade than in the veins. The elemental map of seeds revealed that Ca and K were homogeneously distributed throughout the wing that envelops the seed with a higher signal in the pericarp. A high Ni signal was detected in the pericarp with the maximum intensity in the seed embryo.

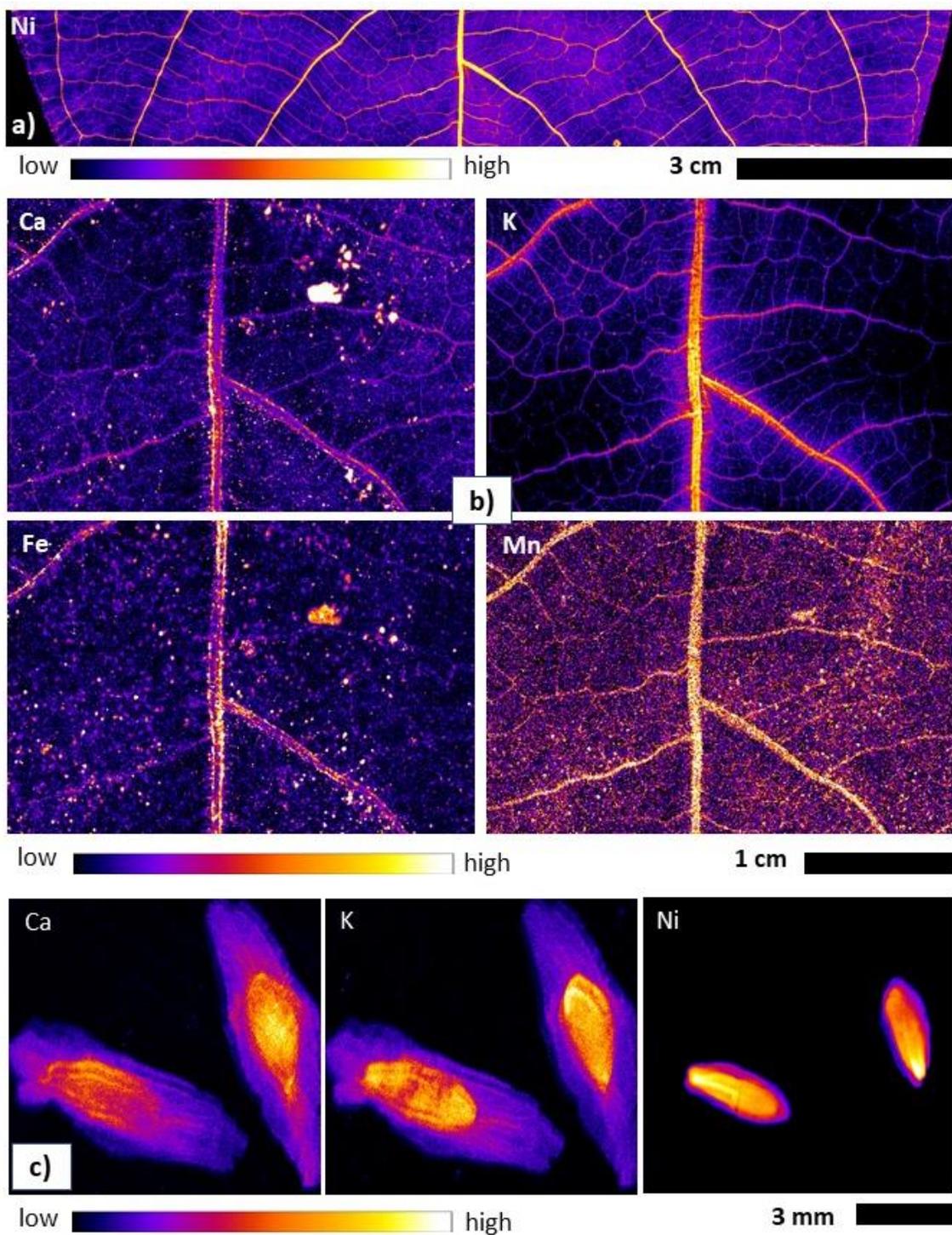


Figure 16. Elemental speciation of: a mature leaf (air-dried), b leaf detail and c seeds of *Blepharidium guatemalense*. Maps were generated using micro-XRF (20 μm resolution). Mean concentrations in the leaf: 14 100 $\mu\text{g g}^{-1}$ Ni, 3800 $\mu\text{g g}^{-1}$ Ca, 17 900 $\mu\text{g g}^{-1}$ K, 140 $\mu\text{g g}^{-1}$ Fe and 190 $\mu\text{g g}^{-1}$ Mn. Mean concentrations in seeds: 3280 $\mu\text{g g}^{-1}$ Ca, 9680 $\mu\text{g g}^{-1}$ K, 19 300 $\mu\text{g g}^{-1}$ Ni.

3.6. Elemental localization in plant tissues by XFM

Maps generated by XFM technology enabled us to elucidate the elemental localization in the organs of the different plant tissues (root, stem, petiole and leaf midrib) of *Blepharidium guatemalense*. The results showed that Ni is exceptionally highly concentrated in the phloem of all plant tissues. In the leaf midrib cross-section (**Figure 17 a**), Ni is mainly enriched in the collenchyma, followed by in the primary and secondary veins (phloem) and in the parenchyma of the leaf blade. Zinc follows the same distribution pattern as Ni but with a lower intensity. Calcium spots are probably calcium oxalate crystals in all plant tissues as observed in many plants (van der Ent et al. 2017). The mesophyll is strongly enriched in Ca all along the leaf blade. Potassium is uniformly distributed throughout the leaf. In the petiole cross-section (**Figure 17 b**), Ni is enriched in the phloem but highest in the sclerenchyma cells of the cortex. Zinc occurs similarly to Ni but also in the xylem. Calcium and K are mainly distributed in the phloem and the cortex. In the stem cross-section (**Figure 17 c**) Ni is strongly enriched in the phloem and in the cambium, but only moderately enriched in the cell walls of the cortex, in the primary xylem and in the parenchymal cell walls of the pith. Zinc distribution mirrors (again) that of Ni but with a noticeably higher enrichment in the primary xylem. Potassium is poorly concentrated in the primary xylem and secondary phloem. There is strong enrichment of Ca in the phloem and in the parenchyma cells of the pith. In the roots cross-section (**Figure 17 d**), the Ni is particularly highly enriched in the phloem, in the endoderm and in the epidermis. Nickel is also present in the xylem but in a lower concentration. Potassium is moderately enriched in the epidermis. Zinc is localized at a low concentration in the phloem being highest in the epidermis. Calcium is depleted in the cortex but strongly enriched in the phloem and in the epidermis.



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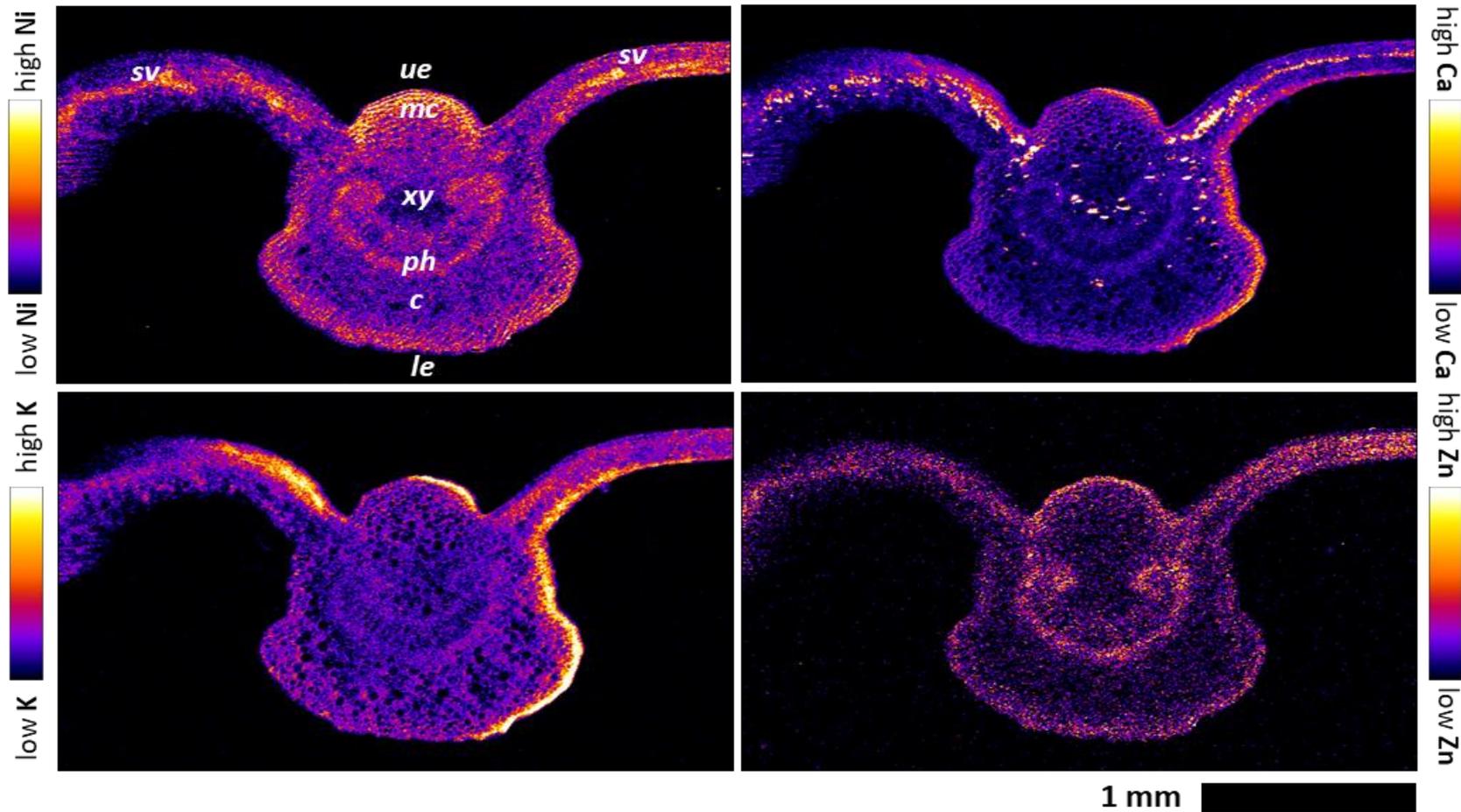


Figure 17a. X-ray fluorescence microscopy (XFM) elemental maps of leaf midrib cross-sections of *Blepharidium guatemalense*. Scan area is 3 x 1.8 mm (601 x 361 pixels) with a resolution of 5 microns and a per-pixel dwell of 9ms. Abbreviations of anatomical features: **xy**, xylem; **ph**, phloem; **c**, cortex; **sv**, secondary veins; **ue**, upper epidermis; **le**, lower epidermis; **mc**, midrib collenchyma.

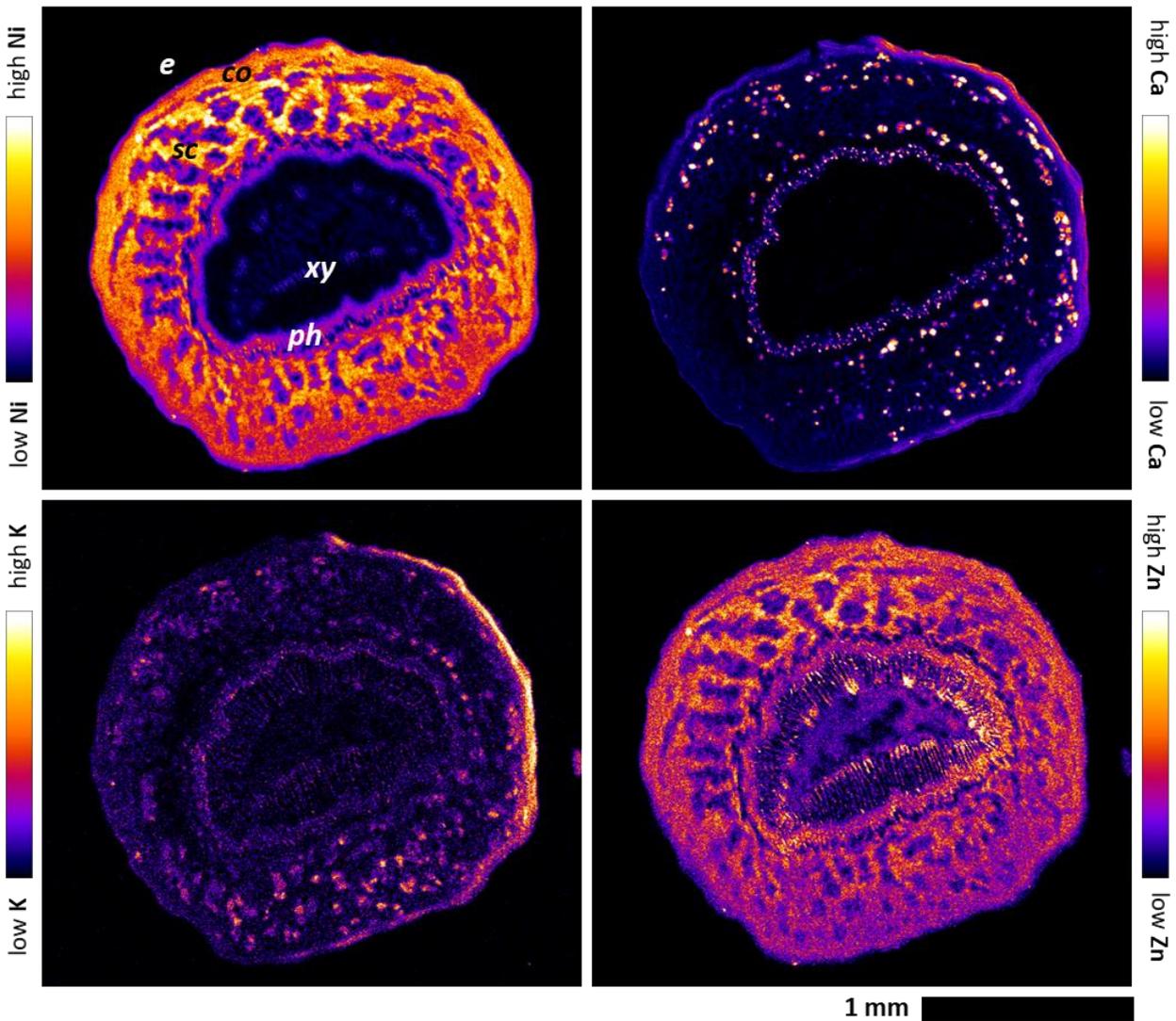


Figure 17b. X-ray fluorescence microscopy (XFM) elemental maps of a petiole cross-sections of *Blepharidium guatemalense*. Scan area is 2.5 x 2.3 mm (340 x 306 pixels) with a resolution of 3 microns and a per-pixel dwell of 4ms. Abbreviations of anatomical features: **xy**, xylem; **ph**, phloem; **co**, collenchyma; **sc**, sclerenchyma; **e**, epidermis.

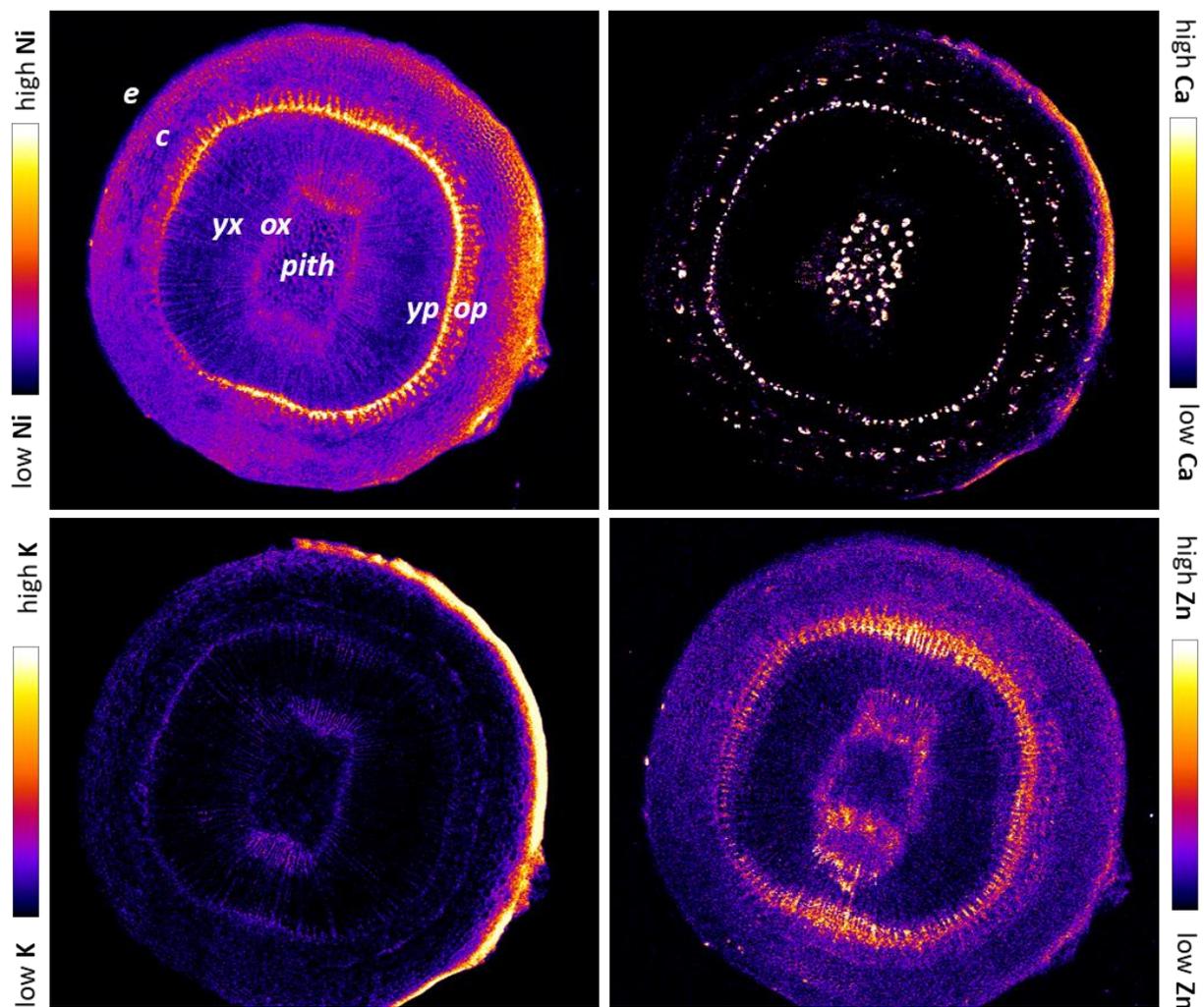


Figure 17c. X-ray fluorescence microscopy (XFM) elemental maps of a stem cross-sections of *Blepharidium guatemalense*. Scan area is 4.6 x 4.2 mm (782 x 692 pixels) with a resolution of 6 microns and a per-pixel dwell of 7ms. Abbreviations of anatomical features: **ox**, older xylem; **yx**, younger xylem; **op**, older phloem; **yp**, younger phloem; **c**, cortex; **e**, epidermis.

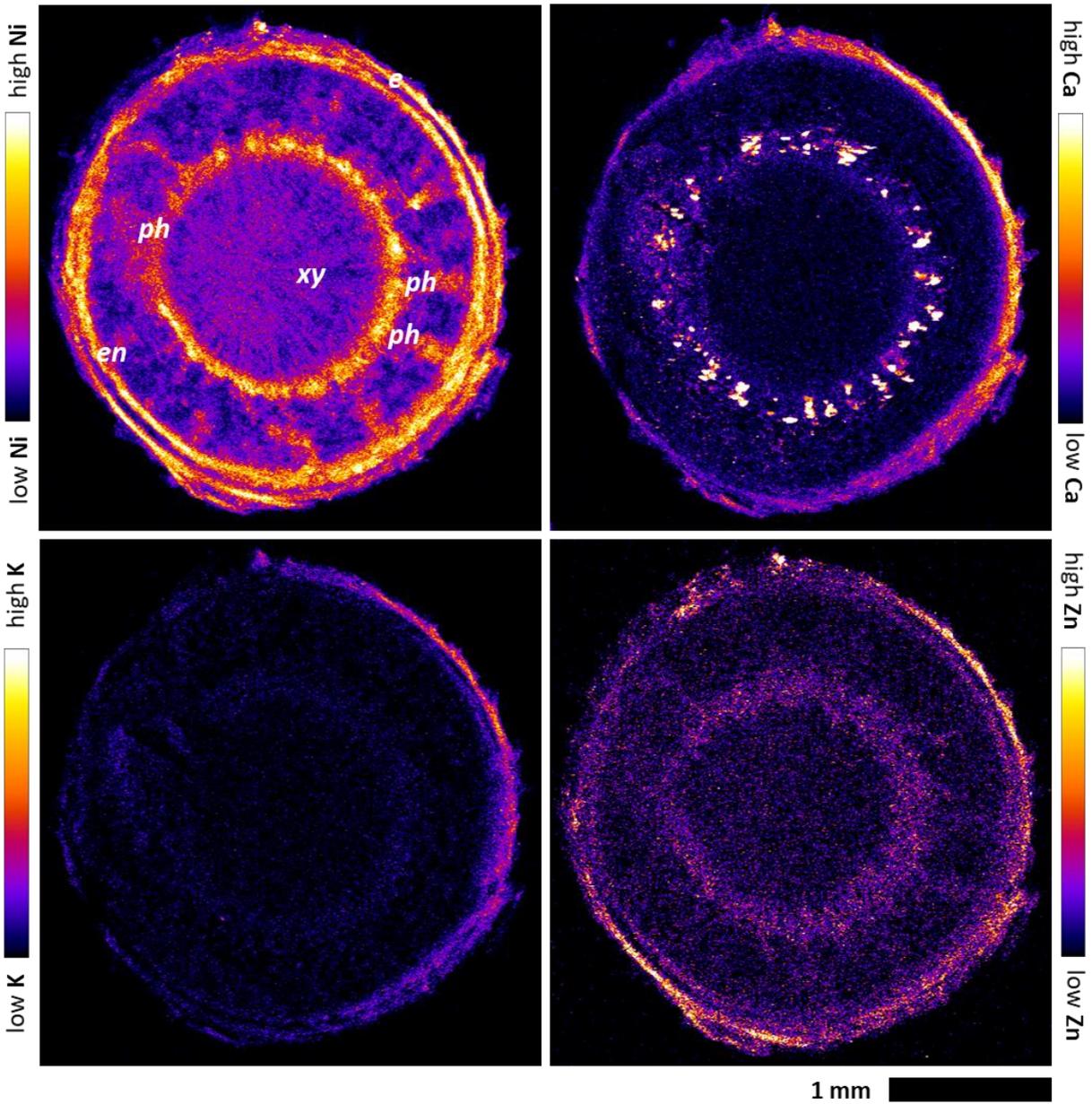


Figure 17d. X-ray fluorescence microscopy (XFM) elemental maps of a root cross-section of *Blepharidium guatemalense*. Scan area is 2.7 x 2.5 mm (278 x 291 pixels) with a resolution of 5 microns and a per-pixel dwell of 5ms. Abbreviations of anatomical features: **xy**, xylem; **yx**, younger xylem; **ph**, phloem; **e**, epidermis; **en**, endodermis.

4. Discussion

4.1. Ecology and occurrence of *B. guatemalense* in Mexico

Many sites in Tabasco (*e.g.* Balancan region), where *B. guatemalense* was often collected three decades before, suffered a dramatic land-use changes. The evergreen rainforest cleared to develop cattle grassland, reducing its geographic distribution to the areas where traditional agriculture co-exists with secondary and sometimes primary forests. For this reason, the survey of the species in the lowlands of Campeche was abandoned as it was impossible to find the species at any of the sampling locations of herbarium specimens in the Balancan Region or any other lowland region of Tabasco. However, *B. guatemalense* still occurs over a broad range of ecosystems across the state of Chiapas, from Palenque down to the Usumacinta valley and the Lacandona Forest and the extreme southern part of Tabasco close to the border with Chiapas where the landscape becomes hilly and soils are developed on limestone (Tapijulapa region). Its current distribution advocates for removing this species from the IUCN Red List as it is still very widely distributed and very common in the occurring areas which cover thousands of sq. km. Moreover, it is commonly used by local communities for roofing and selectively maintained in pastures to harvest the tree for poles.

Although all the soils where this species was sampled were highly enriched in Ni, Fe and Mn, as it is probably the case in this entire lowland region of Mexico (Hernández-Quiroz *et al.* 2012), they did not form from ultramafic bedrock (Ortega-Gutiérrez, 1992; SGM, 2006). The soils have no apparent deficiency in Ca or in the other essential macronutrients (N, K, P), by contrast with ultramafic soils (Proctor, 2003). Mean total Ni concentration in the first cm of the soils ($651 \mu\text{g g}^{-1}$) is well above the limit of Ni concentration recommended in soils from the USA ($210 \mu\text{g g}^{-1}$) but below the toxicity threshold specified by the Mexican norm NOM-147-SEMARNAT/SSA1-2004 ($1600 \mu\text{g g}^{-1}$). A potential source of Ni in these soils could be lateritic material transported by water from the ultramafic complexes occurring throughout the Polochic-Motagua fault systems in Guatemala. Hernández-Quiroz *et al.* (2012) proposed that main sources of Ni ($39\text{--}318 \mu\text{g g}^{-1}$) in similar soils collected near a petrochemical facility in Tabasco were the volcanic material deposits, and the lateritic nodular fragments (associated with crystalline Fe oxides and the residual fraction) transported from preexisting soils (pedorelicts) possibly formed out of ultramafic rocks situated in

the margins of the Caribbean plate. Another significant contributor to the current Ni concentration in soils could be the volcanic ash deposits derived from the constant activity of the nearest volcanos, El Chichón and Tacaná. Nevertheless, further mineralogical studies are needed to answer these questions and to better characterize these unusual soils. In any case the presence of elevated soil Ni in localities scattered throughout this region probably explains the extension of *B. guatemalense* and other associated Ni-hyperaccumulators (e.g. *Psychotria costivenia*, *P. lorenciana* and *P. papantlensis*) (McCartha et al., 2019).

4.2. Nickel hyperaccumulation in *B. guatemalense* and its significance

Non-destructive hand-held XRF technology is an excellent example of these promising new tools which has permitted the addition of many hyperaccumulator taxa to the global inventory in recent years (Gei et al., 2018). Extensive systematic screenings of mounted specimens in different herbaria have been performed including at the FRC Herbarium in Sabah (Malaysia) and the IDR Herbarium in Nouméa (New Caledonia) with the discovery of 85 and 91 new hyperaccumulator taxa respectively (van der Ent et al. 2019a, b; Gei 2020).

Data obtained from the XRF herbarium scanning confirmed the obligate hyperaccumulation trait in this species. Thus, only 10 specimens, out of the 182 analyzed in total, recorded Ni concentrations below the hyperaccumulation threshold ($1000 \mu\text{g g}^{-1}$). Mean Ni concentrations in the leaves of *B. guatemalense* scanned in the herbaria (mean value of $4022 \mu\text{g g}^{-1}$) differ considerably from those collected in the field (mean value of $13\,500 \mu\text{g g}^{-1}$). All parts of the plant (seeds, flowers, fruits, bark, roots, leaves) had Ni concentrations above $2600 \mu\text{g g}^{-1}$ with the highest value reported in leaves ($42\,900 \mu\text{g g}^{-1}$). In field-collected plants, *Blepharidium guatemalense* was therefore able to accumulate among the highest Ni concentrations reported in hyperaccumulator leaves globally, which places it in the group of ‘hypernickelophores’ plants (i.e. $>1\%$ wt) (Boyd and Jaffré, 2009; R. D. Reeves et al., 2018a).

Until now, Ni hyperaccumulator species belonging to the Rubiaceae family have been only reported in the well-known tropical hotspots of biodiversity such as the neighbouring territory of Cuba (11 spp), New Caledonia (3 spp) and Malaysia (3 spp). Within the Rubiaceae, the tribe Rondeletieae (Manns and Bremer, 2010; Rova et al., 2009) includes *Blepharidium* along with at

least three other genera of hyperaccumulators reported from Cuba by Reeves et al. (1999): *Rondeletia*, *Phyllomelia* and *Mazanea* (= *Ariadne*). Further herbarium surveys on close relatives of known hyperaccumulators in Mexico could lead to the discovery of new hyperaccumulator taxa.

4.3. Level of uptake of Ni and Co by *B. guatemalense* from surrounding soils

The heterogeneous distribution of DTPA-extractable Ni in soils could explain the high variability in Ni concentrations within foliar samples collected in the field as those two variables were positively correlated ($r = 0.510$). No other soil characteristics seemed to influence Ni uptake. A maximum DTPA-extractable Ni concentration of almost $200 \mu\text{g g}^{-1}$ was recorded in an old pasture with a high density of *B. guatemalense* clustered in patches of around 10 m in diameter. Because of the high heterogeneity of Ni availability in soils resulting from similar geological and pedological situations, one can question whether the presence of the species itself influences the variability of Ni levels. The highly concentrated large leaves from fast-growing trees suggest, among other factors, that litter produced by *B. guatemalense* could contribute significantly to the available Ni pool in the surface soils. These changes in Ni concentrations could, in turn, offer a suitable habitat for other Ni hyperaccumulator plants. Indeed, in primary and secondary forests in our study area, *B. guatemalense* was frequently observed accompanied by the facultative Ni-hyperaccumulator *Psychotria costivenia* as an understory plant beneath it. Preliminary observations suggested that shrubs of *P. costivenia* growing under *B. guatemalense* trees have much higher Ni concentrations than those that are not under such a canopy.

Simultaneous Ni and Co hyperaccumulation ($> 300 \mu\text{g g}^{-1}$ for the latter) was reported in 27 mounted specimens from the MEXU and MO herbaria with a ratio of $\sim 8:1$ Ni:Co. Similar cases of simultaneous Ni and Co hyperaccumulation have been observed in the Malaysian tree *Glochidion cf. sericeum* (Phyllanthaceae) with an unusual ratio of 1:1 Ni:Co. Another example is *Rinorea bengalensis* (Wall.) Kuntze (Violaceae) with Co values up to $630 \mu\text{g g}^{-1}$, also found by XRF scanning or *Berkheya coddii* (Asteraceae), with Co values higher than $600 \mu\text{g g}^{-1}$ (Lange et al., 2017). Nevertheless, the Co concentration in foliar samples of *B. guatemalense* collected in the field barely reached $149 \mu\text{g g}^{-1}$. This may be due to the almost neutral pH in rhizosphere soils, since it is known that plants accumulate Co mostly in acidic substrata (Kukier et al., 2004b), but

also to the competition between Ni and Co for uptake mechanisms, as described for *Alyssum* species and *B. coddii* (Homer et al., 1991; Keeling et al., 2003).

4.4. Elemental distribution in *B. guatemalense* and physiological aspects

On sufficiently old trunks, the inner bark of the tree that is in direct contact with wood (phloem tissues) on trunks that are old enough shows a particular turquoise green-blue colour with Ni concentrations reaching more than 1.8 wt%. Such an intense green colour is one of the most outstanding features of woody Ni hyperaccumulators that has been also observed in some SE Asian and Pacific species (*e.g. Phyllanthus balgooyi* Petra Hoffm. & A.J.M.Baker, and *R. bengalensis*), and is due to the very high concentrations of Ni²⁺ ions in this tissue (van der Ent et al., 2017b). Nickel-enriched phloem tissues on the stem of the South African Ni hyperaccumulator *Senecio coronatus* (Thunb.) Harv. have also been reported (Mesjasz-Przybylowicz, 1997). Desktop micro-XRF showed rather heterogeneous distributions of Ni across the leaf in both veins and leaf blades. The XFM elemental maps confirmed the extremely high Ni concentrations not only in the phloem of the primary (leaf midrib) and secondary leaf veins, but also in the phloem of the root, stem and petiole. As for other species which show this specific feature, *e.g. P. balgooyi* (van der Ent et al., 2017a) or *R. bengalensis* (Zelano et al. *in press*), *Blepharidium guatemalense* appears to redistribute Ni within the plant *via* the phloem. Phloem active transport of Ni was confirmed in another Ni hyperaccumulator *Noccaea caerulescens* by using Ni stable isotope analysis (Deng et al., 2018, 2016).

Another fact that confirms the re-distribution of Ni *via* phloem in *B. guatemalense* is the high Ni concentrations reported in flowers and fruits (mean concentrations 5930 µg g⁻¹ and 2650 µg g⁻¹ respectively), and extremely high values in seeds (mean concentration 18 400 µg g⁻¹). Indeed, the only pathway for Ni (and eventually other heavy metals) to reach sink organs (maturing fruits, seeds, flowers, *etc.*) is through the mass movement of phloem fluids coming from the source organs (senescing leaves) (Bhatia et al., 2003; Page and Feller, 2015). Leaf epidermal cells of *B. guatemalense* were not Ni enriched as opposed to most hyperaccumulator species; this trait was also observed for the following species: *Berkheya coddii* and *P. balgooyi* (van der Ent et al. 2017).

According to the updated knowledge on Ni hyperaccumulators which redistribute Ni through the phloem, we could hypothesize that Ni²⁺ could be loaded into the xylem as the free cation and transported to the shoots where it may be complexed by carboxylic acids (mainly citrate), and partly stored in the leaf midrib collenchyma (van der Pas and Ingle, 2019). Since a slight Ni accumulation is observed in the leaf parenchyma, transport may also occur from the xylem to mesophyll cells in the transpiration stream (Page and Feller, 2015). In minor leaf veins, Ni may also be transferred directly from the xylem to the phloem through companion cells (Page and Feller, 2015; van Bel, 1990). In many Ni hyperaccumulators globally, Ni²⁺ is complexed predominantly by citrate or malate in leaves, phloem or latex and its chemical speciation does not substantially change among different plant tissues (van der Ent et al. 2017). Sugars, amino acids and organic acids are expected to be the most predominant metabolites in the phloem sap (Braun et al., 2014). The dominant Ni-citrate complexes in the phloem tissues of *P. balgooyi* seem to be associated with low sugar contents (mainly sucrose) (van der Ent et al. 2017). In this case, higher sucrose concentration in the mesophyll cell cytoplasm may enhance the passive loading of the phloem moving by diffusion down its concentration gradient to the sieve-tube elements. Such a process could explain the higher Ni concentration in the phloem tissues than in the leaves in this species, as it could be the case in *B. guatemalense*. Conversely, *P. rufuschaneyi* accumulated higher Ni accumulation in leaves with respect to phloem tissues (van der Ent et al. 2017). This highlights that further studies on the Ni chemical speciation in the different tissues and fluids of *B. guatemalense*, including phloem sap, are crucial to better understand these processes at the cellular level.

In the leaf samples taken in the field, cobalt was accumulated at close-to-hyperaccumulation levels in the leaf samples taken in the field for micro-XRF analysis. In Co hyperaccumulators, Co is sometimes found in the form of lesions in the leaf surface as a result of an extracellular detoxification mechanism *via* exudation of guttation liquids (van der Ent et al., 2018), whereas it is found accumulated at the leaf tip in *Alyssum murale* (Tappero et al., 2007). No evidence of these lesions or of tip accumulation was observed in the micro-XRF distribution map of *B. guatemalense* because of the low Co concentration in the foliar samples collected in the field. Pearson correlation coefficient (*r*) of foliar elemental concentrations showed a strong positive correlation between Ni

and Zn (Spearman test, p value < 0.0001). Both elements can, therefore, enter through the plasma membrane of root cells *via* the same poorly selective cation transporter members of the ZIP gene family (van der Pas and Ingle, 2019). Also, the distribution of Zn mirrors that of Ni within the different plant tissues, as observed in the XFM elemental maps.

4.5. Potential applications for *B. guatemalense*

Blepharidium guatemalense possess the required characteristics to be an excellent candidate for Ni agromining (phytomining), such as Ni concentrations in shoots above 1 wt%, high biomass, apparent fast-growth rate and easy reproduction (Nkrumah et al., 2018b). The fact that the wood of this species is currently used for building (*e.g.* rafters, beams and supports of houses) by local populations in Chiapas and Tabasco supports the idea that the wood of hyperaccumulator plants may be durable against termites by acting as natural insecticide/fungicide (van der Ent and Mulligan, 2015). Our observations also suggest the possibility of further agromining implementation in secondary forest and pasture lands where the tree is grown (or left to grow) in a deliberate way. Sustainable agromining systems using *B. guatemalense* could contribute to the preservation of this unique species. Research is currently underway to study the agronomy of this species and the environmental, social and economic implications of any implementation of agromining in those regions.

5. Conclusions

Blepharidium guatemalense, a new hypernickelophore species able to accumulate up to 4.29 wt.% Ni in its leaves, was discovered in south-eastern Mexico. Field surveys together with XRF herbarium screenings revealed its geographic distribution, which includes the States of Tabasco, Chiapas and Campeche. The maximum Ni concentration reported in herbarium specimens was 1.88 wt.% and Ni concentrations were above the hyperaccumulation threshold ($1000 \mu\text{g g}^{-1}$) for 95% of the specimens. The obligate character of Ni hyperaccumulation in this species is therefore confirmed. Micro-XRF mapping in the leaves of *B. guatemalense* revealed that the Ni is uniformly distributed in the leaf with higher concentrations in the midrib and in the secondary veins. Elemental maps generated by XFM confirms that *B. guatemalense* has a physiology that results in an extremely Ni-rich phloem tissue, suggestive of internal re-distribution mechanisms similar to

those reported in other extreme tropical Ni-hyperaccumulator species (including *P. balgooyi*, *P. rufuschaneyi* and *R. bengalensis* from Malaysian Borneo).

Prior this research, no plant had ever been reported to exceed the ‘hypernickelophore’ concentration of 1.0 wt.% when growing in soil that was neither ultramafic nor enriched by anthropogenic pollutants. *Blepharidium guatemalense* does so consistently, over a vast region of south-eastern Mexico. Moreover, in the natural lowland forests of Chiapas, stands of *B. guatemalense* may create Ni-rich ‘oases’ for other Ni hyperaccumulator species (e.g. *P. costivenia*) likely by recycling Ni through the decay of Ni-rich litter in the topsoil. This finding shifts the paradigm and enables the exploration of ‘natural’ Ni hyperaccumulator habitats beyond the constraints of ultramafic geology. Pioneering investigations are being carried out to study the application of *B. guatemalense* for Ni agromining (phytomining).

Chapter IV

Nickel agromining in Mexico

Among the discovered hyperaccumulator species, *B. guatemalense* had an evident potential for Ni agromining based on its valuable traits such as high foliar Ni concentrations and biomass, fast-growing large biomass tree, perennial tree which resists coppicing and apparently easy to reproduce. Even though the species has been used by local populations for centuries, it is not yet cultivated as such. Thus, the agronomy of this species remains unexplored.

This chapter is dedicated to the assessment of the potential of *B. guatemalense* for Ni agromining in Mexico. Different agronomic practices such as fertilization and harvesting were tested for this plant species. The selection of suitable sites for agromining implementation as well as the different agronomic treatments and cropping systems that could be implemented in Mexico are also discussed. This article has been submitted to the journal “*International Journal of Phytoremediation*”.

The potential of *Blepharidium guatemalense* for nickel agromining in Mexico and Central America

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Abstract

The aim of this study was to assess the potential of the woody nickel hyperaccumulator species *Blepharidium guatemalense* (Standl.) Standl. for agromining in southeastern Mexico. Pot trials consisting of nickel dosing (0, 20, 50, 100 and 250 mg Ni kg⁻¹), and synthetic and organic fertilization were conducted. Field trials were also undertaken with different harvesting regimes of *B. guatemalense*. Foliar nickel concentrations increased significantly with rising nickel additions, with a 300-fold increase in the 250 mg Ni kg⁻¹ treatment relative to the control. Synthetic fertilization strongly increased nickel uptake without any change in plant growth or biomass, whereas organic fertilization enhanced plant shoot biomass with a negligible effect on foliar nickel concentrations. A 5-year-old stand which was subsequently harvested twice per year produced the maximum nickel yield tree⁻¹ yr⁻¹, with an estimated total nickel yield of 142 kg ha⁻¹ yr⁻¹. *Blepharidium guatemalense* is a prime candidate for nickel agromining on account of its high foliar Ni concentrations, high bioconcentration (180) and translocation factors (3.3), fast growth rate and high shoot biomass production. Future studies are needed to test the outcomes of the pot trials in the field. Extensive geochemical studies are needed to identify potential viable agromining locations.

Keywords: *hyperaccumulation, agronomy, fertilization, neotropics, Ni yield, phytomining.*

1. Introduction

Metal hyperaccumulation is an unusual phenomenon that occurs in at least 720 plant species belonging to 130 genera and 52 families distributed worldwide (R. Reeves et al., 2018). These species have evolved physiological mechanisms enabling them to take up and accumulate one or more metallic (or metalloid) elements in their leaves to concentration levels two to three orders of magnitude higher than is found in plants growing on non-metalliferous soils (Baker and Whiting, 2002; van der Ent et al., 2013; Merlot et al., 2018). About 75% of hyperaccumulator species are for nickel (Ni) (Reeves 2003; Reeves et al. 2017) and this can be explained by the large extent of Ni-enriched ultramafic outcrops covering approximately 1% of the Earth's land surface (Garnier et al., 2009; Echevarria, 2018). Hyperaccumulator plants are of particular interest for both fundamental research (*e.g.* metal regulation in plants) and practical application in prospecting ore-bodies, phytoremediation (Baker and Brooks, 1988) and phytomining (Chaney et al., 2007, 2018a; van der Ent et al., 2015).

Agromining, a type of phytomining, is a complete chain of agro-metallurgical processes that aims to recycle strategic metals from secondary resources (*e.g.* contaminated or naturally metal-enriched soils) by means of the cultivation of selected hyperaccumulator plants ('metal crops') to provide valuable products (Echevarria et al., 2015; Morel et al., 2015; van der Ent et al., 2015). This 'green' technology is also a cost-effective and environmentally friendly approach for rehabilitating degraded mine sites (Erskine et al., 2018).

Most agromining research to date has focused on nickel because of the relatively high value of this metal on the market (US\$ 15 kg⁻¹ on the LME, August 28, 2020), and the existence of many Ni-enriched soils and Ni-hyperaccumulator plants around the world. Optimal agronomic practices, based on laboratory and field experiments, have significantly increased the Ni yields of some 'metal crops'. The improvement of the agronomy of the Eurasian species *Odontarrhena muralis sensu lato* (synonym *Alyssum murale s.l.*) resulted in a maximum yield of 112–145 kg Ni ha⁻¹ in Albania (Bani et al., 2015b; Bani and Echevarria, 2019), and 400 kg Ni ha⁻¹ in the USA (Yin-M Li et al., 2003; Chaney et al., 2007). Furthermore, pilot-scale agromining trials in western, central and southern Europe have been set-up to improve plant yields whilst simultaneously enhancing soil quality (Kidd et al., 2018). Thus, fertilization regimes, herbicide application, soil tillage, plant

density planting, etc. are all currently being tested for high performance Mediterranean ‘metal crops’ such as *O. muralis* and *Bornmuellera emarginata* (Kidd et al., 2018).

Research on tropical Ni agromining has rapidly progressed in recent years, specifically in Southeast Asia (Sabah, Malaysia). Two promising species, *Phyllanthus rufuschaneyi* and *Rinorea* cf. *bengalensis*, have been tested in pots and in the field to evaluate their response to synthetic fertilization, pH adjustment and organic amendments addition, among other treatments (Nkrumah et al., 2019b, 2019c, 2019d). A maximum Ni yield of 250 kg Ni ha⁻¹ has been estimated for a large-scale agromining operation using *P. rufuschaneyi* under field conditions in Sabah (Nkrumah et al., 2019a).

The neotropical region is a major hot-spot for hyperaccumulator plants, with Cuba and Brazil recording ~30% of all currently known Ni hyperaccumulator species (R. Reeves et al., 2018; Reeves et al., 1999). Species from the genera *Buxus* and *Leucocroton* in Cuba have been identified as potential ‘metal crops’ (> 2% wt Ni in dry leaves) (Reeves et al., 1996; Nkrumah et al., 2016). However, agromining in this region has remained unexplored until recent years. Our field and herbarium surveys have led to the discovery of Ni hyperaccumulation in at least 13 plant species belonging to three families (Oleaceae, Rubiaceae and Violaceae) distributed mainly in the tropical forests of Mexico, Central and South America (McCartha et al., 2019; Nkrumah et al., in press). Among the discovered species, there is one ‘hypernickelophore’ (>1 wt% Ni in dry leaves) that seems to be the most promising candidate for Ni agromining in the neotropical regions. *Blepharidium guatemalense* (Standl.) Standl. (Rubiaceae) or ‘Popiste’, as it is known in the Mayan local language, is a ligneous species mainly distributed in the tropical perennial and sub-perennial rainforests of southern Mexico (Chiapas, Tabasco and Campeche) and Guatemala. This high-biomass tree is able to concentrate up to 4.3 wt% Ni in its dry leaves and has highly Ni-enriched phloem which may have significant role in Ni distribution in this species (Navarrete et al., 2020, submitted). *Blepharidium guatemalense* also grows in fallow fields under ‘slash-and-burn’ agriculture and pasturelands and has long been used for building traditional houses (poles for roofing), and as live fences by the local communities (Jimenez-Ferrer et al., 2008). The high density of this wild species observed in some fallow fields is indicative of the current management of this tree, which is referred to by the local people as “tolerated/promoted”. In this plant

management approach, local people seek to maintain or even increase the abundance of useful species without protecting (*e.g.* removal of competitors) or cultivating (*e.g.* fertilization practices) it (Casas et al., 2017). Field observations and testimonials from local people suggest that the species grows fast and can be easily propagated via seeds, which are desirable traits of a commercially viable ‘metal crop’ (Chaney et al., 2007; Nkrumah et al., 2018a). Considering the high potential of *B. guatemalense* as a ‘metal crop’ for economic Ni agromining in suitable neotropical regions, it is critical to develop the agronomic systems to optimize Ni yield. This study is the first to assess the response of *B. guatemalense* to different agronomic systems at both laboratory- and field-scale. The specific objectives of this study were to elucidate the effect of Ni (and other elements) uptake and shoot biomass production of *B. guatemalense* in response to: i) soluble Ni additions, ii) synthetic fertilization, iii) organic fertilization and iv) harvesting schedules.

2. Materials and Methods

2.1. Nickel dosing trial

The Ni tolerance and accumulation potential of *B. guatemalense* was assessed through a pot experiment under controlled conditions in the Laboratoire Sols et Environnement (LSE) in Nancy, France (growth chamber at 25°C, 85% relative humidity and a 12 hr daylength). Seeds were collected from one individual in the surroundings of the Universidad Tecnológica de la Selva in Chiapas, Mexico (374 m. a.s.l. 16°49'21.2"N, 91°07'21.5"W) and germinated in pots using a mix of 50% compost and 50% native soil. A well characterized soil from Champenoux, France was collected from the A horizon (5-15 cm depth), air-dried, sieved to < 2 mm and 1 kg of each placed into 5 plastic bags. The soils were dosed with Ni salt (NiSO₄ 6H₂O): 0 (control), 20 (T1), 50 (T2), 100 (T3) and 250 (T4) mg Ni kg⁻¹, mixed in the plastic bags to avoid Ni losses and then placed in 1kg pots. Plant seedlings of about 2.5 cm height were carefully transplanted into each pot with 3 replicates per treatment, using a fully-randomized design. Plants were watered daily to 80% of the soil water-holding capacity with deionized water and harvested after three months.

2.2. Nutrient dosing trial

The response of *B. guatemalense* to synthetic fertilization was evaluated in a pot experiment conducted in a well-ventilated greenhouse at the Universidad Tecnológica de la Selva, Mexico. Soils were collected from the A horizon (5–15 cm depth) at the native habitat, air-dried and sieved to 2 mm. Forty 8-month-old seedlings obtained from seeds mentioned in the Ni dosing trial were carefully transplanted into 1 kg pots. The seedlings were allowed to establish for one month and then assigned to the treatments using a fully-randomized design. Three doses of macronutrients nitrogen, phosphorus and potassium (NPK) and one control with ten replicates each were tested: i) 0:0:0 ii) 50:40:40; iii) 100:80:80 and iv) 120:120:120 kg ha⁻¹. Fertilizers used were urea (46:0:0) and a granulated mixture of NPK (17:17:17) divided into two doses, the first one applied immediately after transplanting, and the second a month later to avoid losses by leaching. An organic treatment of 100 g of vermicompost per pot was also tested. Plants were watered daily with local tap water over a period of 5 months after which they were harvested.

2.3. Bulk analysis of harvested plants

Harvested plants were separated into different fractions (roots, stems and leaves), washed with deionized water and placed in a drying oven at 70°C for three days. The dried plant samples were then weighed, ground to 150 µm and digested using 1 mL HNO₃ (65%) and 2 mL H₂O₂ (30%). After 24 h, samples were heated for 2 hrs at 95°C in a block system (DigiPREP), left to cool at least for 3 hrs, filtered to < 0.45 µm and adjusted in volume to 10 mL with ultrapure water. The resulting extracts were analyzed by ICP-AES (Thermo Fisher CAP 6300 Duo) for minor elemental quantification. An extra 1:10 dilution was needed for the quantification of major elements. Three blanks and three reference samples of the known hyperaccumulator plant *Noccaea caerulea*, were used in each analytical series for the quality control of data.

2.4. Bulk analysis of soil samples

Soil samples were dried at 70°C for 3 days in a drying oven and sieved to < 2 mm. Subsamples of 0.5 g (± 0.001 g) were finely ground (< 250 µm) and digested by adding 6 mL of 37% HCl and 2 mL of 70% HNO₃ per sample. After allowing to stand for 24 hrs, digests were heated for 3 hrs at 100°C in a block system (DigiPREP MS), and then left to cool down to a minimum temperature of 40°C. Solutions were adjusted in volume to 45 mL with ultrapure water, filtered < 0.45 µm, and then readjusted to 50 mL. A final dilution of 1:20 was necessary for major elements. The resulting

soil digests/extracts were analyzed by ICP-AES for the quantification of major and minor elements. Trace element availabilities in soil samples were also determined by ICP-AES using diethylenetriaminepentaacetic acid (DTPA-extractant) according to the method of Lindsay and Norvell (1978). Cation exchange capacity (CEC) of soils was determined using cobalt-hexamine trichloride extraction by adding 50 mL of solution (4.458 g of $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ with 700 mL of distilled water). After 1 hr shaking, 10 ml of each sample was filtered at 0.45 μm and measured by spectrophotometrically at a wavelength of 475 nm (BioRad, SmartSpec Plus). Available phosphorus (P) was determined according to the Olsen method (Olsen et al., 1954; ISO 11263, 1994). Soil pH was measured with in a 1:5 soil:water (5 mg soil with 25 mL ultrapure water) suspension after 1 hr shaking at 16 rpm and 1 hr standing.

2.5. Harvesting schedule/ nickel yield estimation

The potential biomass and Ni yields were estimated by means of a one-year field experiment. The experimental site was a pastureland, formerly a tropical rainforest located 10 km northeast of Bonampak archaeological site in Chiapas, Mexico (16°44'20.4"N, 90°59'46.2"W). The site was selected due to the high DTPA-available Ni in the soils (Navarrete et al., 2020, submitted), and the presence of many 5-8-year-old trees of *B. guatemalense* distributed in patches of > 8 individuals. Three plots of 15 m x 15 m were delimited, and 6 five-year-old trees (diameter at 1.3 m height > 20 cm) were selected per plot, identified, measured and coppiced at a height of 1.5 m. Prior to harvesting, soil (5–10, 10–25, 25–30 cm depths) and plant samples (three leaves per tree) were collected from each plot for elemental analysis. Total and DTPA-available Ni concentrations in soils as well as foliar Ni concentrations of selected trees are shown in **Table 13**.

Experimental plot	Horizon (cm)	pH	Total Ni (mg kg ⁻¹)	DTPA-extractable Ni (mg kg ⁻¹)	Foliar Ni (g kg ⁻¹)
C1	5-10	6.3	1030	131	22.5 ± 1.4
	10-25	6.6	544	8.6	
	25-30	6.7	571	5.4	
C2	5-10	6.3	1130	97	15.2 ± 0.5
	10-25	6.5	1000	41	
	25-30	6.6	1010	34	
C3	5-10	6.2	815	114	12.3 ± 0.2
	10-25	6.4	571	30	
	25-30	6.5	626	42	

Table 13. Total and DTPA-available Ni in composite soil samples (n = 1) of each experimental plot and average Ni concentrations in leaves of 6-years old trees of *B. guatemalense* (n = 18) prior to harvesting trial.

Two harvesting frequency scenarios were tested with 9 replicates each, one and two times a year, harvesting only leaves and branches. Growth (height) of coppiced trees was measured every 15 days during the first 6 months. In addition, an 8-year-old tree was coppiced to a height of 35 cm to consider a third scenario with a harvesting of the whole biomass (leaves, branches, fruits, flowers and bark) after 8-years cropping. Fresh harvested biomass was separated into the different fractions and weighed. Dried biomass was re-weighed after a two-week period of sun-drying. Leaf samples of each tree were taken for chemical analysis.

2.6. Statistical analyses

Descriptive analyses, boxplots and one-way analyses of variance (ANOVA) with a confidence level of 95% were performed using XLSTAT 2019.1.1 version ‘Ecology’ in Microsoft Excel 365. Normality and homoscedasticity of data were verified using Shapiro and Levene tests. When these two assumptions were fulfilled, t-tests or ANOVA followed by Newman Keuls tests were performed. In specific cases (*e.g.* data ranging over several orders of magnitude), data were log-transformed. Non-parametric tests (Mann-Whitney, Kruskal-Wallis tests) were performed when assumptions of a parametric test were not met.

3. Results

3.1. Nickel dosing trial

Nickel concentrations in the leaves of *B. guatemalense* increased significantly with increasing DTPA-available Ni concentrations (Kruskal-Wallis test, $p < 0.05$), with no significant decrease in shoot (leaves and stem) biomass production (ANOVA, Newman-Keuls test, $p > 0.9$) (**Figure 18**).

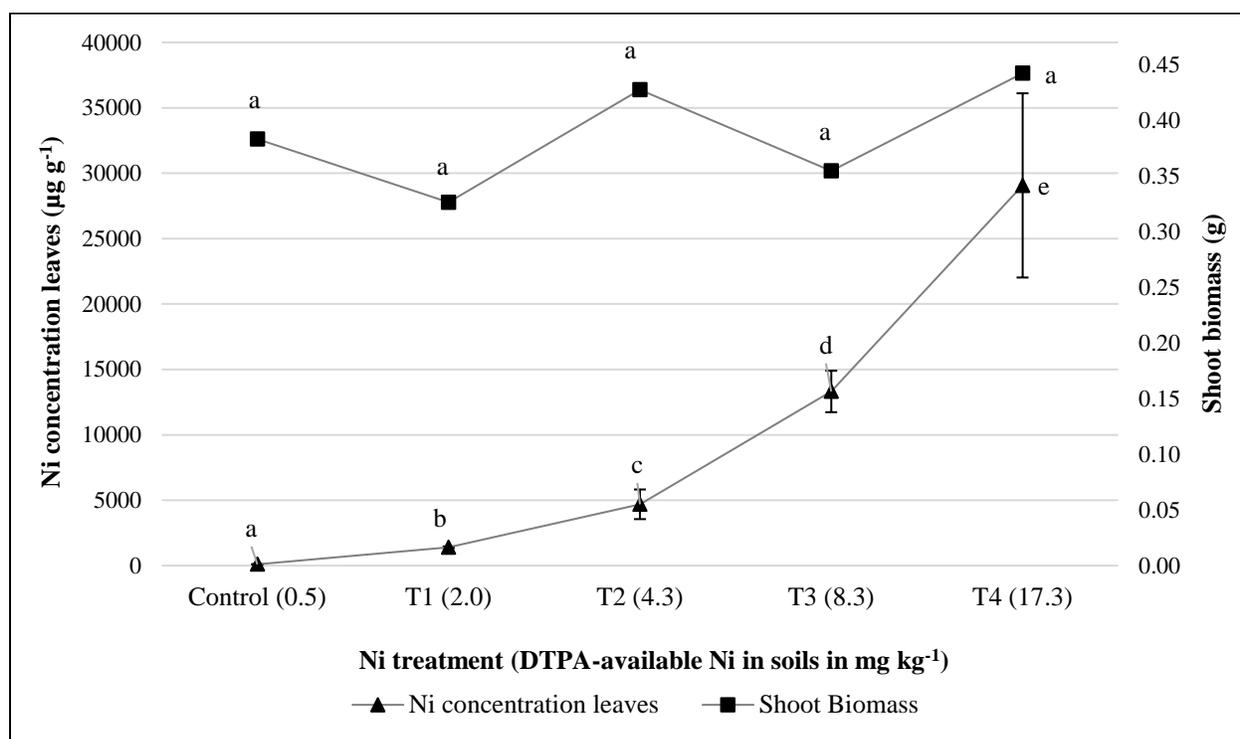


Figure 18. Nickel concentrations in leaves and biomass production of *B. guatemalense* under different doses of Ni in soils. Nickel concentrations in leaves with different letters indicate significant differences according to Kruskal-Wallis test ($\alpha = 0.05$). For shoot biomass, same letters indicate no significant differences according to ANOVA, Newman-Keuls test, ($\alpha = 0.05$).

Nickel concentrations in the leaves of plants from the T4 treatment were almost 300-fold higher than in the control. There were no significant differences in plant growth (height) and leaf count among treatments (ANOVA, Newman-Keuls, $p > 0.8$). Nickel concentrations in stems and roots of the plant increased significantly and steadily with Ni addition (**Figure 19**).

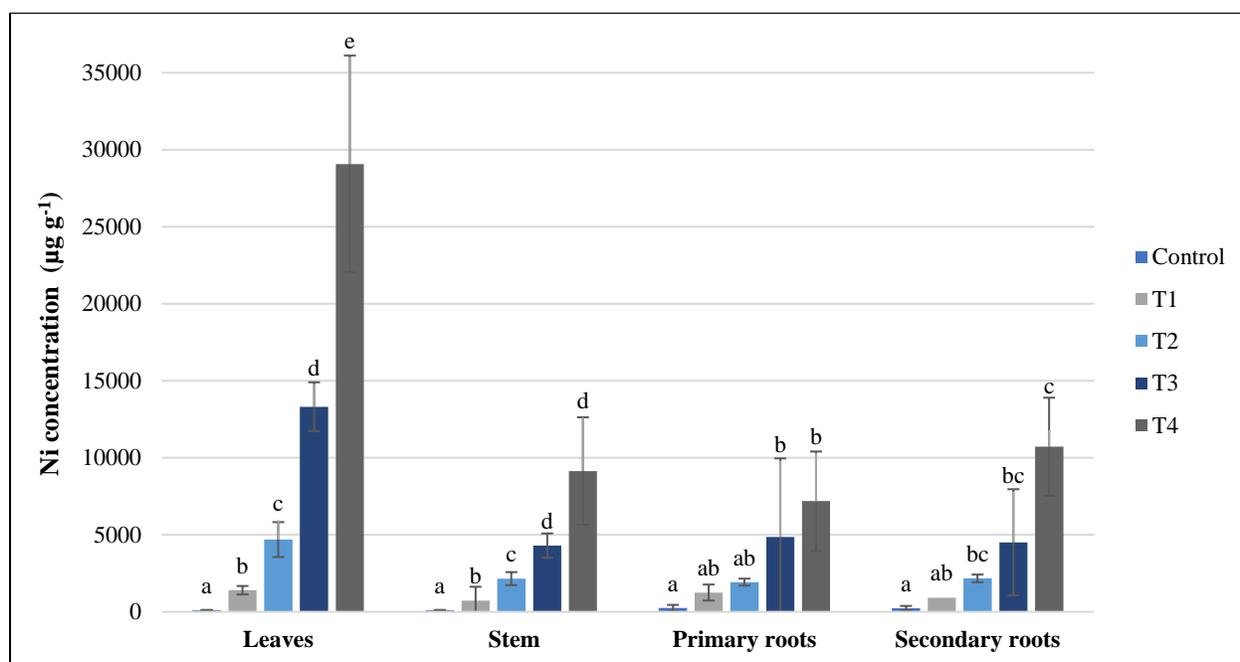


Figure 19. Nickel concentrations in the different parts of *B. guatemalense* under different Ni additions to soils. Values with different letters indicate significant differences according to Kruskal-Wallis test ($\alpha = 0.05$, Conover-Iman as post-hoc test).

Nickel accumulation in primary roots was not significantly different from that found in secondary roots (t-test, $p > 0.9$; data transformed to \log_{10}). The bioconcentration factor (BAF = ratio of the metal concentration in leaves to the total Ni concentration in soils, (Mackay and Fraser, 2000)) of the T4 treatment (180), was 70-fold higher than that in the control (**Figure 20**). The translocation factor (TF = ratio of the Ni concentration in leaves to that in roots, (Mattina et al., 2003)) of the T4 treatment also showed an 8-fold increase (3.3) compared to the control.

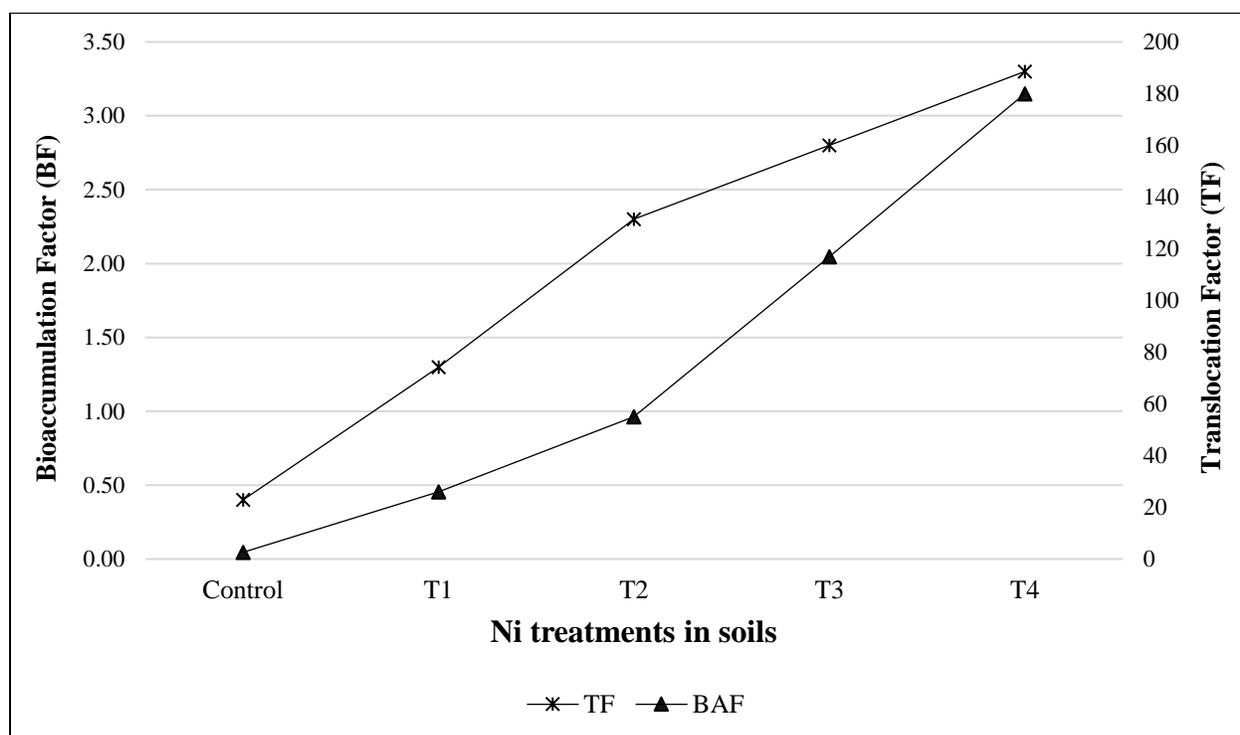


Figure 20. Bioconcentration and translocation factors of *B. guatemalense* under different Ni doses in soils.

3.2. Nutrient dosing trial

The effect of the different NPK additions on shoot dry biomass, plant growth and Ni concentrations in the different parts of *B. guatemalense* are shown in **Figure 21**. Although no significant differences were observed in the shoot biomass and in the plant height among treatments (ANOVA, Newman-Keuls, $p > 0.05$) (**Figures 21a, b**), there was a two-fold increase in the number of leaves in the 120:120:120 treatment compared to the control (t-test, $p < 0.05$). There was a slight decrease in Ni concentrations in the leaf fraction of the plants in the 50:40:40 treatment with respect to the control, but it was not statistically significant (Mann-Whitney test, $p > 0.6$). Nickel concentrations in leaves, stems and roots were significantly higher in the 100:80:80 and 120:120:120 treatments with respect to the control (**Figures 21c, d, e**). There was a three-fold increase in the average foliar Ni concentration in the 120:120:120 treatment compared to that in the control. The shoot Ni yields were significantly different among treatments with an almost five-fold increase for the 120:120:120 treatment with respect to the control (Kruskal-Wallis test – Conover-Iman test, $p < 0.05$).

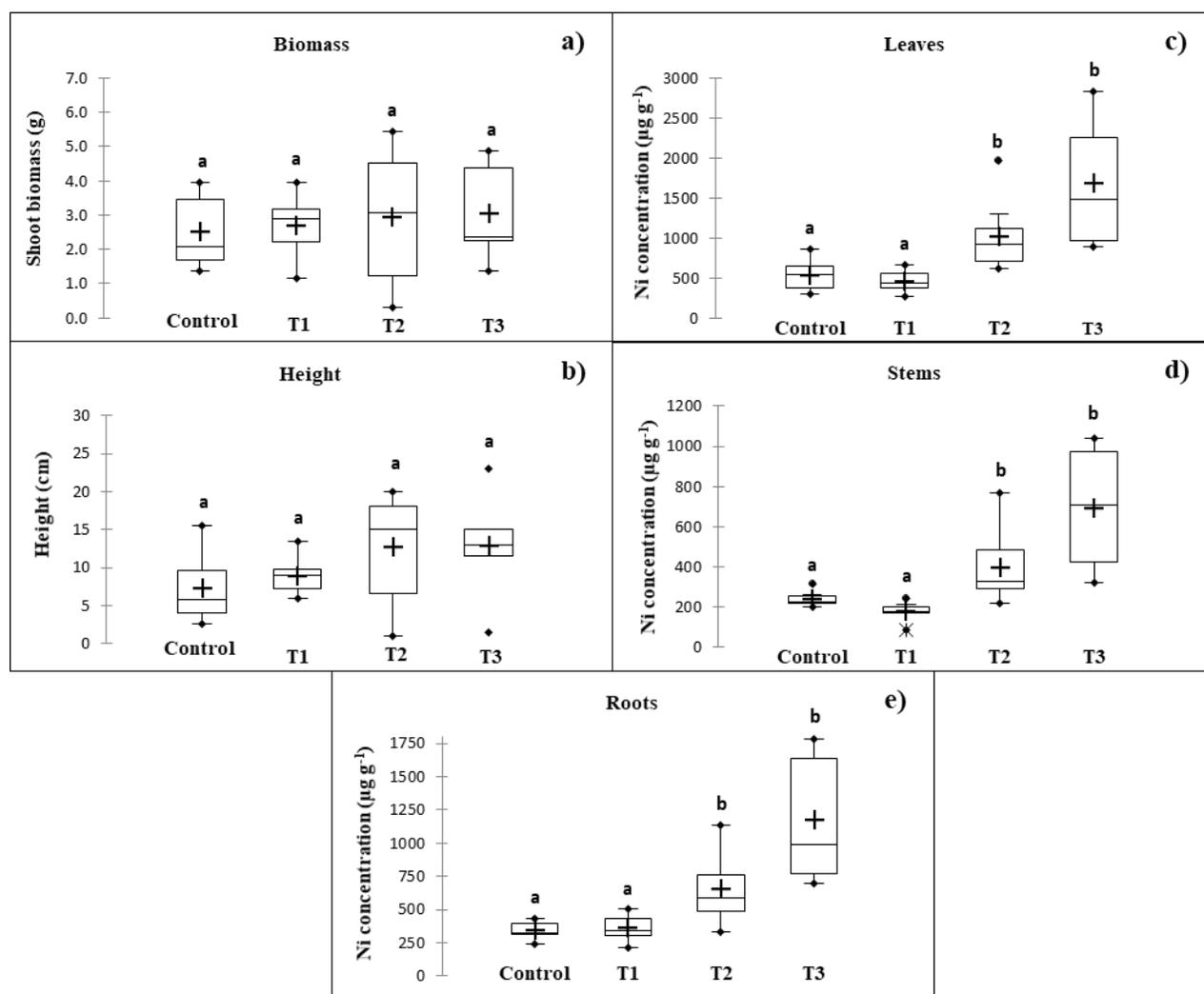


Figure 21. Response of *B. guatemalense* to synthetic fertilization after 5-months pot experiment. For **a)** biomass and **b)** height, same letters represent no significant differences (ANOVA, Newman-Keuls test, $\alpha = 0.05$). For Ni concentrations in leaves, stems and roots (**c**, **d**, **e**), significant differences are indicated by different letters (Kruskal-Wallis test, $\alpha = 0.05$).

Concentrations of elements in plant leaves in response to the nutrient dosing are shown in **Table 14**. Most of the elements did not show any significant difference among treatments, except for magnesium (Mg), iron (Fe), manganese (Mn) and zinc (Zn). However, the differences in Mn and Zn foliar concentrations between the 120:120:120 treatment and the control were not significant (Mann-Whitney test, $p > 0.3$). However, in contrast the foliar Mg and Fe concentrations in the 120:120:120 treatment were significantly lower compared to the control (Mann-Whitney test, $p < 0.05$). There were significant differences in the foliar nitrogen (N) concentration within treatments

although they did not show an upward trend. No significant differences were found in foliar P and K concentrations in the 120:120:120 treatment with respect to the control (Mann-Whitney test, $p > 0.05$).

Treatment (NPK)	N (%)	P	K	Ca	S	Mg	Fe	Mn	Zn	Ni
T0, Control (0:0:0)	3.22 ± 0.36 ab	1430 ± 400 a	19 000 ± 5570 a	6700 ± 1670 a	2170 ± 637 a	3240 ± 1409 b	7880 ± 4073 b	238 ± 53 b	107 ± 130 ab	541 ± 204 a
T1 (50:40:40)	3.17 ± 0.34 ab	1500 ± 354 a	24 300 ± 3420 a	4890 ± 720 a	1990 ± 414 a	1810 ± 346 ab	981 ± 930 a	81 ± 28 a	42 ± 20 a	463 ± 140 a
T2 (100:80:80)	3.35 ± 0.37 a	1770 ± 424 a	23 200 ± 2900 a	5770 ± 980 a	2530 ± 1160 a	1840 ± 356 a	714 ± 612 a	104 ± 60 a	130 ± 94 b	1020 ± 431 b
T3 (120:120:120)	2.77 ± 0.37 b	2340 ± 1110 a	23 100 ± 2730 a	5070 ± 623 a	2490 ± 351 a	1590 ± 385 a	2070 ± 1524 ab	206 ± 89 b	93 ± 70 ab	1690 ± 844 b

Table 14. Elemental concentrations in leaves ($\mu\text{g g}^{-1}$) of *B. guatemalense* under different synthetic fertilization treatments. Values followed by the different letters are significantly different (p value < 0.05) according to Kruskal-Wallis test. Nitrogen values are given as a percentage (%) (ANOVA Newman-Keuls test, $p < 0.05$).

The chemical properties of soils (before and after the experiment) are shown in **Table 15**. DTPA-extractable Ni did not significantly change with nutrient addition (ANOVA, Newman-Keuls test, $p > 0.05$). A slight increase in total and extractable K and P concentrations in soils due to the NPK addition was observed. Total N concentrations did not significantly change within treatments (ANOVA, Newman-Keuls test, $p > 0.05$). A slight increase in the total concentrations of Ni, Mg and Fe was observed.

Treatments	Ni	Zn	Ca	Mg	K	P	Mn	Fe	S
<i>Total element (mg kg⁻¹)</i>									
PS	294	42	7920	5500	2590	250	2910	62 900	658
T0	244 ± 24	42 ± 4.1	7580 ± 1410	4510 ± 605	2440 ± 195	200 ± 46	2440 ± 481	53 350 ± 3244	695 ± 116
T1	273 ± 31	54 ± 12	8670 ± 981	5360 ± 775	2710 ± 293	406 ± 106	2250 ± 358	57 700 ± 4277	745 ± 50
T2	284 ± 9	57 ± 11	9210 ± 1410	5620 ± 408	2860 ± 143	451 ± 114	2220 ± 238	58 200 ± 3182	773 ± 100
T3	285 ± 15	47 ± 3.2	8170 ± 639	5950 ± 771	3050 ± 361	402 ± 76	2230 ± 391	60 700 ± 4232	708 ± 45
T Org	276 ± 10	56 ± 4.9	10 400 ± 644	5700 ± 367	2920 ± 228	457 ± 74	2470 ± 265	58 500 ± 1724	865 ± 51
<i>DTPA-extractable element (mg kg⁻¹)</i>									
PS	11	3.0	-	245	22	1.4	39	44	-
T0	10 ± 1.7	3.3 ± 1.2	-	255 ± 18	21 ± 5.6	2.1 ± 1.6	36 ± 5.0	43 ± 4.5	-
T1	13 ± 2.5	9.0 ± 5.3	-	269 ± 28	41 ± 22	13 ± 5.6	38 ± 4.9	51 ± 4.6	-
T2	13 ± 1.4	8.3 ± 3.8	-	258 ± 26	50 ± 13	13 ± 7.3	35 ± 7.1	49 ± 6.1	-
T3	13 ± 2.4	4.8 ± 1.2	-	242 ± 19	58 ± 14	17 ± 8.1	40 ± 4.6	50 ± 8.4	-
T Org	11 ± 2.0	8.6 ± 2.7	-	252 ± 27	49 ± 9.4	27 ± 11	27 ± 5.3	37 ± 5.3	-
<i>CEC (cmol+ kg⁻¹)</i>							<i>pH</i>		
PS	-		39	5.7	0.5		6.8		
T0	-		37 ± 4.7	5.6 ± 0.1	0.5 ± 0.4		6.7		
T1	-		40 ± 2.2	6.3 ± 0.3	0.9 ± 0.9		6.7		
T2	-		40 ± 3.9	6.4 ± 0.2	1.0 ± 0.7		6.7		
T3	-		37 ± 5.6	5.8 ± 0.3	1.1 ± 0.5		6.9		
T Org	-		42 ± 0.9	6.4 ± 0.2	1.0 ± 0.5		6.8		

Table 15. Elemental contents in soil prior to fertilization treatment application (PS), and in soils under the different treatments at the end of the experiment. Available P was obtained through Olsen method.

The shoot biomass (leaves and stems) and growth (height) significantly increased in response to vermicompost amendment (t-test, $p < 0.05$). Foliar biomass was three-fold higher than that of the control. However, the Ni concentrations in leaves and roots did not significantly change with respect to the control (t-test, $p > 0.05$), but decreased significantly in the stem fraction (**Figure 23**).

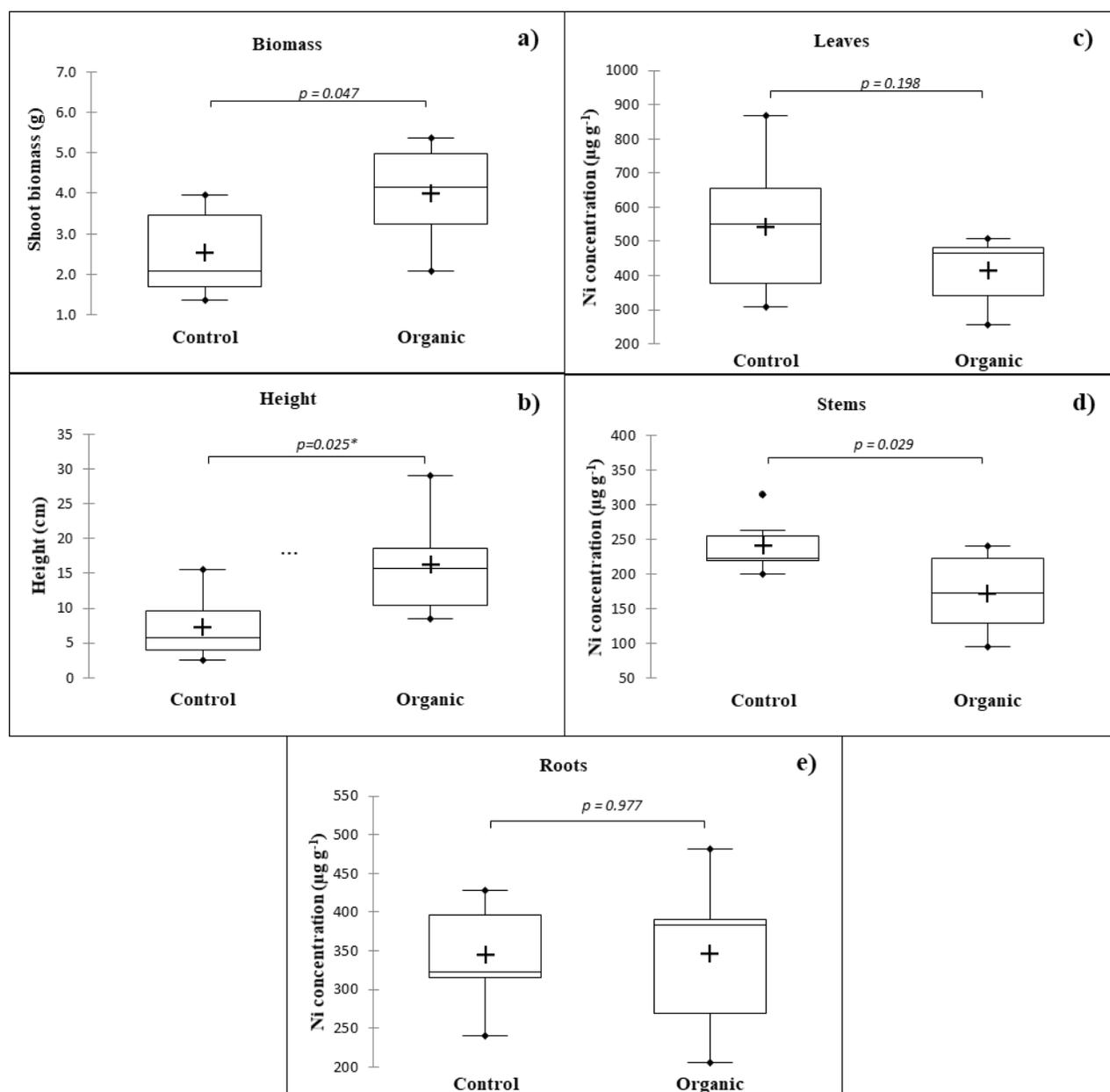


Figure 22. Shoot biomass a), height b) and Ni concentrations in leaves, stems and roots (c, d, e) of *B. guatemalense* in response to organic fertilization after 5-months pot experiment, p values < 0.05 are significantly different (t-test, alpha = 0.05).

The overall shoot Ni yield for the organic treatment was not significantly different to that of the control (t-test, $p > 0.1$), but significantly lower with respect to the NPK 120:120:120 treatment (Mann-Whitney test, $p < 0.05$). Foliar elemental concentrations in both the organic and control treatments are shown in **Table 16**. There was a significant increase in the K concentration in leaves in the organic treatment compared to that in the control (ANOVA, Newman-Keuls test, $p < 0.05$).

Iron and Mn concentrations were significantly lower in the organic treatment than in the control (Mann-Whitney test, $p < 0.05$). There was no significant effect on DTPA-extractable Ni concentrations in soils after vermicompost addition (t -test, $p > 0.7$).

Treatment (NPK)	Ni	Zn	N (%)	P	K	Ca	S	Mg	Fe	Mn
T0, Control (0:0:0)	541 ± 204 a	107 ± 130 α	3.22 ± 0.36 a	1430 ± 400 a	19 000 ± 5570 b	6700 ± 1670 a	2170 ± 637 a	3240 ± 1409 α	7880 ± 4070 a	238 ± 53 a
T4, Organic	414 ± 106 a	39 ± 7.0 †	3.09 ± 0.35 a	1480 ± 206 a	25 900 ± 4520 a	5800 ± 721 a	1960 ± 212 a	2070 ± 303 †	1397 ± 1790 b	79 ± 61 b

Table 16. Elemental concentrations in leaves ($\mu\text{g g}^{-1}$) of *B. guatemalense* under organic fertilization. Values followed by the different letters are significantly different (p value < 0.05) according to ANOVA Newman-Keuls test. Values followed by different symbols mean significant differences between the position according to Mann-Whitney test (p value < 0.05).

3.3. Harvesting schedule

The foliar biomass resulting from the harvesting frequency test is shown in **Table 17**. There was a significant difference between the annual average yield per tree produced by the two scenarios (t -test, $p < 0.05$). Harvesting twice per year produced three-fold more dry leaf biomass than only harvesting once per year. For the twice per year harvesting scenario, there was no significant difference between the pre-harvest average leaf biomass (3.2 kg tree^{-1}) and that obtained the year after (3.4 kg tree^{-1}) (t -test, $p > 0.05$). In contrast, the once per year frequency scenario had a significant reduction of about 30% in the average leaf biomass produced in the final harvest (1.0 kg tree^{-1}) with respect to the pre-harvest (3.5 kg tree^{-1}) (t -test, $p < 0.05$). The once per year harvesting scenario had a significantly higher dry biomass of the branches (3.1 kg tree^{-1}) compared to that obtained from the twice per year harvesting regime (1.4 kg tree^{-1}) (t -test, $p < 0.05$). The coppiced trees had an average height of 1.20 m after 6 months with no significant differences within the three plots (ANOVA, Newman-Keuls test, $p > 0.05$).

Harvesting frequency	Leaf biomass yield (kg tree ⁻¹)	Leaf biomass yield (t ha ⁻¹)	Ni phytoextraction yield (kg ha ⁻¹)
Once per year	1.02 ± 0.8 b*	2.55	43
Twice per year	3.40 ± 1.3 a*	8.49	142
Single coppicing after 8 th year	5.68	14.2	237

Table 17. Biomass values refer to dry weight (* average value, n = 9). Different letters express significant differences (ANOVA, Newman-Keuls test after data transformation to log, p < 0.001). Mean Ni concentration in leaves from trees in the experimental site was of 16.7 g kg⁻¹. Leaf biomass yield was calculated for a density of 2500 trees per hectare.

The third scenario which was of an 8-year crop followed by the harvesting of the whole biomass including all the parts of the tree (leaves, trunk, branches, flowers and fruit capsules) had a total Ni phytoextraction yield of 154 g per tree. The part that most contributed to the Ni yield was the foliar biomass followed by the trunk and the branches (**Table 18**). Assuming a density of 2500 8-year old trees per hectare, it could be possible to obtain a Ni yield of 240 kg ha⁻¹ when harvesting only the foliar biomass and 385 kg ha⁻¹ when considering the whole biomass.

Plant part	Proportion of the tree (%)	Dry biomass weight (kg)	Ni concentration in biomass* (g kg ⁻¹)	Ni phytoextracted (g)
Leaves	18	5.68	13.5	77
Trunk	51	16.8	2.55	43
Bark	25	0.60	4.36	2.6
Branches	0.3	8.43	3.29	28
Flowers	2.5	0.06	5.93	0.4
Fruits	3.0	1.06	2.65	2.8
<i>Total</i>	<i>100</i>	<i>32.6</i>		<i>154</i>

Table 18. Biomass obtained from an eight-year-old tree of *B. guatemalense* in a cattle pasture. (*) Mean Ni concentration values obtained from field samples (number of samples n > 9) (Navarrete et al. 2020, submitted), except for the trunk (n = 1).

4. Discussion

4.1. Agronomy of *Blepharidium guatemalense*

The ability of *B. guatemalense* to take up Ni from soils is strongly dependent on the DTPA-extractable Ni in soils. The latter is an approximation of the phytoavailable (i.e. isotopically-exchangeable or labile) Ni pool in soil (Echevarria et al., 1998; Massoura et al., 2004). These two variables were previously reported to be positively correlated (Pearson, $r = 0.5$) from foliar and rhizosphere soil of *B. guatemalense* samples collected in the field (Navarrete et al., 2020, submitted). Although Ni uptake and Ni concentrations in the soil solution are generally correlated, many other soil and plant factors can also influence plant Ni uptake such as soil pH (Kabata-Pendias and Pendias, 2001). The maximum Ni accumulation of *B. guatemalense* could not be attained in this study since the maximum concentration in leaves was 3.5 wt% at a DTPA-available Ni of 17 mg kg⁻¹ resulting from the highest Ni treatment (addition of 250 mg Ni kg⁻¹). In its natural habitat, *B. guatemalense* is able to accumulate 4.3 wt% Ni and 3.3 wt% Ni from soils with 190 mg kg⁻¹ and 460 mg kg⁻¹ DTPA-available Ni, respectively (Navarrete et al 2020, submitted). Thus, the Ni tolerance peak could be 4–5 wt% Ni at 200–300 mg kg⁻¹ available Ni in soils. *Blepharidium guatemalense* has a TF >1 when growing in soils with DTPA-available Ni > 2.0 mg kg⁻¹, which is indicative of its effectiveness to translocate Ni from roots to leaves (Baker and Brooks, 1989).

4.2. NPK and organic fertilization

Blepharidium guatemalense responded strongly to synthetic fertilization with a three-fold increase in foliar Ni concentrations. This surprising result contrasts with most previous research which reports a negligible effect in shoot Ni concentrations in Ni hyperaccumulators after mineral fertilization (Shallari et al., 2001; Kidd and Monterroso, 2005; Bani et al., 2015a; Nkrumah et al., 2019a). However, this increase on the shoot Ni concentrations has been reported in *B. coddii* with a two-fold increase as a response to N additions (B. H. Robinson et al., 1997). Magnesium and Fe concentrations in plant leaves from the three NPK treatments significantly decreased compared to the non-treated ones but they were within normal ranges for plant growth (Campbell et al., 2012). Shoot biomass of *B. guatemalense* did not significantly change upon NPK additions. There is usually an increase in shoot biomass of Ni hyperaccumulators in response to synthetic fertilization

growing in ultramafic substrates (e.g. *P. rufuschaneyi* (Nkrumah et al., 2019a), and *Odontarrhena* spp. (Bani et al., 2015a; Álvarez-López et al., 2016)).

Soils from this region in Chiapas and from other humid-tropical environments in south-eastern Mexico are mostly calcareous (Mendoza-Vega and Messing, 2005). Moreover, these soils are not generally deficient in essential macronutrients as are many ultramafic soils (Proctor and Woodell, 1975), which could partly explain the neutral response in growth of *B. guatemalense* to synthetic fertilization. However, the available P is relatively low in the soils in Chiapas (9.4 mg kg⁻¹) (Navarrete et al., submitted), which is a common feature in this type of limestone calcareous soils where P is probably adsorbed by calcium carbonate or precipitated as calcium phosphate (von Wandruszka, 2006; Falkowski et al., 2016). Synthetic fertilization is therefore recommended to increase P availability in soils, at least to compensate for plant nutrient uptake over time. Split NPK fertilizer application is recommended to minimize N leaching to the groundwater (Kidd et al., 2015; Nkrumah et al., 2018b). Further experiments should be conducted to evaluate the effect of each nutrient separately, as has been undertaken in Sabah (Nkrumah et al. 2019b, c, d). Considering the large amounts of Ca taken up by *B. guatemalense*, amendments of this element must be made in order to avoid soil depletion over time (Bani et al., 2015a).

Organic fertilization (vermicompost) increased the foliar biomass of *B. guatemalense* three-fold, with no significant reduction in the foliar Ni concentrations. Similarly, higher total Ni yields with composted sewage sludge compared to NPK fertilization have been obtained due to a substantial increase of the biomass in some species of *Odontarrhena* (Álvarez-López et al., 2016). Biochar amendments increased *O. muralis* growth by improving the fertility of ultramafic and constructed soils (Rue et al., 2019). On the other hand, manure compost did not show any positive effect on the biomass of the Ni hyperaccumulator *O. chalcidica* (Broadhurst and Chaney 2016). Fertilization of *B. guatemalense* using vermicompost could be preferred to NPK fertilization when no increase in foliar Ni concentration is needed or when soils are not nutrient deficient. There is evidence that organic matter amendments enhance the growth of ‘metal crops’ by improving soil drainage and water holding capacity (Angle et al., 2003; Nkrumah et al., 2016, 2018b). In Ni-rich ultramafic soils, organic matter reduces phytoavailable Ni concentrations and may lead to increased or decreased Ni yield depending on shoot Ni concentrations which may depend on soil Ni availability

(Bani and Echevarria 2019; Nkrumah et al. 2019b). Therefore, field trials are needed to assess the effects of organic amendments on the Ni yield of *B. guatemalense* in Ni-rich substrates.

4.3. Harvesting scenarios

A natural tree density of 2500 trees per hectare was calculated by measuring the canopy of 15 *B. guatemalense* adult trees (diameter at 1.3 m height > 15 cm) and the distances between them in three different fallow fields with a high tree density in Chiapas (Mexico). The mean Ni concentration in leaves from the trees at the end of our experiment was 16.7 g kg⁻¹. Thus, the Ni phytoextraction yield from the foliar biomass could be 43 kg ha⁻¹ yr⁻¹ when harvesting once a year and 142 kg ha⁻¹ yr⁻¹ when harvesting biannually. In the case of combined leaves and branches, it could be possible to obtain 10.3 t and 12 t ha⁻¹ yr⁻¹ of dry biomass, corresponding to 68 and 154 kg ha⁻¹ yr⁻¹ Ni yields from the once per year and twice per year harvesting scenarios, respectively. Finally, the third scenario (8-year crop followed by the harvesting of the whole biomass) would generate a Ni yield of 193 kg ha⁻¹ when harvesting only the foliar biomass and 385 kg ha⁻¹ when considering the whole biomass. Nevertheless, *B. guatemalense* trees can be harvested from the 5th year of culture and still obtain high Ni phytoextraction yields. Harvesting twice per year in monoculture is therefore the best regime for profitable Ni agromining in this context. However, the foliar Ni concentrations of *B. guatemalense* in the experimental site (16.7 g kg⁻¹) were above its previously reported average Ni concentration (13.5 g kg⁻¹, n = 78) (Navarrete et al., submitted) and may not be representative. Notwithstanding, most pastures in the region have similar available soil Ni concentrations and *B. guatemalense* foliar Ni concentrations, hence, Ni yields within a range of 23–364 kg Ni ha⁻¹ (mean of 115 kg Ni ha⁻¹) could be attained considering foliar Ni concentrations of 0.3–4.3 wt% Ni. Comparable high Ni yields of 250 kg ha⁻¹ have been estimated for large-scale tropical agromining using *P. rufuschaneyi* in Sabah, Malaysia (Nkrumah et al., 2019d).

The pastureland used for the harvesting trial had relatively higher total and DTPA-available Ni concentrations in the superficial soil (5–10 cm) compared to that in deeper soil (10–30 cm). Clearly, Ni-rich litter from *B. guatemalense* trees seems to be substantially contributing to the total and available Ni concentrations in the topsoil as it was observed for *O. muralis* in Albania and other Ni hyperaccumulator species (Estrade et al., 2015; Echevarria, 2018). A detailed

biogeochemical investigation is required to assess recycling of Ni through Ni-rich leaf litter degradation and the impact of Ni-rich biomass harvesting in an agromining operation on the Ni biogeochemistry.

The phenological cycle of *B. guatemalense* provides valuable information for the better management of the crop. For example, the first harvesting should preferably be done in March, just before the partial leaf fall period (April–May), whereas the second harvest could be in October, four months after the growth of new leaves. Seed collection could be performed during the fruit maturing stage (April–May) based on field observations taken during one year in the native habitat in Ocosingo, Chiapas (April 2018–2019). However, the phenological patterns could be different between populations and may be influenced by local climate (Mantovani et al., 2003). Therefore, specific studies must be performed in different regions to identify possible differences in these patterns and to adjust the management practices accordingly.

4.4. Potential sites for agromining

The selection of potential sites is of great importance for the success of Ni agromining (Nkrumah et al., 2018b). A high phytoavailable Ni concentration is always a desirable property of soils for effective Ni agromining (Nkrumah et al. 2016). Considering the strong response of *B. guatemalense* to Ni additions, a high Ni phytoavailability in soils is key to obtaining optimum yields for economic Ni agromining (Massoura et al., 2004). Soils in the natural habitat of *B. guatemalense* in southeastern Mexico (Chiapas and Tabasco) are not ultramafic but are rather derived from limestone sedimentary rocks (lutite, limonite) (Müllerried, 1957; SGM, 2017). Unpublished data from recent field surveys revealed total Ni concentrations range from 30–1600 mg kg⁻¹ in Chiapas and 200–500 mg kg⁻¹ in Tabasco, relatively lower than that in ultramafic soils (Kabata-Pendias and Pendias, 2001). The origin of Ni is uncertain but it could be related to volcanic ash deposition from the nearest volcano (*i.e.* El Chichón) (Hernández-Quiroz et al., 2012). Our unpublished data indicates that Ni in Chiapas is found in ferromagnesian clays, mainly associated with Mn oxides. The average phytoavailable Ni in Chiapas is relatively high (50 mg kg⁻¹ DPTA-Ni), reaching 200 mg kg⁻¹ DPTA-Ni in some cattle farms with a high a density of *B. guatemalense* trees. Thus, a resource 415 kg Ni ha⁻¹ is estimated assuming one hectare of soil with an average DTPA-available Ni of 50 mg kg⁻¹ to a depth of 1 m and a bulk density of 0.83 g cm⁻³.

It is expected that the subsequent cropping of *B. guatemalense* would progressively decrease Ni toxicity in soils through the reduction of the phytoavailable Ni pool in soils (Morel, 2012). Therefore, the above-mentioned Ni resource in soils per hectare would be completely depleted in 3 years of culture considering an annual yield of 142 kg Ni yr⁻¹ after which the site will be suitable for other types of cropping. Research is currently underway to better understand the role of Ni hyperaccumulators in the geochemical cycle of Ni and the replenishment of Ni labile pools in soils over time (Echevarria, 2018; Ratié et al., 2019). Intensive geochemical investigations are needed to determine the total extent of Ni-enriched substrates and to select the most suitable sites for Ni agromining in southeastern Mexico.

4.5. Local adaptation of Ni agromining into existing agroforestry systems

The Ni yields mentioned above are expected under a monocropping regime in open pastures or degraded/abandoned land with low food crop productivity. However, *B. guatemalense* is currently part of some agroforestry systems from which it could currently be harvested. For example, it is commonly found in backyard gardens of Tabasco, Yucatan and Campeche (Mariaca Méndez, 2012; van der Wal et al., 2011) as well as in silvo-pastoral systems in Chiapas and Tabasco as dispersed trees (Grande et al., 2010; Pignataro et al., 2016, 2017). In eastern Chiapas and in Izabal (Guatemala), the species is used in live fences for cattle pastures (Ramírez-Marcial et al., 2012) (**Figure 23f**). Indeed, *B. guatemalense* is used by local communities due to the hardness of its wood which they use for building houses or for sale in the local market (Standley, 1918; Jimenez-Ferrer et al., 2008; Marinidou and Jimenez-Ferrer, 2010) (**Figure 23g**). Considering a scenario where *B. guatemalense* is used as live fencing in pasturelands (232 trees ha⁻¹, 13.5 g Ni kg⁻¹ in leaves, 2 harvest per year), a yield of 11 kg Ni ha⁻¹ yr⁻¹ could be expected. The economic benefit may seem less than in monoculture, but it will depend on the number of hectares under this system and it would also represent an extra income for local farmers. Future studies must investigate how Ni agromining could be properly integrated into the existing local agroforestry systems.



Figure 23. Details of a), b), c) and e) different harvesting frequencies of *B. guatemalense* tested in the field in Chiapas, Mexico, d) green Ni-rich phloem in the bark, f) trees used as living fences in Izabal, Guatemala, and g) trunks used as house roofs in Tabasco, Mexico.

General Conclusions

Agromining takes advantage of hyperaccumulator plants for the recovery of valuable metals from sub-economic mineral resources. This technology is currently in the transition from pilot-scale to large-scale investigations in some specific places in Europe (Albania, Greece, Spain, etc.) and in Southeastern Asia (Malaysia). To enable agromining to continue to expand worldwide, the search of new species of metal “hyperaccumulator” plants in potential unexplored regions and their consequently potential assessment for metal agromining are essential steps. Mexico could potentially host plant metal hyperaccumulation given its large extents of metalliferous (e.g. ultramafic) soils as well as its great diversity of vascular plants species. Therefore, the main objective of this thesis work was to evaluate the potential of Mexican natural resources for the development of agromining. Therefore, the secondary objectives were divided into two important approaches of research:

1. Fundamental approach:

The aims were: i) to identify some metal hyperaccumulator plant species in Mexico and ii) to provide valuable information about these species (ranges of metal hyperaccumulation in plant tissues, geographic and phylogenetic distribution of metal hyperaccumulation, ecology, biochemistry, metal localization within plant tissues, etc.).

To successfully find plant metal hyperaccumulators, two methodologies of screening were used in parallel: i) geochemical anomalies (ultramafic regions and other Ni-enriched soils) through field surveys completed with XRF herbarium screening, and ii) plant phylogeny as a tool to predict metal hyperaccumulation and verified by XRF herbarium screening.

To generate substantial and useful information about the new discovered hyperaccumulator species, XRF data from herbarium screening was corrected and analyzed, plant and soil samples collected in the field were described, observed and chemically analyzed, and the resulting information was subsequently summarized using descriptive statistic tools, tables, and maps. XRF spectroscopy and microscopy were used to decipher elemental distribution in different plant tissues of some of the discovered hyperaccumulator plants as a first step in understanding their mechanisms of metal hyperaccumulation.

2. Practical approach :

The main goals were to select one hyperaccumulator species with suitable traits for a potential “metal crop” and to assess its potential for Ni agromining. Different laboratory and field tests were then performed to evaluate its response to different agronomic practices (Ni addition, organic and synthetic fertilization and harvesting schedules). A first evaluation of the Ni yield of the best cropping systems was obtained.

Metal hyperaccumulator plants research in Mexico: ultramafic regions

Soils from the tree ultramafic complexes: Cuicatlán-Concepción Pápalo (Oaxaca), Tehuitzingo-Tecomatlán (Puebla), and San Juan de Otates (Guanajuato) presented typical characteristics of ultramafic soils. However, no nickel hyperaccumulation was found in any of these regions, despite the availability of soil and climatic conditions, and only two Mexican endemic species were identified although they were not serpentine endemics.

This lack of hyperaccumulation in ultramafic regions of Mexico may suggest potential geoclimatic characteristics that could be determinant for the occurrence of nickel hyperaccumulation in the flora of ultramafic regions. In the ultramafic complex of Guanajuato, a very low available Ni is probably the main factor impeding Ni hyperaccumulation occurrence. Indeed, high phytoavailability of Ni, Ca and Mg and circum-neutral pH in ultramafic soils from Sabah (Malaysia) seem to be indicative edaphic factors of metal hyperaccumulation (van der Ent et al., 2016). Longer times of exposure for plant colonization of ultramafic regions may have been associated to higher numbers of plant hyperaccumulator species in Cuba and New Caledonia (Reeves et al., 1999, 1996). However, the ultramafic complex of Tehuitzingo-Tecomatlán (Puebla) dates from the Late Paleozoic (~ 350 Ma), much older than ultramafics in Cuba (~30 Ma). The ultramafic complex of Cuicatlán-Concepción Pápalo (Oaxaca) is also an old complex (Cretaceous, ~130 Ma) and displays very high available Ni concentrations in soils. Nevertheless, the particular disposition of the serpentines in narrow patches as well as the accidental topography of serpentines exposed to soil erosion may have impeded the evolution of metal hyperaccumulation in that flora. Thus, a long and continuous exposure to plant colonization, high available Ni in soils, and a mild

topography may be required characteristics for the occurrence of Ni hyperaccumulation in the flora of ultramafic regions. However, these are only suppositions based on field explorations and need to be experimentally proved at a global scale. Other unstudied ultramafic complexes in Northern Mexico and Southeastern Chiapas may be hosting metal hyperaccumulation.

Metal hyperaccumulator plants research in Mexico: other metalliferous regions in Mexico

Metal hyperaccumulation was found in Ni-enriched soils (non-ultramafic) under tropical and subtropical climates. In total, ten plant metal hyperaccumulator species were identified during this research, four of them belonging to the Rubiaceae (genera *Blepharidium* and *Psychotria*) family and six to the Violaceae (genera *Orthion* and *Mayanaea*) family. The geographic distribution of hyperaccumulation in these species included southeastern Mexico (Veracruz, Chiapas, Tabasco, and Campeche), Guatemala, and Belize. Therefore, the phylogenetic approach revealed to be powerful in identifying new metal hyperaccumulator plants.

This study revealed two of the strongest (and obligatory) hypernickelophores reported so far: *Blepharidium guatemalense* (4.3 %wt) and *Orthion subsessile* (4.2 %wt). The fact that metal hyperaccumulation has been found in moderate Ni-enriched soils influenced by volcanic activity and non in the typical serpentine soils breaks a paradigm and open new perspectives in terms of metal hyperaccumulation prospection.

Blepharidium guatemalense could be an excellent resource for genetic engineering. The genetic material of this species could be introduced in other species to improve their phytoextraction potential. Indeed, new sequencing technologies such as transcriptome sequence can be used to identify the genes involved in nickel hyperaccumulation and tolerance.

Orthion and *Mayanaea* are the first reports of Ni hyperaccumulation in the Violaceae family in the American continent. The two genera are situated in the same clade as three strong Ni hyperaccumulators of the *Hybanthus* genus from Australia and New Caledonia. Thus, the phylogeny of Ni hyperaccumulation is consistent in the Violaceae, despite the geographical dispersion of species globally.

This study revealed that *B. guatemalense* has similar re-distribution mechanism (via the phloem) than those reported in other tropical Ni-hyperaccumulator species (e.g. *P. balgooyi* and *R. bengalensis*). Indeed, the phloem on leaves, roots, stems and petioles of this plant are the richest in Ni and not the leaf epidermal cells like in most temperate and Mediterranean Ni hyperaccumulators. Modern metabolomic techniques, such as GC-MS and LC-MS, can serve to reveal the chemical speciation of Ni in the different tissues of *B. guatemalense* to better understand the physiological mechanisms involved on Ni hyperaccumulation. DNA and RNA sequencing and the characterization of targeted gene expression (e.g. Iron Transporter) would help characterizing the molecular mechanisms involved in these species and especially among phylogenetically related taxa.

X-ray fluorescence handheld spectrometer is an efficient method for the detection of Ni hyperaccumulators through systematic screening of specimens in herbaria. However, for elements other than Ni, and especially when measuring low concentrations, this technique may be not very precise. A recent publication proposed to consider only high-range values as a confidence threshold for the detection of hyperaccumulators through XRF method (Gei et al., 2020a). In the specific case of Co, this high-range value is fixed to < 1000. Therefore, *B. guatemalense* and *O. subsessile* can be also considered as facultative Co hyperaccumulators.

During this investigation, XRF herbarium screening was very focused on to the species with a higher probability of Ni hyperaccumulation based on molecular phylogeny information or on field discoveries. However, massive systematic screenings can be performed in MEXU herbarium (and other Mexican herbaria) to increase the number of hyperaccumulator species in Mexico.

Nickel agromining in Mexico: perspectives

This species was selected to assess its potential for Ni agromining because of its suitable traits such as a wide geographic distribution (including a variety of habitats such as primary and secondary forest and pasturelands), extremely high Ni concentrations in shoots, high biomass production, woody, perennial, currently used for local populations for house building and as live fences for paddocks.

One important factor to consider when assessing the potential of a species for agromining is the tendency of its bioaccumulation factor (shoot/soil concentration ratio) when facing raising metal contents in soils. In controlled conditions, the bioaccumulation factor of *B. guatemalense* increased with increasing Ni in soils, thus highlighting its extremely efficient Ni uptake.

Synthetic fertilization is a recommendable agronomic practice for *B. guatemalense* since it strongly increased Ni uptake without affecting plant growth or biomass. Furthermore, fertilization may prevent nutrient depletion over time caused by plant uptake. However, it must be carefully applied to avoid N leaching.

Organic vermicompost enhanced plant shoot biomass with a negligible effect on foliar nickel concentrations. Thus, it could be also a good management practice for this “metal crop”. However, elemental analysis of this organic amendments must be carried out to ensure an accurate application according to the soil needs and to avoid nutrient losses.

The results of this study partly revealed the potential of *B. guatemalense* for Ni agromining. Nevertheless, larger and longer studies in the field are needed to verify these results and to test other agronomic practices.

The maximum estimated yield $142 \text{ kg ha}^{-1} \text{ yr}^{-1}$ is under a monocropping agromining scenario of a 5-year-old crop harvested twice per year. However, there is evidence that *B. guatemalense* is currently part of different agroforestry systems in southeastern Mexico and Guatemala. These systems could be promoted to other areas or other systems could be also adapted to the needs to the local people, evidencing its great potential for Ni agromining in Mexico.

Assuming a biomass yield of $142 \text{ kg Ni ha}^{-1}$, a Ni price of 14 USD per kg (5 years average, Info Mine), a metal recovery cost of 20% of the Ni value, \$ 250 ha^{-1} of production costs and \$150 ha^{-1} of rental cost. The estimated net value of an annual phytomining crop would be $1190 \text{ USD ha}^{-1} \text{ yr}^{-1}$. However, this is a first estimation and deeper studies using techno economic models are needed to obtain a more realistic estimation of the net profit value (Kidd et al., 2018).

For large-scale agromining, large mineralized areas are required. Until now, the overall extent of mineralized soils in southeastern Mexico is unknown. To this, extensive studies are needed to identify potential suitable sites for Ni agromining. Other technical and social aspects must be also

considered such as proper storage and incineration sites, the organization of farmers (e.g. cooperatives), in-situ and ex-situ conservation of seeds, etc.

Orthion subsessile seems to be another potential “metal crop” for Ni agromining due to its high biomass, high shoot Ni concentrations, and its apparently fast growth (observations on several field sites after coppicing of several year-old plants). Further studies are needed to evaluate its potential for agromining.

Agromining, as any other type of agriculture, depends on soil functions (productive, environmental and social), for the sustainable provisioning of several ecosystem services (provisioning, regulation-maintenance and socio-cultural) (Morel et al., 2015). Therefore, a parallel aim of agromining is the maintaining and/or improvement of soil functions for the optimization of the ecosystem services (Echevarria et al., 2015; Kidd et al., 2018).

Visualizing an agroforestry agromining system including *B. guatemalense*, a wide range of ecosystem services could be provided: i) provisioning services (cattle production, valuable Ni products, and energy production for local communities), ii) regulation-maintenance (soil quality and nutrient availability improvement, soil Ni detoxification, carbon sequestration, species conservation, co-culture with local legumes for N fixation) and iii) socio-cultural (education - research, crop management training, life-quality improvement of farmers).

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Annexes

Annex 1a Correlation matrix (Pearson) based on the foliar elemental concentrations of *B. guatemalense*. The values in bold are different from 0 at an alpha = 0.05 significance level ($n = 21$). For soil variables, we used DTPA-extractable elements for Mg, Mn, Ni and Zn.

	Ca	K	Mg	Mn	P	S	B	Co	Cu	Ni	Zn
Ca	1										
K	0.005	1									
Mg	0.807	0.053	1								
Mn	0.597	0.136	0.419	1							
P	-0.034	-0.066	0.066	0.370	1						
S	0.428	0.382	0.286	0.602	0.243	1					
B	0.178	-0.074	0.132	-0.194	-0.468	-0.134	1				
Co	0.245	0.574	0.150	0.331	-0.155	0.299	0.080	1			
Cu	0.186	0.022	0.317	0.036	0.262	0.165	0.018	0.120	1		
Ni	0.413	0.036	0.467	0.367	0.211	0.344	0.136	-0.097	0.326	1	
Zn	0.426	-0.098	0.508	0.334	0.268	0.227	0.169	-0.105	0.400	0.970	1

Appendix 1b Correlation matrix (Pearson) based on total elements contents from rhizosphere soils of *B. guatemalense*. The values in bold are different from 0 at an alpha = 0.05 significance level ($n = 19$).

Variables	Al	Ca	Fe	K	Mg	Mn	P	S	As	B ₋	Cd	Co	Cr	Cu	Ni	Pb	Zn
Al	1																
Ca	0.540	1															
Fe	0.586	0.160	1														
K	0.616	-0.029	0.246	1													
Mg	0.039	-0.205	-0.035	0.504	1												
Mn	-0.153	-0.413	0.169	0.214	0.155	1											
P	0.268	0.602	0.023	-0.105	-0.229	-0.389	1										
S	0.589	0.918	0.198	0.021	-0.372	-0.448	0.704	1									
As	0.915	0.588	0.682	0.528	0.053	-0.148	0.300	0.572	1								
B ₋	0.486	0.108	0.024	0.837	0.442	0.195	-0.311	0.106	0.365	1							
Cd	0.802	0.505	0.521	0.569	-0.003	-0.014	0.329	0.491	0.873	0.351	1						
Co	-0.472	-0.605	-0.148	-0.097	0.138	0.775	-0.659	-0.657	-0.519	0.060	-0.475	1					
Cr	-0.620	-0.347	0.044	-0.647	0.111	0.026	-0.096	-0.470	-0.469	-0.752	-0.515	0.230	1				
Cu	0.798	0.346	0.541	0.711	0.178	0.120	-0.020	0.348	0.722	0.657	0.793	-0.193	-0.603	1			
Ni	-0.476	-0.319	-0.231	-0.310	-0.053	0.041	-0.318	-0.376	-0.367	-0.253	-0.258	0.288	0.391	-0.299	1		
Pb	0.406	0.458	0.591	-0.202	-0.605	0.120	0.206	0.534	0.358	-0.143	0.285	-0.096	-0.106	0.308	-0.284	1	
Zn	0.352	-0.168	-0.031	0.509	0.188	0.109	-0.069	-0.061	0.209	0.404	0.148	0.117	-0.417	0.263	0.166	-0.263	1

Appendix 1c Correlation matrix (Pearson) based on the DTPA-extractable elemental contents and other variables from rhizosphere soils of *B. guatemalense*. The values in bold are different from 0 at an alpha = 0.05 significance level ($n = 19$).

<i>Variab les</i>	<i>Al</i>	<i>Cd</i>	<i>Co</i>	<i>Cu</i>	<i>Fe</i>	<i>K</i>	<i>Mg</i>	<i>Mn</i>	<i>Na</i>	<i>Ni</i>	<i>Pb</i>	<i>Zn</i>	<i>CEC</i>	<i>P_s</i>	<i>N</i>	<i>C</i>	<i>Ph</i>
<i>Al</i>	1																
<i>Cd</i>	-0.114	1															
<i>Co</i>	0.070	0.426	1														
<i>Cu</i>	-0.040	0.687	0.325	1													
<i>Fe</i>	0.147	0.652	0.789	0.695	1												
<i>K</i>	0.049	0.731	0.291	0.824	0.575	1											
<i>Mg</i>	-0.167	0.193	-0.080	0.272	0.313	0.269	1										
<i>Mn</i>	-0.026	0.710	0.761	0.435	0.712	0.603	0.015	1									
<i>Na</i>	-0.127	0.254	0.015	0.399	0.389	0.125	0.450	0.011	1								
<i>Ni</i>	-0.210	0.112	0.278	0.396	0.229	0.249	-0.053	0.061	0.062	1							
<i>Pb</i>	-0.189	0.722	0.364	0.740	0.753	0.580	0.542	0.498	0.718	0.219	1						
<i>Zn</i>	-0.257	0.426	0.689	0.208	0.605	0.150	0.297	0.537	0.137	0.186	0.501	1					
<i>CEC</i>	-0.368	0.725	0.265	0.412	0.554	0.387	0.463	0.485	0.519	-0.068	0.817	0.480	1				
<i>P_s</i>	-0.022	0.644	0.678	0.846	0.783	0.732	0.052	0.680	0.208	0.510	0.668	0.400	0.362	1			
<i>N</i>	-0.234	0.721	0.343	0.377	0.511	0.387	0.274	0.583	0.583	0.094	0.732	0.506	0.766	0.371	1		
<i>C</i>	-0.256	0.700	0.271	0.363	0.468	0.435	0.379	0.590	0.578	0.031	0.755	0.499	0.775	0.349	0.969	1	
<i>Ph</i>	-0.482	0.011	-0.500	-0.283	-0.553	-0.241	-0.272	-0.166	-0.200	-0.287	-0.202	-0.312	0.164	-0.366	-0.007	0.025	1

