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## **Blepharidium guatemalense, an obligate nickel hyperaccumulator plant from non-ultramafic soils in Mexico**

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1                    ***Blepharidium guatemalense*, an obligate nickel**  
2                    **hyperaccumulator plant from non-ultramafic soils in Mexico**

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27

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49

50 **DECLARATIONS**

51 The Authors declare that there is no conflict of interest. The content of the manuscript has not been  
52 published or submitted for publication elsewhere in any language. All authors have contributed  
53 significantly to the realization of the manuscript in different ways.

54 **Abstract**

55 *Aims*

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

60 *Methods*

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

65 *Results*

66 *Blepharidium guatemalense* is distributed throughout Chiapas, Tabasco and Campeche, reaching the  
67 maximum nickel concentration in leaves (4.3 wt%) followed by roots and seeds (2.0 wt%) and bark  
68 (1.8 wt%). Simultaneous hyperaccumulation of cobalt and nickel was found in 15% of the herbarium  
69 specimens. Phloem has the highest nickel-enriched tissue from all parts of the plant (from roots to  
70 leaves). A high total nickel (mean of 610  $\mu\text{g g}^{-1}$ ) was found in rhizosphere soils even though no  
71 evidence of ophiolite emplacement in that area has been reported.

72 *Conclusions*

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

76

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

78

79

## 80 INTRODUCTION

81

82 Hyperaccumulator plants actively take up and accumulate metals or metalloids to extremely high  
83 concentrations in their above-ground tissues (mostly leaves) when growing in their natural habitats  
84 (Baker et al. 1992; van der Ent et al. 2013; Dar et al. 2018). To date, 731 hyperaccumulator species  
85 are reported, of which 532 are nickel (Ni) hyperaccumulators (Reeves et al. 2018a; van der Pas and  
86 Ingle 2019). The nominal threshold values in plant dry matter depend on the element, *e.g.* 100  $\mu\text{g g}^{-1}$   
87 for cadmium (Cd), 300  $\mu\text{g g}^{-1}$  for cobalt (Co), 1000  $\mu\text{g g}^{-1}$  for Ni, 3000  $\mu\text{g g}^{-1}$  for zinc (Zn) and 10 000  
88  $\mu\text{g g}^{-1}$  for manganese (Mn) (van der Ent et al. 2013). Among these plants, there is a distinct group of  
89 about 50 species termed ‘hypernickelophores’, which can accumulate Ni to concentrations exceeding  
90 1 wt% (10 000  $\mu\text{g g}^{-1}$ ) in their shoots (Boyd and Jaffré 2009; Jaffré and Schmid 1974).  
91 Hyperaccumulator plants growing on metalliferous soils are classified as ‘obligate  
92 hyperaccumulators’, meanwhile those that are reported to grow (without exhibiting metal  
93 hyperaccumulation) in non-metalliferous soils are ‘facultative hyperaccumulators’ (Pollard et al.  
94 2014). The primary habitats of these hyperaccumulator plants comprise soils developed from the  
95 weathering of surficial ore deposits or naturally enriched metalliferous rocks. Most Ni  
96 hyperaccumulators grow on soils issued from the weathering of ultramafic rocks, which are the most  
97 significant reservoirs of Ni and Co for terrestrial ecosystems (Estrade et al. 2015; Reeves et al. 2018).  
98 Since the discovery of the first Ni hyperaccumulator *Odontarrhena* (syn. *Alyssum*) *bertolonii* (Desv.)  
99 Jord. & Fourr. (Minguzzi and Vergnano 1948), series of studies of the ultramafic flora provided  
100 numerous data on the distribution and number of hyperaccumulator taxa worldwide (Reeves et al.  
101 2018). Most Ni hyperaccumulator plants occur in: i) ultramafic regions around the Mediterranean  
102 Basin, especially in Turkey and the Balkans (Brooks et al. 1979; Reeves and Adigüzel 2008), and ii)  
103 humid tropical and subtropical ultramafic areas, with three essential hotspots which are Cuba, New  
104 Caledonia, and Southeast Asia (Galey et al. 2017; Reeves 2003). According to the Global  
105 Hyperaccumulator Database (Reeves et al. 2017), the eight most diverse families in terms of Ni  
106 hyperaccumulator species are the Phyllanthaceae (130 spp.), Brassicaceae (104 spp.), Asteraceae (68  
107 spp.), Cunoniaceae (55 spp.), Euphorbiaceae (45 spp.), Salicaceae (39 spp.), Fabaceae (29 spp.) and  
108 Rubiaceae (23 spp.).

109

110 Mexico is the country with the fourth-largest floristic richness globally, with a record of 23 314  
111 vascular plant species (Villaseñor 2016). These species belong to different families including those  
112 also present in the most diverse Ni hyperaccumulator families, such as the Asteraceae (3057 spp.),  
113 Fabaceae (1903 spp.), Euphorbiaceae (714 spp.), Rubiaceae (707 spp.), and 10 Brassicaceae (2 spp.)

114 (Villaseñor 2016). Such diversity suggested the possibility of searching for native metal  
115 hyperaccumulator plants in Mexico. New techniques for the discovery of hyperaccumulators, such as  
116 the non-destructive elemental screening of herbarium specimens through the use of a portable X-ray  
117 fluorescence (XRF) instrument, have provided the opportunity to use herbarium specimens to discover  
118 new hyperaccumulators (van der Ent et al. 2019a) and new occurrences of mineralized soils.

119

120 After several recent attempts to identify Ni hyperaccumulators on ultramafic soils in central and  
121 southern Mexico through field surveys, Navarrete Gutiérrez et al. (2018) concluded that Ni  
122 hyperaccumulation did not occur in this part of the world. However, a recent XRF screening of  
123 herbarium specimens at Missouri Botanical Garden (MO) in Saint Louis, Missouri, USA, revealed for  
124 the first time Ni hyperaccumulation in Mexico occurring in several woody species of the genus  
125 *Psychotria* (Rubiaceae), namely *Psychotria costivenia* Griseb., *Psychotria lorenciana* C.M. Taylor  
126 and *Psychotria papantlensis* (Oerst.) Hemsl., collected in the states of Chiapas, Tabasco and Veracruz  
127 (McCartha et al. 2019). These findings led us to undertake new field surveys in these regions and to  
128 discover that *Blepharidium guatemalense* Standl. (Rubiaceae) occurs in some of the same communities  
129 with the above-mentioned *Psychotria* species, and that also hyperaccumulates Ni (Figure 1).  
130 *Blepharidium* is a monospecific genus which was first described in 1918 by Paul Carpenter Standley  
131 based upon a type collected in the Department of Alta Verapaz in Guatemala by Henry Pittier in 1905  
132 (Standley 1918). Years later, the species *Blepharidium mexicanum* was reported as a separate species  
133 based on taxonomic features such as smaller flowers and sparse pubescence over the lower leaf surface  
134 (Standley 1940). Despite morphological differences, *B. mexicanum* is now considered a synonym of  
135 *B. guatemalense* (WCSP 2019). The latter is distributed mainly in primary tropical perennial and sub-  
136 perennial rainforest of southern Mexico and Guatemala at altitudes ranging from 200 to 500 meters  
137 above sea level (m.a.s.l.). ‘Popiste’ or ‘Sayaxché’ (as it is called in the local languages), is a hardwood  
138 tree up to 70 m in height (Ricked et al. 2013) with a diameter up to 70 cm, and a straight trunk and  
139 rounded crown. The outer bark is blue-grey and scaly whereas the inner bark is cream and granular  
140 with a total thickness from 5 to 10 cm. Leaves are decussate, oblanceolate 10–30 cm long by 5–12 cm  
141 wide. Long and acute stipules occur on younger leaves. The inflorescences are in axillary panicles 10–  
142 25 cm long with white flowers and fruits are erect capsules 2–3.5 cm long with abundant brown,  
143 flattened and winged seeds 1–1.5 cm long (Pennington and Sarukhán 1968). *Blepharidium*  
144 *guatemalense* is commonly used for rafters, beams and supports of lowland houses due to the strength  
145 of its wood whereas its leaves and bark are reputed poisonous (Standley and Williams 1975). The  
146 species also grows in secondary forests and on agro-pastoral areas (Ochoa-Gaona et al. 2007;  
147 Villanueva López et al. 2015). In the secondary forest, however, *B. guatemalense* appears to be a

148 pioneer species that colonizes land after many cycles of ‘*slash - and - burn*’ which is the ancient  
149 cultivation agriculture system still used by local people. In contrast to primary forests, *B. guatemalense*  
150 trees are usually between 5–8 m high when growing in secondary forest and in open pasture areas, and  
151 they seldom exceed 20 m in these environments. The species is on the IUCN Red List, mainly because  
152 of the lack of data (Nelson 1988).

153

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

159

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

163

## 164 **MATERIALS AND METHODS**

165

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

172

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

174 Three herbarium collections were surveyed to get a sufficient distribution of the species in Mexico,  
175 these were : MEXU (Mexico City, Mexico), P (Paris, France) and MO (Saint Louis, U.S.A.). Elemental  
176 concentrations in mounted herbarium specimens of *B. guatemalense* were measured at the three  
177 different herbaria with the same procedure and the same material. The scanning was performed by  
178 using a hand-held X-Ray Fluorescence Spectrometer (Thermo Scientific GOLDD+ XL3T-980). The  
179 analyzer uses a miniature X-ray tube [Ag anode (6–50 kV, 0–200  $\mu$ A max] as the primary excitation  
180 source and a Silicon Drift Detector (SDD) with 185 eV, 60 000 cps, 4  $\mu$ s shaping time. It can detect a

181 range of different elements simultaneously within 30–60 s with a detection limit of 140  $\mu\text{g g}^{-1}$  for most  
182 transition elements such as Ni, Cd, Cr, Zn, *etc.* The leaves of dried herbarium specimens were scanned  
183 for 30 s in ‘soils’ mode and by placing a titanium plate (99.999% purity) underneath the herbarium  
184 sheet. During the scanning, specimens are subjected to a beam of high-energy X-rays generated from  
185 the X-ray tube; the resulting spectrum of excited fluorescent X-rays serves to detect the elements and  
186 to quantify their concentrations.

187 To obtain better XRF measurement accuracy, calibration and correction of the data were performed.  
188 Hence, a total of 256 leaf samples of different Ni (and some concomitantly Zn) hyperaccumulator  
189 plant species were collected from different plant species including *B. guatemalense* in the field because  
190 it was not possible to achieve destructive sampling of herbarium specimens. Leaf squares of 1  $\text{cm}^2$  in  
191 size of each sample were cut and analyzed for 30 s in ‘soils’ mode with three replicated readings per  
192 sample. After XRF measurements, leaf samples were digested and analyzed with Inductively Coupled  
193 Plasma Atomic Emission Spectroscopy (ICP-AES) for major and minor elements following the  
194 methodology mentioned below in this section.

195

196 Paired ICP and XRF data were analyzed by logarithmic linear regression. No noise was detected  
197 because the minimum XRF value was about 500  $\mu\text{g g}^{-1}$ , greatly higher than the limit of detection (LOD  
198 < 190) estimated by (Gei et al. 2020; van der Ent et al. 2019b). Residuals (observed vs. predicted  
199 values) were used to detect and remove outliers ( $\pm 3$  SD of the residual). A secondary linear regression  
200 was then derived and transformed to power regression generating the following calibration equation  
201 with an  $R^2$  of 0.87:

$$202 \quad y = 1.1216x^{0.953}$$

203 where:

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

205

206 Nickel XRF measurements were finally corrected by using the resulting equation. A calibration  
207 equation for Zn ( $y = 1.0041x^{0.9316}$  and  $R^2$  of 0.93) was also derived by following the same procedure  
208 described above. For Co and Mn XRF measurements, data were corrected by using the calibration  
209 equations generated by (Gei et al. 2020): for Co  $y = 0.6277x^{1.1706}$  ( $r^2 = 0.84$ ) and for Mn  $y = 1.148x^{0.937}$   
210 ( $r^2 = 0.73$ ).

211

## 212 **Study and collection sites**

213

214 Ocosingo, Chiapas

215 In total, 13 sites from the municipality of Ocosingo in Chiapas were sampled. The sites were located  
216 in the surroundings of the communities of Lacanhá Chanyasab, San Javier and Cojolita, at altitudes  
217 ranging from 100 to 300 m.a.s.l. The area is mainly covered by the “Lacandona” tropical evergreen  
218 rainforest, and dominated by rolling hills made up mainly of limestone sedimentary rocks (lutite,  
219 limonite) from the upper Cretaceous (Mesozoic) with overlapped layers of marine origin from the  
220 lower Tertiary (Cenozoic). Marls and clays could be found on the foot of the slopes and in alluvial  
221 areas (Müllerried 1957; Levy Tacher et al. 2006; SGM 2006). Main soil types are Rendzinas, Gleysols  
222 and Litosols (INIFAP-CONABIO 1995; Mendoza-Vega and Messing 2005).. Climate is humid warm  
223 with abundant rains in summer (Am (i') gw”) (García 2004; Köppen 1936)l. The average annual  
224 temperature is 25 ° C and total annual precipitation of around 2500 mm (García 2004). The  
225 predominant vegetation is evergreen rainforest alternated with secondary vegetation, rain-fed  
226 agriculture, and grassland. The first survey took place in December 2017. Promising results from  
227 herbarium XRF screenings of *B. guatemalense* leaves from all over south-eastern Mexico prompted a  
228 second survey in November 2018 to study some unexplored sites during the first survey in Chiapas.

229

#### 230 Tacotalpa, Tabasco

231 The second survey in November 2018 included the exploration of one site in Tapijulapa, which is a  
232 small village belonging to the municipality of Tacotalpa in the southern state of Tabasco. The climate  
233 is humid warm with abundant rainfall all year round and an average annual temperature of 26 °C (Af  
234 (m) (i) gw”) and total rainfall of 3554 mm (Díaz P. et al. 2006; García 2004). Previous predominant  
235 vegetation was evergreen rainforest, but this has gradually changed to corn crops, coffee plantations  
236 and livestock. Predominant soils are Rendzinas, Humic Acrisols and Eutric Gleysols (CONABIO 2001;  
237 INEGI 2005), mainly composed of sedimentary rocks (lutite, limonite, limestone and sandstone). This  
238 site was also selected for exploration based on previous data from XRF herbarium screening.

239

#### 240 Balancán, Tabasco

241 Balancán is a municipality located in the eastern part of Tabasco. Climate is humid with summer rains  
242 in most of the territory and an average annual temperature of 27 °C (Am (f) (i) gw”) (Díaz P. et al.  
243 2006; García 2004; Köppen 1936). Altitude varies from 10 to 50 m.a.s.l. and lands are generally flat  
244 with small hills. Vertisol is the main type of soil, derived from sedimentary rocks (sandstone, alluvial  
245 deposits) (CONABIO 2001; SGM 2017). Even though many herbarium specimens of *B. guatemalense*  
246 presenting high Ni concentrations in their leaves were collected in the municipality of Balancán, no  
247 plant could be found in this municipality during the 2018 survey despite many efforts. Main cause  
248 could be the drastic woodland reduction from 49 % to 14% that Tabasco suffered over the last 60 years

249 (Palma-López and Triano 2007). Large surfaces of evergreen rainforest have been converted to  
250 grasslands for livestock.

251

### 252 **Plant and soil sampling**

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

269

### 270 **Bulk analysis of plant tissues**

271 Plant tissue samples of *B. guatemalense* were dried at 40 °C for 5 days in a drying oven and  
272 subsequently packed for transport to the laboratory for chemical analysis. Dried samples were finely  
273 ground (< 250 µm) and subsamples of 0.05 g (± 0.005) were then pre-digested overnight using 1 mL  
274 HNO<sub>3</sub> (65%) and 2 mL H<sub>2</sub>O<sub>2</sub> (30%). The next day, samples were placed in a heating block (*DigiPREP*)  
275 for 2h at 95°C, left to cool to 40 °C, filtered at 0.45µm, and adjusted in volume to 10 mL for minor  
276 elements quantification. A 1:10 dilution was carried out with ultrapure water for the quantification of  
277 major elements. Finally, digests were analyzed using ICP-AES (Thermo Fisher CAP 6300 Duo). Two  
278 blanks and two reference samples of the known hyperaccumulator *Noccaea caerulea* (J. Presl &  
279 C. Presl) F.K. Mey, were used for each analytical series. The same instrument was used in all  
280 subsequent ICP-AES analyses.

281

### 282 **Bulk analysis of rhizosphere soil samples**

283 Rhizosphere soil samples of *B. guatemalense* collected in the field were air-dried for one week, placed  
284 into identified plastic bags and then transported to the laboratory for chemical analysis. Soil pH was  
285 measured in a 1:5 soil: water (5 mg soil with 25 mL ultrapure water) suspension after 1 h shaking at  
286 16 rpm and 1h standing. Cation exchange capacity (CEC) of soils was determined using cobalt-  
287 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  
288 distilled water). After 1 h shaking, 10 ml of each sample were filtered at 0.45  $\mu\text{m}$  and measured by  
289 spectrophotometry (BioRad, SmartSpec Plus). Available phosphorus (P) was determined according to  
290 the Olsen method. For total major and minor elemental quantification, soil samples were dried at 70°C  
291 for 3 days in a drying oven and sieved (< 2 mm). Subsequently, 0.5 g ( $\pm$  0.001 g) of each sample,  
292 finely ground (< 250  $\mu\text{m}$ ), were digested using 6 mL of 37% HCl and 2 mL of 70%  $\text{HNO}_3$  per sample.  
293 The acid solution containing soil samples were left to stand overnight before placing on a heating block  
294 (*DigiPREP MS*) for 3h at 100°C, and then allowed to cool to a minimum temperature of 40°C.  
295 Solutions were adjusted in volume to 45 mL, filtered at 0.45  $\mu\text{m}$ , and then readjusted to 50 mL with  
296 ultrapure water. The final dilution of 1:20 was necessary for major elements. The resulting soil  
297 digests/extracts were analyzed by ICP-AES for the quantification of major and minor elements. Trace  
298 elements availability in soil samples were determined by using diethylenetriaminepentaacetic acid  
299 (DTPA-extractant) according to the method of Lindsay and Norvell, (1978), and then analysis of soil  
300 extracts by ICP-AES.

301

### 302 **Micro X-ray fluorescence elemental mapping**

303 High spatial resolution (20  $\mu\text{m}$ ) distribution maps for Ni and other elements were obtained using a  
304 Micro-XRF (M4 Tornado, Bruker). The device has a Rh X-ray tube (50 kV and 200  $\mu\text{A}$ ) as a primary  
305 excitation source with a Be side window and polycapillary optics. A large and flat sample is subjected  
306 to an X-ray beam with a diameter of 20–30  $\mu\text{m}$  inside a chamber under controlled 20 mbar vacuum.  
307 X-rays are then detected by two 30  $\text{mm}^2$  Xflash® Silicon Drift detectors (energy resolution of <135  
308 eV at 250 000 cps). A leaf of *B. guatemalense* was collected from its natural habitat in Chiapas and  
309 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  
310 cut and analyzed with micro-XRF in the GeoRessources laboratory (France).

311

### 312 **X-ray fluorescence microscopy (XFM)**

313 X-ray fluorescence microscopy analysis of different plant tissues of *B. guatemalense* was undertaken  
314 at PETRA III (Deutsches Elektronen-Synchrotron; DESY) a 6 GeV synchrotron. The microprobe  
315 beamline P06 is equipped with a cryogenically cooled double-crystal monochromator with Si (111)  
316 crystals and a K/B mirror focusses a beam of  $10^{11}$  photon/s down to 300 nm size in the energy range

317 5–21 keV (Schroer et al. 2010). The XRF was collected using a silicon drift detector (Hitachi Vortex  
318 EM-90, 80 mm<sup>2</sup>, energy-resolution 120 eV) operated in 90-degree geometry). Incident energy of 12  
319 keV was used in order to excite the element of interest with the highest atomic number (*i.e.*, Zn).  
320 Different plant tissues (root, stem, petiole, and leaf) of a three-month-old individual of *B.*  
321 *guatemalense*, which was grown in pot experiments under controlled conditions in a Ni-contaminated  
322 soil, were analyzed. The samples were hand-cut with a stainless-steel razor blade ('dry knife'),  
323 mounted between two sheets of 4 μm 'Ultralene' thin film in a tight sandwich to limit evaporation,  
324 and analyzed within 10 minutes after excision. X-ray micro-fluorescence was performed at high speed  
325 to keep the scan time to a minimum. The fresh and freeze-dried samples were mounted between two  
326 sheets of 'Ultralene' thin film (4 μm) stretched over a perspex frame magnetically attached to the *x-y*  
327 motion stage at atmospheric temperature (~20 °C). The XRF data were fitted and processed using  
328 PyMCA software (Solé et al. 2007), and exported into ImageJ as greyscale 16-bit TIFF files and  
329 displayed using ImageJ's 'Fire' lookup table.

330

### 331 **Statistical analyses**

332 Soils and plants data were summarized by using Microsoft Excel 2010 (XL STAT ECOLOGY version  
333 2019.1.1) for descriptive statistics, Pearson Correlation Coefficient (*r*) and Principal Components  
334 Analysis (PCA). Nickel concentrations are presented as boxplots and in tables. Key symbols: open  
335 boxes are inter-quartile ranges, bold crosses are ± mean, whiskers are ± standard deviation and bold  
336 circles are outliers. Maps were generated using ArcGIS 10.3.

337

## 338 **RESULTS**

339

### 340 **Elemental concentrations from the herbarium XRF screening in relation to geographical** 341 **distribution**

342 A total of 182 specimens of *B. guatemalense* historically collected in the current territory of Mexico  
343 were scanned by using X-ray fluorescence spectroscopy (XRF); 132 from the MEXU herbarium, 47  
344 from MO and 3 from P. The results revealed the 'obligate' hyperaccumulation character of this species  
345 since only 5% of the specimens had Ni concentrations lower than 1000 μg g<sup>-1</sup>. Yet all the values were  
346 > 586 μg g<sup>-1</sup>, a value higher than the threshold considered to define 'hemi-accumulators' and about  
347 3% of the specimens exceeded the hypernickelophore threshold (10 000 μg g<sup>-1</sup>). The mean Ni  
348 concentration in all 182 specimens was 4022 μg g<sup>-1</sup> and the maximum Ni concentration of 18 786 μg  
349 g<sup>-1</sup> was recorded in one specimen collected from Ocozocuautla, Chiapas. The histogram shows an  
350 approximately log-normal distribution of Ni concentration in the species (Figure 2). Specimens with

351 available geographical coordinates were georeferenced in a geological map (Figure 3) which confirms  
352 that the plant grows across different geological basements but never on ultramafic bedrocks.  
353 Ultramafic rock outcrops in Chiapas are found in the southern city of Motozintla, outside the apparent  
354 range of distribution of the plant and in a different climatic zone. Occurrences of the species were  
355 found on sedimentary formations that include limestone, sandstone, lutite, limonite, marls, clays and  
356 alluvial formations derived from the above-mentioned sediments.

357  
358 Cobalt hyperaccumulation ( $>300 \mu\text{g g}^{-1}$ ) was found in 27 specimens (*i.e.* 15 %), from which one was  
359 collected in Campeche, 11 in Tabasco and 15 in Chiapas with the highest Co concentration of 1994  
360  $\mu\text{g g}^{-1}$  being found in a specimen collected in Tacotalpa, Tabasco. The mean concentration of Co was  
361  $446 \mu\text{g g}^{-1}$  for the 83 specimens that showed values above the detection limit ( $140 \mu\text{g g}^{-1}$ ). The mean  
362 Mn concentration for the 121 specimens with values above the detection limit was  $260 \mu\text{g g}^{-1}$  with a  
363 maximum value of  $1220 \mu\text{g g}^{-1}$ . Although they were sometimes high, the concentrations never reached  
364 the hyperaccumulation threshold for this metal (*i.e.*  $10000 \mu\text{g g}^{-1}$ ). In the case of Zn, the maximum  
365 concentration was  $476 \mu\text{g g}^{-1}$ , always well below the hyperaccumulation threshold of  $3000 \mu\text{g g}^{-1}$ . In  
366 general, values found for herbarium specimens were lower than in field collected specimens.

#### 367 368 **Elemental concentrations in plant tissues of *B. guatemalense* collected in the field**

369 The bulk elemental concentrations in the different tissues of *B. guatemalense* are reported for leaves,  
370 bark, flowers, fruits, roots and seeds (Table 1). The results were compared with the concentrations of  
371 a “reference plant” proposed by Markert (1992). Some major element concentrations were in the  
372 expected ranges for most plants in the different plant parts, such as Phosphorus (P), sulphur (S), and  
373 magnesium (Mg). Mean potassium (K) concentrations in leaves, flowers and fruits were slightly low  
374 (mean  $< 1.8 \text{ wt}\%$ ), whereas in the roots K concentration barely reached a mean of  $0.7 \text{ wt}\%$ . The highest  
375 concentration of K was found in the bark with a maximum concentration exceeding  $5.4 \text{ wt}\%$ . Calcium  
376 had typical concentrations in almost all parts of the plant except in the inner bark (with phloem) with  
377 a maximum concentration of  $3.4 \text{ wt}\%$ . Copper (Cu) concentrations in the different plant parts were  
378 within the normal range (mean  $< 12 \mu\text{g g}^{-1}$ ). Roots were particularly enriched in aluminium (Al) (mean  
379  $3910 \mu\text{g g}^{-1}$ ) and iron (Fe) (mean  $4200 \mu\text{g g}^{-1}$ ) compared to other parts of the plant but this could be  
380 due to some minor soil contamination, despite thorough rinsing of roots after collection. Leaves  
381 showed the highest concentrations from all plant parts, reaching  $4.3 \text{ wt}\%$  Ni (Figure 4) with a mean  
382 value of  $1.4 \text{ wt}\%$ , which is beyond the threshold that defines hypernickelophores. The lowest  
383 concentration of Ni in the leaves was of  $2700 \mu\text{g g}^{-1}$ . The other parts of the plant also had quite elevated  
384 concentrations, especially seeds with a mean Ni concentration of  $1.8 \text{ wt}\%$ . Roots showed a mean Ni

385 concentration of 1.2 wt% (ranging from 0.2 to 2 wt%) and bark inner tissues (largely phloem) showed  
386 the same range as roots (*i.e.* from 0.1 to 1.8 wt% Ni). The lowest Ni concentrations were observed in  
387 fruits with a maximum concentration of 4160  $\mu\text{g g}^{-1}$ . Maximum Co accumulation values did not reach  
388 the hyperaccumulation threshold ( $> 300 \mu\text{g g}^{-1}$ ) but exceeded 100  $\mu\text{g g}^{-1}$  in leaves, bark, and roots  
389 which were also the parts with maximum Ni accumulation values. Manganese was not accumulated in  
390 any of the plant parts and ranged from 22–587  $\mu\text{g g}^{-1}$ , and similarly Zn was not accumulated either  
391 with a maximum concentration of 304  $\mu\text{g g}^{-1}$  reported in leaves.

392

### 393 **Soil chemistry in the rhizosphere of *B. guatemalense***

394 Primary habitats of this species correspond mainly to evergreen tropical forests, but it is most often  
395 found in secondary forest and in pasture lands where it can be quite abundant. Rhizosphere soil samples  
396 from *B. guatemalense* presented different physical properties according to the localities they were  
397 collected. Soils from the primary forest were sandy-clay-loam textured, with an olive colour and a  
398 granular structure. In the secondary forest, soils were silty-clay textured, exhibiting a grey colour and  
399 angular blocks structure. In the pasture lands, they were also silty-clay textured, with an olive colour  
400 and a granular structure. The general chemical properties of rhizosphere soil samples were not  
401 consistent with an ultramafic origin. Mean pH values for the primary evergreen forest, secondary forest  
402 and pasture lands were 6.4, 6.6 and 6.9 respectively. The maximum pH in the primary forest was 6.7,  
403 whereas in the secondary forest it was 7.4. Soils revealed a high CEC (mean 40  $\text{cmol}^+ \text{kg}^{-1}$ ) as expected  
404 for tropical clayey soils; the mean total carbon (C) and nitrogen (N) concentrations in soils were 75  $\text{g}$   
405  $\text{kg}^{-1}$  and 6.16  $\text{g kg}^{-1}$ , respectively. Mean exchangeable Ca:Mg ratio was around 4:1. Table 2 shows the  
406 total and available concentrations of major and minor elements. High total concentrations were  
407 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  
408 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  
409 Ni concentration was surprisingly high for non-ultramafic soils (with a mean value of 610  $\mu\text{g g}^{-1}$ ).  
410 Mean DTPA-extractable Ni concentration was also very high (and similar to typical concentrations  
411 found in ultramafic soils) with a mean value of 50  $\mu\text{g g}^{-1}$ , and the maximum value almost reached 200  
412  $\mu\text{g g}^{-1}$ . Total major nutrient elements were relatively high in the case of Ca (7360  $\mu\text{g g}^{-1}$ ) and K (3370  
413  $\mu\text{g g}^{-1}$ ). The available P concentrations were moderate (9.4  $\mu\text{g g}^{-1}$ ).

414

### 415 **Statistical correlations between plant ionomics and rhizosphere soil composition**

416 A Principal Components Analysis (PCA) was performed to reveal the existing relations between the  
417 different elemental concentrations in plant leaves and their associated rhizosphere soils collected in  
418 the sampling sites. The first three principal components together explained 60.4 % of the total variance,

419 with 30.5 % of the variance on the first axis, 18.2 % on the second, and 11.7 % on the third. Projections  
420 of factor scores and loadings for the first and second components are presented in Figure 5. There was  
421 no apparent relation among soil and plant variables, except for a positive association between Ni  
422 concentration in leaves and the available Ni content in soils ( $r = 0.510$ ). Three Pearson correlation  
423 coefficients were obtained separately: i) plant elemental concentrations, ii) total elemental contents in  
424 soils and iii) DTPA-extractable concentrations in soils. Within plant leaves, Ni was highly positively  
425 related with Zn ( $r = 0.970$ ). Other strong positive correlations could be observed, such as Ca-Mg ( $r =$   
426  $0.807$ ). In soils, CEC was positively correlated with the N ( $r = 0.766$ ) and C ( $r = 0.775$ ) contents in  
427 soils. Pearson correlation matrix with all the significant correlations is shown in Appendix 1.

428

### 429 **Desktop fluorescence microscopy on plant tissues**

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

438

### 439 **Elemental localization in plant tissues by XFM**

440 Maps generated by XFM technology enabled us to elucidate the elemental localization in the organs  
441 of the different plant tissues (root, stem, petiole and leaf midrib) of *B. guatemalense*. The results  
442 showed that Ni was highly concentrated in the phloem of all plant tissues. In the leaf midrib cross-  
443 section (Figure 7 a), Ni was mainly enriched in the collenchyma, followed by in the primary and  
444 secondary veins (phloem) and the parenchyma of the leaf blade. Zinc followed the same distribution  
445 pattern as Ni but with a lower intensity. Calcium spots were probably calcium oxalate crystals in all  
446 plant tissues as observed in many plants (van der Ent et al. 2017). The mesophyll was strongly enriched  
447 in Ca all along the leaf blade. Potassium was uniformly distributed throughout the leaf. In the petiole  
448 cross-section (Figure 7 b), Ni was enriched in the phloem but highest in the sclerenchyma cells of the  
449 cortex. Zinc occurs similarly to Ni but was also found in the xylem. Calcium and K were mainly  
450 distributed in the phloem and the cortex. In the stem cross-section (Figure 7 c) Ni was strongly enriched  
451 in the phloem and in the cambium, but only moderately enriched in the cell walls of the cortex, in the  
452 primary xylem and in the parenchymal cell walls of the pith. Zinc distribution matches (again) that of

453 Ni but with a noticeably higher enrichment in the primary xylem. Potassium was poorly concentrated  
454 in the primary xylem and secondary phloem. There was a strong enrichment of Ca in the phloem and  
455 in the parenchyma cells of the pith. In the root cross-section (Figure 7 d), the Ni was particularly highly  
456 enriched in the phloem, in the endoderm and in the epidermis. Nickel was also present in the xylem  
457 but at lower concentrations. Potassium was moderately enriched in the epidermis. Zinc is localized at  
458 a low concentration in the phloem being highest in the epidermis. Calcium is depleted in the cortex  
459 but strongly enriched in the phloem and in the epidermis.

460

## 461 **DISCUSSION**

462

### 463 **Ecology and occurrence of *B. guatemalense* in Mexico**

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

478

479 Although all the soils where this species was sampled were highly enriched in Ni, Fe and Mn, as it is  
480 probably the case in this entire lowland region of Mexico (Hernández-Quiroz *et al.* 2012), they did not  
481 form from ultramafic bedrock (Ortega-Gutiérrez 1992; SGM 2006). The soils have no apparent  
482 deficiency in Ca or in the other essential macronutrients (N, K, P), by contrast with ultramafic soils  
483 (Proctor 2003). Mean total Ni concentration in the first cm of the soils is well above the limit of Ni  
484 concentration recommended in soils from the USA (*i.e.* 210  $\mu\text{g g}^{-1}$ ) and the toxicity threshold specified  
485 by the Mexican norm NOM-147-SEMARNAT/SSA1-2004 (*i.e.* 1600  $\mu\text{g g}^{-1}$ ). A potential source of Ni  
486 in these soils could be lateritic material transported by water from the ultramafic complexes occurring

487 throughout the Polochic-Motagua fault systems in Guatemala. Hernández-Quiroz *et al.* (2012)  
488 proposed that the main sources of Ni (39–318  $\mu\text{g g}^{-1}$ ) in similar soils collected near a petrochemical  
489 facility in Tabasco were either volcanic material deposits or lateritic nodular fragments (associated  
490 with crystalline Fe oxides and the residual fraction). The latter materials were transported from  
491 preexisting soils (pedorelicts), possibly formed out from ultramafic rocks situated in the margins of  
492 the Caribbean plate in ancient times. Another significant contributor to the current Ni concentration in  
493 soils could be the volcanic ash deposits derived from the constant activity of the nearest volcanos, e.g.  
494 El Chichón and Tacaná. Nevertheless, further mineralogical studies are needed to answer these  
495 questions and to better characterize these unusual soils. In any case, the presence of elevated soil Ni  
496 in localities scattered throughout this region probably explains the extension of *B. guatemalense* and  
497 other associated Ni-hyperaccumulators (e.g. *Psychotria costivenia*, *P. lorenciana* and *P. papantlensis*)  
498 (McCartha *et al.* 2019).

#### 500 **Nickel hyperaccumulation in *B. guatemalense* and its significance**

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

507  
508 Data obtained from the XRF herbarium scanning confirmed the obligate hyperaccumulation trait in  
509 this species because only 5.5% of the herbarium specimens had Ni concentrations below the  
510 hyperaccumulation threshold. Mean Ni concentrations in the leaves of *B. guatemalense* scanned in the  
511 herbaria were considerably lower than those collected in the field. In field-collected plants,  
512 *Blepharidium guatemalense* was, therefore, able to accumulate among the highest Ni concentrations  
513 reported in hyperaccumulator leaves globally, which places it in the group of ‘hypernickelophores’  
514 plants (*i.e.* >1% wt) (Boyd and Jaffré 2009; Reeves *et al.* 2018b).

515  
516 Until now, Ni hyperaccumulator species belonging to the Rubiaceae family had been only reported in  
517 the well-known tropical hotspots of biodiversity such as the neighbouring territory of Cuba (11 spp),  
518 New Caledonia (3 spp) and Malaysia (3 spp). Within the Rubiaceae, the tribe *Rondeletieae* (Manns  
519 and Bremer 2010; Rova *et al.* 2009) includes *Blepharidium* along with at least three other genera of  
520 hyperaccumulators reported from Cuba by Reeves *et al.* (1999): *Rondeletia*, *Phyllomelia* and *Mazanea*

521 (= *Ariadne*) or newly discovered hyperaccumulators in the genus *Arachnothryx* (formerly *Rondeletia*)  
522 in central America (Nkrumah et al., 2021). Further herbarium surveys on phylogenetically close  
523 genera/species of known hyperaccumulators in Mexico could lead to the discovery of new  
524 hyperaccumulator taxa.

525

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

527 The heterogeneous distribution of DTPA-extractable Ni in soils could explain the high variability in  
528 Ni concentrations within foliar samples collected in the field as those two variables were positively  
529 correlated. No other soil characteristics seemed to influence Ni uptake. An old pasture which had the  
530 maximum DTPA-extractable Ni concentration showed a high density of *B. guatemalense* clustered in  
531 patches of around 10 m in diameter. Because of the high heterogeneity of Ni availability in soils  
532 resulting from the similar geological and pedological situation, one can question whether the presence  
533 of the species itself influences the variability of Ni levels through biogeochemical recycling. The  
534 highly concentrated large leaves from fast-growing trees suggest, among other factors, that litter  
535 produced by *B. guatemalense* could contribute significantly to the available Ni pool in the surface  
536 soils. These changes in Ni concentrations could, in turn, offer a suitable habitat for other Ni  
537 hyperaccumulator plants. Indeed, in primary and secondary forests in our study area, *B. guatemalense*  
538 was frequently observed accompanied by the facultative Ni-hyperaccumulator *Psychotria costivenia*  
539 as an understory plant beneath it. Preliminary observations suggested that shrubs of *P. costivenia*  
540 growing under *B. guatemalense* trees have much higher Ni concentrations than those that are not under  
541 such a canopy.

542

543 Simultaneous Ni and Co hyperaccumulation was reported in 27 mounted specimens from the MEXU  
544 and MO herbaria with a ratio of ~ 8:1 Ni:Co. Similar cases of simultaneous Ni and Co  
545 hyperaccumulation have been observed in the Malaysian tree *Glochidion* cf. *sericeum*  
546 (Phyllanthaceae) with an unusual ratio of 1:1 Ni:Co. Another example is *Rinorea bengalensis* (Wall.)  
547 Kuntze (Violaceae) with Co values up to 630  $\mu\text{g g}^{-1}$ , also found by XRF scanning or *Berkheya coddii*  
548 (Asteraceae), with Co values higher than 600  $\mu\text{g g}^{-1}$  (Lange et al. 2017). Nevertheless, the Co  
549 concentration in foliar samples of *B. guatemalense* collected in the field did not reach  
550 hyperaccumulation threshold. This may be due to the almost neutral pH in rhizosphere soils, since it  
551 is known that plants accumulate Co mostly in acidic substrata (Kukier et al. 2004), but also to the  
552 competition between Ni and Co for uptake mechanisms, as described for *Alyssum* species and *B. coddii*  
553 (Homer et al. 1991; Keeling et al. 2003).

554

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

556 On sufficiently old trunks, the inner bark of the tree that is in direct contact with wood (phloem tissues)  
557 on trunks that are old enough shows a particular turquoise green-blue colour due to high Ni  
558 concentrations. Such an intense green colour is one of the most outstanding features of woody Ni  
559 hyperaccumulators that has been also observed in some South Eastern Asian and Pacific species (*e.g.*  
560 *Phyllanthus balgooyi* Petra Hoffm. & A.J.M.Baker, and *R. bengalensis*), and is due to the very high  
561 concentrations of Ni<sup>2+</sup> ions in this tissue (van der Ent et al. 2017a). Nickel-enriched phloem tissues on  
562 the stem of the South African Ni hyperaccumulator *Senecio coronatus* (Thunb.) Harv. have also been  
563 reported (Mesjasz-Przybylowicz 1997). Desktop micro-XRF showed rather heterogeneous  
564 distributions of Ni across the leaf in both veins and leaf blades. The XFM elemental maps confirmed  
565 the high Ni concentrations, not only in the phloem of the primary (leaf midrib) and secondary leaf  
566 veins, but also in the phloem of the root, stem and petiole. As for other species which show this specific  
567 feature, *e.g.* *P. balgooyi* (van der Ent et al. 2017b) or *R. bengalensis* (Zelano et al. *in press*), *B.*  
568 *guatemalense* appears to redistribute Ni within the plant *via* the phloem. Phloem active transport of Ni  
569 was confirmed in another Ni hyperaccumulator *Noccaea caerulescens* by using Ni stable isotope  
570 analysis (Deng et al. 2016, 2018).

571  
572 Another fact that confirms the re-distribution of Ni *via* phloem in *B. guatemalense* is the high Ni  
573 concentrations reported in flowers and fruits, and extremely high values in seeds. Indeed, the only  
574 pathway for Ni (and eventually other heavy metals) to reach sink organs (maturing fruits, seeds,  
575 flowers, *etc.*) is through the mass movement of phloem fluids coming from the source organs  
576 (senescing leaves) (Bhatia et al. 2003; Page and Feller 2015). Leaf epidermal cells of *B. guatemalense*  
577 were not Ni enriched as opposed to most hyperaccumulator species; this trait was also observed for  
578 the following species: *Berkheya coddii* and *P. balgooyi* (van der Ent et al. 2017).

579  
580 According to the updated knowledge on Ni hyperaccumulators which redistribute Ni through the  
581 phloem, we could hypothesize that Ni<sup>2+</sup> could be loaded into the xylem as the free cation and  
582 transported to the shoots where it may be complexed by carboxylic acids (mainly citrate), and partly  
583 stored in the leaf midrib collenchyma (van der Pas and Ingle 2019). Since a slight Ni accumulation is  
584 observed in the leaf parenchyma, transport may also occur from the xylem to mesophyll cells in the  
585 transpiration stream (Page and Feller 2015). In minor leaf veins, Ni may also be transferred directly  
586 from the xylem to the phloem through companion cells (Page and Feller 2015; van Bel 1990). In  
587 many Ni hyperaccumulators globally, Ni<sup>2+</sup> is complexed predominantly by citrate<sup>-</sup> or malate<sup>-</sup> in leaves,  
588 phloem or latex and its chemical speciation does not substantially change among different plant tissues

589 (van der Ent et al. 2017). Sugars, amino acids and organic acids are expected to be the most  
590 predominant metabolites in the phloem sap (Braun et al. 2014). The dominant Ni-citrate complexes in  
591 the phloem tissues of *P. balgooyi* seem to be associated with low sugar contents (mainly sucrose) (van  
592 der Ent et al. 2017). In this case, higher sucrose concentration in the mesophyll cell cytoplasm may  
593 enhance the passive loading of the phloem moving by diffusion down its concentration gradient to the  
594 sieve-tube elements. Such a process could explain the higher Ni concentration in the phloem tissues  
595 than in the leaves in this species, as it could be the case in *B. guatemalense*. Conversely, *P.*  
596 *rufuschaneyi* accumulated higher Ni accumulation in leaves with respect to phloem tissues (van der  
597 Ent et al. 2017). This highlights that further studies on the Ni chemical speciation in the different  
598 tissues and fluids of *B. guatemalense*, including phloem sap, are crucial to better understand these  
599 processes at the cellular level.

600

601 In the leaf samples taken in the field, cobalt was accumulated at close-to-hyperaccumulation levels in  
602 the leaf samples taken in the field for micro-XRF analysis. In Co hyperaccumulators, Co is sometimes  
603 found in the form of lesions in the leaf surface as a result of an extracellular detoxification mechanism  
604 *via* exudation of guttation liquids (van der Ent et al. 2018), whereas it is found accumulated at the leaf  
605 tip in *Alyssum murale* (syn. *Odontarrhena chalcidica*) (Tappero et al. 2007). No evidence of these  
606 lesions or of tip accumulation was observed in the micro-XRF distribution map of *B. guatemalense*  
607 because of the low Co concentration in the foliar samples collected in the field. Pearson correlation  
608 coefficient ( $r$ ) of foliar elemental concentrations showed a strong positive correlation between Ni and  
609 Zn (Spearman test,  $p$  value  $< 0.0001$ ). Both elements can, therefore, enter through the plasma  
610 membrane of root cells *via* the same poorly selective cation transporter members of the ZIP gene  
611 family (van der Pas and Ingle 2019). Also, the distribution of Zn matches with that of Ni within the  
612 different plant tissues, as observed in the XFM elemental maps.

613

#### 614 **Potential applications for *B. guatemalense***

615 *Blepharidium guatemalense* possess the required characteristics to be an excellent candidate for Ni  
616 agromining (phytomining), such as being a hypernickelophore, high biomass, apparent fast-growth  
617 rate and easy reproduction (Nkrumah et al. 2018). The fact that the wood of this species is currently  
618 used for building (*e.g.* rafters, beams and supports of houses) by local populations in Chiapas and  
619 Tabasco supports the idea that the wood of hyperaccumulator plants may be durable against termites  
620 by acting as natural insecticide/fungicide (van der Ent and Mulligan 2015). Our observations also  
621 suggest the possibility of further agromining implementation in secondary forest and pasturelands  
622 where the tree is grown (or left to grow) in a deliberate way. Sustainable agromining systems using *B.*

623 *guatemalense* could contribute to the preservation of this unique species. Research is currently  
624 underway to study the agronomy of this species and the environmental, social and economic  
625 implications of any implementation of agromining in those regions.

626

## 627 **CONCLUSIONS**

628 *Blepharidium guatemalense*, a new obligate hypernickelophore species able to accumulate up to 4.3  
629 wt.% Ni in its leaves, was discovered in south-eastern Mexico thanks to herbarium screening and field  
630 sampling. It was found in the States of Tabasco, Chiapas and Campeche. Inner bark samples of older  
631 trees show a typical green colour and Ni was shown to be uniformly distributed in the leaf but with  
632 higher concentrations in the midrib and in the secondary veins. Phloem tissues were identified with  
633 XFM techniques as the major sink of Ni in this tree, suggesting active internal re-distribution  
634 mechanisms similar to those reported in other extreme tropical Ni-hyperaccumulator species with  
635 green phloem sap.

636

637 Prior this research, no ‘hypernickelophore’ had ever been reported on non-ultramafic soils.  
638 *Blepharidium guatemalense* does so consistently, over a vast region of south-eastern Mexico that is  
639 covered by sedimentary geological layers, although showing elevated geochemical backgrounds in Ni  
640 and associated elements. Moreover, in the natural lowland forests of Chiapas, stands of *B.*  
641 *guatemalense* may create Ni-rich habitats for other Ni hyperaccumulator species growing underneath  
642 (e.g. *P. costivenia*) likely by recycling Ni through the decay of Ni-rich litter in the topsoil. This finding  
643 shifts the paradigm and enables the exploration of ‘natural’ Ni hyperaccumulator habitats beyond the  
644 constraints of ultramafic geology. Pioneering investigations are being carried out to study the  
645 application of *B. guatemalense* for Ni agromining (phytomining).

646

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660

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845

846 **Figure 1** Detail of *Blepharidium guatemalense* plants in the tropical forests of southeast Mexico. **a.**  
847 seeds; **b** fruit capsules; **c** inflorescences; **d** mature leaf of approximately 45 cm long and 18 cm wide;  
848 **e** detail of bark cutting showing a positive reaction to the field spot test based on DMG; **f** 25-m high  
849 individual.

850

851 **Figure 2** Histogram indicating the frequency distribution of Ni concentration ( $\mu\text{g g}^{-1}$ ) in herbarium  
852 specimens of *Blepharidium guatemalense* ( $n=182$ ) analysed by X-ray fluorescence spectroscopy in  
853 MEXU, P and MO herbariums. The abscissa is plotted on a logarithmic scale.

854

855 **Figure 3** Map showing the geographic distribution of *B. guatemalense* herbarium specimens analysed  
856 with XRF spectroscopy in the herbariums MEXU, P and MGB (number of voucher specimens with  
857 available geographic coordinates = 83). Ultramafic complexes are shown in black: Motozintla  
858 (Chiapas) and Polochic-Motahua (Guatemala). Geologic map sources: “Surface Geology of the  
859 Caribbean Region” of the USGC (United States Geological Survey) and Sistema Geológico Mexicano  
860 (SGM 2006, 2017).

861

862 **Figure 4** Nickel concentrations in plant tissues of *B. guatemalense* presented as boxplots. Key  
863 symbols: open boxes are interquartile ranges, bold crosses are  $\pm$  mean, whiskers are  $\pm$  standard  
864 deviation and bold circles are outliers.

865

866 **Figure 5** Biplot of the Principal Components Analysis (PCA) based on foliar elemental concentrations  
867 of *B. guatemalense* and the properties of its associated rhizosphere soils,  $n=21$ .

868

869 **Figure 6** Elemental speciation of: **a** mature leaf (air-dried), **b** leaf detail and **c** seeds of *Blepharidium*  
870 *guatemalense*. Maps were generated using micro-XRF (20  $\mu\text{m}$  resolution). Mean concentrations in  
871 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  
872 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.

873

874 **Figure 7a** X-ray fluorescence microscopy (XFM) elemental maps of leaf midrib cross-sections of  
875 *Blepharidium guatemalense*. Scan area is 3 x 1.8 mm (601 x 361 pixels) with a resolution of 5  
876 microns and a per-pixel dwell of 9ms. Abbreviations of anatomical features: *xy*, xylem; *ph*, phloem;  
877 *c*, cortex; *sv*, secondary veins; *ue*, upper epidermis; *le*, lower epidermis; *mc*, midrib collenchyma.

878

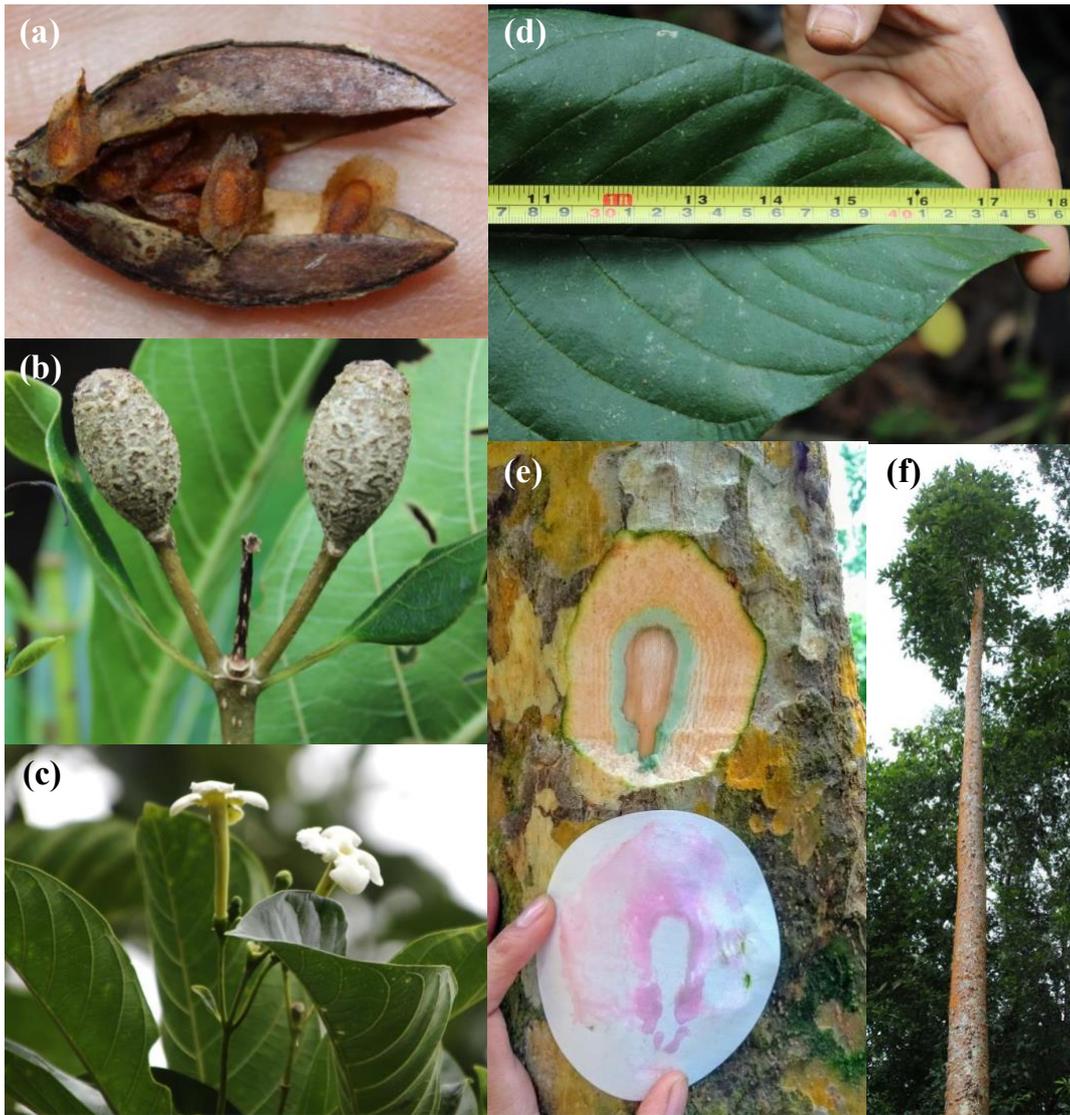
879 **Figure 7b** X-ray fluorescence microscopy (XFM) elemental maps of a petiole cross-sections of  
880 *Blepharidium guatemalense*. Scan area is 2.5 x 2.3 mm (340 x 306 pixels) with a resolution of 3  
881 microns and a per-pixel dwell of 4ms. Abbreviations of anatomical features: *xy*, xylem; *ph*, phloem;  
882 *co*, collenchyma; *sc*, sclerenchyma; *e*, epidermis.

883

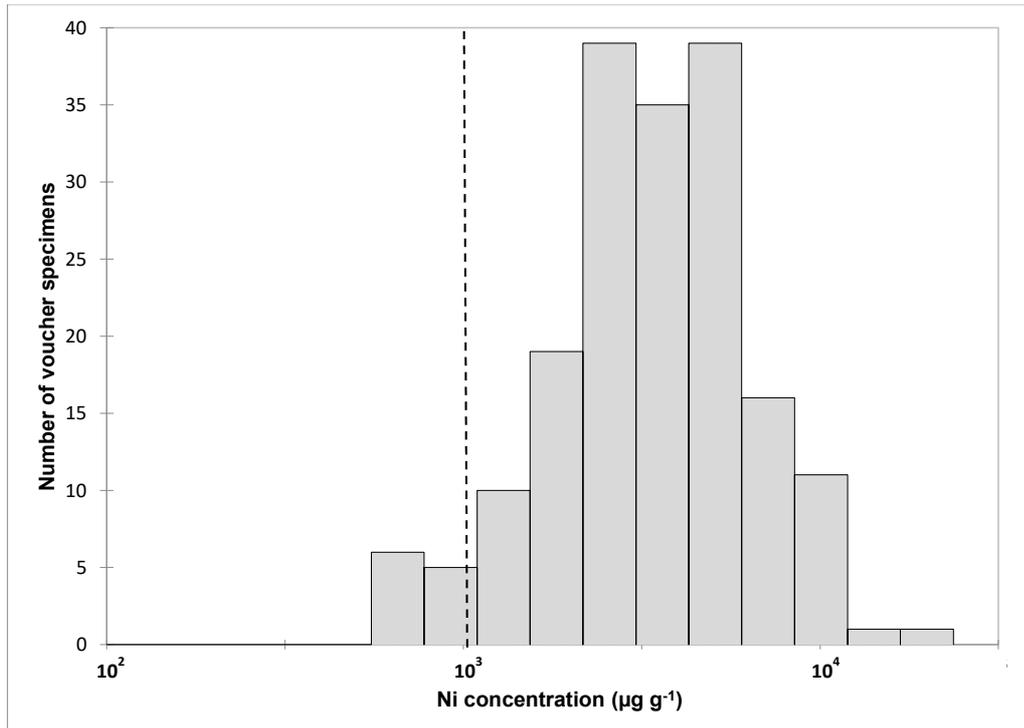
884 **Figure 7c** X-ray fluorescence microscopy (XFM) elemental maps of a stem cross-sections of  
885 *Blepharidium guatemalense*. Scan area is 4.6 x 4.2 mm (782 x 692 pixels) with a resolution of 6  
886 microns and a per-pixel dwell of 7ms. Abbreviations of anatomical features: *ox*, older xylem;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

888

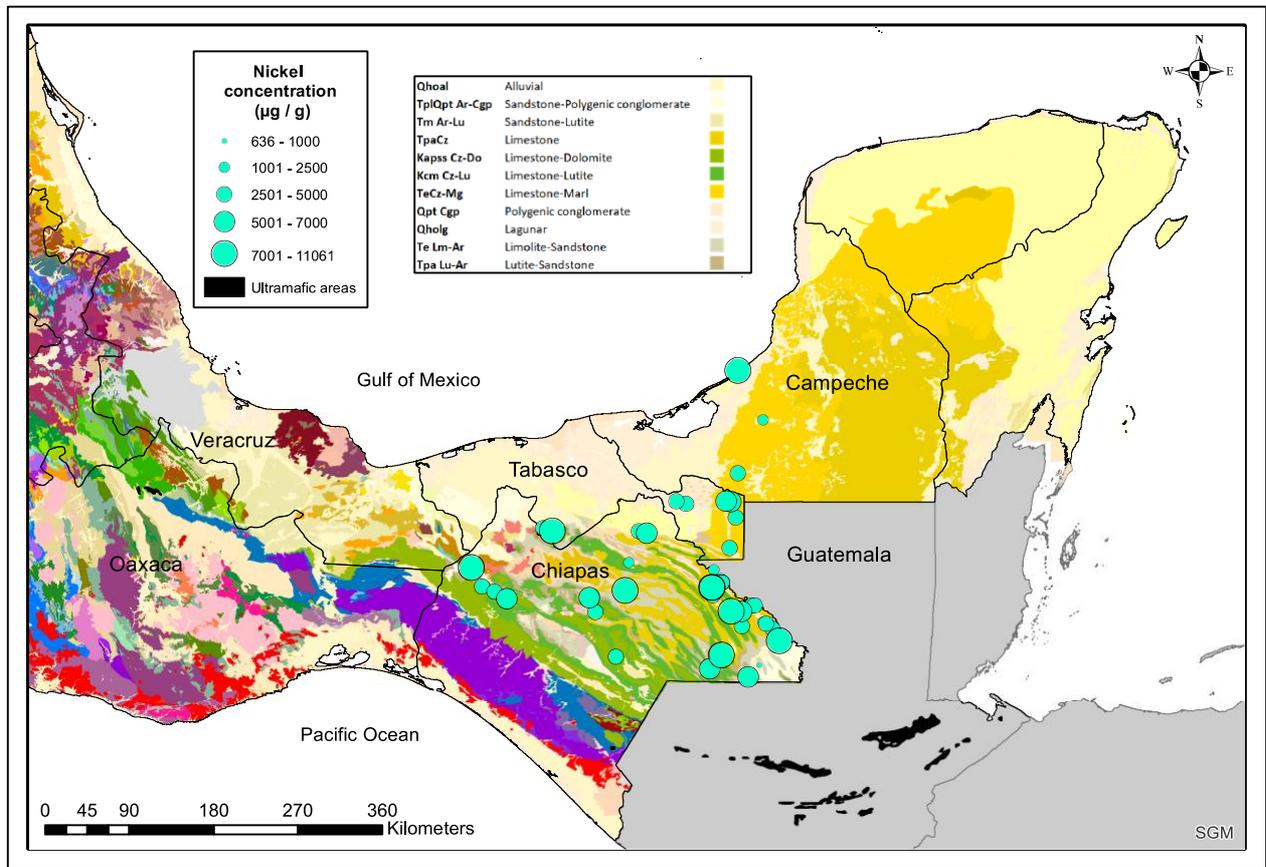
889 **Figure 7d** X-ray fluorescence microscopy (XFM) elemental maps of a root cross-section of  
890 *Blepharidium guatemalense*. Scan area is 2.7 x 2.5 mm (278 x 291 pixels) with a resolution of 5  
891 microns and a per-pixel dwell of 5ms. Abbreviations of anatomical features: *xy*, xylem; *yx*, younger  
892 xylem; *ph*, phloem; *e*, epidermis; *en*, endodermis.



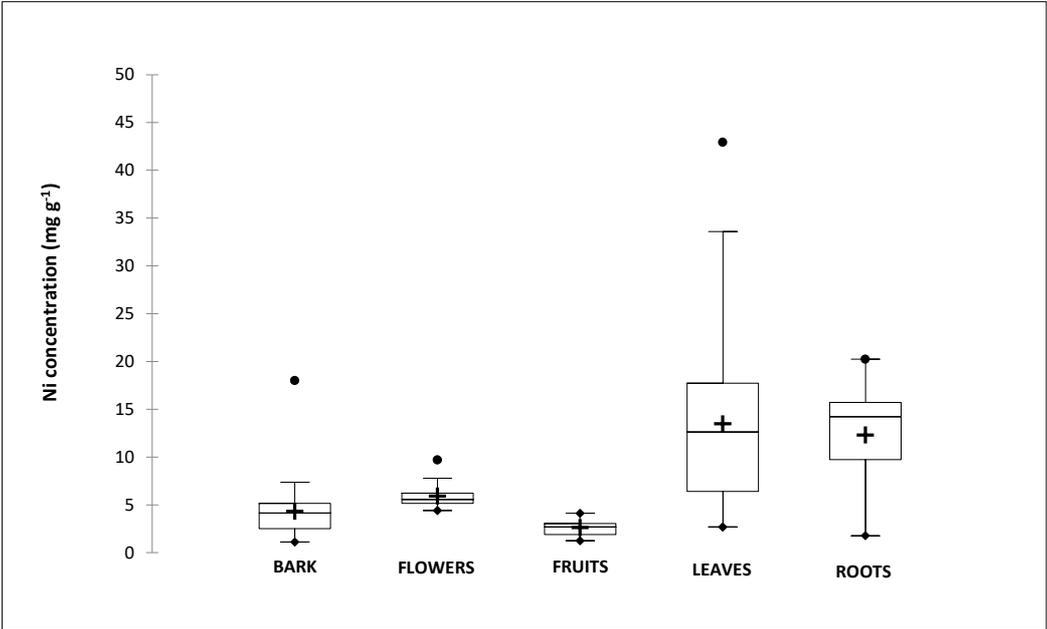
894  
895 Figure 1



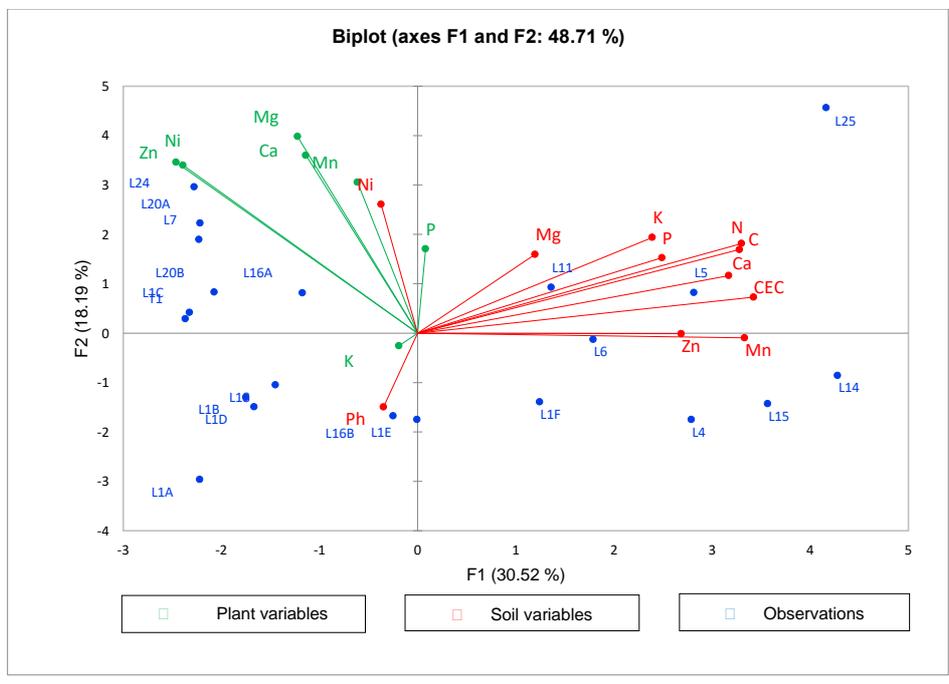
896  
897 Figure 2



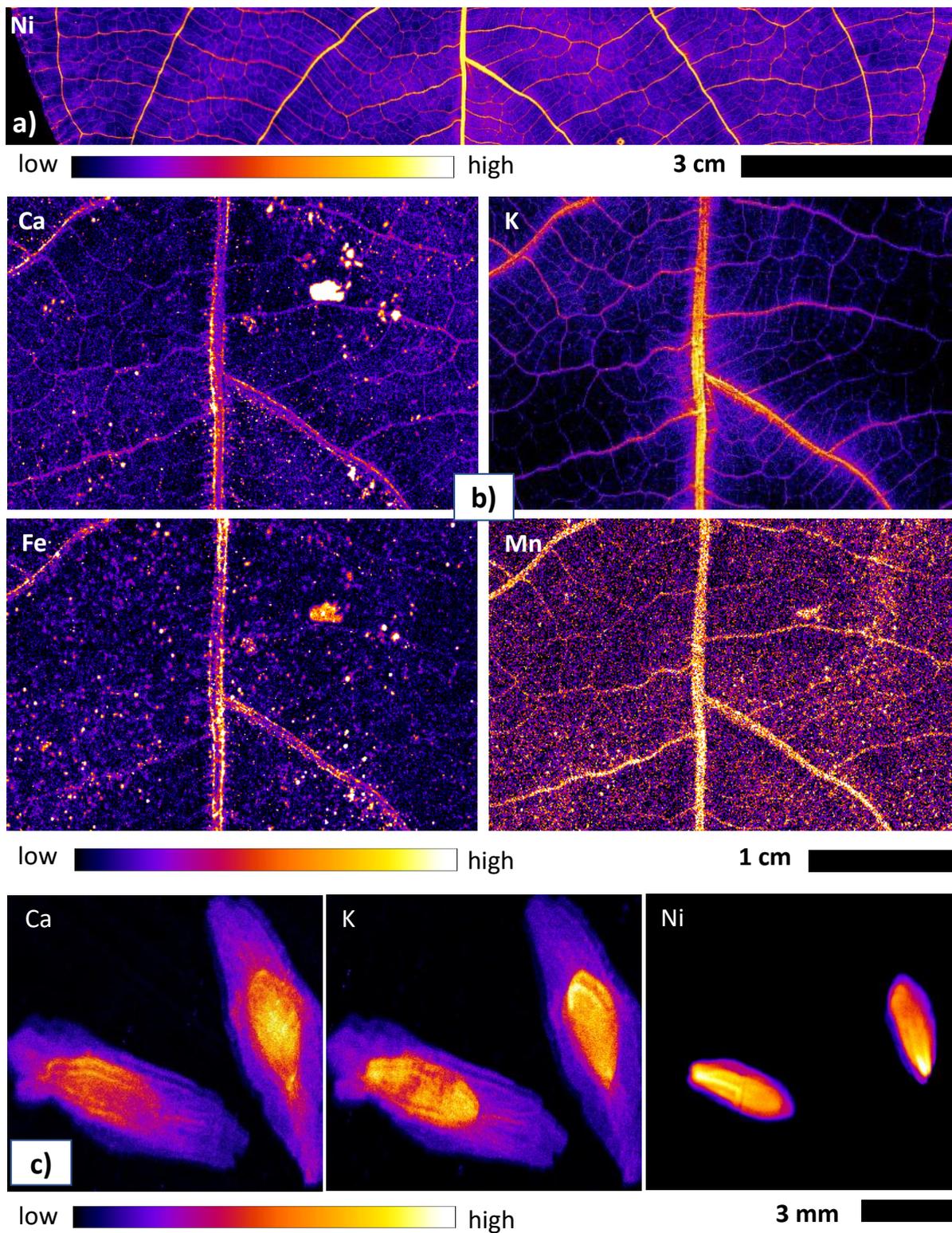
898  
899 Figure 3



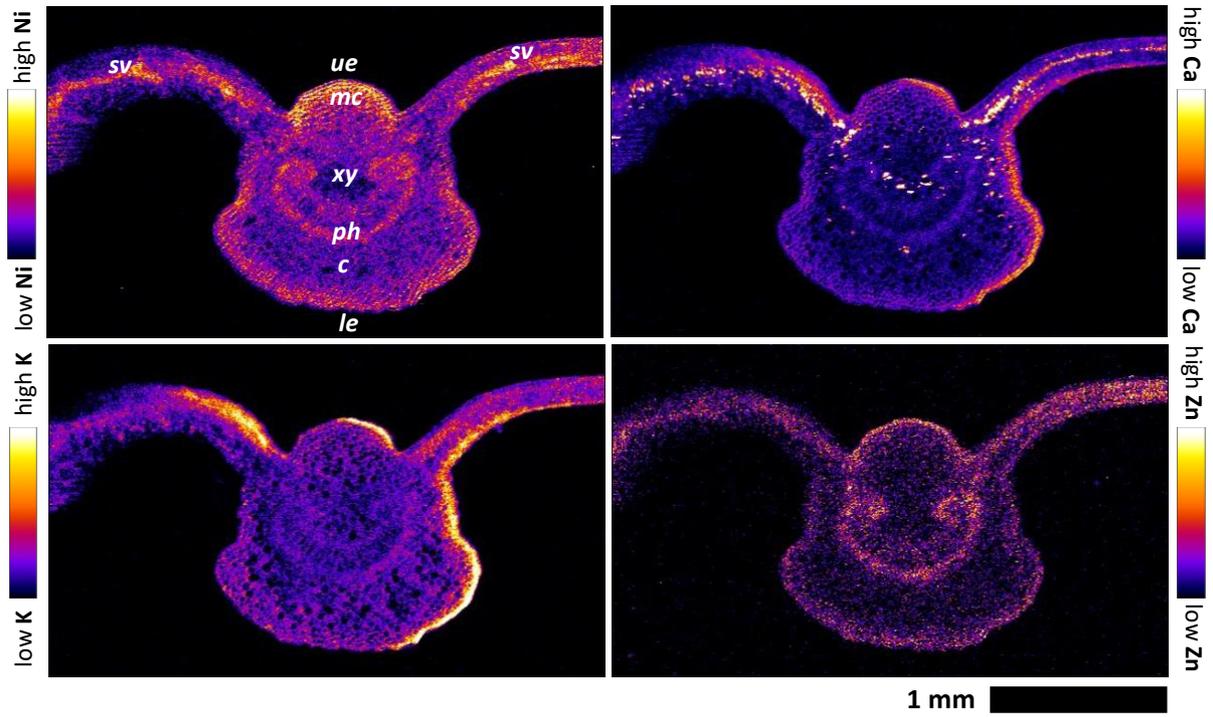
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901 Figure 4



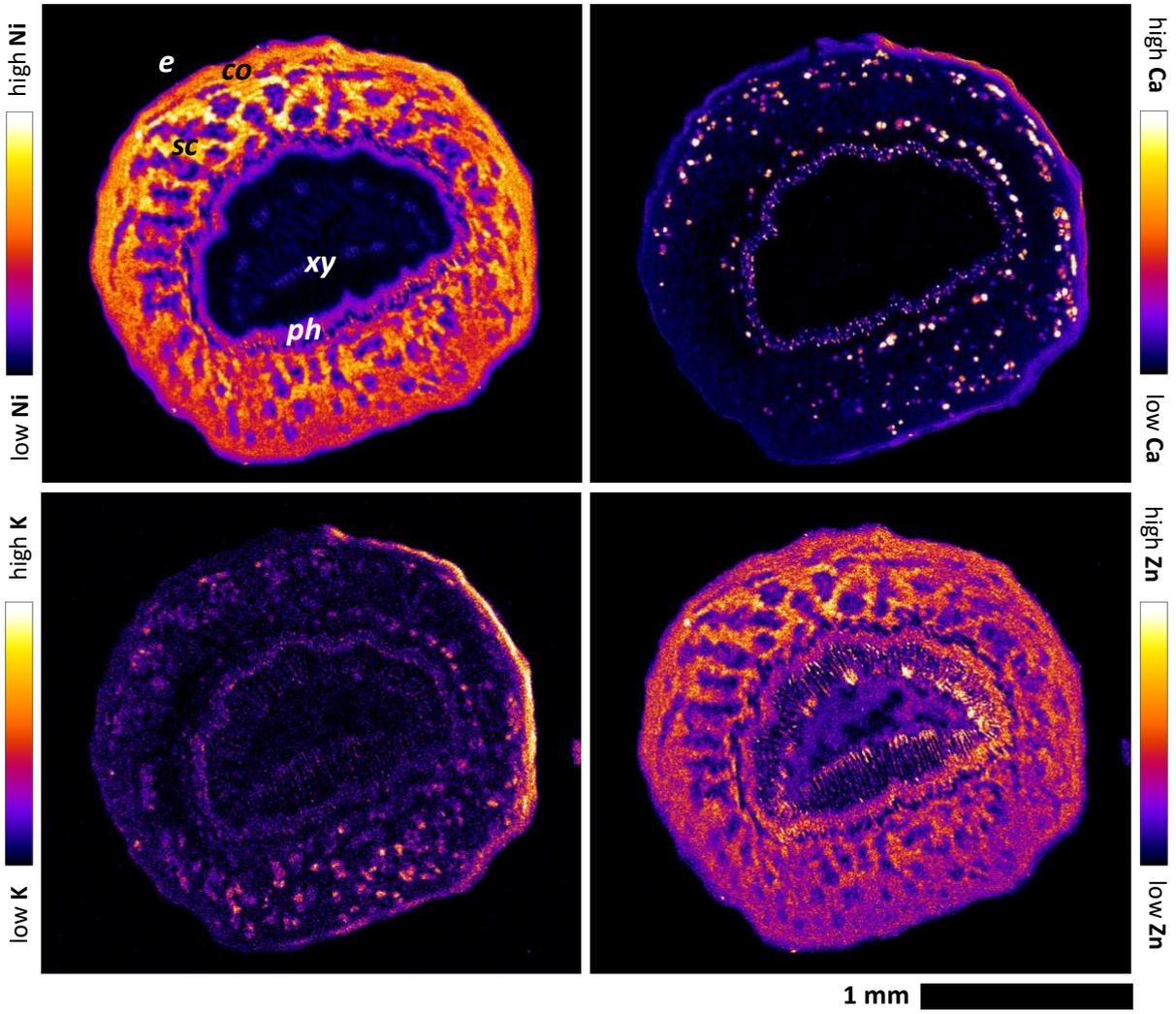
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903 Figure 5



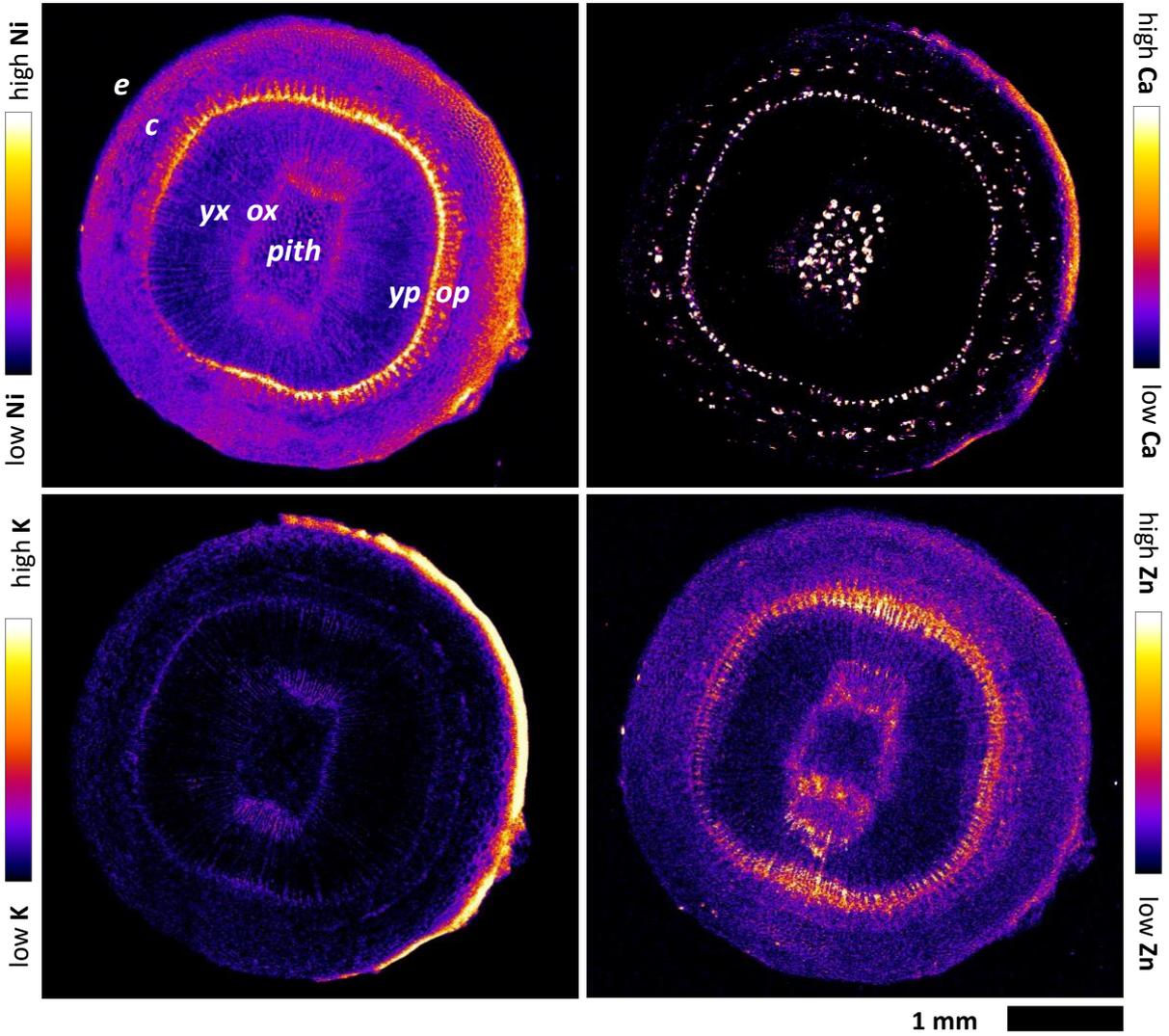
904  
905 Figure 6



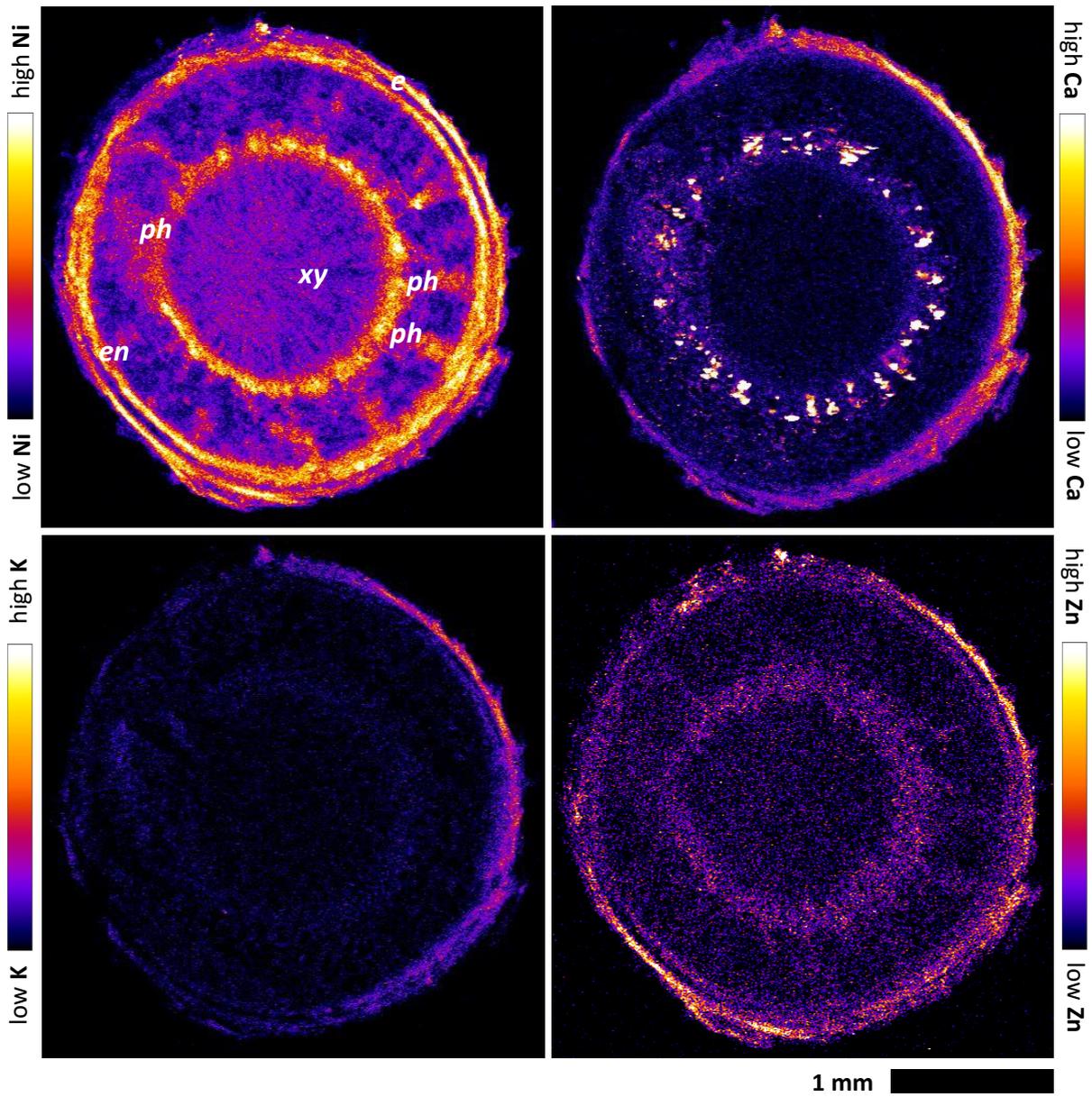
906  
907 Figure 7a



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909 Figure 7b



910  
911 Figure 7c



912  
 913 Figure 7d  
 914

915 **Table 1** Elemental concentrations in plant tissues of *B. guatemalense* from individuals collected in  
 916 their native habitat at Lacandonian rainforest (Chiapas, Mexico) and Tacotalpa (Tabasco, México).  
 917 The elemental concentrations are shown in ranges and means in  $\mu\text{g g}^{-1}$  ( $n$ = number of samples).  
 918

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)	2 990–14 200 (7200)	1730–7 060 (3070)	< 163–227 (85)	877–4700 (2260)
Flower	16	1790–2970 (2480)	13 500–24 200 (17 100)	2700–8850 (4835)	1680–2 660 (2370)	< 163	1650–5 490 (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)	10700–54 600 (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)

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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)

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922 **Table 2** Total and available elements of the rhizosphere soil collected in the native habitat of *B.*  
 923 *guatemalense* at Lacandonian rainforest (Chiapas, Mexico). Concentrations are given in ranges and  
 924 means in  $\mu\text{g g}^{-1}$  and the number of samples is 19.  
 925

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)

926 Soil pH is 5.91 - 7.36 (mean pH 6.53), \*phosphorus using Olsen P extraction.

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929 **Appendix 1a** Correlation matrix (Pearson) based on the foliar elemental concentrations of *B.*  
 930 *guatemalense*. The values in bold are different from 0 at an alpha = 0.05 significance level (*n* = 21).  
 931 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
933 Ca	1										
934 K	0.005	1									
935 Mg	0.807	0.053	1								
936 Mn	0.597	0.136	0.419	1							
937 P	-0.034	-0.066	0.066	0.370	1						
938 S	0.428	0.382	0.286	0.602	0.243	1					
939 B	0.178	-0.074	0.132	-0.194	-0.468	-0.134	1				
940 Co	0.245	0.574	0.150	0.331	-0.155	0.299	0.080	1			
941 Cu	0.186	0.022	0.317	0.036	0.262	0.165	0.018	0.120	1		
942 Ni	0.413	0.036	0.467	0.367	0.211	0.344	0.136	-0.097	0.326	1	
943 Zn	0.426	-0.098	0.508	0.334	0.268	0.227	0.169	-0.105	0.400	0.970	1
944											
945											

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

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951 **Appendix 1c** Correlation matrix (Pearson) based on the DTPA elemental contents and other variables  
 952 from rhizosphere soils of *B. guatemalense*. The values in bold are different from 0 at an alpha = 0.05  
 953 significance level ( $n = 19$ ).  
 954

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

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