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

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28 **Novelty Statement**

29 Our research team is a pioneer in the discovery of metal hyperaccumulator plants in
30 Mesoamerica with at least 13 species discovered in the last 2 years. This study is the first
31 to assess the potential of nickel agromining (phytomining) in Mexico (and in all the
32 American continent), using one of the strongest nickel hyperaccumulators reported so far.
33 The promising results of this study are the basis for optimal agricultural management of
34 *Blepharidium guatemalense*.

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37 **The potential of *Blepharidium guatemalense* for nickel agromining in**
38 **Mexico**

39

40 **Abstract**

41 The aim of this study was to assess the potential of the woody nickel
42 hyperaccumulator species *Blepharidium guatemalense* (Standl.) Standl. for
43 agromining in southeastern Mexico. Pot trials consisting of nickel dosing (0, 20,
44 50, 100 and 250 mg Ni kg⁻¹), and synthetic and organic fertilization were
45 conducted. Field trials were also undertaken with different harvesting regimes of
46 *B. guatemalense*. Foliar nickel concentrations increased significantly with rising
47 nickel additions, with a 300-fold increase at 250 mg Ni kg⁻¹ treatment relative to
48 the control. Synthetic fertilization strongly increased nickel uptake without any
49 change in plant growth or biomass, whereas organic fertilization enhanced plant
50 shoot biomass with a negligible effect on foliar nickel concentrations. A 5-year-
51 old stand which was subsequently harvested twice per year produced the maximum
52 nickel yield tree⁻¹ yr⁻¹, with an estimated total nickel yield of 142 kg ha⁻¹ yr⁻¹.
53 *Blepharidium guatemalense* is a prime candidate for nickel agromining on account
54 of its high foliar Ni concentrations, high bioconcentration (180) and translocation
55 factors (3.3), fast growth rate and high shoot biomass production. Future studies
56 are needed to test the outcomes of the pot trials in the field. Extensive geochemical
57 studies are needed to identify potential viable agromining locations.

58

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

61

62 **Introduction**

63 Soil and freshwater ecosystems can be extremely enriched in some trace elements (e.g.
64 Zn, Cu, Ni, Cr, Cd), resulting from lithogenic, pedogenic or anthropogenic inputs
65 (Kabata-Pendias and Pendias, 2001; Sidhu, 2016; Kumar et al., 2019a, 2019b). Metal
66 hyperaccumulation is an unusual phenomenon that occurs in at least 720 plant species.
67 belonging to 130 genera and 52 families distributed worldwide (Reeves et al., 2018).
68 These plant species have evolved physiological mechanisms of metal tolerance, enabling
69 them to take up and accumulate one or more metallic (or metalloid) elements in their
70 leaves to concentration levels two to three orders of magnitude higher than is found in
71 plants growing on non-metalliferous soils (Baker and Whiting, 2002; van der Ent et al.,
72 2013; Merlot et al., 2018). About 75% of hyperaccumulator species are for nickel (Ni)
73 (Reeves 2003; Reeves et al. 2017) and this can be explained by the large extent of Ni-
74 enriched ultramafic outcrops covering approximately 1% of the Earth's land surface
75 (Garnier et al., 2009; Echevarria, 2018). Hyperaccumulator plants are of particular
76 interest for both fundamental research (e.g. metal regulation in plants) and practical
77 application. Indeed, metal hyperaccumulator plants can be used as indicators of metals in
78 soils for geochemical prospecting (Baker and Brooks, 1988; Nkoane et al., 2005) and for
79 phytoremediation (Baker and Brooks, 1988; Atma et al., 2017; Sidhu et al., 2018a;
80 Korzeniowska and Stanislawski-Glubiak, 2019; Sidhu et al., 2020). Phytoremediation is
81 a family of plant-based technologies to deal with environmental aspects of sites that
82 requires remediation, including phytostabilization (metals are transformed into non
83 phyto/bioavailable forms), phytoextraction (metals are removed from the soil by plants),
84 and phytomining (metals are removed from soils and recovered from the plant biomass)
85 (Chaney and Baklanov, 2017). Several metal hyperaccumulator plant species are

86 currently used for phytoextraction and phytomining (Chaney et al., 2007; van der Ent et
87 al., 2015; Sidhu et al., 2018b; Chaney et al., 2018).

88 Agromining, a type of phytomining, is a complete chain of agro-metallurgical processes
89 that aims to recycle strategic metals from secondary resources (*e.g.* contaminated or
90 naturally metal-enriched soils) by means of the cultivation of selected hyperaccumulator
91 plants ('metal crops') to provide valuable products (Echevarria et al., 2015; Morel et al.,
92 2015; van der Ent et al., 2015, 2018). This 'green' technology is also a cost-effective and
93 environmentally friendly approach for rehabilitating degraded mine sites (Erskine et al.,
94 2018).

95 Most agromining research to date has focused on nickel because of the relatively high
96 value of this metal on the market (US\$ 15 kg⁻¹ on the LME, August 28, 2020), and the
97 existence of many Ni-enriched soils and Ni-hyperaccumulator plants around the world.
98 Optimal agronomic practices, based on laboratory and field experiments, have
99 significantly increased the Ni yields of some 'metal crops'. The improvement of the
100 agronomy of the Eurasian species *Odontarrhena muralis sensu lato* (synonym *Alyssum*
101 *murale s.l.*) resulted in a maximum yield of 112–145 kg Ni ha⁻¹ in Albania (Bani et al.,
102 2015b; Bani and Echevarria, 2019), and 400 kg Ni ha⁻¹ in the USA (Li et al., 2003; Chaney
103 et al., 2007). Furthermore, pilot-scale agromining trials in western, central and southern
104 Europe have been set-up to improve plant yields whilst simultaneously enhancing soil
105 quality (Kidd et al., 2018). Thus, fertilization regimes, herbicide application, soil tillage,
106 plant density planting, etc. are all currently being tested for high performance
107 Mediterranean 'metal crops' such as *O. muralis* and *Bornmuellera emarginata* (Kidd et
108 al., 2018).

109 Research on tropical Ni agromining has rapidly progressed in recent years, specifically
110 in Southeast Asia (Sabah, Malaysia). Two promising species, *Phyllanthus rufuschaneyi*
111 and *Rinorea cf. bengalensis*, have been tested in pots and in the field to evaluate their
112 response to synthetic fertilization, pH adjustment and organic amendments addition,
113 among other treatments (Nkrumah et al., 2019b, 2019c, 2019d). A maximum Ni yield of
114 250 kg Ni ha⁻¹ has been estimated for a large-scale agromining operation using *P.*
115 *rufuschaneyi* under field conditions in Sabah (Nkrumah et al., 2019a).

116 The neotropical region is a major hot-spot for hyperaccumulator plants, with Cuba and
117 Brazil recording ~30% of all currently known Ni hyperaccumulator species (Reeves et
118 al., 2018, 1999). Species from the genera *Buxus* and *Leucocroton* in Cuba have been
119 identified as potential ‘metal crops’ (> 2% wt Ni in dry leaves) (Reeves et al., 1996;
120 Nkrumah et al., 2016). However, agromining in this region has remained unexplored until
121 recent years. Our field and herbarium surveys have led to the discovery of Ni
122 hyperaccumulation in at least 13 plant species belonging to three families (Oleaceae,
123 Rubiaceae and Violaceae) distributed mainly in the tropical forests of Mexico, Central
124 and South America (McCartha et al., 2019; Nkrumah et al., 2021). Among the discovered
125 species, there is one ‘hypernickelophore’ (>1 wt% Ni in dry leaves) that seems to be the
126 most promising candidate for Ni agromining in the neotropical regions. *Blepharidium*
127 *guatemalense* (Standl.) Standl. (Rubiaceae) or ‘Popiste’, as it is known in the Mayan local
128 language, is a ligneous species mainly distributed in the tropical perennial and sub-
129 perennial rainforests of southern Mexico (Chiapas, Tabasco and Campeche) and
130 Guatemala. This high-biomass tree is able to concentrate up to 4.3 wt% Ni in its dry
131 leaves and has highly Ni-enriched phloem which may have significant role in Ni
132 distribution in this species (Navarrete Gutiérrez et al., 2021). *Blepharidium guatemalense*
133 also grows in fallow fields under ‘slash-and-burn’ agriculture and pasturelands and has

134 long been used for building traditional houses (poles for roofing), and as live fences by
135 the local communities (Jimenez-Ferrer et al., 2008). The high density of this wild species
136 observed in some fallow fields is indicative of the current management of this tree, which
137 is referred to by the local people as “tolerated/promoted”. In this plant management
138 approach, local people seek to maintain or even increase the abundance of useful species
139 without protecting (*e.g.* removal of competitors) or cultivating (*e.g.* fertilization practices)
140 it (Casas et al., 2017). Field observations and testimonials from local people suggest that
141 the species grows fast and can be easily propagated via seeds, which are desirable traits
142 of a commercially viable ‘metal crop’ (Chaney et al., 2007; Nkrumah et al., 2018a).
143 Considering the high potential of *B. guatemalense* as a ‘metal crop’ for economic Ni
144 agromining in suitable neotropical regions, it is critical to develop the agronomic systems
145 to optimize Ni yield. This study is the first to assess the response of *B. guatemalense* to
146 different agronomic systems at both laboratory- and field-scale. The specific objectives
147 of this study were to elucidate the effect of Ni (and other elements) uptake and shoot
148 biomass production of *B. guatemalense* in response to: i) soluble Ni additions, ii)
149 synthetic fertilization, iii) organic fertilization and iv) harvesting schedules.

150 **Materials and Methods**

151 *Nickel dosing trial*

152 The Ni tolerance and accumulation potential of *B. guatemalense* was assessed through a
153 pot experiment under controlled conditions in the Laboratoire Sols et Environnement
154 (LSE) in Nancy, France (growth chamber at 25°C, 85% relative humidity and a 12 hr
155 daylength). Seeds were collected from one individual in the surroundings of the
156 Universidad Tecnológica de la Selva in Chiapas, Mexico (374 m. a.s.l. 16°49'21.2"N,
157 91°07'21.5"W) and germinated in pots using a mix of 50% compost and 50% native soil.

158 A well characterized soil from Champenoux, France was collected from the A horizon
159 (5-15 cm depth), air-dried, sieved to < 2 mm and 1 kg of each placed into 5 plastic bags.
160 The soils were dosed with Ni salt ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$): 0 (control), 20 (T1), 50 (T2), 100 (T3)
161 and 250 (T4) mg Ni kg^{-1} , mixed in the plastic bags to avoid Ni losses and then placed in
162 1kg pots. Plant seedlings of about 2.5 cm height were carefully transplanted into each pot
163 with 3 replicates per treatment, using a fully-randomized design. Plants were watered
164 daily to 80% of the soil water-holding capacity with deionized water and harvested after
165 three months.

166 *Nutrient dosing trial*

167 The response of *B. guatemalense* to synthetic fertilization was evaluated in a pot
168 experiment conducted in a well-ventilated greenhouse at the Universidad Tecnológica de
169 la Selva, Mexico. Soils were collected from the A horizon (5–15 cm depth) at the native
170 habitat, air-dried and sieved to 2 mm. Forty 8-month-old seedlings obtained from seeds
171 mentioned in the Ni dosing trial were carefully transplanted into 1 kg pots. The seedlings
172 were allowed to establish for one month and then assigned to the treatments using a fully-
173 randomized design. Three doses of macronutrients nitrogen, phosphorus and potassium
174 (NPK) and one control with ten replicates each were tested: i) 0:0:0 ii) 50:40:40; iii)
175 100:80:80 and iv) 120:120:120 kg ha^{-1} . Fertilizers used were urea (46:0:0) and a
176 granulated mixture of NPK (17:17:17) divided into two doses, the first one applied
177 immediately after transplanting, and the second a month later to avoid losses by leaching.
178 An organic treatment of 100 g of vermicompost per pot was also tested. Plants were
179 watered daily with local tap water over a period of 5 months after which they were
180 harvested.

181 *Bulk analysis of harvested plants*

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

192 ***Bulk analysis of soil samples***

193 Soil samples were dried at 70°C for 3 days in a drying oven and sieved to < 2 mm.
194 Subsamples of 0.5 g (± 0.001 g) were finely ground (< 250 µm) and digested by adding
195 6 mL of 37% HCl and 2 mL of 70% HNO₃ per sample. After allowing to stand for 24 hrs,
196 digests were heated for 3 hrs at 100°C in a block system (*DigiPREP MS*), and then left
197 to cool down to a minimum temperature of 40°C. Solutions were adjusted in volume to
198 45 mL with ultrapure water, filtered < 0.45 µm, and then readjusted to 50 mL. A final
199 dilution of 1:20 was necessary for major elements. The resulting soil digests/extracts were
200 analyzed by ICP-AES for the quantification of major and minor elements. Trace element
201 availabilities in soil samples were also determined by ICP-AES using
202 diethylenetriaminepentaacetic acid (DTPA-extractant) according to the method of
203 Lindsay and Norvell (1978). Cation exchange capacity (CEC) of soils was determined
204 using cobalt-hexamine trichloride extraction by adding 50 mL of solution (4.458 g of
205 Co(NH₃)₆Cl₃ with 700 mL of distilled water). After 1 hr shaking, 10 ml of each sample
206 was filtered at 0.45 µm and measured by spectrophotometrically at a wavelength of 475

207 nm (BioRad, SmartSpec Plus). Available phosphorus (P) was determined according to
208 the Olsen method (Olsen et al., 1954; ISO 11263, 1994). Soil pH was measured with in a
209 1:5 soil:water (5 mg soil with 25 mL ultrapure water) suspension after 1 hr shaking at 16
210 rpm and 1 hr standing.

211 *Harvesting schedule/ nickel yield estimation*

212 The potential biomass and Ni yields were estimated by means of a one-year field
213 experiment. The experimental site was a pastureland, formerly a tropical rainforest
214 located 10 km northeast of Bonampak archaeological site in Chiapas, Mexico
215 (16°44'20.4"N, 90°59'46.2"W). The site was selected due to the high DTPA-available Ni
216 in the soils (Navarrete Gutiérrez et al., 2021), and the presence of many 5-8-year-old trees
217 of *B. guatemalense* distributed in patches of > 8 individuals. Three plots of 15 m x 15 m
218 were delimited, and 6 five-year-old trees (diameter at 1.3 m height > 20 cm) were selected
219 per plot, identified, measured and coppiced at a height of 1.5 m. Prior to harvesting, soil
220 (5–10, 10–25, 25–30 cm depths) and plant samples (three leaves per tree) were collected
221 from each plot for elemental analysis. Total and DTPA-available Ni concentrations in
222 soils as well as foliar Ni concentrations of selected trees are shown in **Table 1**. Two
223 harvesting frequency scenarios were tested with 9 replicates each, one and two times a
224 year, harvesting only leaves and branches. Growth (height) of coppiced trees was
225 measured every 15 days during the first 6 months. In addition, an 8-year-old tree was
226 coppiced to a height of 35 cm to consider a third scenario with a harvesting of the whole
227 biomass (leaves, branches, fruits, flowers and bark) after 8-years cropping. Fresh
228 harvested biomass was separated into the different fractions and weighed. Dried biomass
229 was re-weighed after a two-week period of sun-drying. Leaf samples of each tree were
230 taken for chemical analysis.

231 *Statistical analyses*

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

240

241

242 **Results**

243 *Nickel dosing trial*

244 Nickel concentrations in the leaves of *B. guatemalense* increased significantly with
245 increasing DTPA-available Ni concentrations (Kruskal-Wallis test, $p < 0.05$), with no
246 significant decrease in shoot (leaves and stem) biomass production (ANOVA, Newman-
247 Keuls test, $p > 0.9$) (**Figure 1**). Nickel concentrations in the leaves of plants from the T4
248 treatment were almost 300-fold higher than in the control. There were no significant
249 differences in plant growth (height) and leaf count among treatments (ANOVA,
250 Newman-Keuls, $p > 0.8$). Nickel concentrations in stems and roots of the plant increased
251 significantly and steadily with Ni addition (**Figure 2**). Nickel accumulation in primary
252 roots was not significantly different from that found in secondary roots (t-test, $p > 0.9$;
253 data transformed to \log_{10}). The bioconcentration factor (BAF = ratio of the metal
254 concentration in leaves to the total Ni concentration in soils, (Mackay and Fraser, 2000))
255 of the T4 treatment (180), was 70-fold higher than that in the control (**Figure 3**). The
256 translocation factor (TF = ratio of the Ni concentration in leaves to that in roots, (Mattina
257 et al., 2003)) of the T4 treatment also showed an 8-fold increase (3.3) compared to the
258 control.

259 *Nutrient dosing trial*

260 The effect of the different NPK additions on shoot dry biomass, plant growth and Ni
261 concentrations in the different parts of *B. guatemalense* are shown in **Figure 4**. Although
262 no significant differences were observed in the shoot biomass and in the plant height
263 among treatments (ANOVA. Newman-Keuls, $p > 0.05$) (**Figures 4a, b**), there was a two-
264 fold increase in the number of leaves in the 120:120:120 treatment compared to the
265 control (t-test, $p < 0.05$). There was a slight decrease in Ni concentrations in the leaf

266 fraction of the plants in the 50:40:40 treatment with respect to the control, but it was not
267 statistically significant (Mann-Whitney test, $p > 0.6$). Nickel concentrations in leaves,
268 stems and roots were significantly higher in the 100:80:80 and 120:120:120 treatments
269 with respect to the control (**Figures 4c, d, e**). There was a three-fold increase in the
270 average foliar Ni concentration in the 120:120:120 treatment compared to that in the
271 control. The shoot Ni yields were significantly different among treatments with an almost
272 five-fold increase for the 120:120:120 treatment with respect to the control (Kruskal-
273 Wallis test – Conover-Iman test, $p < 0.05$).

274 Concentrations of elements in plant leaves in response to the nutrient dosing are shown
275 in **Table 2**. Most of the elements did not show any significant difference among
276 treatments, except for magnesium (Mg), iron (Fe), manganese (Mn) and zinc (Zn).
277 However, the differences in Mn and Zn foliar concentrations between the 120:120:120
278 treatment and the control were not significant (Mann-Whitney test, $p > 0.3$). However, in
279 contrast the foliar Mg and Fe concentrations in the 120:120:120 treatment were
280 significantly lower compared to the control (Mann-Whitney test, $p < 0.05$). There were
281 significant differences in the foliar nitrogen (N) concentration within treatments although
282 they did not show an upward trend. No significant differences were found in foliar P and
283 K concentrations in the 120:120:120 treatment with respect to the control (Mann-Whitney
284 test, $p > 0.05$).

285 The chemical properties of soils (before and after the experiment) are shown in **Table 3**.
286 DTPA-extractable Ni did not significantly change with nutrient addition (ANOVA,
287 Newman-Keuls test, $p > 0.05$). A slight increase in total and extractable K and P
288 concentrations in soils due to the NPK addition was observed. Total N concentrations did
289 not significantly change within treatments (ANOVA, Newman-Keuls test, $p > 0.05$). A
290 slight increase in the total concentrations of Ni, Mg and Fe was observed.

291 The shoot biomass (leaves and stems) and growth (height) significantly increased in
292 response to vermicompost amendment (t-test, $p < 0.05$). Foliar biomass was three-fold
293 higher than that of the control. However, the Ni concentrations in leaves and roots did not
294 significantly change with respect to the control (t-test, $p > 0.05$), but decreased
295 significantly in the stem fraction (**Figure 5**). The overall shoot Ni yield for the organic
296 treatment was not significantly different to that of the control (t-test, $p > 0.1$), but
297 significantly lower with respect to the NPK 120:120:120 treatment (Mann-Whitney test,
298 $p < 0.05$). Foliar elemental concentrations in both the organic and control treatments are
299 shown in **Table 4**. There was a significant increase in the K concentration in leaves in the
300 organic treatment compared to that in the control (ANOVA, Newman-Keuls test, $p <$
301 0.05). Iron and Mn concentrations were significantly lower in the organic treatment than
302 in the control (Mann-Whitney test, $p < 0.05$). There was no significant effect on DTPA-
303 extractable Ni concentrations in soils after vermicompost addition (t-test, $p > 0.7$).

304 *Harvesting schedule*

305 The foliar biomass resulting from the harvesting frequency test is shown in **Table 5**.
306 There was a significant difference between the annual average yield per tree produced by
307 the two scenarios (t-test, $p < 0.05$). Harvesting twice per year produced three-fold more
308 dry leaf biomass than only harvesting once per year. For the twice per year harvesting
309 scenario, there was no significant difference between the pre-harvest average leaf biomass
310 (3.2 kg tree^{-1}) and that obtained the year after (3.4 kg tree^{-1}) (t-test, $p > 0.05$). In contrast,
311 the once per year frequency scenario had a significant reduction of about 30% in the
312 average leaf biomass produced in the final harvest (1.0 kg tree^{-1}) with respect to the pre-
313 harvest (3.5 kg tree^{-1}) (t-test, $p < 0.05$). The once per year harvesting scenario had a
314 significantly higher dry biomass of the branches (3.1 kg tree^{-1}) compared to that obtained
315 from the twice per year harvesting regime (1.4 kg tree^{-1}) (t-test, $p < 0.05$). The coppiced

316 trees had an average height of 1.20 m after 6 months with no significant differences within
317 the three plots (ANOVA, Newman-Keuls test, $p > 0.05$).

318 The third scenario which was of an 8-year crop followed by the harvesting of the whole
319 biomass including all the parts of the tree (leaves, trunk, branches, flowers and fruit
320 capsules) had a total Ni phytoextraction yield of 154 g per tree. The part that most
321 contributed to the Ni yield was the foliar biomass followed by the trunk and the branches
322 (**Table 6**). Assuming a density of 2500 8-year old trees per hectare, it could be possible
323 to obtain a Ni yield of 240 kg ha⁻¹ when harvesting only the foliar biomass and 385 kg
324 ha⁻¹ when considering the whole biomass.

325 **Discussion**

326 *Agronomy of Blepharidium guatemalense*

327 The ability of *B. guatemalense* to take up Ni from soils is strongly dependent on the
328 DTPA-extractable Ni in soils. The latter is an approximation of the phytoavailable (*i.e.*
329 isotopically-exchangeable or labile) Ni pool in soil (Echevarria et al., 1998; Massoura et
330 al., 2004). These two variables were previously reported to be positively correlated
331 (Pearson, $r = 0.5$) from foliar and rhizosphere soil of *B. guatemalense* samples collected
332 in the field (Navarrete Gutiérrez et al., 2021). Although Ni uptake and Ni concentrations
333 in the soil solution are generally correlated, many other soil and plant factors can also
334 influence plant Ni uptake such as soil pH (Kabata-Pendias and Pendias, 2001). The
335 maximum Ni accumulation of *B. guatemalense* could not be attained in this study since
336 the maximum concentration in leaves was 3.5 wt% at a DTPA-available Ni of 17 mg kg⁻¹
337 resulting from the highest Ni treatment (addition of 250 mg Ni kg⁻¹). In its natural
338 habitat, *B. guatemalense* is able to accumulate 4.3 wt% Ni and 3.3 wt% Ni from soils
339 with 190 mg kg⁻¹ and 460 mg kg⁻¹ DTPA-available Ni, respectively (Navarrete et al 2020,

340 submitted). Thus, the Ni tolerance peak could be 4–5 wt% Ni at 200–300 mg kg⁻¹
341 available Ni in soils. *Blepharidium guatemalense* has a TF >1 when growing in soils with
342 DTPA-available Ni > 2.0 mg kg⁻¹, which is indicative of its effectiveness to translocate
343 Ni from roots to leaves (Baker and Brooks, 1989).

344 ***NPK and organic fertilization***

345 *Blepharidium guatemalense* responded strongly to synthetic fertilization with a three-fold
346 increase in foliar Ni concentrations. This surprising result contrasts with most previous
347 research which reports a negligible effect in shoot Ni concentrations in Ni
348 hyperaccumulators after mineral fertilization (Shallari et al., 2001; Kidd and Monterroso,
349 2005; Bani et al., 2015a; Nkrumah et al., 2019a). However, this increase on the shoot Ni
350 concentrations has been reported in *B. coddii* with a two-fold increase as a response to N
351 additions (B. H. Robinson et al., 1997). Magnesium and Fe concentrations in plant leaves
352 from the three NPK treatments significantly decreased compared to the non-treated ones
353 but they were within normal ranges for plant growth (Campbell et al., 2012). Shoot
354 biomass of *B. guatemalense* did not significantly change upon NPK additions. There is
355 usually an increase in shoot biomass of Ni hyperaccumulators in response to synthetic
356 fertilization growing in ultramafic substrates (*e.g.* *P. rufuschaneyi* (Nkrumah et al.,
357 2019a), and *Odontarrhena* spp. (Bani et al., 2015a; Álvarez-López et al., 2016)).

358 Soils from this region in Chiapas and from other humid-tropical environments in south-
359 eastern Mexico are mostly calcareous (Mendoza-Vega and Messing, 2005). Moreover,
360 these soils are not generally deficient in essential macronutrients as are many ultramafic
361 soils (Proctor and Woodell, 1975), which could partly explain the neutral response in
362 growth of *B. guatemalense* to synthetic fertilization. However, the available P is relatively
363 low in the soils in Chiapas (9.4 mg kg⁻¹) (Navarrete et al., submitted), which is a common

364 feature in this type of limestone calcareous soils where P is probably adsorbed by calcium
365 carbonate or precipitated as calcium phosphate (von Wandruszka, 2006; Falkowski et al.,
366 2016). Synthetic fertilization is therefore recommended to increase P availability in soils,
367 at least to compensate for plant nutrient uptake over time. Split NPK fertilizer application
368 is recommended to minimize N leaching to the groundwater (Kidd et al., 2015; Nkrumah
369 et al., 2018b). Further experiments should be conducted to evaluate the effect of each
370 nutrient separately, as has been undertaken in Sabah (Nkrumah et al. 2019b, c, d).
371 Considering the large amounts of Ca taken up by *B. guatemalense*, amendments of this
372 element must be made in order to avoid soil depletion over time (Bani et al., 2015a).

373 Organic fertilization (vermicompost) increased the foliar biomass of *B. guatemalense*
374 three-fold, with no significant reduction in the foliar Ni concentrations. Similarly, higher
375 total Ni yields with composted sewage sludge compared to NPK fertilization have been
376 obtained due to a substantial increase of the biomass in some species of *Odontarrhena*
377 (Álvarez-López et al., 2016). Biochar amendments increased *O. muralis* growth by
378 improving the fertility of ultramafic and constructed soils (Rue et al., 2019). On the other
379 hand, manure compost did not show any positive effect on the biomass of the Ni
380 hyperaccumulator *O. chalcidica* (Broadhurst and Chaney 2016). Fertilization of *B.*
381 *guatemalense* using vermicompost could be preferred to NPK fertilization when no
382 increase in foliar Ni concentration is needed or when soils are not nutrient deficient. There
383 is evidence that organic matter amendments enhance the growth of ‘metal crops’ by
384 improving soil drainage and water holding capacity (Angle et al., 2003; Nkrumah et al.,
385 2016, 2018b). In Ni-rich ultramafic soils, organic matter reduces phytoavailable Ni
386 concentrations and may lead to increased or decreased Ni yield depending on shoot Ni
387 concentrations which may depend on soil Ni availability (Bani and Echevarria 2019;

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

390 *Harvesting scenarios*

391 A natural tree density of 2500 trees per hectare was calculated by measuring the canopy
392 of 15 *B. guatemalense* adult trees (diameter at 1.3 m height > 15 cm) and the distances
393 between them in three different fallow fields with a high tree density in Chiapas (Mexico).
394 The mean Ni concentration in leaves from the trees at the end of our experiment was 16.7
395 g kg⁻¹. Thus, the Ni phytoextraction yield from the foliar biomass could be 43 kg ha⁻¹ yr⁻¹
396 ¹ when harvesting once a year and 142 kg ha⁻¹ yr⁻¹ when harvesting biannually. In the case
397 of combined leaves and branches, it could be possible to obtain 10.3 t and 12 t ha⁻¹ yr⁻¹ of
398 dry biomass, corresponding to 68 and 154 kg ha⁻¹ yr⁻¹ Ni yields from the once per year
399 and twice per year harvesting scenarios, respectively. Finally, the third scenario (8-year
400 crop followed by the harvesting of the whole biomass) would generate a Ni yield of 193
401 kg ha⁻¹ when harvesting only the foliar biomass and 385 kg ha⁻¹ when considering the
402 whole biomass. Nevertheless, *B. guatemalense* trees can be harvested from the 5th year of
403 culture and still obtain high Ni phytoextraction yields Harvesting twice per year in
404 monoculture is therefore the best regime for profitable Ni agromining in this context.
405 However, the foliar Ni concentrations of *B. guatemalense* in the experimental site (16.7
406 g kg⁻¹) were above its previously reported average Ni concentration (13.5 g kg⁻¹, *n* = 78)
407 (Navarrete et al., submitted) and may not be representative. Notwithstanding, most
408 pastures in the region have similar available soil Ni concentrations and *B. guatemalense*
409 foliar Ni concentrations, hence, Ni yields within a range of 23–364 kg Ni ha⁻¹ (mean of
410 115 kg Ni ha⁻¹) could be attained considering foliar Ni concentrations of 0.3–4.3 wt% Ni.
411 Comparable high Ni yields of 250 kg ha⁻¹ have been estimated for large-scale tropical
412 agromining using *P. rufuschaneyi* in Sabah, Malaysia (Nkrumah et al., 2019d).

413 The pastureland used for the harvesting trial had relatively higher total and DTPA-
414 available Ni concentrations in the superficial soil (5–10 cm) compared to that in deeper
415 soil (10–30 cm). Clearly, Ni-rich litter from *B. guatemalense* trees seems to be
416 substantially contributing to the total and available Ni concentrations in the topsoil as it
417 was observed for *O. muralis* in Albania and other Ni hyperaccumulator species (Estrade
418 et al., 2015; Echevarria, 2018). A detailed biogeochemical investigation is required to
419 assess recycling of Ni through Ni-rich leaf litter degradation and the impact of Ni-rich
420 biomass harvesting in an agromining operation on the Ni biogeochemistry.

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

431 *Potential sites for agromining*

432 The selection of potential sites is of great importance for the success of Ni agromining
433 (Nkrumah et al., 2018b). A high phytoavailable Ni concentration is always a desirable
434 property of soils for effective Ni agromining (Nkrumah et al. 2016). Considering the
435 strong response of *B. guatemalense* to Ni additions, a high Ni phytoavailability in soils is
436 key to obtaining optimum yields for economic Ni agromining (Massoura et al., 2004).

437 Soils in the natural habitat of *B. guatemalense* in southeastern Mexico (Chiapas and
438 Tabasco) are not ultramafic but are rather derived from limestone sedimentary rocks
439 (lutite, limonite) (Müllerried, 1957; SGM, 2017). Unpublished data from recent field
440 surveys revealed total Ni concentrations range from 30–1600 mg kg⁻¹ in Chiapas and 200–
441 500 mg kg⁻¹ in Tabasco, relatively lower than that in ultramafic soils (Kabata-Pendias and
442 Pendias, 2001). The origin of Ni is uncertain but it could be related to volcanic ash
443 deposition from the nearest volcano (*i.e.* El Chichón) (Hernández-Quiroz et al., 2012).
444 Our unpublished data indicates that Ni in Chiapas is found in ferromagnesian clays,
445 mainly associated with Mn oxides. The average phytoavailable Ni in Chiapas is relatively
446 high (50 mg kg⁻¹ DPTA-Ni), reaching 200 mg kg⁻¹ DPTA-Ni in some cattle farms with a
447 high a density of *B. guatemalense* trees. Thus, a resource 415 kg Ni ha⁻¹ is estimated
448 assuming one hectare of soil with an average DTPA-available Ni of 50 mg kg⁻¹ to a depth
449 of 1 m and a bulk density of 0.83 g cm⁻³. It is expected that the subsequent cropping of *B.*
450 *guatemalense* would progressively decrease Ni toxicity in soils through the reduction of
451 the phytoavailable Ni pool in soils (Morel, 2012). Therefore, the above-mentioned Ni
452 resource in soils per hectare would be completely depleted in 3 years of culture
453 considering an annual yield of 142 kg Ni yr⁻¹ after which the site will be suitable for other
454 types of cropping. Research is currently underway to better understand the role of Ni
455 hyperaccumulators in the geochemical cycle of Ni and the replenishment of Ni labile
456 pools in soils over time (Echevarria, 2018; Ratié et al., 2019). Intensive geochemical
457 investigations are needed to determine the total extent of Ni-enriched substrates and to
458 select the most suitable sites for Ni agromining in southeastern Mexico.

459 *Local adaptation of Ni agromining into existing agroforestry systems*

460 The Ni yields mentioned above are expected under a monocropping regime in open
461 pastures or degraded/abandoned land with low food crop productivity. However, *B.*

462 *guatemalense* is currently part of some agroforestry systems from which it could currently
463 be harnessed. For example, it is commonly found in backyard gardens of Tabasco,
464 Yucatan and Campeche (Mariaca Méndez, 2012; van der Wal et al., 2011) as well as in
465 silvo-pastoral systems in Chiapas and Tabasco as dispersed trees (Grande et al., 2010;
466 Pignataro et al., 2016, 2017). In eastern Chiapas and in Izabal (Guatemala), the species is
467 used in live fences for cattle pastures (Ramírez-Marcial et al., 2012) (**Figure 6f**). Indeed,
468 *B. guatemalense* is used by local communities due to the hardness of its wood which they
469 use for building houses or for sale in the local market (Standley, 1918; Jimenez-Ferrer et
470 al., 2008; Marinidou and Jimenez-Ferrer, 2010) (**Figure 6g**). Considering a scenario
471 where *B. guatemalense* is used as live fencing in pasturelands (232 trees ha⁻¹, 13.5 g Ni
472 kg⁻¹ in leaves, 2 harvest per year), a yield of 11 kg Ni ha⁻¹ yr⁻¹ could be expected. The
473 economic benefit may seem less than in monoculture, but it will depend on the number
474 of hectares under this system and it would also represent an extra income for local
475 farmers. Future studies must investigate how Ni agromining could be properly integrated
476 into the existing local agroforestry systems.

477

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489 **Declaration of interest statement**

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745 matter on its mobility. *Geochem. Trans.* 7, 6. <https://doi.org/10.1186/1467-4866-7-6>
746

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

750
 751

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	

752

753 Table 2. Elemental concentrations in leaves ($\mu\text{g g}^{-1}$) of *B. guatemalense* under different synthetic fertilization treatments. Values followed by the
 754 different letters are significantly different (p value < 0.05) according to Kruskal-Wallis test. Nitrogen values are given as a percentage (%)
 755 (ANOVA Newman-Keuls test, p < 0.05).
 756
 757

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

758

759 Table 3. Elemental contents in soil prior to fertilization treatment application (PS), and in soils under the different treatments at the end of the
 760 experiment. Available P was obtained through Olsen method.
 761

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		

762

763 Table 4. Elemental concentrations in leaves ($\mu\text{g g}^{-1}$) of *B. guatemalense* under organic fertilization. Values followed by the different letters are
 764 significantly different (p value < 0.05) according to ANOVA Newman-Keuls test. Values followed by different symbols mean significant
 765 differences between the position according to Mann-Whitney test (p value < 0.05).
 766

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

767 Table 5. Biomass values refer to dry weight (* average value, $n = 9$). Different letters express
 768 significant differences (ANOVA, Newman-Keuls test after data transformation to log, $p <$
 769 0.001). Mean Ni concentration in leaves from trees in the experimental site was of 16.7 g kg^{-1} .
 770 Leaf biomass yield was calculated for a density of 2500 trees per hectare.
 771

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

772
 773

774 Table 6. Biomass obtained from an eight-year-old tree of *B. guatemalense* in a cattle pasture.
 775 (*) Mean Ni concentration values obtained from field samples (number of samples $n > 9$)
 776 (Navarrete et al. 2020, submitted), except for the trunk ($n = 1$).
 777

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	0.3	0.60	4.36	2.6
Branches	25	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>

778

779

780 **Figure captions**

781 Figure 1. Nickel concentrations in leaves and biomass production of *B. guatemalense* under
782 different doses of Ni in soils. Nickel concentrations in leaves with different letters indicate
783 significant differences according to Kruskal-Wallis test ($\alpha = 0.05$). For shoot biomass,
784 same letters indicate no significant differences according to ANOVA, Newman-Keuls test,
785 ($\alpha = 0.05$).

786 Figure 2. Nickel concentrations in the different parts of *B. guatemalense* under different Ni
787 additions to soils. Values with different letters indicate significant differences according to
788 Kruskal-Wallis test ($\alpha = 0.05$, Conover-Iman as post-hoc test).

789 Figure 3. Bioconcentration and translocation factors of *B. guatemalense* under different Ni
790 doses in soils.

791 Figure 4. Response of *B. guatemalense* to synthetic fertilization after 5-months pot experiment.
792 For a) biomass and b) height, same letters represent no significant differences (ANOVA,
793 Newman-Keuls test, $\alpha = 0.05$). For Ni concentrations in leaves, stems and roots (c, d, e),
794 significant differences are indicated by different letters (Kruskal-Wallis test, $\alpha = 0.05$).

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

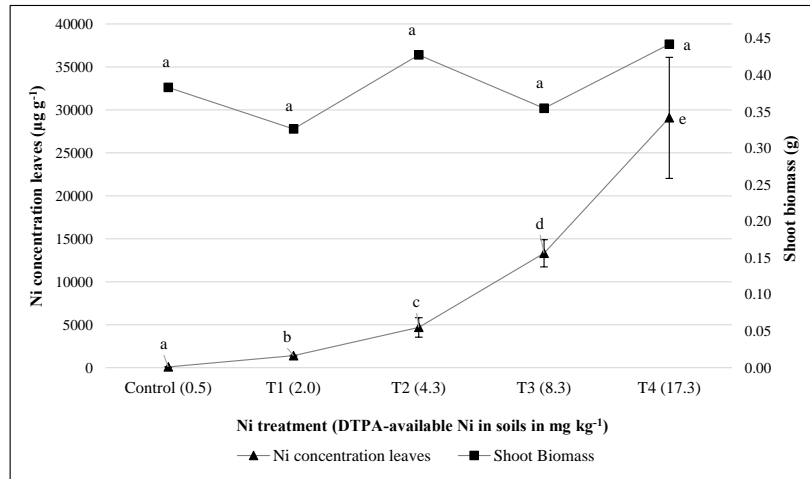
798 Figure 6. Details of a), b), c) and e) different harvesting frequencies of *B. guatemalense* tested
799 in the field in Chiapas, Mexico, d) green Ni-rich phloem in the bark, f) trees used as living
800 fences in Izabal, Guatemala, and g) trunks used as house roofs in Tabasco, Mexico.

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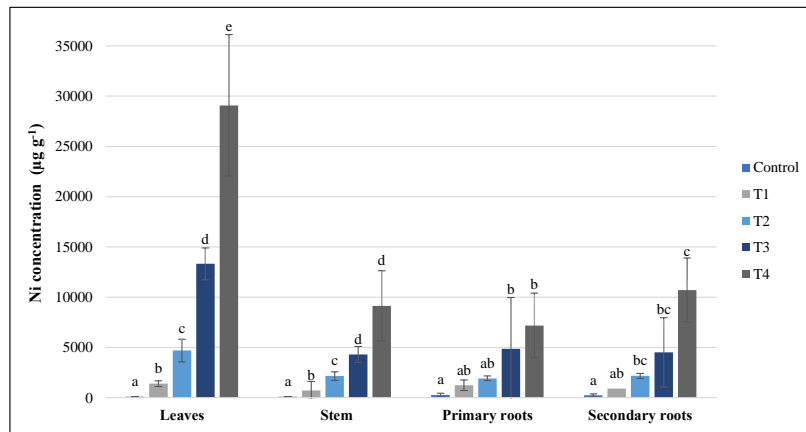
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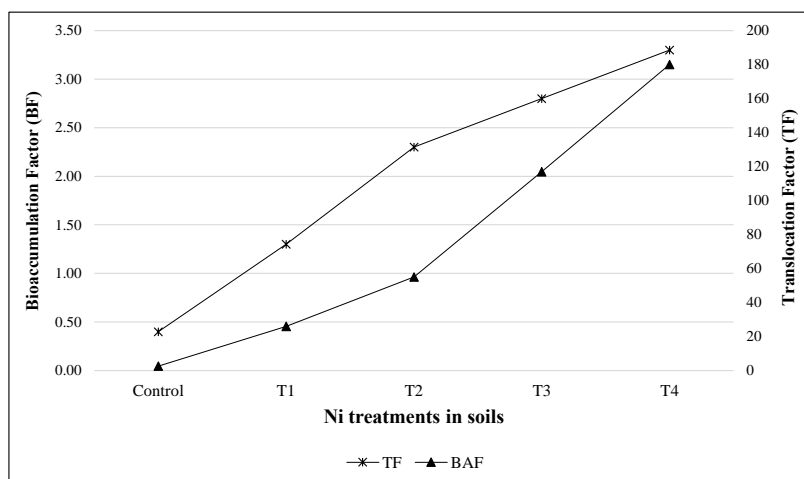
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805 **Figure 1**

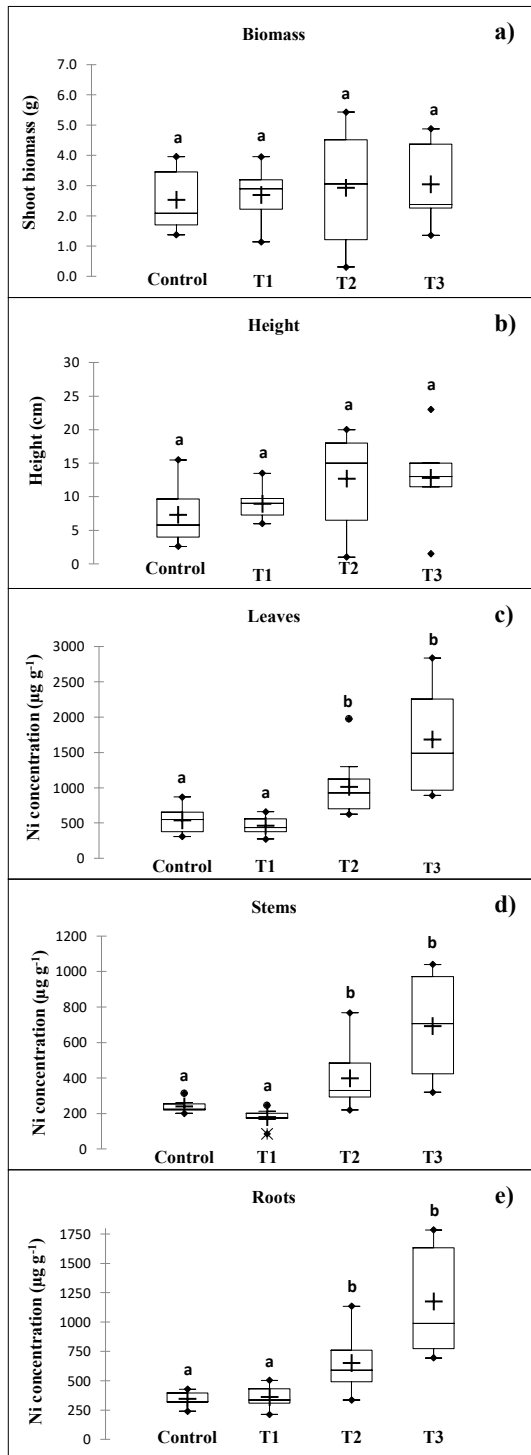


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807 **Figure 2**
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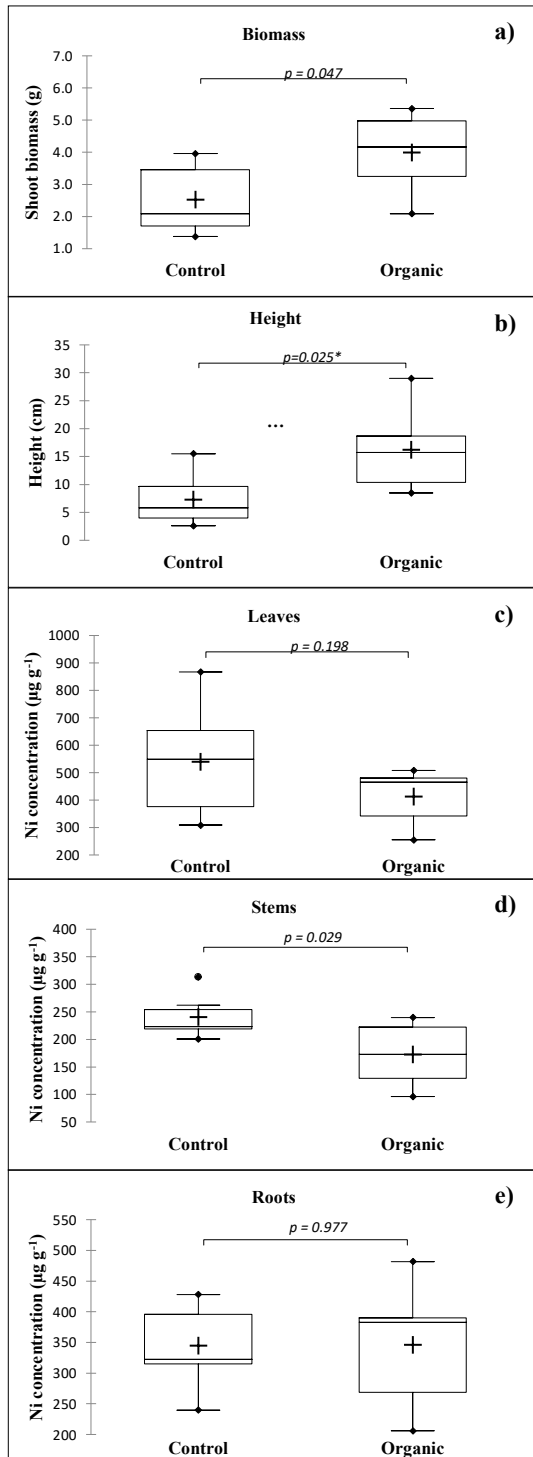


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810 **Figure 3**





811
812 **Figure 4**



813
814 **Figure 5**



815
816 **Figure 6**