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# Spatially-resolved localization and chemical speciation of nickel and zinc in *Noccaea tymphaea* and *Bornmuellera emarginata*

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1 **Spatially-resolved localization and chemical speciation of nickel**  
2 **and zinc in *Noccaea tymphaea* and *Bornmuellera emarginata***  
3  
4

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27 **ABSTRACT**

28 Hyperaccumulator plants present the ideal model system for studying the physiological regulation  
29 of the essential (and potentially toxic) transition elements nickel and zinc. This study used  
30 synchrotron X-ray Fluorescence Microscopy (XFM) elemental imaging and spatially resolved X-  
31 ray Absorption Spectroscopy (XAS) to elucidate elemental localization and chemical speciation of  
32 nickel and zinc in the hyperaccumulators *Noccaea tymphaea* and *Bornmuellera emarginata*. It turns  
33 out that in the leaves of *N. tymphaea* nickel and zinc have contrasting localization, and whereas  
34 nickel is present in vacuoles of epidermal cells, zinc occurs mainly in the mesophyll cells. In the  
35 seeds Ni and Zn are similarly localized and strongly enriched in the cotyledons in *N. tymphaea*. Ni  
36 is strongly enriched in the tip of the radicle of *B. emarginata*. *Noccaea tymphaea* has an Fe-rich  
37 provascular strand network in the cotyledons of the seed. The chemical speciation of Ni in the intact  
38 seeds of *N. tymphaea* is unequivocally associated with carboxylic acids, whereas Zn is present as  
39 the phytate species. The spatially resolved spectroscopy did not reveal any spatial variation in  
40 chemical speciation of Ni and Zn within the *N. tymphaea* seed. The dissimilar ecophysiological  
41 behaviour of Ni and Zn in *N. tymphaea* and *B. emarginata* raises questions about the evolution of  
42 hyperaccumulation in these species.

43  
44 **Key words:** *capsule, cotyledons; hyperaccumulator; seed; translocation.*

45

46 **1. INTRODUCTION**

47

48 Hyperaccumulators are unusual plants that accumulate particular metals or metalloids in their living  
49 tissues to levels that may be hundreds to thousands of times greater than is normal for most  
50 plants<sup>1,2</sup>. Hyperaccumulator plants have the unique ability to take up and detoxify exceptional  
51 concentrations of metals without any signs of toxicity. Plants have been found to hyperaccumulate a  
52 wide range of elements, including nickel (Ni), manganese (Mn), cadmium (Cd), copper (Cu), cobalt  
53 (Co), selenium (Se), arsenic (As), thallium (Tl) and Zn<sup>2,3</sup>. This contrasts with 'normal plants' that  
54 have a tightly controlled regulation of essential transition elements (Cu, Fe, Ni, Mn, Zn) to avoid  
55 either deficiency or toxicity. Hyperaccumulator plants represent the most extreme example of  
56 adaptation to a surplus of metal transition elements in their environment, and are therefore ideal  
57 model systems for understanding the physiological regulation of essential and potentially toxic,  
58 non-essential transition elements<sup>4,5,6</sup>. In many Ni hyperaccumulator species, Ni occurs in mixtures  
59 of citrate and malate complexes that vary in different parts of the plants<sup>7,8,9</sup>. Hyperaccumulation  
60 results from adaptations of the metal regulation mechanisms shared by all higher plants<sup>10</sup>. Hence,  
61 insights into the mechanisms of hyperaccumulation may be applied to improve the uptake and  
62 accumulation of deficient elements, such as iron (Fe) and zinc (Zn), in economically important food  
63 crops. These insights may also be applied to limit uptake of potentially phytotoxic elements, such as  
64 nickel (Ni) in food crops. The extreme metal accumulation capability of hyperaccumulator plants  
65 spawned the concept of phytoextraction for remediating contaminated soils, which has attracted  
66 much research effort<sup>11</sup>. Hyperaccumulator plants also have potential for use in phytotechnologies  
67 such as biofortification, phytoremediation and phytomining, the latter utilizes hyperaccumulators as  
68 'metal crops' to sequester Ni or other metals in harvestable biomass that can then be used to  
69 produce fine Ni chemicals<sup>12,13</sup>.

70

71 Nickel is the most recent element shown to be essential for higher plants<sup>14,15</sup>, due to its key role for  
72 the activity of urease, an enzyme widely distributed in higher plants<sup>16</sup>, and playing a crucial role in  
73 nitrogen remobilization from senescing leaves and during seed germination. However, excess Ni  
74 induces oxidative and genotoxic stresses that are deleterious to plant growth<sup>17</sup>. Therefore, every  
75 plant species needs to regulate Ni homeostasis according to its needs. Apart from its function in  
76 urease activation, other physiological functions of Ni are poorly understood in higher plants<sup>6,18</sup>.  
77 Although Ni is an essential micronutrient, its physiological requirement is extremely low. It is  
78 shown that 0.1 mg Ni kg<sup>-1</sup> is sufficient for seed germination and plant growth<sup>15,19</sup>. Hence, Ni  
79 deficiency in naturally-grown plants rarely occurs, and the only known case is for pecan<sup>20</sup>. The  
80 molecular mechanisms involved in the regulation of Ni homeostasis are not well known even in

81 model species such as *Arabidopsis thaliana*. Nickel can be transported from the soil and inside  
82 plants by several families of metal transporters (e.g. ZIP/IRT, IREG, YSL<sup>21,22,23</sup>). Since plants  
83 normally only require minute amounts of Ni, no Ni specific transporter has so far been identified to  
84 account for enhanced Ni uptake from soil. In non-accumulator species, this function is most likely  
85 performed by one of the ZIP family Zn/Fe/Mn uptake transporters e.g. the Zn-deficiency induced  
86 expression of AtZIP4 can be repressed by supplying Ni<sup>2+</sup>. Preliminary results suggest that one of  
87 these transporters has developed more affinity for Ni<sup>2+</sup> in Ni hyperaccumulators than in non-  
88 accumulators<sup>21,23</sup>. The transporter TgMTP1 of the CDF family was originally suspected to mediate  
89 Ni storage in the vacuole of the Ni hyperaccumulator *Noccaea goesingense*<sup>24</sup>. More recent studies  
90 suggested that transporters of the IREG/Ferroportin family, localized on the vacuolar membrane,  
91 are involved in the storage of Ni in non-accumulators and hyperaccumulators<sup>21,25</sup>. Studies on Zn  
92 and Cd hyperaccumulation in Brassicaceae species (e.g. *Arabidopsis halleri* and *N. caerulescens*)  
93 revealed that Zn and Cd hyperaccumulation traits are correlated with high and constitutive  
94 expression of genes involved in metal transport, in the biosynthesis of metal chelators and in  
95 cellular defences to oxidative stresses<sup>26,27,28</sup>. These changes in gene expression are often the  
96 combined effect of gene duplication and altered promoter activity<sup>29,30</sup>.

97  
98 The genus *Noccaea* has at least 23 species that hyperaccumulate Ni, a further 10 that  
99 hyperaccumulate Zn, three that hyperaccumulate Cd and one that hyperaccumulates Pb<sup>31,32,33,34,35</sup>.  
100 *Noccaea caerulescens* (J.Presl & C.Presl) F.K.Mey. (*Thlaspi caerulescens* J.Presl & C.Presl) is  
101 unique in consisting of different ecotypes with distinct metal tolerance and hyperaccumulation  
102 abilities<sup>36</sup>. While calamine, ultramafic and non-metallicolous populations can hyperaccumulate Zn  
103 and Ni, or Cd, when supplied<sup>36</sup>, they differ in their ability to tolerate these metals, often depending  
104 on the metal concentrations at their site of origin<sup>37,38</sup>. Zinc is taken up by ZIP-like plasma  
105 membrane located Zn-transporters. Rather than storing excess Zn in vacuoles of root cells, which  
106 most non-accumulator species do, the Zn<sup>2+</sup> is loaded into the xylem, by HMA4, as in *Arabidopsis*  
107 *halleri* (L.) O'Kane & Al-Shehbaz<sup>29</sup>, thus translocated to the leaves, where it is stored in mesophyll  
108 and epidermal vacuoles. Ultramafic populations are known<sup>39</sup> and converted into genetically  
109 homogeneous lines by recurrent inbreeding for characterization of Zn, Ni and Cd accumulation and  
110 tolerance properties. The variation in metal tolerance and hyperaccumulation is heritable and  
111 independent of each other<sup>40,41,42</sup>. The calamine and non-metallicolous populations have been  
112 investigated in more detail than the ultramafic populations. So far most of the analysis of ultramafic  
113 *N. caerulescens* involved the accession from Monte Prinzera, in the Italian Apennine mountain  
114 range<sup>36</sup>. This accession has been subject to several proteomics studies, trying to correlate local  
115 adaptation to specific protein expression<sup>44</sup> and was included in transcriptome studies as well<sup>45</sup>. Ni,

116 and also Zn, xylem loading in *N. caerulescens* is facilitated by high histidine levels<sup>43</sup> although the  
117 genes involved have not yet been identified.

118 *Noccaea tymphaea* (Hausskn.) F.K.Mey. (synonyms: *Thlaspi tymphaeum* Hausskn. and *Thlaspi*  
119 *goesingense* Halácsy) is distributed in Albania, Bosnia and Herzegovina, Greece, Macedonia. It  
120 occurs on montane ultramafic soils, where it can accumulate high foliar Ni (up to 11 800  $\mu\text{g g}^{-1}$ ) and  
121 relatively low foliar Zn (up to 179  $\mu\text{g g}^{-1}$ )<sup>31</sup>. *Bornmuellera emarginata* (Boiss.) Rešetnik  
122 (synonyms: *Leptoplax emarginata* (Boiss.) O. E. Schulz, *Peltaria emarginata* (Boiss.) Hausskn.)<sup>46</sup>  
123 is endemic to ultramafic soils in Greece, with a discontinuous distribution from Pindus mountains,  
124 Mt. Smolikas, and the island of Euboea. Some specimens were also sampled in Syria and are kept at  
125 Paris Herbarium (P) showing a disjunct distribution pattern across the Eastern Mediterranean. It is a  
126 strong Ni hyperaccumulator that can accumulate up to 34 400  $\mu\text{g g}^{-1}$  foliar Ni<sup>47</sup>.

127  
128 Currently little is known on storage and acquisition of Ni in seeds and during germination. In  
129 *Noccaea praecox* (Wulfen) F.K.Mey. (synonym: *Thlaspi praecox* Wulfen.) Cd was mobilised to the  
130 shoots during germination, but not to the roots<sup>48</sup>. Scanning electron microscopy with energy  
131 dispersive spectroscopy (SEM-EDS) was undertaken on the seeds of *Noccaea pindica* (Hausskn.)  
132 Holub (synonym of *Thlaspi pindicum* Hausskn.) and the results showed that Ni accumulated in the  
133 micropylar area opposite the radicle and in the epidermis of cotyledons<sup>49</sup>. Little information is  
134 available about the elemental distribution in other hyperaccumulating genera of the Brassicaceae,  
135 and no study yet has focussed on *B. emarginata* except the SEM-EDS observation of herbarium  
136 specimen air-dried leaves which showed accumulation of Ni in the epidermis cells except in the  
137 vicinity of stomata<sup>50</sup>. Knowledge about the ecophysiology of Ni and Zn in reproductive organs of  
138 hyperaccumulator plants is especially scarce. In order to provide a more general view about the  
139 ecophysiology of hyperaccumulation, including the reproduction organs and first phases of life of  
140 these plants, this study aimed to elucidate the distribution and chemical speciation of Ni and Zn in  
141 the seeds and siliques of *N. tymphaea* and *B. emarginata* originating from their native habitats in  
142 Greece. X-ray Fluorescence Microscopy (XFM) has substantial explanatory power for advancing  
143 the understanding of the ecophysiology of hyperaccumulation<sup>9</sup>. In order to determine the  
144 distribution and spatially-resolved chemical speciation of Ni and Zn in both species, we make use of  
145 the singular ability of the Maia detector system<sup>51,52</sup> to perform ultra-rapid X-ray elemental mapping  
146 and spatially resolved X-ray Absorption Spectroscopy (XAS) on live/fresh samples.

147

## 148 **2. MATERIALS AND METHODS**

149

### 150 **2.1 Collection of plant tissues and soils**

151 Whole, live plants of *N. tymphaea* were collected in Greece (at the Katara Pass, 39°48'00.5"N  
152 21°10'60.0"E, altitude 1690 m. a.s.l.) growing in natural ultramafic soils. Intact seeds capsules were  
153 collected from *B. emarginata* in their native habitat Greece (near Trigona, 39°47'29"N, 21°25'32"E,  
154 altitude 830 m. a.s.l.). The soils of the collection locality are described in detail elsewhere<sup>53</sup>. The  
155 plants were potted in natural soil from the habitat and brought alive to the P06 beamline (PETRA  
156 III Synchrotron, DESY Campus, Hamburg Germany) for the experiments described below.

157

## 158 **2.2 Chemical bulk analysis of tissue samples**

159 Plant tissue samples for bulk chemical analysis were first dried on silica gel and then dried at 70°C  
160 for five days in a drying oven. They were subsequently ground and digested using 4 mL HNO<sub>3</sub>  
161 (70%) in a microwave oven (Milestone Start D) for a 45-minute programme and diluted to 30 mL  
162 with ultrapure water (Millipore 18.2 MΩ·cm at 25°C). Finally, they were analysed with ICP-AES  
163 (Thermo iCAP 7400) for Cd, Ni, Co, Cr, Cu, Zn, Mn, Fe, Mg, Ca, Na, K, S, P.

164

## 165 **2.3 Preparation of tissue samples for X-ray fluorescence microscopy**

166 The seeds and seed capsules could be investigated in their native state without any sample  
167 preparation. The intact seeds were mounted between two sheets of Ultralene thin film (4 μm)  
168 stretched over a Perspex frame magnetically attached to the *x-y* motion stage at atmospheric  
169 temperature (~20°C). However, in order to reveal the internal distribution of Ni, Zn and other  
170 elements inside roots, stems and leaves, cross-sections were prepared. The samples were hand cut  
171 with a stainless-steel razor blade ('dry knife'), mounted between two sheets of 4 μm Ultralene thin  
172 film in a tight sandwich to limit evaporation, and analysed within 15 minutes after excision. X-ray  
173 micro-fluorescence was performed at high speed to keep the scan time to a minimum. Since the  
174 penetration depth of the X-rays is greater than the thickness of a cell layer, the information obtained  
175 from thick sections is a combined distribution originating from more or less superimposed cell  
176 layers. The semi-thick sections (~200 μm) correspond to 3–4 cell layers. As such the obtained data  
177 do not reveal subcellular distribution, but nevertheless show the tissue-level distribution (*e.g.*  
178 epidermal cells, mesophyll, vascular bundles, *etc.*).

179

## 180 **2.4 X-ray fluorescence microscopy**

181 The X-ray fluorescence microscopy (XFM) experiments were undertaken at beamline P06 at  
182 PETRA III at DESY (Deutsches Elektronen-Synchrotron). The undulator beam was  
183 monochromatised using either a Si(111) channel-cut crystal or a double-crystal monochromator,  
184 depending on beamline mode for each part of the experiment. A Kirkpatrick-Baez mirror pair was  
185 used to focus the incident beam. The X-ray flux of the focussed beam was in the order of 10<sup>10</sup>

186 photons/s<sup>54</sup>. X-ray fluorescence was detected using the Maia detector system in backscatter  
187 geometry<sup>52,55</sup>. The large solid-angle (1.2 steradian) of the Maia detector is particularly suited to  
188 biological samples such as these as it allows detection of a good proportion of the fluoresced signal,  
189 allowing a reduction of the radiation dose and thus reducing potential damage to a specimen<sup>51</sup>.

190

191 The 2D  $\mu$ XRF measurements carried out in the microprobe of P06 at DESY were performed with a  
192 beam size of  $720 \times 780$  nm at an incident energy of 11 keV. The single-slice tomography  
193 measurements of the *N. tympheae* seed were carried out with a beamsite of  $400 \times 450$  nm at a  
194 photon energy of 15 keV using the same beamline endstation and general setup. Scanning  
195 parameters were a step size of 2  $\mu$ m, a dwell time of 1 ms and an angular range of 452 projections  
196 covering  $360^\circ$  in 2 subscans. As the seed was naturally dehydrated a cryo-stream was not employed.  
197 For the XRF 2D and tomography scans, the pixel size chosen was larger than the focused beam size  
198 as a result of necessary compromises due to time constraints.

199

## 200 **2.5 Synchrotron X-ray Absorption Spectroscopy (XAS)**

201 Ni and Zn K-edge XAS spectra of the plant tissue samples and standards were recorded in  
202 fluorescence mode with the Maia detector. The X-ray beam energy was calibrated using either a Ni  
203 or Zn metal foil recorded in transmission, where the first peak of the first derivative was assumed to  
204 be 8333 or 9659 eV, respectively. The seeds ( $\sim 1.5 \times 0.3$  mm) were scanned at 1.6  $\mu$ m pixels with a  
205 20 ms per pixel dwell time. In addition to these  $\mu$ XRF elemental images on these seeds, spatially  
206 resolved XANES spectra were collected as image ‘stacks’ of  $\mu$ XRF maps, each with 15  $\mu$ m pixels  
207 and a 12 ms per pixel dwell time, at 170 increasing energies, spanning the energy range 8183–9082  
208 eV over the Ni K-edge at 8.333 keV, and spanning the energy range 9486–9858 eV over the Zn K-  
209 edge at 9659 eV.

210

211 Several Ni<sup>2+</sup> and Zn<sup>2+</sup> standards were prepared by adding organic ligands in calculated molar excess  
212 (1:5) to Ni<sup>2+</sup> and Zn<sup>2+</sup> to ensure the formation of organo-metallic complexes. The selection of the  
213 ligands was based on previous reports of Ni and Zn complexation in hyperaccumulator plants<sup>7,9,57,58</sup>.  
214 Aqueous standards were prepared from Ni(NO<sub>3</sub>)<sub>2</sub> or Zn(NO<sub>3</sub>)<sub>2</sub> salts respectively in ultrapure water  
215 (Millipore) with the following ligands: malate, citrate, oxalate, phytate, and histidine.  
216 Supplementary references from previous experiments were also used to increase the number of  
217 reference spectra, such as Ni in aqueous solution (Ni-aqueous), Ni-citrate with a metal:ligand ratio  
218 equal to 1 (Ni-citrate) and Zn sulfate. The solutions were diluted to 10 mM [Ni or Zn<sup>2+</sup>] before  
219 analysis. The pH of the standards was checked, and adjusted to 6. The aqueous standards were then  
220 applied to filter paper (Whatmann), allowed to dry and enveloped in Kapton tape before scanning.



221

222

## 223 **2.6 Data processing and analysis**

224 The XRF event stream was analysed using the Dynamic Analysis method<sup>59,60</sup> as implemented in  
225 GeoPIXE<sup>61,62</sup>. GeoPIXE provides quantitative first-order self-absorption corrected maps of  
226 projected areal elemental density – maps of elemental content. Conversion of X-ray counts to  
227 concentration was performed through analysis of Ni and Co XRF reference foil scans (Micromatter,  
228 Canada). The samples were each considered of a uniform thickness and either hydrated or dry, with  
229 respective thicknesses and compositions of 1000  $\mu\text{m}$  and  $\text{C}_{7.3}\text{O}_{33}\text{H}_{59}\text{N}_{0.7}\text{S}_{0.15}$  for the (hydrated)  
230 whole leaf and leaf, stem and root cross-sections, and 1500  $\mu\text{m}$  and  $\text{C}_{31}\text{O}_{15}\text{H}_{51}\text{N}_2\text{S}_{0.8}$  for the (dry)  
231 whole seeds and capsules. Assuming a uniform thickness for the seeds introduces further  
232 approximations to the measurements of seeds and seed capsules, however, as these have a generally  
233 flattened oblate cross-section, the approximation was considered appropriate.

234

235 Reconstruction of the single-slice tomographic data was performed using a maximum-likelihood  
236 expectation-maximization (MLEM) algorithm.

237

238 PCA analysis was performed on the XANES stacks using the MANTiS package. The extracted  
239 XANES data were reduced using standard normalization procedures performed with Bruce Ravel  
240 and Matthew Newville programs ATHENA and ARTEMIS<sup>63,64</sup>. Spectra were background  
241 subtracted and normalized. The XANES signals obtained were fitted as linear combinations of the  
242 standard spectra collected on solutions to evidence the main organic ligands involved in metal  
243 complexation. The number of standards was constrained to be at a maximum of three. The sum of  
244 components was released and not forced to be equal to 1. The selection of the linear combination  
245 was made on the basis of the indicators of fitting quality ( $\chi^2$ , r-factor and reduced  $\chi^2$ ), and the  
246 number of components was finally set to 2 as a third component did not improve the fitting quality.

247

## 248 **3. RESULTS**

249

### 250 **3.1 Localization of Ni and Zn in *N. tympheae* root, stem and leaf cross-sections**

251 Elemental maps of the root cross-sections (Fig. 1) show that Ni is concentrated mainly in the  
252 epidermis, in phloem bundles and pericycle. Zinc is mainly enriched in the phloem bundles. In the  
253 stem cross-section (Fig. 2) Ni is enriched in the collenchyma and the phloem bundles, as well as in  
254 the xylem.

255

256 In the whole leaves of *N. tympheae*, Ni is mainly localised in the leaf blade, with increasing  
257 concentrations towards the margins. The whole leaf elemental map of Ni (Fig. 3 and Suppl. Fig 1)

258 provides intriguing insight in the distribution of Ni at the tissue-level. The circular features are  
259 suggestive of major enrichment in the apoplast surrounding large epidermal cells. In contrast, Zn is  
260 distributed primarily surrounding the secondary vasculature across the leaf blade. Calcium is clearly  
261 depleted in the veins, but high in the interveinal regions. The distribution of Co (not shown) mirrors  
262 that of Ni, with enrichment in the leaf margins, and towards the leaf apex. Potassium is enriched  
263 throughout the leaf blade, but highest near the petiole.

264

265 In the leaf cross-section of *N. tymphaea* Ni and Zn have contrasting localizations (Fig 4 and Suppl.  
266 Fig 2). Whereas Ni is present in vacuoles of epidermal cells, Zn occurs mainly in the mesophyll  
267 cells, especially on either side of the central vascular bundles (Fig. 4). Calcium is depleted in the  
268 vascular bundles, but strongly enriched in the mesophyll and also in the epidermal region.  
269 Potassium is enriched in the epidermal cells and in the phloem bundles of the primary vein and also  
270 in secondary veinlets.

271

### 272 **3.2 Localization of Ni and Zn in seed capsules of *N. tymphaea* and *B. emarginata***

273 The intact *N. tymphaea* siliques contain 8–10 seeds attached to the central ovary (Fig. 5). Nickel is  
274 strongly enriched in vascular bundles of the ovary connected via the hilum onto the seeds. Nickel  
275 occurs also at the base of the style (micropyle). Zinc is highest in the micropylar region, in the  
276 vascular bundles, and in the seeds in radicles. Nickel and Zn appear to be similarly highly enriched  
277 in the cotyledons (Fig. 7). Calcium is highest in the margins of the seed capsules and in the style,  
278 whereas K is highest in the central vascular bundles of the ovary. These patterns are similar in  
279 another *N. tymphaea* silique (Suppl. Fig. 3), but Ni and Zn differ in that Zn is particularly  
280 accumulated in the radicles of the seeds, whereas Ni is more broadly enriched in the seeds. The  
281 distribution of Co (not shown) again mirrors that of Ni with enrichment mainly in the vascular  
282 bundles of the ovary, and in connecting tissues to the seeds.

283

284 The intact silique of *B. emarginata* contains just a single seed (Fig. 6 and Suppl. Figs. 4 and 5).  
285 Nickel is strongly enriched on the outer margins of the capsules, as well as in the micropylar region  
286 and style. Nickel appears enriched in the whole of the seeds, as well as in the hilum. As *B.*  
287 *emarginata* is not a Zn hyperaccumulator, the Zn content is low and its distribution is  
288 unremarkable. Calcium is strongly enriched in a peripheral region around the seed margin, likely  
289 the wings. Finally, K is especially high in the hilum and vascular bundles leading into the seed  
290 capsule and seed.

291

292

293 **3.3 Localization of Ni and Zn in the seeds of *N. tymphaea* and *B. emarginata***

294 The distribution of Ni and Zn is similar and strongly enriched in the cotyledons in *N. tymphaea* as  
295 shown in the 2D maps (Fig. 7 and Suppl. Fig. 6). In contrast, in *B. emarginata* they are strongly  
296 enriched in the tip of the radicle (Fig. 8). The enrichment of Fe in the provascular strands of the  
297 cotyledons and in the hypocotyl) is clearly visible in the network, and Fe is highest in hotspots in  
298 the hilum of the micropylar area.

299

300 The tomographic reconstructions of the *N. tymphaea* seeds confirm the observations from the 2D  
301 maps and show that Ni is localised in the vacuoles (the round solid outlines of vacuoles are clearly  
302 visible) of the cotyledon epidermal cells, and similarly in the epidermal cells of the hypocotyl (Fig.  
303 9). The virtual slice is looking tangentially showing the two cotyledons on either side (*i.e.* from the  
304 narrow plane) and the hypocotyl on the top right (Suppl Figs. 7, 8 and 9). Nickel is also enriched in  
305 the testa (seed coat). Nickel is depleted in the vascular bundles in the cotyledons. In contrast, Zn is  
306 enriched more or less evenly throughout the cotyledons, albeit slightly higher in the hypocotyl. It  
307 cannot be ascertained whether Zn is present on the vacuoles. The distribution of Fe in the  
308 provascular strands (note 'hollow' features) of the hypocotyl and cotyledons is clearly visible.

309

310 **3.4 Spatially-resolved chemical speciation of Ni and Zn in the seeds of *N. tymphaea***

311 The chemical speciation of Ni in the intact seeds of *N. tymphaea* is unequivocally associated with  
312 carboxylic acids (Fig. 10). The spectra extracted from the different regions of the seed (regions  
313 determined on the basis of PCA) were strictly identical to each other. Qualitative comparison with  
314 reference spectra suggested the predominance of Ni-malate species, confirmed by the linear  
315 combination results (Ni evidenced being at 80% complexed with malate). A smaller contribution of  
316 Ni-histidine complex can be discerned with the fitting. In the case of Zn (Fig. 11), Zn-phytate  
317 species were dominating Zn XANES spectra. In both Ni and Zn, spatially resolved spectroscopy did  
318 not reveal any spatial variation in chemical speciation within the seed.

319

320 **4. DISCUSSION**

321

322 Until recently detection systems for synchrotron XFM were not sufficiently fast to analyse fresh  
323 and hydrated plant tissue because the long dwell times caused excessive radiation damage<sup>66</sup>. The  
324 unparalleled ability of the Maia X-ray detection system to undertake very fast measurements (per-  
325 pixel dwell times as low as 1 ms and total scan times of less than 20 minutes for leaf cross-sections)  
326 makes it possible to analyse live plants and fresh plant materials<sup>65</sup>. There are limitations to this  
327 approach, however, including the fact that the elemental maps give information from different

328 depths combined into one plane (in the case of whole plant leaves). The only approach that avoids  
329 most or all sample preparation artefacts are cryotechniques which preserve both the distribution, the  
330 chemical form and the concentration of all elements *in situ*<sup>66</sup>. However, such techniques are not  
331 always available or not operable due to technical constraints, for example a cryo-stream can only  
332 cool samples smaller than 2 mm in diameter, and only one synchrotron facility (the BioNano  
333 Probe<sup>67</sup>) has a fully enclosed cryogenic chamber for large samples. In order to map elemental  
334 distribution within tissues, cross-sectioning is necessary, either physical or virtual by using  
335 tomographic methods. Sectioning of fresh plant material using a ‘dry knife’ method (as done in this  
336 study) avoids the loss of water-soluble ions (Ni<sup>2+</sup>, Zn<sup>2+</sup>), but may result in smearing of cell sap over  
337 the sample surface. Such artefacts were, however, not observed in this study and the elemental maps  
338 show intact inflated vacuoles (interpreted from the K maps). Seeds and seed capsules are unique  
339 among plant organs/tissues in that they are inherently dehydrated, and therefore, can be analysed “as  
340 is” in microanalytical experiments.

341  
342 Previous studies on the distribution of Ni and Zn in hyperaccumulator plants have shown that in  
343 *Hybanthus floribundus* subsp. *adpressus* (Violaceae) seeds the highest Ni concentrations were in  
344 the cotyledons, followed by the embryonic axis. In *Pimelea leptospermoides* (Thymelaeaceae)  
345 seeds Ni was preferentially localised in the embryonic axis, and in *N. caerulescens*, Zn was highest  
346 in cotyledons<sup>68</sup>. Nickel was also concentrated in the epidermis of the cotyledons in *N. caerulescens*  
347 seeds<sup>69</sup>, whereas in *N. pindica* seeds Ni was concentrated in the micropylar area and in the  
348 epidermis of cotyledons<sup>50</sup>. In *Stackhousia tryonii* (Celastraceae) seeds the highest Ni concentrations  
349 were in the pericarp<sup>70</sup>. In *Pycnanandra acuminata* (Sapotaceae) seeds the highest Ni concentration  
350 were in the endosperm and mesocarp<sup>71</sup>. Similarly, in *Biscutella laevigata* (Brassicaceae) seeds, the  
351 highest concentration of Zn was in the endosperm<sup>72</sup>. Finally, in *Berkheya coddii* (Asteraceae) Ni  
352 was localised in the lower epidermis, margins of cotyledons, and the pericarp in the micropylar  
353 area<sup>73,74</sup>. The diversity in location of Ni and Zn seeds of various hyperaccumulator plants reflects  
354 the variety of phylogenetic origins and distinct physiologies of hyperaccumulator plants. In this  
355 study we observed Ni to be localised in the vacuoles of the cotyledon and hypocotyl epidermal cells  
356 in *N. tymphaea* seeds, which agrees with the findings for other *Noccaea* species previously studied.  
357 During germination the seedling relies primarily on its Fe stores before it develops a root to take up  
358 Fe from the soil<sup>75</sup>. *Arabidopsis thaliana* stores Fe in vacuoles of the root endodermis and around the  
359 pro-vasculature in the cotyledons<sup>76</sup>. The Fe-rich provascular strand network of the cotyledons  
360 occurs in *N. tymphaea* too.

361

362 The European *Noccaea caerulescens* is among the most intensively-studied hyperaccumulator taxa  
363 globally, and used as a model for the genetics, ecology and molecular biology of metal  
364 hyperaccumulation<sup>38,39,45,77</sup>. The other taxa in the genus, such as *N. goesingense* and *N. praecox*, but  
365 also the various taxa in the *Alyssum* genus, are much less studied<sup>79,80, 81, 82</sup>. *N. tymphaea*, and taxa  
366 from other genera (*B. emarginata*), have so far obtained little attention, mostly because they grow in  
367 a rather confined and remote area in the Balkan region of Europe. Most *Noccaea* species can  
368 hyperaccumulate Zn, whereas many taxa also hyperaccumulate Ni<sup>5,83,84</sup>, but since most species have  
369 only been sampled in the field and not been re-grown on Zn or Ni containing soil, this issue is far  
370 from resolved. We set out to determine whether the distribution and chemical speciation of Ni and  
371 Zn differed in *N. tymphaea* and *B. emarginata*, both sampled from ultramafic soil. Although we  
372 expected important differences, due to the differing physiological functions (potentially toxic for Ni  
373 and essential for Zn) of these elements, the results show that Ni and Zn behave remarkably similar  
374 in *N. tymphaea* and *B. emarginata*. The chemical speciation of Ni is univocally associated with low  
375 molecular weight carboxylic ligands (likely malate), as in most hyperaccumulator species studied to  
376 date<sup>7,9,58,85</sup>. Specifically, in *N. caerulescens* and *B. emarginata* X-ray absorption spectroscopy  
377 showed that citrate was found as the predominant ligand for Ni in stems, whereas in the leaves  
378 malate was predominant<sup>7</sup>. In contrast, Zn was associated with phytates in the seeds. In the  
379 ultramafic soils of which *N. tymphaea* and *B. emarginata* grow, Ni is present at 20–50-fold higher  
380 concentrations, which explains concentrations differences in the plant shoots. Under these  
381 conditions *N. tymphaea* is not a Zn hyperaccumulator (foliar Zn reaches up to 362  $\mu\text{g g}^{-1}$ ). Based on  
382 the predominant Ni and Zn hyperaccumulation properties found in the current *Noccaea* spp., we  
383 hypothesize that the genus evolved from a Ni adapted and probably Ni hyperaccumulating ancestor.  
384 Some species managed to escape from ultramafic soil and develop as Zn hyperaccumulators on  
385 non-metallicolous soils, with a few, e.g. *N. caerulescens* later adapting to and (re-)colonizing  
386 calamine and ultramafic soils. *Noccaea tymphaea* may represent a taxon that never left the  
387 ultramafic conditions and remained adapted to Ni hyperaccumulation.

388

389 This study has shown that XFM can successfully be applied to help answering questions about the  
390 mechanisms of trace element hyperaccumulation, providing elemental distribution and chemical  
391 speciation in fresh/live hyperaccumulator plant tissues. . The dissimilar ecophysiological behaviour  
392 of Ni and Zn in *N. tymphaea* and *B. emarginata* raises questions about the evolution of  
393 hyperaccumulation in these species. Given that Zn accumulation is constitutive in *Noccaea* spp.  
394 occurring in non-metalliferous populations, Ni hyperaccumulation may have evolved as an  
395 adaptation when plants colonised ultramafic soils. In comparison, *B. emarginata* is not a Zn  
396 hyperaccumulator, and only hyperaccumulates (Ni) on metalliferous soils.

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714 **FIGURE CAPTIONS**

715

716 **Figure 1.** Elemental  $\mu$ XRF maps of fresh *Noccaea tymphaea* root hand cut section. The maps  
717 measure  $4.6 \times 3.2$  mm ( $460 \times 316$  pixels). The elemental image was acquired in 10- $\mu$ m step size  
718 with 5 ms dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps. Abbreviations  
719 annotated of anatomical features: *C* cortex, *Xy* xylem, *Ph* phloem.

720

721 **Figure 2.** Elemental  $\mu$ XRF maps of fresh *Noccaea tymphaea* stem hand cut section. The maps  
722 measure  $1.72 \times 1.78$  mm ( $430 \times 444$  pixels). The elemental image was acquired in 4- $\mu$ m step size  
723 with 7 ms dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps. Abbreviations  
724 annotated of anatomical features: *C* cortex, *Xy* xylem, *Ph* phloem.

725

726 **Figure 3.** Elemental  $\mu$ XRF maps of fresh *Noccaea tymphaea* whole mature leaf. The maps measure  
727  $12.55 \times 9.28$  mm ( $502 \times 371$  pixels). The elemental image was acquired in 25- $\mu$ m step size with 10  
728 ms dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.

729

730 **Figure 4.** Elemental  $\mu$ XRF maps of fresh *Noccaea tymphaea* leaf hand cut section. The maps  
731 measure  $4.45 \times 0.91$  mm ( $890 \times 181$  pixels). The elemental image was acquired in 5- $\mu$ m step size  
732 with 12m ms dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.  
733 Abbreviations annotated of anatomical features: *UE* epidermis, *LE* epidermis, *PM* palisade  
734 mesophyll, *SM* spongy mesophyll, *Xy* xylem, *Ph* phloem.

735

736 **Figure 5.** Elemental  $\mu$ XRF maps of *Noccaea tymphaea* intact silique. The maps measure  $4.91 \times$   
737  $10.02$  mm ( $327 \times 668$  pixels). The elemental image was acquired in 15- $\mu$ m step size with 10 ms  
738 dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.

739

740 **Figure 6.** Elemental  $\mu$ XRF maps of *Bornmuellera emarginata* intact silique. The maps measure  $7.5$   
741  $\times 6.52$  mm ( $375 \times 326$  pixels). The elemental image was acquired in 20- $\mu$ m step size with 20 ms  
742 dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.

743

744 **Figure 7.** Elemental  $\mu$ XRF maps of *Noccaea tymphaea* intact whole seed. The maps measure  $1.83$   
745  $\times 1.15$  mm ( $1143 \times 717$  pixels). The elemental image was acquired in 1- $\mu$ m step size with 20 ms  
746 dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.

747 **Figure 8.** Elemental  $\mu$ XRF maps of *Bornmuellera emarginata* intact whole seed. The maps  
748 measure  $4.52 \times 3.73$  mm ( $903 \times 746$  pixels). The elemental image was acquired in 5- $\mu$ m step size  
749 with 20 ms dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.

750

751 **Figure 9.** Single-slice tomography  $\mu$ XRF maps of *Noccaea tymphaea* intact whole seed. The  
752 elemental image was acquired in 2- $\mu$ m step size with 1 ms dwell per pixel, with 15.0 keV as the  
753 energy of the incident beam, showing Compton, Fe, Ni and Zn K maps.

754

755 **Figure 10.** Nickel speciation within the *Noccaea tymphaea* seed. A Principal Component Analysis  
756 (PCA) was performed on the stack of fluorescence scans, deciphering 4 regions of interest (A and  
757 B) from which XANES spectra were extracted. 2 supplementary spectra were extracted from the  
758 whole seed (white dotted line on picture A) and from the tip of the hypocotyl (black dotted line on  
759 picture A). Panel C shows the corresponding XANES, compared to Ni-malate and Ni-histidine  
760 spectra. Panel D displays the linear combination fitting (red dotted line) for one spectrum.

761

762 **Figure 11.** Zinc speciation within the *Noccaea tymphaea* seed. A PCA was performed on the stack  
763 of fluorescence scans, deciphering 4 regions of interest (A and B) from which XANES spectra were  
764 extracted. 2 supplementary spectra were extracted from the whole seed (white dotted line on picture  
765 A) and from the tip of the hypocotyl (black dotted line on picture A). Panel C presents the different  
766 spectra and compares them to Zn-phytate spectrum recorded in the same conditions on P06.  
767 Spectrum 3 displays a high background level, preventing a correct interpretation and was discarded.  
768 Panel D shows the linear combination fitting (red dotted line) and the fitting residual (green line) for  
769 one spectrum.

**Table 1.** Macro and trace element concentrations in roots, stems and flowers of *Bornmuellera emarginata* (values in  $\mu\text{g g}^{-1}$  dry weight) with ICP-AES. <LQ is lower than the limit for quantification.

Species	Part	Al	Ca	K	Mg	Mn	Na	P	S
<i>Bornmuellera emarginata</i>	Roots	<LQ	7900	11 325	1818	32	5570	1068	3559
	Roots	875	6349	10 070	3335	73	5416	1871	6492
	Stems	<LQ	14 430	23 535	3576	51	3037	2806	10 735
	Stems	1336	4583	13 350	4565	212	3669	1632	4531
	Flower	<LQ	21 010	15 350	6154	95	<LQ	2372	13870

  

Species	Part	Fe	Mn	Zn	Co	Cr	Cu	Ni	Zn
<i>Bornmuellera emarginata</i>	Roots	62	32	193	2.1	1.1	18	539	200
	Roots	1743	73	177	8	43	35	289	166
	Stems	43	51	251	4.9	1.0	20	1030	259
	Stems	2936	212	<LQ	16	66	21	499	90
	Flower	<LQ	95	311	43	<LQ	47	3641	306

**Table 2.** Macro and trace element concentrations in leaves, fruits, and seeds of the actual *Noccaea tymphaea* and *Bornmuellera emarginata* samples used for XFM elemental mapping (values in  $\mu\text{g g}^{-1}$  dry weight) with ICP-AES. <LQ is lower than the limit for quantification.

<b>Species</b>	<b>Organ</b>	<b>P</b>	<b>S</b>	<b>Mg</b>	<b>K</b>	<b>Ca</b>	<b>Fe</b>	<b>Mn</b>	<b>Zn</b>	<b>Ni</b>
<i>Noccaea tymphaea</i>	Leaves	616	2284	3496	4059	9549	218	26	362	12 410
<i>Bornmuellera emarginata</i>	Fruits	1639	8564	2996	7742	6317	38	7.8	96	10 345
<i>Noccaea tymphaea</i>	Seeds	3834	5738	1628	6087	3701	<LQ	50	112	2504
<i>Bornmuellera emarginata</i>	Seeds	2926	15 159	1775	7800	4419	24	12	69	6242