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1 **Cytotoxicity and genotoxicity of lanthanides for *Vicia faba* L. are mediated by their**
2 **chemical speciation in different exposure media**

3

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18 **Abstract**

19 A comprehensive study of the toxicity of lanthanides (LN) in relation to the media
20 composition will enhance the prediction of their potential adverse effects for living
21 organisms. Here we examined the effect of different media on the *V. faba* root elongation
22 and on the cytotoxic (mitotic index) and the genotoxic (micronucleated cell number)
23 effects from toxicity tests with Ce, Gd and Lu (100, 800 and 6400 $\mu\text{g L}^{-1}$). Three different
24 exposure media were selected: the standard Hoagland media (SH); an alternative SH,
25 without phosphates (SH-P); and distilled water (DW). In the SH no cyto-genotoxic effects

26 were observed and even, for low LN content, potential root elongation stimulation was
27 reported. The absence of toxic effects was explained by a drastic decrease of the total
28 dissolved LN concentration due to the presence of phosphates causing LN precipitation.
29 In SH-P, LN remained largely soluble and inhibition of root elongation was observed
30 mainly for the highest treatments. While in the tests done in DW, toxic effects were
31 obtained for all treatments. Our results showed that in absence of phosphorous, LN appear
32 mainly as free form and complexed in carbonates and sulphates, and can cause toxic
33 effects, whereas toxicity is not expected when phosphorous is available in aquatic media.
34 The highest LN root contents were observed for the tests using distilled water, possibly
35 due to the absence of competition by Ca^{2+} for uptake. The present work demonstrated that
36 media composition has a great impact in assessing the ecotoxicology of lanthanides.

37

38 **Keywords:** rare earth elements, mitotic index, phosphate, cerium, gadolinium, lutetium

39

40 **1. Introduction**

41

42 The relevance of lanthanides (LN), which are also known as Rare Earth Elements (REE),
43 in agricultural, industrial and, especially in high-tech applications has increased in the last
44 decades (Lambert and Ledrich, 2014). This growing demand in the global market has
45 caused an explosive increase in lanthanide mining activities. Subsequently, there is an
46 unavoidable release of LN into the surrounding environment, posing a potential threat to
47 aquatic and terrestrial ecosystem, and ultimately, to human health (Gong et al., 2021).
48 Therefore, it will be essential to enhance the knowledge in their potential hazards in
49 organism accumulation (Pagano et al., 2015) and the future adverse ecotoxicological
50 effects in biosphere. However, ecotoxicological information regarding these elements is
51 still scarce (Pagano et al., 2016), especially when compared with other more common
52 metals.

53 In recent years, the environmental behaviour of LN has attracted increasing scientific
54 attention, principally through the study of their cycling and geochemistry (Aksentov and
55 Sattarova, 2016). In aquatic systems, solution and interface chemistry appear to be the
56 major factor controlling their concentrations and they can form strong complexes with
57 several ligands present in the environment (Davranche et al., 2015). Previous studies have
58 shown that LN may precipitate with carbonates, phosphates, hydroxides, fluorides and
59 oxalates (Evans, 1990). González et al. (2015) studying ecotoxicity in a battery of acute
60 and chronic toxicity tests with aquatic organisms observed that, depending on test media,
61 insoluble LN species can be formed resulting in a considerable decrease in their
62 availability. Gong et al. (2021) observed in a test with wheat (*Triticum aestivum*) that P
63 addition reduce free ion activities of Y and Ce in exposure media, and decreases their
64 uptake concentration in plant roots. This implied that the increase of P concentrations in

65 exposure media induced a greater decrease in LN toxicity. In aquatic systems, the free
66 ionic form of many metals is considered most responsible for toxicity and the potential
67 formation of complexes of these ions in the media may result in decreased toxicity
68 (Peijnenburg and Vijver, 2007). The mentioned observations imply that for LN toxicity,
69 a good knowledge of the composition of the exposure media will be decisive, in order to
70 be able to calculate the chemical speciation of the studied elements (Twiss et al., 2001)
71 and to predict realistic toxic effects.

72 On the other hand, even though LN show rather similar behaviour among them in natural
73 processes owing to their similar atomic and chemical properties (Aksentov and Sattarova,
74 2016), there is no consensus about a predictable pattern of their toxicity across the
75 lanthanide series (González et al., 2014). The bioaccumulation potential of the different
76 elements of the LN group has been shown to differ. However, current observations are
77 still controversial, and the reported divergences about their effects and their behaviour
78 are still under discussion (González et al., 2014; Romero-Freire et al., 2019). In addition,
79 it has also been reported that LN and major cations (e.g. Ca^{2+} or Mg^{2+}) can show
80 competition effects for cellular internalization into organisms due to their chemical
81 similarity (Tan et al., 2017). One important aspect of LN bioaccumulation is their
82 similarity to Ca^{2+} (Sherry et al., 2009) which can disturb the Ca^{2+} metabolism in
83 organisms, as was shown for skeleton formation in sea urchins (Martino et al., 2016), the
84 shell composition of crabs (Bosco-Santos et al., 2017), and the proton and calcium
85 competition for the uptake sites in a freshwater algae (El-Akl et al., 2015). This observed
86 similarity/competition with Ca^{2+} was suggested before to promote the translocation or
87 replacement of calcium in different cell functions (Das et al., 1988; González et al., 2014;
88 Tan et al., 2017; Joonas et al., 2017) but the real existence of LN functions in the operating
89 of living organisms is not yet defined.

90 Higher plants are considered excellent living models to study the toxicity and
91 accumulation of various pollutants, and are extensively used to detect and evaluate the
92 toxicity of various environmental stressors (Jiang et al., 2019), therefore toxicological
93 assessments using them will help in the understanding of the LN effects. In addition,
94 higher plants are usually more ecologically relevant than commonly used species for
95 mutagenicity such as *Salmonella* spp. (Corrêa et al., 2016). The species *Vicia faba* L.
96 (broad bean) has been used in several studies for environmental toxicity monitoring,
97 highlighting the high sensitivity of this species and its efficiency in the study of the
98 mutagenic activity of metals (Dong and Zhan, 2010, Iqbal, 2016). Using higher plants
99 such as *V. faba* for genotoxicity evaluation of environmental samples has been
100 recommended as rapid, convenient, sensitive and reliable in vivo test system (Basu and
101 Tripura, 2021). Moreover, *V. faba* is cultivated and used for both human and livestock
102 nutrition, consequently, because of its direct introduction in food chains, it provides a
103 reliable framework for studying ecotoxicity and bioaccumulation. Several studies dealing
104 with *V. faba* and lanthanides have been identified in a literature review. Lanthanum (La^{3+})
105 can bind to Ca^{2+} absorption sites and thus have an effect in the uptake of nutrient ions
106 through Ca^{2+} channels for *V. faba* (Wang et al., 2012). Holium (Ho) at low dosage (4 mg
107 L^{-1}) could accelerate the growth of root tips of *V. faba*, however with the rise of
108 concentrations certain cytotoxic and genotoxic effects were observed (Qu et al., 2004).
109 The use of Pr and Nd also showed an increase in cytogenetic effects (clastogenic effect)
110 with the increase in their concentrations (Jha and Singh, 1994, Singh et al., 1997).
111 Small amounts of LN have been described to be beneficial for plants (Pang et al., 2001)
112 and bacteria (Wehrmann et al., 2017), even being used as fertilizers in agriculture (Zhang
113 et al., 2001). Their accumulation in plants has been studied, deducing that i) it used to be
114 greater in roots than in other parts of the plants (Lingott et al., 2016), ii) the accumulation

115 has been reported to be independent of the concentration present in the substrate used
116 (Markert, 1987), and iii) the penetration of LN has been shown to be facilitated by
117 endocytosis in plant cells (Wang et al., 2014). However, there is not a clear indication in
118 literature that they are essential to living organisms. Their potential effects in plants have
119 been studied for last decades (d'Aquino et al., 2009), and both positive and negative
120 effects of crop production following treatments with them have been reported (Hu et al.,
121 2004). Hence, all this previous knowledge warrants further investigation on LN effects
122 and their potential (eco)toxicity effects in plants and other organisms.

123 The gaps in the knowledge that currently exist in relation to the toxicity of LN and the
124 effect of the studied media, if not addressed, could become a serious barrier to predict
125 future adverse effects in the biosphere. The research hypothesis is that the presence of P
126 in the media will modify the potential effects of lanthanides in living organisms.
127 Therefore, in the present work, the cytotoxic and genotoxic effects of three lanthanides
128 (Ce, Gd and Lu) representatives of the three main groups (light, middle and heavy rare
129 earth elements respectively) were investigated using *V. faba*. Toxicity tests were carried
130 out in hydroponic systems with three different media in order to investigate the lanthanide
131 speciation and distribution and to reveal their relationship with the potential toxic effect.
132 The selection of different exposure media for investigating LN toxicity will hold up to
133 the light the mechanisms of action of these elements in plants and will help to identify the
134 effects of P presence in the media and the lanthanides uptake through Ca^{2+} channels.

135

136 **2. Materials and methods**

137

138 **2.1. Exposure media and scenarios**

139

140 The lanthanide solutions were prepared individually by using commercially available
141 chemicals (SIGMA Aldrich; purity >99.99%): cerium chloride ($\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$),
142 gadolinium chloride ($\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$) and lutetium chloride ($\text{LuCl}_3 \cdot 6\text{H}_2\text{O}$). The lanthanide
143 salts were diluted in milliQ and pH adjusted to prepare stock solutions. The following
144 nominal concentrations: 100, 800 and 6400 $\mu\text{g L}^{-1}$ were selected in order to ensure
145 toxicological response, according to previous studies done by González et al. (2015). The
146 study concentrations were prepared for each element by diluting stock solution in the
147 exposure media. In this experiment, three different exposure scenarios were studied: (1)
148 standard Hoagland media (SH) (5 mM KNO_3 ; 5 mM $\text{Ca}(\text{NO}_3)_4 \cdot 4\text{H}_2\text{O}$; 5 mM
149 $\text{MgSO}_4 \cdot \text{K}, 7\text{H}_2\text{O}$; 1 mM KH_2PO_4 ; 9 μM Iron tartrate); (2) modified Hoagland media,
150 similar to the standard Hoagland media but without phosphate (SH-P); and, (3) distilled
151 water (DW). Prior to the toxicity experiments, the ability of plants to grow in the three
152 different media was tested.

153

154 **2.2. *V. faba* root elongation and micronucleus test**

155

156 Ecotoxicological tests were carried out using seeds of *Vicia faba* L. (var. Aguadulce,
157 Fabres) following the standardized protocol ISO (2013) with minor modifications. For
158 the pre-germination of seeds, they were dipped in distilled water for 24h; then, the outer
159 covers were removed and seeds were placed between two layers of wet cotton for
160 germination at 22 °C for 3 days. Before the test, the primary root tips were cut off (2 mm)
161 and seeds were suspended in aerated Hoagland's solution at 22 °C for 4 days (96 h) to
162 encourage the development of secondary roots. For the toxicity test, glass pots were filled
163 with 100 mL of exposure solution for each treatment and one *V. faba* plant was placed in
164 each pot. Secondary roots were identified and measured in each plant at the beginning

165 and at the end of the test (96 h), to compare different rates of elongation. Maleic hydrazide
166 (CAS n°. 123-33-1) (MH) at the concentration of 10^{-5} M was used as a positive control
167 and aerated standard Hoagland's solution was used as negative control. Each treatment
168 was tested in triplicate. All test samples were incubated in the dark at 24 ± 1 °C for 96 h.
169 After exposure, secondary roots were washed twice with distilled water and dried with a
170 paper towel. Fresh weight (analytical balance in mg) and root elongation were
171 determined. Root elongation inhibition with respect to the control (REI) was calculated
172 according to Bellani et al. (2014), as follows: $REI = (C-T) / C$, being C: root length in
173 control seeds, and T: root length in treated seeds.

174 For the micronucleus test, some root tips were excised and fixed in Carnoy solution
175 (absolute ethanol:glacial acetic acid, 3:1 v/v) at 4°C overnight, and transferred to 70%
176 ethanol for storage. Root tips were placed in distilled water for 10 min, hydrolyzed in 1
177 N HCl at 60° for 6 min and retransferred into distilled water. After that, roots were stained
178 with 1 % aceto-orcein. The number of cells in mitotic division and the micronuclei
179 formation were scored under a 400X magnification. Two slides were prepared for each
180 replicate, giving a total of 6000 root tip cell observation per treatment. The number of cell
181 division (mitotic index, MI) as cytotoxicity indicator, and the number of micronuclei
182 (micronucleated cell number, MCN) as genotoxicity indicator, were determined (Cotelle
183 et al., 2015) and expressed as the average based on 1000 interphase cells per root tip.

184

185 **2.3. Lanthanide concentration in test media**

186

187 Lanthanide concentrations in studied media were evaluated in all dilutions at t 0 h and at
188 t 96 h of the exposition by inductively coupled plasma-mass spectrometry (ICP-MS;
189 Hewlett Packard 4500 STS spectrometer); for that, samples of the solution were

190 previously filtered (Millex®; 0.22 µm). Measurements were carried out in triplicate using
191 Rh as internal standard. For calibration, two sets of multi-element standards containing
192 the three analytes of interest at five different levels of concentration were prepared using
193 ICP single-element standard solutions (Merck, Darmstadt, Germany). The accuracy of
194 the method was confirmed with analysis of the Standard SPS-SW1 Batch 120 (LGC
195 standard, UK). For all elements (Ce, Gd and Lu) analyzed, average recoveries (n=6)
196 ranged between 95 and 102 % of the certified reference values. The pH in media solutions
197 was measured at the beginning of the test (t 0 h) and at the end (t 96 h) in all exposure
198 conditions (pHmeter edge®blu, Hanna Instruments, USA).

199

200 **2.4. Speciation calculation**

201

202 Lanthanide speciation in the exposure media was calculated in the three studied media
203 using the chemical speciation model ORCHESTRA (Meeussen, 2003). The speciation
204 was calculated from the total concentrations of the lanthanides and all chemical
205 components present in the considered exposure media (Table SI.1) and pH. The solutions
206 were assumed to be in equilibrium with atmospheric CO₂ (pCO₂ = 3.8E⁻⁴ atm.). The
207 calculations were done for three cases: (i) nominal concentrations of the lanthanides (100,
208 800 and 6400 µg L⁻¹), (ii) from the measured lanthanide concentrations and pH at the start
209 (t=0 h), and (iii) from the measured pH and lanthanide concentrations at the end of the
210 experiment (t= 96 h). We used the standard Minteq-V4 thermodynamic database for all
211 species. Thermodynamic constants of lanthanide species were added according to NIST.
212 Similar to Janssen and Verweij (2003), we used additional thermodynamic constants for
213 Ln-CO₃⁺ and Ln-(CO₃)₂⁻ from Lee and Byrne (1993), for Ln-HCO₃²⁺ from Millero
214 (1992), for Ln-PO₄, Ln-(PO₄)₂⁻³ from Lee and Byrne and for Ln-HPO₄⁺, Ln-(HPO₄)₂⁻ and

215 Ln-H₂PO₄²⁺ from Millero (1992). Lanthanide complexation with tartarate was calculated
216 using lanthanum-tartarate constants (Dunsmore and Midgley, 1972) for all three
217 lanthanides because they were absent for the considered LN. Activity corrections were
218 calculated using the Davies equation using calculated ionic strength (Stumm and Morgan,
219 1995).

220

221 **2.5. Lanthanide uptake and micro-observation**

222

223 For internal concentration analysis, *V. faba* roots at the end of the test were washed
224 thoroughly with 10 mmol L⁻¹ EDTA solution at 4°C for 30 min under stirring and twice
225 with distilled water to remove adsorbed metals from the surface of roots (Wang et al.,
226 2012). Thereafter, roots were dried and digested in strong acid (HNO₃) and H₂O₂ using
227 microwave and closed digestion vessels (Kingston and Jassie, 1986; Sah and Miller,
228 1992). The content of Ce, Gd and Lu in the digestate was determined in triplicate by ICP-
229 MS (ICP-MS; Hewlett Packard 4500 STS spectrometer) according to the methodology
230 previously described for determination of lanthanides in the media dilutions.

231 For chemical mapping, secondary roots, of the highest treatments (6400 µg L⁻¹) in the
232 modified Hoagland media (SH-P) which had been washed as previous explained, were
233 frozen at -80°C. Lanthanide (Ce, Gd or Lu) as well as other major elements (e.g. P, Ca,
234 and K) distributions were determined by laser ablation-inductively coupled plasma-mass
235 spectrometry (LA-ICP-MS) using a Nd:YAG laser operating at 213 nm (CETAC LSX-
236 213 G2+) and the HeEx 2-volume sample chamber. Analyses with millisecond washouts
237 are routinely achievable and the baseline returns below 0.1% of the signal very far below
238 1 s. We operated with a pulse rate of 20 Hz in raster mode and single line scan, a 10 µm

239 spot size and a $10 \mu\text{m s}^{-1}$ scan rate. The system is coupled with an ICP-MS (PerkinElmer,
240 Nexion 300X) to analyse ^{140}Ce , ^{158}Gd , and ^{175}Lu .

241

242 **2.6. Statistical analysis**

243

244 Descriptive statistics were calculated to check the normality and Levene's test to check
245 the homogeneity of variances for all analyzed parameters. Significant differences were
246 analyzed by one-way ANOVA (Tukey's test, $p < 0.05$) for the individual means of REI,
247 MI and MCN variables. For the root elongation, root weight and MI and MCN frequency,
248 the values obtained in the treatments were compared to the values obtained in the
249 respective controls using the Dunnett's test ($p < 0.05$). The Spearman's correlation was also
250 performed to determine the effect of lanthanide speciation for the different obtained
251 endpoints. All analyses were performed using SPSS v.20.0 (SPSS Inc. Chicago, USA).
252 All data are reported as means \pm SD.

253

254 **3. Results**

255 **3.1. Response of *V. faba* roots and toxicity assessment**

256

257 **3.1.1. Root development**

258

259 Root elongation in controls, without LN addition, did not show significant differences
260 between the SH (2.53 ± 0.35 cm) and SH-P media (3.34 ± 0.88 cm); however, a significant
261 decrease ($F = 6.38$; $p < 0.05$; d.f = 8) in root elongation was observed when control
262 without LN was performed in DW (1.67 ± 0.32 cm) (Table SI.2). For an easier
263 interpretation of the results, root elongation inhibition indexes (REI) in respect to controls
264 were calculated (Figure 1). In SH, REI values were negative for all elements and

265 treatments, therefore no inhibition in root elongation was observed. In SH-P, values of
266 REI increased with the increase in nominal concentrations, except for the treatment with
267 Lu where the highest REI was obtained for the lowest nominal content ($100 \mu\text{g L}^{-1}$). In
268 DW media it was observed that an increase in nominal LN concentration increased REI
269 for the three studied lanthanides. According to root weight, no significant differences (F
270 $= 1.58$; $p > 0.05$; d.f. = 8) were observed among the three studied media for controls
271 (average $57.02 \pm 15.08 \mu\text{g}$, $n=9$) (Table SI.2).

272

273 **3.1.2. Cytotoxicity and genotoxicity**

274

275 The validation criteria for the cytotoxicity and genotoxicity tests are a mean mitotic index
276 (MI) (number of cells in division) for the negative controls (SH, SH-P and DW without
277 LN addition) greater than 20 for 1000 cells, and a positive response obtained for the
278 positive control (maleic hydrazide, MH) (ISO 29200). When MI was lower than 20%,
279 micronuclei frequency (MCN) was not recorded according to the standard. In this study,
280 MI for the controls of the studied exposure media were higher than 26 in all cases and did
281 not show significant differences among them; whereas the positive control showed a
282 positive MI (19.5 ± 3.1 , $n=3$) which narrowly satisfies the validation criteria of the ISO
283 protocol (Table 1). The micronucleus frequency (MCN) showed a decrease in the
284 negative controls for the SH-P and DW compared to SH, however it was not significantly
285 different ($F = 3.05$; $p > 0.05$; d.f. = 8), thus the specific effects observed in MCN
286 frequency cannot be directly attributed to the use of different media compositions.

287 In SH media, cytotoxicity and/or genotoxicity were not observed and even less MCN
288 formation at the treatment with $100 \mu\text{g L}^{-1}$ of Lu was observed, suggesting a potential
289 beneficial effect at low Lu concentrations. The highest concentrations of Ce and Gd in

290 the SH-P media induced a decrease of the mitotic index (MI) in *V. faba* roots, but this
291 was not observed for Lu. While in the DW, cytotoxicity was observed for the three
292 lanthanides at all concentrations. An increase in MCN formation (genotoxicity),
293 comparing to the corresponding controls was reported only in SH-P for the highest
294 treatment of Lu, and for Ce at 100 $\mu\text{g L}^{-1}$. When MI inhibition was reported, MCN
295 genotoxic effect was not calculated.

296

297 **3.2. Lanthanide concentrations and speciation in test media**

298

299 **3.2.1. Lanthanide concentrations**

300

301 Measured lanthanide concentrations (Ce, Gd and Lu) at the start of the test (time 0 h) in
302 the standard Hoagland media (SH) decreased strongly before reaching values lower than
303 13% of the nominal concentration (Table 2). In the other two media used, the modified
304 Hoagland media without phosphates (SH-P), and distilled water (DW), the LN
305 concentrations measured at t 0h were >80% of the nominal concentration. In the SH
306 media, recoveries decreased with increasing nominal concentration in the order
307 Lu>Gd>Ce. Whereas for the SH-P and DW media, the LN recovery increased with an
308 increase in nominal concentration, with different trends in recoveries for the studied LN
309 which decrease in the order Ce>Gd>Lu for SH-P and Gd>Ce>Lu for DW.

310 In the SH media at the end of the test (t 96 h), Ce and Gd recoveries decreased even more
311 compared to recoveries at t 0 h, except in the treatments with a nominal value of 800 μg
312 L^{-1} , which showed the highest recoveries. In the SH-P and DW media recoveries in
313 general decreased compared to t 0 h except in the lowest treatment (100 $\mu\text{g L}^{-1}$) for Ce
314 and Lu in SH-P and for Gd in DW, where recoveries were greater.

315

316 **3.2.2. Lanthanide speciation**

317

318 Model calculations with ORCHESTRA predict precipitation of LN-phosphates for the
319 SH-media in all cases. Figure 2 shows the distribution of the lanthanides over the various
320 chemical species in the different media as obtained with the ORCHESTRA model
321 calculated from the measured LN content at the end of the experiment (t 96 h). The
322 distribution calculated for t 0 h and the nominal content can be found in Figure SI.1 of
323 the supporting information.

324 In the SH media, lanthanides are predicted to be mostly present as LN-phosphates. The
325 largest fractions of LN-phosphates were predicted in the order P-Lu>P-Gd>P-Ce due to
326 the increasing affinities of LN with phosphates with the increase in their atomic numbers.
327 The remaining dissolved LN appear mainly complexed with sulphate or as the free LN
328 ion.

329 In the SH-P media, without P, 25-39% of dissolved LN is calculated to be present as a
330 free ion and 41-66% as LN-sulphates, with the remaining LN species being carbonate and
331 hydroxyl complexes.

332 In the distilled water (DW), and for the lower concentrations studied (100 and 800 $\mu\text{g L}^{-1}$)
333 ¹), dissolved Ce and Lu were predicted to be present mainly complexed with carbonates
334 (>80% of dissolved LN present), whereas gadolinium was predicted mainly as a free ion
335 in all treatments (>90%). At the highest studied concentration (6400 $\mu\text{g L}^{-1}$), Ce and Lu
336 also were predicted to be present as a free ion.

337

338 **3.3. Root internal concentration**

339

340 In all exposure media, internal root concentrations were significantly higher than the
341 controls, which were 0.17, 0.43, 0.22 mg kg⁻¹ for Ce, Gd and Lu, respectively. Root
342 internal concentration increased with increasing nominal content for the 3 studied
343 lanthanides. However, Gd appeared more accumulated in the highest treatments (6400 µg
344 L⁻¹) indiscriminately of the studied media (Figure 3). Lanthanide content in roots showed
345 the lowest concentrations for SH media, higher in SH-P and the highest content in DW.
346 Comparing the studied media, for roots exposed to SH and SH-P, Gd showed the highest
347 internal concentrations with a more than 2-fold content of Gd than Ce or Lu for the
348 organisms exposed to the lowest treatment (100 µg LN L⁻¹). The internal root
349 accumulation in DW expositions showed, for the 3 studied LN, similar values for the
350 lowest treatments. The highest accumulations were observed in all media for the highest
351 treatments, especially in the roots exposed to Gd which showed the highest internal
352 content. In the case of SH, accumulation was 124 ± 1 mg Gd kg⁻¹, and for SH-P internal
353 accumulation was 7 times higher (874 ± 4 mg Gd kg⁻¹). The highest internal root
354 concentration was observed in DW, reaching up to 6838 ± 65 mg Gd kg⁻¹.
355 LN distributions obtained with LA-ICP-MS for EDTA-cleaned roots exposed to 6400 µg
356 L⁻¹ in the SH-P media are shown in Figure 4. The three studied lanthanides (Ce, Gd and
357 Lu) appeared to be distributed evenly over all parts of the roots. Only Gd showed a higher
358 intensity in the root apex. Graphical representation of LN/Ca ratios used as a qualitative
359 approximation of the distribution of both elements in the totally of roots (wall distribution
360 and internalization) showed a potential trend of increasing according to the decrease in
361 ionic radii for LN (Lu>Gd>Ce).

362

363 **3.4. Effect of lanthanide species on toxicity**

364

365 For understanding the LN accumulation in roots and their potential toxic effect, regarding
366 their speciation, Spearman correlation analysis was done with the main endpoints studied
367 (REI, MI, MCN and LN root content) and the LN content and species obtained for the
368 different studied media. In SH-P, the media which showed clear toxic effects associated
369 with LN (Table 3), correlation was not observed for MCN endpoint, and only REI and
370 MI had negative correlation. Root LN content was directly correlated with the measured
371 LN concentrations in the media at t 0h and t 96 h, whereas MI was inversely correlated
372 with LN content at 96 h and the LN content in roots (inverse correlation means higher
373 toxicity). The LN content in roots showed positive correlation with all species
374 concentrations of LN calculated with the geochemical model, whereas for the MI it was
375 only inversely affected by the LN dissolved content, free LN forms and by the LN
376 complexes with $-(\text{SO}_4)_2^-$ and $-\text{tartarate}$. For SH media, without observed toxic effects,
377 and DW media, with toxicity in all treatments, Spearman correlations were done with root
378 LN content and LN content in media (data not shown), analysis results showed a positive
379 correlation of the plant uptake with LN in media at t 0 h for SH and with LN in media at
380 t 0 h and t 96 h for DW.

381

382 **4. Discussion**

383

384 **4.1. *Vicia faba* response to lanthanide exposure**

385

386 In this study root elongation inhibition (REI) was calculated to better understand the
387 potential effect of the different exposure media studied. In SH inhibition was not
388 recorded, for SH-P it was observed, in general, a potential inhibition for the highest
389 concentrations, while in DW inhibition was observed in all treatments. In this last case,

390 the stronger effect could be due to a nutrient shortage or to a direct effect of LN on roots
391 (Bellani et al., 2014), which was also reflected in the higher internal concentration for the
392 roots exposed in this media (Figure 3). No inhibition observed in the SH could be
393 explained due to low LN availability which can favour plant growth and productivity.
394 However, the physiological mechanisms are still not well understood (Tyler, 2004).
395 Toxic effects were not found in SH. In SH-P they were observed in the highest
396 concentration, whereas DW showed a high cytotoxicity for all concentrations studied.
397 According to the phosphorous content in the SH media, the absence of effects can be
398 explained because the highest dose used in this experiment ($6400 \mu\text{g L}^{-1}$) precipitated
399 with ca. 5% of the PO_4 initially present in the solution, and therefore did not induce a
400 nutrient sequestration (Blinova et al., 2018). This is in accordance with Gong et al. (2021),
401 who observed that P addition can reduce LN uptake concentration in roots. In this study,
402 SH media did not show cytotoxicity and even showed a decrease in micronuclei induction
403 (MCN) for the lower Lu content ($100 \mu\text{g L}^{-1}$). This positive effect could be explained due
404 to some kind of growth stimulation of LN at low doses (Cao et al., 2001; Carpenter et al.,
405 2015). Wang et al. (2012) suggested that LN exert low-dose promotion and high-dose
406 inhibition on plant growth, therefore instead of observing toxicity, in SH, due to the low
407 availability of LN, hormesis effects could have happened with the addition of Lu at low
408 concentration. On the other hand, for SH-P media at higher concentrations of Ce and Gd,
409 and for DW media, in all treatments, cytotoxicity effects were reported.
410 In SH media genotoxicity was not observed, while for SH-P media, toxicity was found
411 for the 3 lanthanides in the highest treatment, even for Lu where no cytotoxicity was
412 observed. Cytogenetic effects of Ho (Oral et al., 2010), Nd and Pr (Jha and Singh, 1994)
413 and La (Wang et al., 2012) were already reported for *Vicia faba* roots. However, toxicity
414 in the cells of *V. faba* roots due to LN are still not fully understood (Wang et al., 2012).

415 Lanthanide compounds, without a complete knowledge of their effects, are being used as
416 seed treatment to stimulate growth (Carpenter et al., 2015). But according to our results,
417 both LN content in roots and LN content in media, as well as free LN forms and the LN
418 complexes with –sulphates and –tartarate could explain the toxic effects observed for the
419 mitotic index in roots exposed to SH-P media (Table 3). However, no correlations were
420 observed for MCN, although metal genotoxicity was reported before to be mainly
421 associated with the free ionic forms (Corrêa et al., 2016). The toxicity results obtained
422 could only be explained by LN content in the Hoagland media without phosphates (SH-
423 P) and the correlations obtained with the free LN forms and other LN complexes support
424 the potential LN toxicity in root development, when P in the study media is not presented.
425 In DW media toxicity was observed for all lanthanides at all concentrations. In general,
426 lanthanide effects in root growth in other species, e.g. *T. aestivum*, was observed by
427 decreasing the MI at higher LN concentrations (d’Aquino et al., 2009). However, the
428 toxic effect obtained in the DW media could be also explained by the lack of nutrients
429 (Joonas et al., 2017), but no toxicity was observed in the controls of distilled water, which
430 implies that toxic effects could be also the result of LN presence. The positive correlation
431 observed with the plant uptake (root accumulation) with LN content present in the three
432 studied media help to justify the observed LN effects in root plants.

433 Lanthanide can be taken up from the roots and their internal content showed that LN
434 concentration increased with increasing nominal content for all the media studied (Figure
435 3). Plants have previously shown LN uptake through roots (Lingott et al., 2016), and the
436 increase in root accumulation with the increase in the LN content in exposition media has
437 been also reported (Carpenter et al., 2015). However, Markert (1987) suggested that LN
438 accumulation in plants can be independent of the concentration present in the substrate
439 used. The penetration of LN has been shown before to be facilitated by endocytosis in

440 plant cells (Wang et al., 2014). But there is not a clear indication in literature that LN are
441 essential to plants, and their potential benefits or damages have been studied for many
442 years (d'Aquino et al., 2009). Positive and negative effects of crop production following
443 treatments with LN have been reported (Hu et al., 2004) showing both beneficial and
444 toxic effects for seed and plant growth (d'Aquino et al., 2009). Lanthanides have similar
445 ionic radius to Ca^{2+} , therefore can bind to Ca-located sites in cell membranes, block Ca
446 channels or disturb the uptake of mineral ions, which implies that LN could have a role
447 in the regulation of plant growth (Wang et al., 2011). Wang et al. (2012) deduced from a
448 test performed with lanthanum that La^{3+} can bind to Ca^{2+} absorption sites and thus have
449 an effect on the uptake of nutrient ions through Ca^{2+} channels. El-Akl et al. (2015) also
450 reported competition with calcium for the uptake sites with the lanthanide Ce for
451 freshwater algae. Gong et al. (2019) observed that Ca^{2+} (and Mg^{2+}) significantly
452 attenuated the degree of the toxicity of trivalent lanthanides for the plant *Triticum*
453 *aestivum*. In the present study, Ca concentration in roots was not determined, however
454 the LN concentrations observed in the roots exposed to SH-P were similar to the content
455 in roots exposed to SH for the lowest treatments ($<800 \mu\text{g L}^{-1}$) (only 1.2-1.6 times higher
456 for SH-P, Figure 3). On the other hand, for DW, with Ca absence in the media, uptake of
457 LN was higher, consequently, we can suppose that the presence of Ca (and possibly also
458 other divalent cations) decreases the uptake of LN via competition effects. Our results
459 showed LN content homogeneously distributed in the first mm from the roots apex
460 (Figure 4). This is in agreement with Lingott et al. (2016) who observed a homogenous
461 distribution of Gd in the roots of the plant *Lepidium sativum*. Total chemical analysis
462 showed greater accumulation of Gd and Lu than Ce. Lanthanides with lower ionic radii
463 (heavy rare earth elements i.e. for our study Lu) had higher affinity to translocate with Ca
464 in the roots (González et al., 2014). Lutetium has the most similar ionic radius to Ca from

465 the three LN studied (0.99 Å); and this similarity was suggested to promote the
466 translocation or replacement of calcium in different cell functions (Das et al., 1988;
467 González et al., 2014; Tan et al., 2017; Joonas et al., 2017).

468

469 **4.2. Lanthanide availability and speciation**

470

471 The selection of the two exposure media different from the standard Hoagland (SH) was
472 based on the knowledge of decreasing LN availability under exposure with phosphorous
473 (González et al., 2015). As we expected the dissolved LN content in SH showed the
474 lowest values, whereas no marked difference was found between SH-P and DW (Table
475 2). At the starting time of any toxicity test the recoveries of the studied elements are
476 expected to not differ strongly with nominal values, whereas differences between nominal
477 and measure concentration at the end of the test were previously widely reported
478 (Hermann et al., 2016). In long term tests, without media renewal, the availability of LN
479 can decrease drastically, and the majority of the metals lost at the end of the test are in
480 colloidal forms, as occurred for Ce (El-Akl et al., 2015). Complexes of LN with
481 phosphates are expected to be formed first, due to the higher LN affinity towards
482 phosphates (Joonas et al., 2017), which explains the decrease of LN availability observed
483 in SH. The modified Hoagland (SH-P) was performed without phosphates but other
484 groups (e.g.-SO₄) and nutrients remained (Ca, Mg...) in the media. After the 96 h of
485 exposition the dissolved LN measured also showed some decrease in the SH-P even with
486 the absence of phosphates to precipitate, and the same trend was observed in the poorest
487 media used (DW, distilled water). Therefore, the decrease observed in these both media,
488 could be due to a) plant uptake or b) potential loss of LN by adsorption to the wall of used
489 glass pots. It is interesting to point that in the SH media the recoveries decreased with the

490 increase in the nominal content added (more LN added more precipitation), while for SH-
491 P and DW recoveries increased with the increase in the LN nominal content. Although
492 the equilibrium concentration in solution, was possibly not reached because of the
493 shortness of the test, it is expected to be equal for the three LN concentrations used. In
494 case of the SH-P and DW media no precipitation is predicted to occur. Therefore,
495 according to the results observed for the tests performed in this study, the dissolved LN
496 decreased in all studied media with time which should be taken in consideration when
497 toxicity tests are planned to be done with these elements, independently of the nutrient
498 richness of the used media.

499 Results obtained from the geochemical model ORCHESTRA showed that in the presence
500 of PO_4 (SH media) the solubility of lanthanides is negligible due to the formation of LN-
501 phosphate precipitates, which support the low dissolved lanthanide concentrations
502 measured in this experiment at time 0 h (0.24-13% of the nominal concentration).
503 Geochemical modelling even predicts lower dissolved LN concentrations than measured,
504 indicating that chemical equilibrium is not yet reached. Lanthanide-phosphate complexes
505 are the most important lanthanide species in the presence of PO_4 . In the absence of PO_4
506 (SH-P and DW media) the free lanthanide ion and the sulphate (SH-P) and carbonate
507 (DW) complexes become more relevant. El-Akl et al. (2015), observed in a study with
508 Ce, that trivalent lanthanides are poorly soluble in the natural environments due to the
509 presence of phosphates, therefore free ions often account for a small fraction in water
510 systems, however they have generally been found as the main predictors of LN toxicity
511 (Lingott et al., 2016; Tan et al., 2017). In addition to the free forms, carbonate groups
512 (mainly LNCO_3^+) observed both in SH-P and DW, were important dissolved species for
513 all LN in our study, which was also found in González et al. (2015). Tartaric acid is added
514 to keep Fe dissolved but apparently it is not such a strong Fe-chelator that it keeps the

515 added Fe fully dissolved (Reed and Haas, 1924) and therefore lets Fe-LN complexes
516 form. The use of this geochemical model identifies that equilibrium is not reached during
517 our experiment (96 h) and that even in poorest media (DW) LN form complexes. LN have
518 high affinity for complexing with PO_4 , and in the P absence with sulphates and
519 carbonates. This model prediction, if done before performing any ecotoxicological test,
520 will help obtain the correct selection of stabilization time and media used.

521

522 **5. Conclusion**

523

524 In this study, we observed that lanthanide availability decreased drastically in the
525 presence of phosphate within the media. In that case, no toxic effects were reported, and
526 even a potential stimulation of *V. faba* root growth was recorded following low exposure
527 to Lu. The study of LN toxicity in a media without phosphates showed higher LN
528 availability during the test, and toxic effects were obtained mainly in the treatments with
529 the highest LN contents. Observed toxic effects could be related with a detriment of Ca
530 instead of LN, with higher affinity for LN with higher ionic radii, and/or directly for an
531 increase in the lanthanides content. In distilled water, without nutrient addition, toxicity
532 was found in all treatments with LN addition but not in controls, suggesting that not only
533 the lack of nutrients is causing the toxicity, therefore assuming some potential LN
534 detrimental effect. Although LN function in plants is still an unanswered question, studied
535 species have absorbed them, and we can assume that, at least, when phosphorous are
536 present in the studied media, the LN addition probably will not show any toxic effect.
537 However, in media without P, the LN free content and the different species formed will
538 be responsible for potential detrimental effects for root growth. Lanthanide interactions

539 with Ca in roots and the potential benefits or damages of applied LN in root growth must
540 be further studied.

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710

**Cytotoxicity and genotoxicity of lanthanides for *Vicia faba* L. are mediated by their
chemical speciation in different exposure media**

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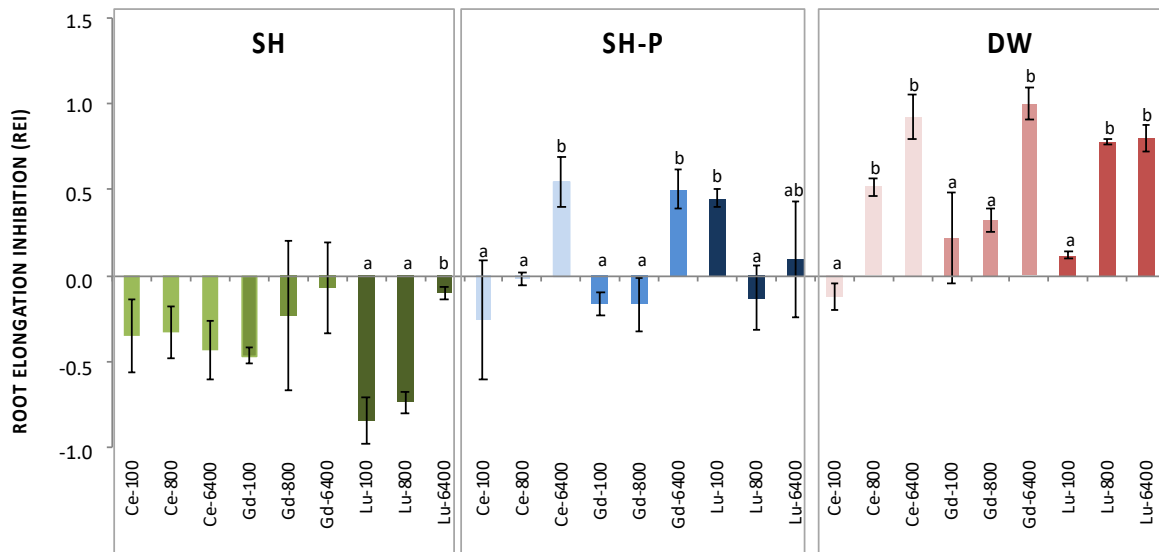


Figure 1. Root elongation inhibition with respect to the control (REI) in the three studied media (SH: standard Hoagland media; SH-P: modified Hoagland media without phosphates; and DW: distilled water) for the three studied lanthanides (Ce, Gd, Lu) exposed at different LN concentrations (100, 800, 6400 µg L⁻¹). REI was calculated as follows: $REI = (C-T) / C$; being C: root length in control seeds, and T: root length in treated seeds. Lowercase letters indicate significant differences (Tukey's test, $p < 0.05$) among each lanthanide concentration for each studied media (N=3). Vertical error bars represent the standard deviations. Root elongation data (cm) for each control and treatment can be consulted in SI Table 1.

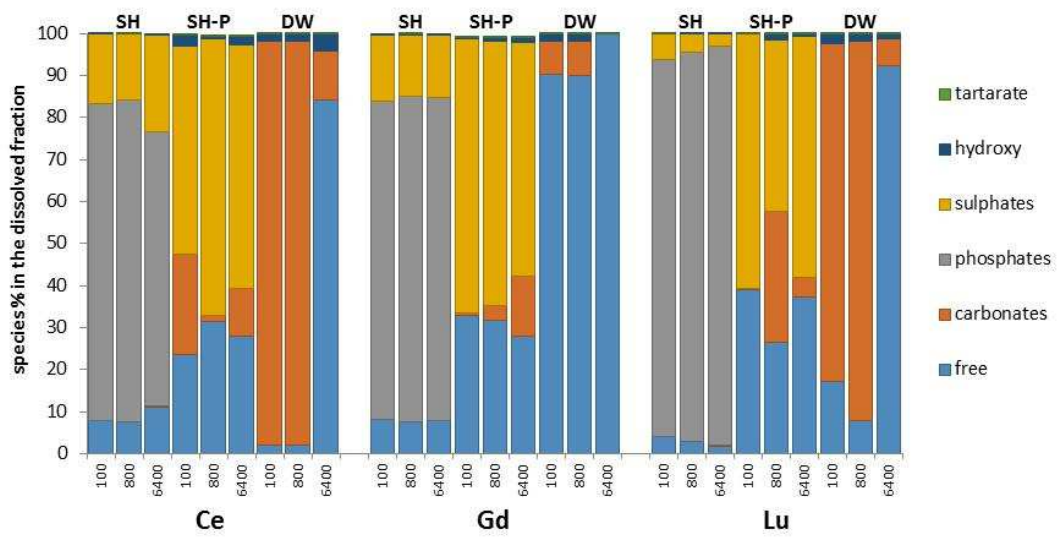


Figure 2. Distribution of lanthanide species in percentage for the 3 studied media (SH: standard Hoagland media; SH-P: modified Hoagland media without phosphates; and DW: distilled water) for the three studied lanthanides (Ce, Gd, Lu) and for the three different concentrations (100, 800, 6400 µg L⁻¹) for the measured LN content at the end (t 96 h) of the *V. faba* L. exposure.

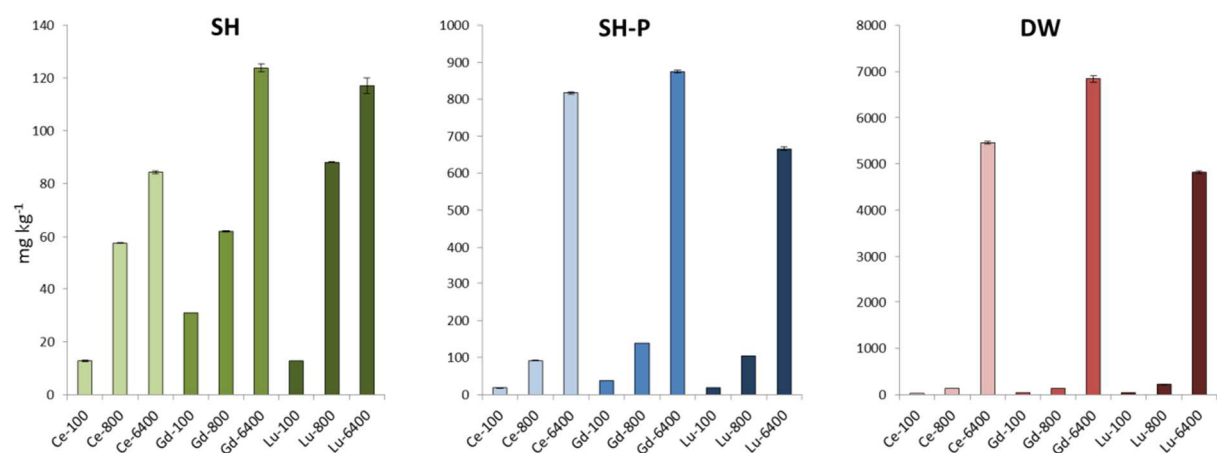


Figure 3. Content of Ce, Gd and Lu in roots exposed to the three studied media (SH: standard Hoagland media; SH-P: modified Hoagland media without phosphates; and DW: distilled water) at different LN concentrations (100, 800, 6400 µg L⁻¹). Vertical error bars represent the standard deviations, (N=3).

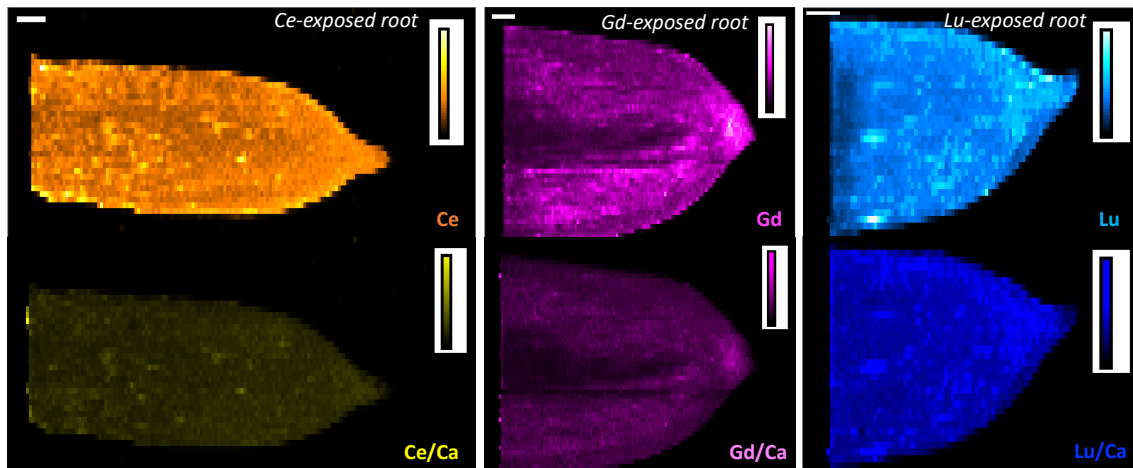


Figure 4. Laser Ablation-Inductively Coupled Plasma Mass Spectrometry (ICP-MS-LA) on the secondary roots exposed to lanthanide (Ce, Gd, or Lu) at $6400 \mu\text{g L}^{-1}$ in the SH-P media (**modified Hoagland media without phosphates**). In the top images are represented the Ce, Gd, and Lu elementary maps and in the bottom the corresponding ratio Ce/Ca, Gd/Ca, and Lu/Ca. Obtained information is a qualitative approximation of the LN whole content in roots, therefore arbitrary scales are used. White scale bar: 0.1 mm. Color intensity bar: black corresponds to low intensity, and yellow, pink, blue to high intensity.

Cytotoxicity and genotoxicity of lanthanides for *Vicia faba* L. are mediated by their chemical speciation in different exposure media

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Table 1. Mitotic index (MI: number of cells in division/1000 cells) and micronucleus frequency (MCN: number of micronuclei/1000 observed cells) of *Vicia faba* roots (\pm standard deviation) (N=3) exposed in the three studied media (SH: standard Hoagland media; SH-P: modified Hoagland media without phosphates; and DW: distilled water) and with three different lanthanides (Ce, Gd, Lu) at different concentrations (100, 800, 6400 $\mu\text{g L}^{-1}$).

Media	Control -	Ce			Gd			Lu			
		100	800	6400	100	800	6400	100	800	6400	
MI	SH	33.5 \pm 12.6	27.7 \pm 6.3	19.6 \pm 2.8	26.3 \pm 6.7	Control + (MH) = 19.5 \pm 3.2			24.0 \pm 6.5	30.0 \pm 3.5	31.0 \pm 16.2
	SH-P	35.7 \pm 13.4	37.2 \pm 19.4	38.17 \pm 7.1	8.5 \pm 5.3*	26.8 \pm 5.5	29.0 \pm 5.5	32.2 \pm 15.1	22.8 \pm 6.7	34.3 \pm 1.8	23.5 \pm 8.0
	DW	26.3 \pm 8.5	5.3 \pm 2.6*	7.2 \pm 4.3*	3.3 \pm 1.5*	41.7 \pm 10.1	30.7 \pm 7.3	8.2 \pm 5.3*	5.2 \pm 2.1*	2.8 \pm 1.6*	5.2 \pm 1.4*
						4.2 \pm 2.6*	4.3 \pm 0.3*	4.3 \pm 2.9*			
MCN	SH	2.2 \pm 0.6	1.7 \pm 1.6	1.0 \pm 1.0	1.5 \pm 0.9	Control + (MH) = 21.8 \pm 5.4 [#]			0.3 \pm 0.3*	2.2 \pm 1.8	1.2 \pm 0.8
	SH-P	0.8 \pm 1.0	3.8 \pm 0.8*	2.2 \pm 1.5	n.d.	2.2 \pm 1.3	1 \pm 0.9	2.8 \pm 2.8	3.3 \pm 2.8	1.8 \pm 1.0	3.8 \pm 1.0*
	DW	0.8 \pm 0.6	n.d.	n.d.	n.d.	3.2 \pm 1.6	1.8 \pm 0.6	n.d.	n.d.	n.d.	n.d.
						n.d.	n.d.	n.d.			

* Statistically significant difference from negative control and treatments (Dunnett's test, $p < 0.05$).

[#] Statistically significant difference among controls (Tukey's test, $p < 0.05$).

n.d. No data calculated for treatments with MI inhibition.

Table 2. Percentage of recovery compared to the nominal content (\pm standard deviation) to assess the effect of exposure media and time in LN availability for the 3 lanthanides (Ce, Gd, Lu) at the 3 concentrations (100, 800, 6400 $\mu\text{g L}^{-1}$) in the 3 studied media (SH: standard Hoagland media; SH-P: modified Hoagland media without phosphates; and DW: distilled water). LN_N : nominal LN added in the solutions; LN_T0 : measured LN in time 0 h; LN_T96 : in time 96 h.

Nominal LN (LN_N)	SH			SH-P			DW		
	100	800 $\mu\text{g L}^{-1}$	6400	100	800 $\mu\text{g L}^{-1}$	6400	100	800 $\mu\text{g L}^{-1}$	6400
%Ce _(T0)	3.3 \pm 0.12	0.48 \pm 0.13	0.24 \pm d.l.	87 \pm 2.1	99 \pm 0.46	100 \pm 1.0	90 \pm 4.3	94 \pm 5.9	102 \pm 5.7
%Gd _(T0)	9.5 \pm 0.26	1.0 \pm d.l.	0.48 \pm d.l.	85 \pm 2.9	92 \pm 0.05	99 \pm 0.35	92 \pm 11	96 \pm 4.3	100 \pm 7.4
%Lu _(T0)	13 \pm 0.33	1.7 \pm 0.06	1.2 \pm d.l.	81 \pm 1.5	89 \pm 2.7	97 \pm 3.8	84 \pm 17	93 \pm 11	94 \pm 3.0
%Ce _(T96)	0.64 \pm d.l.	0.67 \pm 0.08	d.l.	92 \pm 0.64	43 \pm 0.80	67 \pm 0.34	68 \pm 15	33 \pm 10	67 \pm 18
%Gd _(T96)	0.59 \pm d.l.	1.5 \pm 0.05	0.12 \pm d.l.	83 \pm 1.2	75 \pm 0.68	66 \pm 0.25	98 \pm 1.1	49 \pm 0.24	61 \pm 19
%Lu _(T96)	13 \pm 0.06	1.9 \pm d.l.	0.28 \pm d.l.	123 \pm 0.23	66 \pm 0.13	77 \pm 0.49	56 \pm 0.18	28 \pm 5.7	47 \pm 0.09

d.l. data lower than 0.05%

Table 3. Spearman's correlation for the media SH-P (modified Hoagland media without phosphates) with the LN content in roots (LN_{root} , $mg\ g^{-1}$), the mitotic index (MI, number of cells in division/1000 cells) and the Root elongation inhibition (REI) for *Vicia faba* roots, and the LN content and main LN group species in the studied media. Correlation done for the three different lanthanides studied together (Ce, Gd, Lu) and for the 3 different LN concentration used (100, 800, 6400 $\mu g\ L^{-1}$).

		SH-P	
		LN_{root}	MI
	LN t 0 h	0.883**	
	LN t 96 h	0.883**	-0.700*
	LN_{root}	1	-0.667*
	MI	-0.667*	1
	REI		-0.767*
t 96 h	LN^{+3} [free]	0.867**	-0.667*
	LN-dissolved	0.900**	-0.733*
	LN-carbonates	0.867**	
	LN-sulphates	0.883**	-0.700*
	LN-hydroxide	0.900**	
	LN-tartarate	0.867**	-0.667*

* $p < 0.05$, ** $p < 0.01$.

New Graphical Abstract

