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1 **Copper and cobalt mobility in soil and accumulation in a metallophyte as influenced by**
2 **experimental manipulation of soil chemical factors**

3

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22 **Highlights**

23 -Organic matter supply decreased Cu mobility and accumulation in *Anisopappus chinensis* at 500 mg kg⁻¹ Cu.

24 -Oxides of Fe and Mn supplies had little effect on Cu–Co mobility in soil and accumulation by plants.

25 -*Anisopappus chinensis* maintains high foliar Cu–Co without effect on growth while increasing Cu–Co mobility.

26 -*Anisopappus chinensis* is added to the short list of candidate cuprophytes for Cu–Co phytoremediation.

27

28 **Abstract**

29 The influence of Fe oxides, Mn oxides and organic matter (OM) on the Cu and Co mobility in soil and
30 accumulation in the metallophyte *Anisopappus chinensis* (*Ac*), as compared with *Helianthus annuus* (*Ha*), was
31 experimentally investigated. Growth and accumulation response when increasing the exchangeable Cu and Co
32 concentrations in soil were also investigated. Plants were cultivated on soil where concentrations of Cu, Co, Fe
33 oxides, Mn oxides and OM content were varied according to 36 treatments. The OM supply decreased the Cu
34 mobility and increased the Co mobility, resulting in decreasing the foliar Cu of *Ac* and increasing the foliar Co of
35 *Ha*. The Fe oxides supply could increase the Cu accumulation for *Ac*, but was not verified for *Ha*. Compared with
36 *Ha*, *Ac* increasingly accumulated Cu and Co without negative effect on plant growth while increasing Cu and Co
37 mobility to phytotoxic concentrations. The results revealed promising perspectives for the use of *Ac* in Cu-
38 contaminated environment phytoremediation applications.

39

40 **Keywords**

41 heavy metals, metal availability, organic matter, oxides, phytoremediation

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52 **1. Introduction**

53 Soil contamination by metals is critically increasing and has become a major environmental issue
54 (Alloway 1995; Smith and Huyck 1999; Baize and Tercé 2002). The Democratic Republic of Congo (DR Congo)
55 is a region of intensive mining activities, especially in the province of Katanga, where a succession of natural Cu
56 and Co outcrops occurs. These remarkable geological formations, where soil Cu and Co concentrations can reach
57 tens of thousands of mg kg⁻¹ (Duvigneaud 1958), have become an epicentre of Cu and Co extraction. Katanga
58 accounts for 5% and 47.5% of the world production of Cu and Co, respectively (USGS, 2014). Mining and ore-
59 processing activities have contaminated the environment over huge areas, with negative impacts on human health
60 (Banza et al. 2009).

61 In this context, new types of metalliferous habitats have appeared where the soil can be 1,000 times more
62 concentrated in Cu and Co than “normal” soils (Ernst 1974; Baker et al. 2000; Reeves and Baker 2000). These
63 secondary metalliferous sites represent important sources of pollution, especially in sub-tropical regions. Indeed,
64 the risk of metallic transfers through runoff and erosion is increased due to high rainfall (1,200 mm) and the long
65 dry season. It is therefore essential to find solutions to remediate these pollutions, and phytoremediation processes
66 represent innovative solutions. Such techniques require the use of highly metal-tolerant plants. The vegetation of
67 copper hills in Katanga hosts a large number of metal-tolerant species, referred to as cuprophytes (Duvigneaud
68 and Denaeyer-De Smet 1963; Ernst 1974, 1990; Faucon et al. 2012a; Ilunga wa Ilunga et al. 2013; Séleck et al.
69 2013) and cobaltophytes (Duvigneaud 1959). Among them, some are able to hyperaccumulate Cu and/or Co in
70 their natural habitats (Brooks et al. 1986; Baker and Brooks 1989; Reeves and Baker 2000; Reeves 2006; Faucon
71 et al. 2007; Faucon et al. 2009; Lange et al. 2014). Recently reviewed hyperaccumulation thresholds classified Cu
72 and Co hyperaccumulators as species able to accumulate actively these metals in leaves at a level above 300 mg
73 kg⁻¹ (van der Ent et al. 2013).

74 Cuprophytes and cobaltophytes represent a fruitful pool of plant diversity for Cu and/or Co
75 phytoremediation applications. However, in-depth knowledge of Cu and Co tolerance and accumulation are
76 essential when envisaging phytoremediation of Cu- and Co-contaminated soils. Copper tolerance has been
77 extensively studied, but little is known about Cu and Co tolerance among metallophytes from Cu- and Co-rich
78 soils (Brooks and Malaisse 1990; Harper et al. 1997, 1998; Faucon et al. 2012b). Moreover, Cu tolerance has been
79 experimentally demonstrated only for cuprophytes from Katanga: *Haumaniastrum katangense*, *H. robertii*,
80 *Aeolanthus biformifolius* (Lamiaceae) (Morrison et al. 1979; Chipeng et al. 2009; Peng et al. 2012); *Mimulus*
81 *guttatus* (Scrophulariaceae) (Allen and Sheppard 1971; Macnair 1983); *Silene cobalticola* and *S. vulgaris*

82 (Caryophyllaceae) (Baker et al. 1983; Song et al. 2004); and China: *Elsholtzia haichowensis* and *E. splendens*
83 (Lamiaceae) (Jiang et al. 2004; Lou et al. 2004; Song et al. 2004), and has been demonstrated for Co only for *S.*
84 *cobalticola* (Baker et al. 1983).

85 The ecology and evolution of Cu and Co accumulation and tolerance in these metallophytes remains
86 poorly understood. In particular, many cuprophytes show extensive phenotypic variation of Cu and Co
87 accumulation in their natural sites (Faucon et al. 2009; Lange et al. 2014) and some can be defined as ‘facultative
88 hyperaccumulators’ (i.e. that hyperaccumulate metal when occurring on metalliferous soils, yet also grow on non-
89 metalliferous soils) (Pollard et al. 2014). For example, Cu and Co levels in shoots of *Crepidiorhpalon tenuis*
90 (pseudometallophyte from Katanga) range from 80 to 1,400 mg kg⁻¹ and 61 to 1,105 mg kg⁻¹, respectively (Faucon
91 et al. 2009). Such variations for Cu can be genetic, especially due to genetic differentiations among populations
92 (Faucon et al. 2012b; Peng et al. 2012). However, this also suggests an influence of soil factors on Cu and Co
93 accumulation. The soil factors governing the accumulation patterns of Cu and Co in cuprophytes are poorly
94 understood. Soil chemical parameters like pH, redox potential, OM quality and quantity, oxides, clays, sulfides
95 and carbonates are known to influence metal mobility in soils (Kabata-Pendias and Pendias 2001). In metalliferous
96 soils, Cu may be adsorbed by OM and Fe oxides (FeOx) (Pourret et al. 2015a). The affinity of Cu for organic
97 matter (OM) is well documented and it has been reported that insoluble OM with high molecular weight can reduce
98 the Cu mobility in acidic conditions (Chirenje and Ma 1999; Kumpiene et al. 2008). The stability of Cu in soils is
99 also known to be more efficient in FeOx-amended soils (Kumpiene et al. 2008). In contrast to Cu, Co in
100 metalliferous soils is mostly bound to Mn oxides (MnOx) (Lange et al. 2014; Pourret et al. 2015a); previous studies
101 reported this strong affinity in acidic conditions (McKenzie 1970; Childs 1975; Li et al. 2004; Tongtavee et al.
102 2005; Luo et al. 2010).

103 Therefore, the availability of Cu and Co in soil can be indirectly driven by the complex interplay of many
104 soil chemical parameters, including the mineralogical context, and ecological processes driving OM concentration
105 in the soil. Variation in metal accumulation in plants may thus be strongly dependent on a broad range of factors,
106 beyond the concentration of Cu and Co themselves. A previous study (Lange et al. 2014) explored the phenotypic
107 variation of Cu and Co accumulation in *Anisopappus chinensis* (range: 4 to 2,821 mg kg⁻¹ and 3 to 1,334 mg kg⁻¹,
108 respectively), collected *in natura* among strongly pedogeochemically contrasted sites. It was found that metal
109 accumulation in plants was influenced not only by the free Cu and Co concentration in the soil solution. Copper
110 bound to MnOx and Co bound to OM represented a significant pool of available Cu and Co for plants. The

111 concentrations of Cu and Co bound to FeOx also had a positive influence upon the Cu and Co accumulation
112 variations, respectively.

113 This study aimed to test experimentally the influence of FeOx, MnOx and OM supply on (i) Cu and Co
114 mobility in soil and (ii) Cu and Co foliar accumulation in *Anisopappus chinensis*. We also examined (iii) the plants'
115 response, in terms of shoot biomass production and Cu and Co accumulation according to an increase of the Cu-
116 or Co-exchangeable soil concentration. Our working hypotheses are as follows: (i) the OM and FeOx supply
117 negatively affect the Cu mobility; (ii) the MnOx supply negatively affects the Co mobility; (iii) the MnOx and OM
118 supply positively affects the Cu and Co accumulation in plants, respectively; (iv) variation in mobility of Cu and
119 Co will translate into corresponding variation in accumulation in leaves; (v) increasing Cu or Co in soil increases
120 Cu or Co in leaves, respectively, without plant growth inhibition.

121

122 2. Materials and methods

123 2.1. Plants and soil origin

124 *Anisopappus chinensis* L. Hook.f. & Arn. (Asteraceae, subfam. Asteroideae, tribe Anthemidae) is widely
125 distributed in tropical Africa. It is a perennial pseudometallophyte (i.e. occurring both on metalliferous and non-
126 metalliferous soils), widespread on the copper hills of Katanga and in the surrounding Miombo woodlands. The
127 metalliculous populations exhibit broad variation in the Cu and Co concentrations in leaves *in natura*, as described
128 in the introduction. The seeds of *A. chinensis* were collected from the Niamumenda copper hill population
129 (GCSWGS84 DD: S 11.60492°; E 27.29400°) (Katanga, DR Congo). This region of South-Central Africa is
130 characterized by a subtropical humid climate including a rainy (November to March) and a dry season (May to
131 September). *Helianthus annuus* L. var. Sunspot (Asteraceae, subfam. Asteroideae, tribe Anthemidae), was chosen
132 as a well-known non-tolerant control species (Chakravarty and Srivastava 1992); seeds were commercially
133 purchased (Sluis Garden).

134 The soil for the pot experiment was obtained from the upper 20 cm of a Luvisol from homogeneous old
135 forest area (Beauvais, France, coordinates: N 49°28'13.88", E 2°4'0.45"). The soil was sieved (5 mm) and mixed
136 with river sand to obtain 20% sand. The soil was analysed by Acme Analytical Laboratories Ltd. (Vancouver
137 Canada), accredited under ISO 9002. Briefly, 0.25 g soil was heated in HNO₃-HClO₄-HF to fuming and taken to
138 dryness; the residue was further dissolved in HCl solution and diluted with ultra-pure water before Inductively
139 Coupled Plasma-Mass Spectrometry (ICP-MS) measurements. According to data quality result assessment, the
140 measurement accuracy was estimated at ±5% for all the considered elements. The total concentrations of Cu, Co,

141 Fe₂O₃ and MnO were respectively 5.5; 6.2; 24,400 and 850 mg kg⁻¹. The soil pH (water) was 4.5 ± 0.05 and OM
142 content (loss on ignition, 500 °C for 8 h) was 6.0 ± 0.15%. The CaCl₂ (0.01 M)-extractable Cu and Co
143 concentrations (Van Ranst et al. 1999) were 0.07 ± 0.002 mg kg⁻¹ (n = 6) and 0.06 ± 0.008 mg kg⁻¹ (n = 6),
144 respectively.

145

146 2.2. Experimental Design

147 A glasshouse pot experiment was conducted from March to July 2013. Both species were cultivated in
148 the same conditions and received the same treatments. The mass of soil in each pot was 500 g. Two soil
149 concentrations of Cu (200 and 500 mg kg⁻¹), and two concentrations of Co (20 and 100 mg kg⁻¹), were prepared
150 as follows. The Cu and Co supply was performed per pot using 30 mL of pH-adjusted solutions (pH = 4.5) of
151 copper (II) sulfate (CuSO₄•5H₂O, *Sigma-Aldrich*[®]) and/or cobalt (II) sulfate (CoSO₄•7H₂O, *Sigma-Aldrich*[®]).
152 Concentrations of solutions were 0.05 and 0.13 M CuSO₄ for 200 and 500 mg kg⁻¹ Cu treatments, respectively;
153 and 0.0056 and 0.028 M CoSO₄ for 20 and 100 mg kg⁻¹ Co treatments, respectively. For each Cu and Co
154 concentration, the concentrations of MnOx, FeOx and OM content were modified according to several treatments
155 as follows: control, +OM, +MnOx, +FeOx, +OM+FeOx, +OM+MnOx, +FeOx+MnOx, +FeOx+MnOx+OM. For
156 Cu, two additional treatments were tested: +Co and +MnOx+Co. A non-treated soil, hereafter referred to as
157 “control-0” in the paper, was also included. Each treatment was carried out in six replicates, divided in three blocks,
158 each block containing two replicates. For treatments with MnOx and FeOx supply, 130 mg kg⁻¹ pyrolusite (MnO₂,
159 *Sigma-Aldrich*[®]) (65 mg per pot) and 3,000 mg kg⁻¹ hematite (Fe₂O₃, *Sigma-Aldrich*[®]) (1,500 mg per pot) were
160 added in their commercial forms. These are the most widespread oxides (Pourret et al. 2015b) in the Cu- and Co-
161 rich soils in Katanga. The concentrations were chosen to get an increase of up to more than 10% of the total oxide
162 concentrations. For the study of the OM impact, 4% peat moss (*Sphagnum*) were added, to work with an acidic
163 recalcitrant OM (pH = 4.5 ± 0.05) with the same pH as the study soil, and low decomposition kinetics (Moore and
164 Basiliko 2006). A level of +4% OM was chosen to get the OM content close to that usually found in soils in
165 Katanga (Lange et al. 2014). One week before sowing, the soil of each pot was carefully homogenized. During the
166 experiment, all pots were watered with deionized water by filling up saucers every two days, and pots of each
167 block were randomized weekly.

168

169 2.3. Plant and soil analyses

170 After harvesting, plants were carefully brushed (whole shoots), washed with Alconox® 1% in deionized
171 water, dried at 65 °C for 48 h and weighed based on the procedure by Faucon et al. (2007). The Cu and Co
172 concentrations in leaves of *A. chinensis* and *H. annuus* were determined using ICP–MS following digestion using
173 a closed microwave system. Briefly, 0.2 g (accurately weighed) of leaf powder was mixed with 8 mL concentrated
174 HNO₃ and 2 mL concentrated HCl (Avula et al. 2010) directly in a microwave Teflon vessel (Lavilla et al. 2009).
175 After homogenization of the mixture, the vessels were placed in the microwave system (Mars 5, CEM Corporation,
176 USA) according to the protocol of Avula et al. (2010). The digest was then diluted to approximately 30 g
177 (accurately weighted) and then stored at 4 °C. The Cu and Co concentrations in digested samples were measured
178 by ICP–MS (Thermo Scientific XSERIES2). Quality control was performed using the SRM1573a CRM (tomato
179 leaves, Gills 1995). For Cu and Co in this case, the bias was below 5% (Lange et al. 2014). For the soil, pH (water)
180 and OM content (loss on ignition, 500 °C for 8 h) were measured at the end of the experiment for each treatment.
181 The OM content did not vary while the pH of soils enriched with OM were slightly lower (pH = 4.7 ± 0.05) than
182 those of soils without added OM (pH = 4.9 ± 0.05). Then, the soil of each pot was dried at room temperature,
183 sieved (2 mm), and then Cu and Co were extracted with CaCl₂ (0.01 M) (Van Ranst et al. 1999). Copper and Co
184 concentrations were measured using Inductively Coupled Plasma–Optical Emission Spectrometry (ICP–OES;
185 Varian Vista MPX). The precision and accuracy of analysis were determined using in-house standards for soils;
186 biases of ± 5% were obtained in this case. In this study, variations of Cu and Co mobility were assessed by
187 investigating variations of CaCl₂-extractable Cu and Co concentrations, which indicate variations of Cu and Co
188 exchangeable concentration in soils (Van Ranst et al. 1999). Exchangeable Cu and Co in the control-0 soils at the
189 end of the experiment were 0.07 ± 0.004 mg kg⁻¹ (*n* = 6) and 0.06 ± 0.006 mg kg⁻¹ (*n* = 6), respectively for Cu and
190 Co. Exchangeable Cu and Co in the control-0 soils did not vary from those of the soil of origin.

191

192 2.4. Statistical analysis

193 Copper and Co concentrations in soil and leaves were analysed by ANOVAs. The Cu and Co soil
194 concentrations and the treatment were considered as fixed factors, and block as a random factor. The differences
195 between each treatment and the control were analysed with Student's *t*-test on log-transformed data. The response
196 of plants (shoot biomass and accumulation) according to an increase of soil exchangeable concentrations of Cu or
197 Co was tested using the Kruskal–Wallis test due to unequal number of replications between exchangeable ranges.
198 Mann–Whitney pairwise comparisons were used to test differences between ranges. The analyses were performed
199 using R software (3.0.2).

200

201 3. Results and discussion

202 3.1. Influence of MnOx, FeOx and OM on Cu and Co mobility

203 The results showed an effect of Cu concentration, treatment and a significant interaction concentration ×
204 treatment (Table 1). Exchangeable Cu increased from ca. 10 mg kg⁻¹ in the low Cu treatment to ca. 60 mg kg⁻¹ in
205 the high Cu treatment. At 200 mg kg⁻¹ Cu, treatments did not significantly affect exchangeable Cu (Fig. 1a). In
206 contrast, at 500 mg kg⁻¹ Cu, exchangeable Cu was significantly lower in the +OM soil than in the control (42 ± 6
207 mg kg⁻¹ and 57 ± 10 mg kg⁻¹, respectively). A decrease was also observed for OM combined with oxides
208 (+OM+FeOx, +OM+MnOx, +FeOx+MnOx+OM), but this was not significant. The results demonstrated that Cu
209 mobility was mainly influenced by the OM quantity in soil in the way that increasing soil OM content decreased
210 Cu mobility. Copper in soils is known to have a particular affinity for OM (Stevenson, 1982; Temminghoff et al.
211 1997; Gupta et al. 2006; Lange et al. 2014; Pourret et al. 2015), explained by the strong capacity of OM to form
212 chelate complexes with cations (Stevenson 1991) and the high complex stability of Cu (Stumm and Morgan 1996).
213 Moreover, OM was supplied in the form of peat, which has a high capacity to adsorb Cu (Kumpiene et al. 2008).
214 In contrast, Cu mobility was not decreased by FeOx amendment, contrary to our expectations (Fig. 1a, b). Copper
215 adsorption onto oxides would mainly occur as chemisorption (Davis and Leckie 1978; Dzombak and Morel 1990,
216 Peacock and Scherman 2004), and hence would strongly limit its mobility. This type of adsorption differs from
217 physical adsorption (i.e. physisorption, with electrostatic interactions) in the way that high strength chemical bonds
218 are created at the adsorbant surface (Hudson 1998). This unexpected result would be mainly explained by the well-
219 known preferential adsorption of Cu onto OM compared with oxides in acidic conditions (Chirenje and Ma
220 1999). In this way, Cu–oxides associations would have been limited compared with the Cu–OM associations,
221 substantially limiting the influence of FeOx enrichment on Cu mobility.

222 For Co, the results showed a concentration effect on the exchangeable concentration (Table 1).
223 Exchangeable Co increased from ca. 5 mg kg⁻¹ in the low Co treatment to ca. 40 mg kg⁻¹ in the high Co treatment.
224 At 20 mg kg⁻¹ Co, the treatment affected the exchangeable Co (Fig. 2), while it did not at 100 mg kg⁻¹ Co (data
225 not graphically represented). Contrary to expectations, MnOx supply did not influence Co mobility, while a
226 reduction of mobility was expected due to the high affinity between Co and MnOx (Lange et al. 2014, Pourret et
227 al. 2015). This negative result might be explained by the low amount of MnOx added in the +MnOx treatments (+
228 65 mg, i.e. an increase of more than 10% of the total MnOx) compared with the total amount of soil in pots (500
229 g). The influence of MnOx supply on the Cu and Co mobility should be tested with higher MnOx enrichment. At

230 20 mg kg⁻¹ Co, exchangeable Co was higher in +OM, +FeOx, +OM+FeOx, +OM+MnOx and +FeOx+MnOx+OM
231 soils (6.3 ± 0.3 mg kg⁻¹), than in the control (4.8 ± 0.3 mg kg⁻¹) (Fig. 2). The increase in response to OM could be
232 explained by the fact that Co desorption is easier when bound to humic acids than to oxides (McLaren et al. 1986).
233 Moreover, at 20 mg kg⁻¹ Co, OM also had an effect on pH (from 4.9 to 4.7 ± 0.05), which may have increased Co
234 mobility (McLaren and Crawford 1973; Alloway 1995; Kabata-Pendias and Pendias 2001; Chaignon et al. 2002;
235 Faucon et al. 2011). Surprisingly, FeOx addition increased the Co mobility (Fig. 2). The fraction of Co adsorbed
236 onto oxides is not easily desorbed into the soil solution (McLaren et al. 1986; Collins and Kinsela 2010). However,
237 in acidic conditions, iron oxides are able to uptake complexing ligands, and especially Co complexed by natural
238 organic matter (Davis and Leckie 1978; Gu et al. 1994). These associations are known to occur as surface binding
239 (Davis and Leckie 1978), which are hence readily desorbed into soil solution compared with oxide-ions
240 chemisorption, i.e. more easily extracted by CaCl₂ extraction. In our case, iron oxides might have also adsorbed
241 Co-OM ligands at their surfaces, explaining the increase of Co mobility in soils enriched with FeOx.

242

243 3.2. Influence of MnOx, FeOx and OM on the foliar Cu and Co accumulation

244 The two plant species showed contrasting patterns in terms of Cu and Co accumulation. Foliar Cu ranged
245 from 2 to 98 mg kg⁻¹ in *A. chinensis* and from 10 to 302 mg kg⁻¹ in *H. annuus*. Foliar Co ranged from 0.1 to 54
246 mg kg⁻¹ in *A. chinensis* and from 1 to 377 mg kg⁻¹ in *H. annuus*. *Helianthus annuus* exhibited symptoms of toxicity
247 (chlorosis) at 200 mg kg⁻¹ Cu and 20 mg kg⁻¹ Co. Only a few plants of *H. annuus* survived at 500 mg kg⁻¹ Cu.
248 Only a few plants of *A. chinensis* and no plants of *H. annuus* survived at 100 mg kg⁻¹ Co. Consequently, statistical
249 analyses were not performed for *H. annuus* at 500 mg kg⁻¹ Cu, and for both species at 100 mg kg⁻¹ Co. For *A.*
250 *chinensis*, a pseudometallophyte previously defined as facultative Cu and Co hyperaccumulator species because
251 of the large variations of foliar concentrations observed in its natural habitat (Faucon et al. 2009; Lange et al.
252 2014), the plants did not accumulate Cu and Co up to the hyperaccumulation threshold of 300 mg kg⁻¹ (Krämer
253 2010; van der Ent, et al. 2013). In the same way, the few studies regarding Cu and Co hyperaccumulation by plants
254 under controlled conditions did not demonstrate the phenomenon (Morrison et al. 1979, 1981; Baker et al. 1983;
255 Chipeng et al. 2010; Faucon et al. 2012). The Cu and Co hyperaccumulation may express in very specific *in natura*
256 conditions, which are difficult to reproduce.

257 For foliar Cu in *A. chinensis*, there was an effect of the concentration, the treatment and the interaction
258 concentration × treatment, while no treatment effect was found for *H. annuus* (Table 2). At 200 mg kg⁻¹ Cu, foliar
259 Cu of *A. chinensis* was higher on +FeOx and +Co soils (13 ± 0.7 mg kg⁻¹ and 14.2 ± 1.3 mg kg⁻¹, respectively).

260 Interestingly, this behaviour was not associated with an increase of exchangeable Cu (Fig. 1a). In a previous study
261 conducted by Lange et al. (2014), the concentration of Cu bound to FeOx (Cu–FeOx) positively influenced foliar
262 Cu of *A. chinensis* ($r = 0.28, p < 0.05$), and has been identified as a potential Cu available concentration. Root-
263 induced processes, and especially soil acidification, may induce metal mobilization from the non-mobile soil
264 fraction (Marschner 1995; Harter and Naidu 2001; Hinsinger 2001; Hinsinger et al. 2003; Houben and Sonnet
265 2012). In this study, *A. chinensis* may have mobilized Cu from the Cu–FeOx fraction. Nevertheless, this result
266 must be carefully considered because it was observed only for a single treatment. For the 500 mg kg⁻¹ Cu soils,
267 foliar Cu of *A. chinensis* was strongly reduced in plants growing on all treatments with OM (from 40 ± 5 mg kg⁻¹
268 to 22 ± 1 mg kg⁻¹) (Fig. 1b). For these treatments, a strong correlation was observed between the exchangeable Cu
269 and the foliar Cu ($r = 0.84, p < 0.01$), which suggests that the decreased uptake reflects the corresponding decrease
270 in mobility. The hypothesis on the positive influence of MnOx supply on the Cu accumulation was not verified,
271 regardless of the Cu soil concentration and treatment. This lack of effect might be due to the low volume of MnOx
272 added in pots treated with MnOx, despite an increase of more than 10% of the total MnOx concentration.

273 For foliar Co, at 20 mg kg⁻¹ Co, there was an effect of the treatment for *A. chinensis*, not verified for *H.*
274 *annuus* (Table 2). However, for *A. chinensis*, foliar Co for each treatment did not significantly vary from the
275 control. Interestingly, variations in the Co mobility observed between treatments did not lead to variations of Co
276 accumulation for *A. chinensis* (Fig. 2). The hypothesis of a higher Co accumulation in plants growing on soils
277 treated with OM was thus not verified for *A. chinensis*. Conversely, it was verified for *H. annuus* on +OM and
278 +FeOx+MnOx+OM soils (Fig. 3). This result is in agreement with Lange et al. (2014), where Co bound to the OM
279 fraction was shown to be a potential pool of available Co. Similarly, both McLaren et al. (1987) and Li et al. (2004)
280 highlighted that organically bound Co could positively influence Co availability for ryegrass, a Co non-tolerant
281 species. However, this result must also be carefully considered because OM supply increased the Co accumulation
282 only for two treatments and one species.

283 In the same way as for Cu, the results obtained for Co raised the complexity of metal availability, which
284 results in species-specific soil–plant processes, strongly influenced by rhizosphere chemistry and biology (Wenzel
285 2009; Alford et al. 2010). For instance, microbial activity in the rhizosphere could influence the availability of
286 chemical elements in soils (Hinsinger et al. 2005) and the patterns of metal accumulation in plants (Khan et al.
287 2000; Fomina et al. 2005; Toler et al. 2005; Barzanti et al. 2007; Andreatza et al. 2010; Kabagale et al. 2010).
288 Several studies on *E. splendens* highlighted that rhizosphere bacteria inoculation could increase Cu accumulation
289 (Chen et al. 2005), as well as arbuscular mycorrhizal fungi (Wang et al. 2005).

290

291 3.3. Response of growth and accumulation to metal mobility

292 Response of shoot biomass

293 Shoot biomass varied depending on soil exchangeable Cu for both species ($H = 28.3$ and $H = 15.9$,
294 $p < 0.001$; for *A. chinensis* and *H. annuus* respectively) (Fig. 4a, b). The control-0 soils had an exchangeable Cu of
295 0.07 mg kg^{-1} ($\pm 0.004 \text{ mg kg}^{-1}$). Increasing exchangeable Cu to $10\text{--}13 \text{ mg kg}^{-1}$ had no impact on *A. chinensis*
296 shoot biomass, whereas the shoot biomass of *H. annuus* was negatively affected (Fig. 4b). Increasing exchangeable
297 Cu from $10\text{--}13$ to $40\text{--}50 \text{ mg kg}^{-1}$ had a positive effect on the shoot biomass of *A. chinensis*. Apparent growth
298 stimulation by elevated soil Cu concentrations has already been reported in other Katangan cuprophytes (Faucon
299 et al. 2009, 2012b). In sharp contrast, growth was completely inhibited in *H. annuus* and only a few plants survived
300 (number of replicates < 3). Increasing exchangeable Cu to $50\text{--}60 \text{ mg kg}^{-1}$ decreased the shoot biomass of *A.*
301 *chinensis*. However, this decrease was not significant compared with the control-0 plants, and the plants did not
302 show any toxicity symptoms. No plants of *H. annuus* survived at that concentration. A particularly high capacity
303 to grow and survive at high levels of mobile Cu in the soil is here demonstrated for the first time in a population
304 of *A. chinensis*. A comparison with a population from normal soil is needed to assess if a higher Cu tolerance has
305 evolved in the metallicolous population used in this study.

306 Exchangeable Co in the control-0 soils was 0.06 mg kg^{-1} ($\pm 0.006 \text{ mg kg}^{-1}$). Increasing exchangeable Co
307 to $4\text{--}6$, and to $6\text{--}7 \text{ mg kg}^{-1}$, had no impact on *A. chinensis* growth ($H = 3.5$, *ns*) while *H. annuus* growth decreased
308 steadily compared with the control-0 plants ($H = 16.3$, $p < 0.001$) (Fig. 5a, b). These results represent the first
309 demonstration of the capacity to grow and survive at high levels of mobile Co in the soil in a population of *A.*
310 *chinensis*. Few plants of *A. chinensis* survived at 40 to 50 mg kg^{-1} (number of replicates < 3) (not presented on Fig.
311 5a). No plants of *H. annuus* survived at 40 to 50 mg kg^{-1} . The plants of *A. chinensis* from that population suffered
312 from Co toxicity when exchangeable Co reached $40\text{--}50 \text{ mg kg}^{-1}$. In its native site, the Niamumenda population
313 grows on soil with total Co concentration much lower than other Katangan metalliferous sites ($168 \pm 54 \text{ mg kg}^{-1}$),
314 and mostly bound to MnOx (71%) (Lange et al. 2014). Descendants may have suffered from toxicity stemming
315 from higher Co mobility in this experiment than in the native soil (free Co concentration in the rhizosphere was
316 $29.1 \pm 7 \text{ mg kg}^{-1}$) (Lange et al. 2014).

317 Ongoing work explores intraspecific variation of Cu and Co tolerance using metallicolous and non-
318 metallicolous populations of *A. chinensis*, to select the best candidate population(s) for phytoremediation
319 applications, especially phytostabilization of Cu- and/or Co-contaminated sites.

320

321 *Response of accumulation*

322 Leaf Cu concentrations of *A. chinensis* and *H. annuus* increased while increasing exchangeable Cu ($H =$
323 89.7 and $H = 12.9$, $p < 0.001$; respectively) (Fig. 4a, b). *Anisopappus chinensis* accumulated Cu increasingly when
324 increasing exchangeable Cu to 10–13, 40–50 and 50–60 mg kg^{-1} . Plants of *A. chinensis* on the highest Cu range
325 had a concentration of Cu in the leaves six times higher than control-0 plants ($36 \pm 2 \text{ mg kg}^{-1}$). For *H. annuus*, an
326 increased accumulation was also observed ($163 \pm 7 \text{ mg kg}^{-1}$ at 10–13 mg kg^{-1}) but with strong growth inhibition
327 and toxicity symptoms, reflecting metal-induced stress behaviour. Interestingly, in its native site, at a similar range
328 of Cu in soil (10 to 50 mg kg^{-1}), leaf Cu of *A. chinensis* was almost 20 times higher (mean = $251 \pm 123 \text{ mg kg}^{-1}$)
329 (Lange et al. 2014) compared with our plants cultivated in pots (mean = $14.5 \pm 5.6 \text{ mg kg}^{-1}$).

330 Leaf Co concentrations increased while increasing soil-exchangeable Co for the two species ($H = 14.1$
331 and $H = 16.4$, $p < 0.001$; for *A. chinensis* and *H. annuus*, respectively) (Fig. 5a, b). *Anisopappus chinensis*
332 accumulated Co from 0.2 to $20 \pm 3 \text{ mg kg}^{-1}$ when exchangeable Co increased from 0–1 to 4–6 mg kg^{-1} . For *H.*
333 *annuus*, an increased accumulation was also observed (from 1 to $165 \pm 28 \text{ mg kg}^{-1}$) but the plants exhibited
334 chlorosis and growth inhibition. Curiously, for an equivalent range of Co in soil (3 to 7 mg kg^{-1}), plants of that
335 population *in natura* accumulated less Co (mean = $7.7 \pm 3.3 \text{ mg kg}^{-1}$) (Lange et al. 2014) than in our study (17.8
336 $\pm 5.4 \text{ mg kg}^{-1}$).

337 Huge contrasts between Cu and Co concentrations in plant leaves in the field and in our study have been
338 observed, especially for Cu, raising the complexity of soil–plant processes (Wenzel 2009; Alford et al. 2010). The
339 processes governing metal mobilization and availability in the rhizosphere and accumulation in
340 pseudometallophytes from natural Cu- and Co-rich soils require further study, especially in the context of
341 phytoremediation. In particular, future work should investigate the role of microbial processes in the rhizosphere
342 (Hinsinger et al. 2005; Andreatza et al. 2010).

343

344 **4. Conclusion**

345

346 This study attempted to test experimentally the influence of FeOx, MnOx and OM supply on the Cu and
347 Co mobility in soil, and accumulation in a metallophyte as compared with a well-known non-tolerant control
348 species from the same family. Variations in the amount of added Cu and Co had more influence than amendments
349 on concentrations of mobile Cu and Co and on plant accumulation. Organic matter lowered Cu mobility and

350 increased Co mobility. Future work is needed to understand better the factors governing Cu and Co mobility and
351 accumulation in the naturally Cu- and Co-rich soils in Katanga, with a particular attention to biological interactions
352 at the rhizosphere level.

353 Second, this study is the first to report the capacity of the pseudometallophyte *Anisopappus chinensis* to
354 grow and survive at concentrations of Cu and Co in the soil that are extremely toxic to a normal plant. Significant
355 growth was observed in that species even at foliar concentrations of Cu and Co that are well above the normal
356 range in plants. Future work will test if there is a variation in tolerance and accumulation among different
357 populations from soils with different Cu and Co mobility. Our results point to *Anisopappus chinensis* as an
358 interesting candidate in Cu and Co phytoremediation applications. Characterized by a perennial life cycle and a
359 growth phenology in wet and dry seasons, *A. chinensis* is now an addition to the short list of candidate cuprophytes
360 for Cu and Co phytoremediation, especially phytostabilization, to limit environmental risks stemming from metal
361 transfers (e.g. runoff and erosion), in an expanding context of soil and water pollution.

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399 **Table 1. ANOVAs on the Cu and Co exchangeable concentrations in soils**

400 Exchangeable Cu and Co concentrations were determined by CaCl₂ extraction (0.01 M). The ‘Concentration’
401 factor (two levels for each metal) corresponds to the soil metal concentration: 200 or 500 mg kg⁻¹ for Cu and 20
402 or 100 mg kg⁻¹ for Co. The ‘Treatment’ factor (10 levels for Cu and eight levels for Co) corresponds to soil
403 treatments. For Cu and Co, the treatments were: control, +OM, +MnOx, +FeOx, +OM+FeOx, +OM+MnOx,
404 +FeOx+MnOx, +FeOx+MnOx+OM, with two additional treatments for Cu: +Co and +Co+MnOx.

405 ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; *ns*, not significant.

406

407 **Table 2. ANOVAs on the Cu and Co leaf concentrations of *Anisopappus chinensis* and *Helianthus annuus***
408 The 'Concentration' factor (two levels for Cu and one level for Co) corresponds to the soil metal concentration:
409 200 or 500 mg kg⁻¹ for Cu and 20 mg kg⁻¹ for Co. The 'Treatment' factor (10 levels for Cu and eight levels for
410 Co) corresponds to soil treatments. For Cu and Co, the treatments were: control, +OM, +MnOx, +FeOx,
411 +OM+FeOx, +OM+MnOx, +FeOx+MnOx, +FeOx+MnOx+OM, with two additional treatments for Cu: +Co and
412 +Co+MnOx. For *H. annuus*, no results were obtained for plants growing on the 500 mg.kg⁻¹ Cu soils.
413 ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

414

415 **Fig. 1. Copper exchangeable in soils (CaCl₂ 0.01 M) and accumulated in *Anisopappus chinensis* according**
416 **to soil treatments**

417 **a.** Total Cu added: 200 mg kg⁻¹ **b.** Total Cu added: 500 mg kg⁻¹

418 Each treatment ($n = 6$) was compared with the control ($n = 6$) using Student's *t*-test on log-transformed data. [Cu]
419 exchangeable and [Cu] accumulated were tested separately. Treatments with symbols were significantly different
420 from the control. Error bars are standard errors.

421

422 **Fig. 2. Cobalt exchangeable in soils (CaCl₂ 0.01 M) and accumulated in *Anisopappus chinensis* according to**
423 **soil treatments**

424 Each treatment ($n = 6$) was compared with the control ($n = 6$) using Student's *t*-test on log-transformed data. [Co]
425 exchangeable and [Co] accumulated were tested separately. Treatments with symbols were significantly different
426 from the control. Error bars are standard errors.

427

428 **Fig. 3. Cobalt exchangeable in soils (CaCl₂ 0.01 M) and accumulated in *Helianthus annuus* according to Co**
429 **treatments**

430 Each treatment ($n = 6$) was compared with the control ($n = 6$) using Student's *t*-test on log-transformed data. [Co]
431 exchangeable and [Co] accumulated were tested separately. Treatments with symbols were significantly different
432 from the control. Error bars are standard errors.

433

434 **Fig. 4. Shoot biomass and foliar concentrations of Cu as a function of exchangeable Cu in soil**

435 **a.** *Anisopappus chinensis* **b.** *Helianthus annuus*

436 Mean exchangeable Cu ($n = 6$) deriving from the 20 treatments were associated according to four exchangeable
437 ranges: 0–1 (control-0); 10–13 ($n = 10$); 40–50 ($n = 4$) and 50–60 mg kg⁻¹ ($n = 6$). Mean shoot biomass, or foliar
438 concentrations, per range are graphically represented. Error bars are standard errors. Mean values with the same
439 letter are not significantly different (Mann–Whitney pairwise comparisons). Shoot biomass and foliar
440 concentration were tested independently. For *H. annuus*, plants growing on 40–50 mg kg⁻¹ exchangeable Cu in
441 soil were not considered for statistics due to less than three replications. No plants of *H. annuus* survived at 50–60
442 mg kg⁻¹ exchangeable Cu.

443

444 **Fig. 5. Shoot biomass and foliar concentrations of Co as a function of exchangeable Co in soil**

445 **a.** *Anisopappus chinensis* **b.** *Helianthus annuus*

446 Mean exchangeable Co ($n = 6$) deriving from 20 mg kg⁻¹ Co treatments ($n = 8$) were associated according to three
447 exchangeable ranges: 0–1 (control-0); 4–6 ($n = 4$) and 6–7 ($n = 4$). Mean shoot biomass, or foliar concentrations,
448 per range are graphically represented. Error bars are standard errors. Mean values with the same letter are not
449 significantly different (Mann–Whitney pairwise comparisons). Shoot biomass and foliar concentration were tested
450 independently.

451

452 ----

453

454 **Table 1.**

| Source | [exchangeable Cu] | | | | [exchangeable Co] | | | |
|---------------|-------------------|--------|-------|-----|-------------------|--------|-------|-----|
| | df | MS | F | p | df | MS | F | p |
| Concentration | 1 | 53 708 | 1 411 | *** | 1 | 30 366 | 985 | *** |
| Treatment | 9 | 167 | 4.38 | *** | 7 | 29 | 0.946 | ns |
| Conc*Treat | 9 | 146 | 3.85 | *** | 7 | 14 | 0.463 | ns |
| Residuals | 100 | 38 | | | 79 | 31 | | |

455

456 **Table 2.**

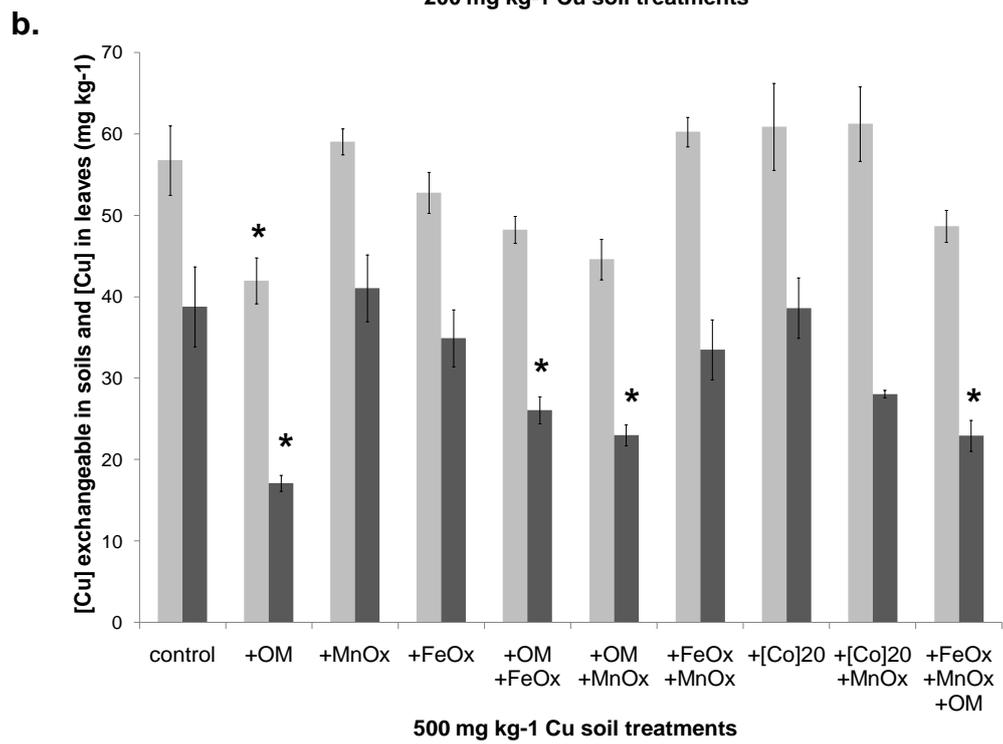
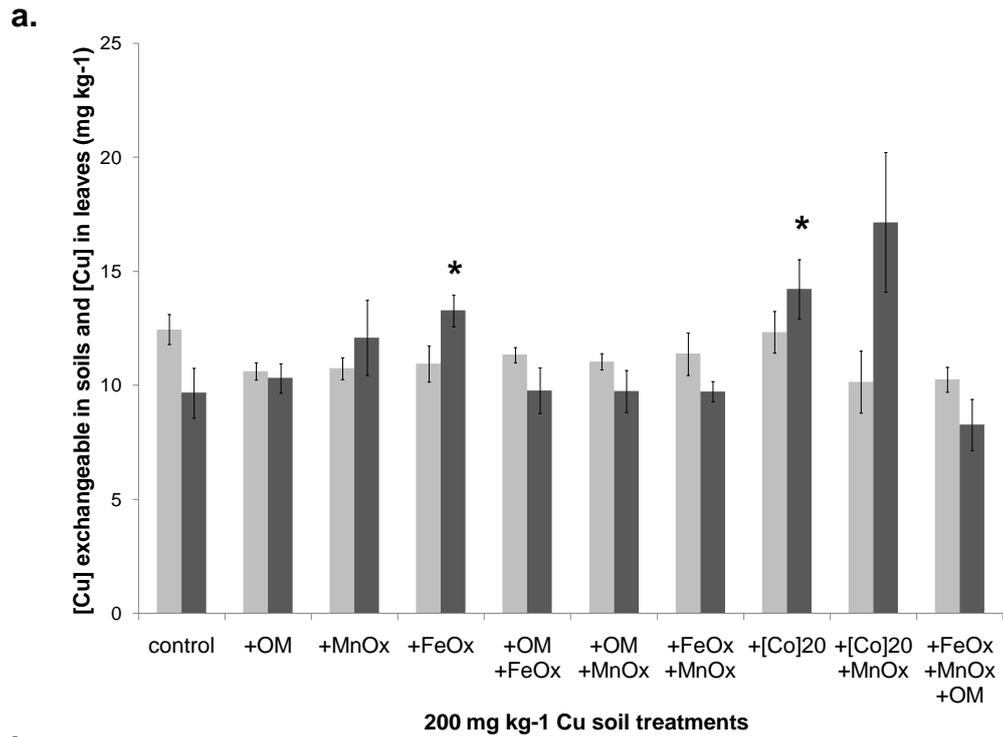
| Sources | <i>Anisopappus chinensis</i> | | | | <i>Helianthus annuus</i> | | | |
|-----------------------|------------------------------|------|-------|-----|--------------------------|------|------|----|
| | df | MS | F | p | df | MS | F | p |
| [Cu] in leaves | | | | | | | | |
| Block | 2 | 100 | 1.83 | ns | 2 | 59 | 0.02 | ns |
| Concentration | 1 | 9983 | 182.6 | *** | – | – | – | – |
| Treatment | 9 | 243 | 4.44 | *** | 9 | 1897 | 0.73 | ns |
| Block×Conc | 2 | 75 | 1.37 | ns | – | – | – | – |
| Block×Treat | 18 | 25 | 0.45 | ns | 16 | 4551 | 1.74 | ns |
| Conc×Treat | 9 | 164 | 3 | ** | – | – | – | – |
| Block×Conc×Treat | 18 | 13 | 0.23 | ns | – | – | – | – |
| Residuals | 51 | 55 | | | 25 | 2608 | | |
| [Co] in leaves | | | | | | | | |
| Block | 2 | 133 | 6.64 | ** | 2 | 442 | 0.17 | ns |
| Treatment | 7 | 169 | 9.6 | *** | 7 | 5584 | 2.12 | ns |
| Block×Treat | 14 | 41 | 2.06 | ns | 14 | 2724 | 1.04 | ns |
| Residuals | 15 | 20 | | | 19 | 2629 | | |

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460 **Fig. 1**



■ [Cu] exchangeable in soils (mg kg⁻¹) ■ [Cu] in leaves (mg kg⁻¹)

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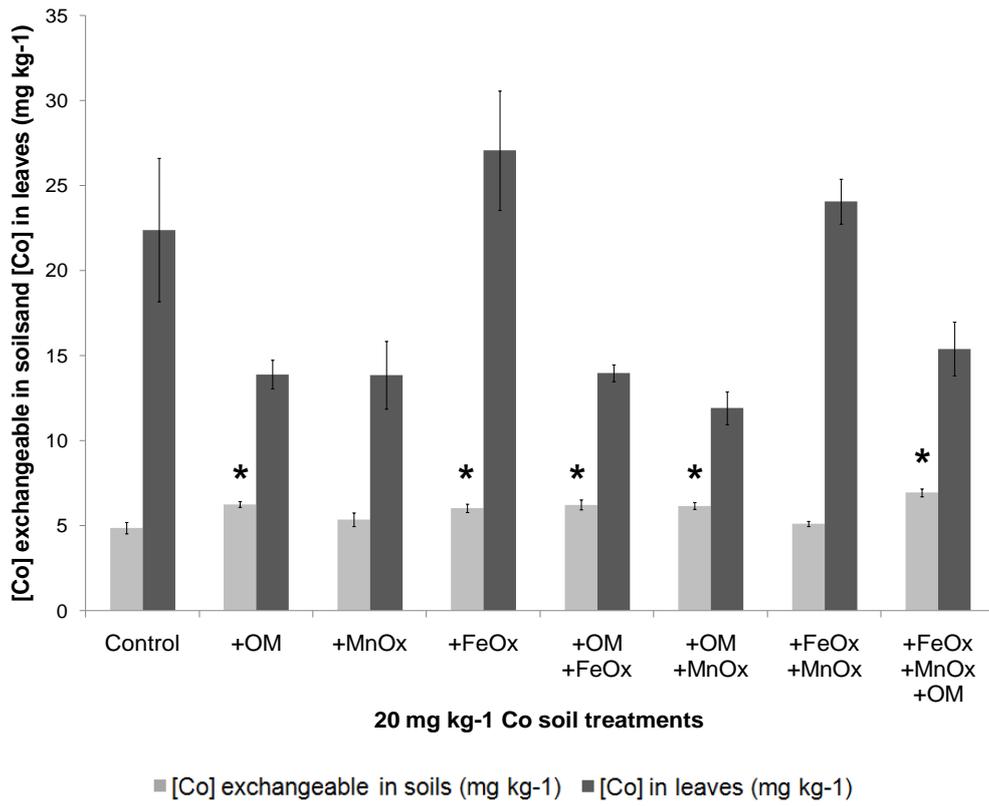
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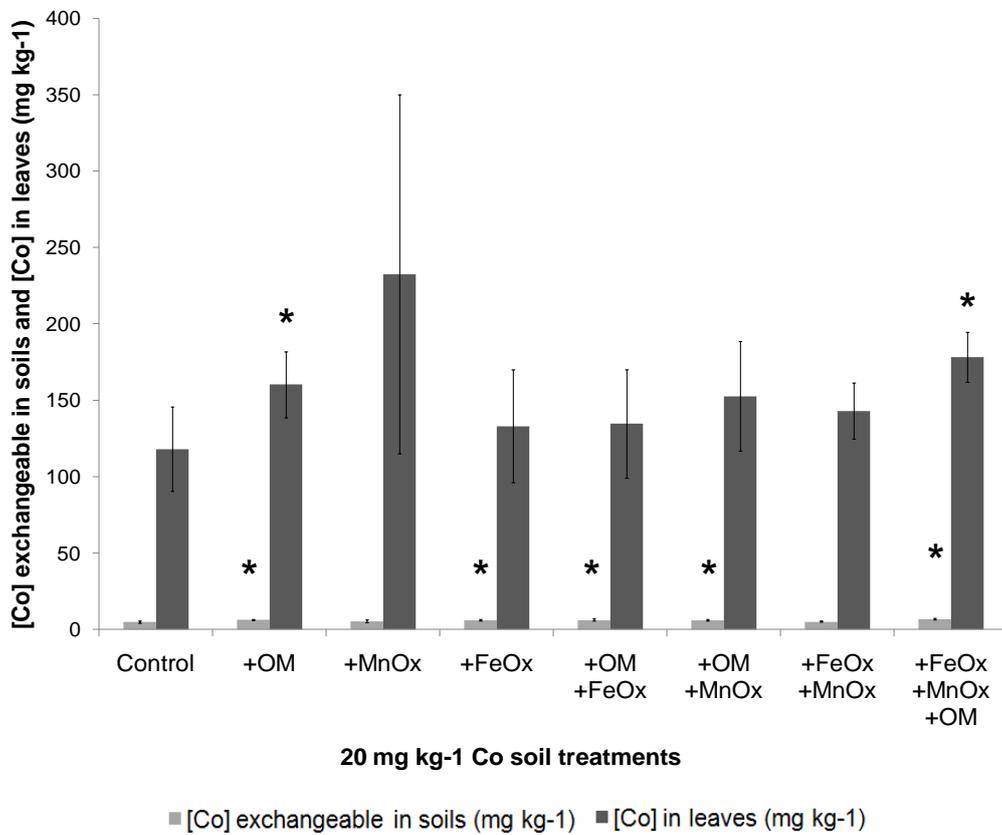
466 Fig. 2



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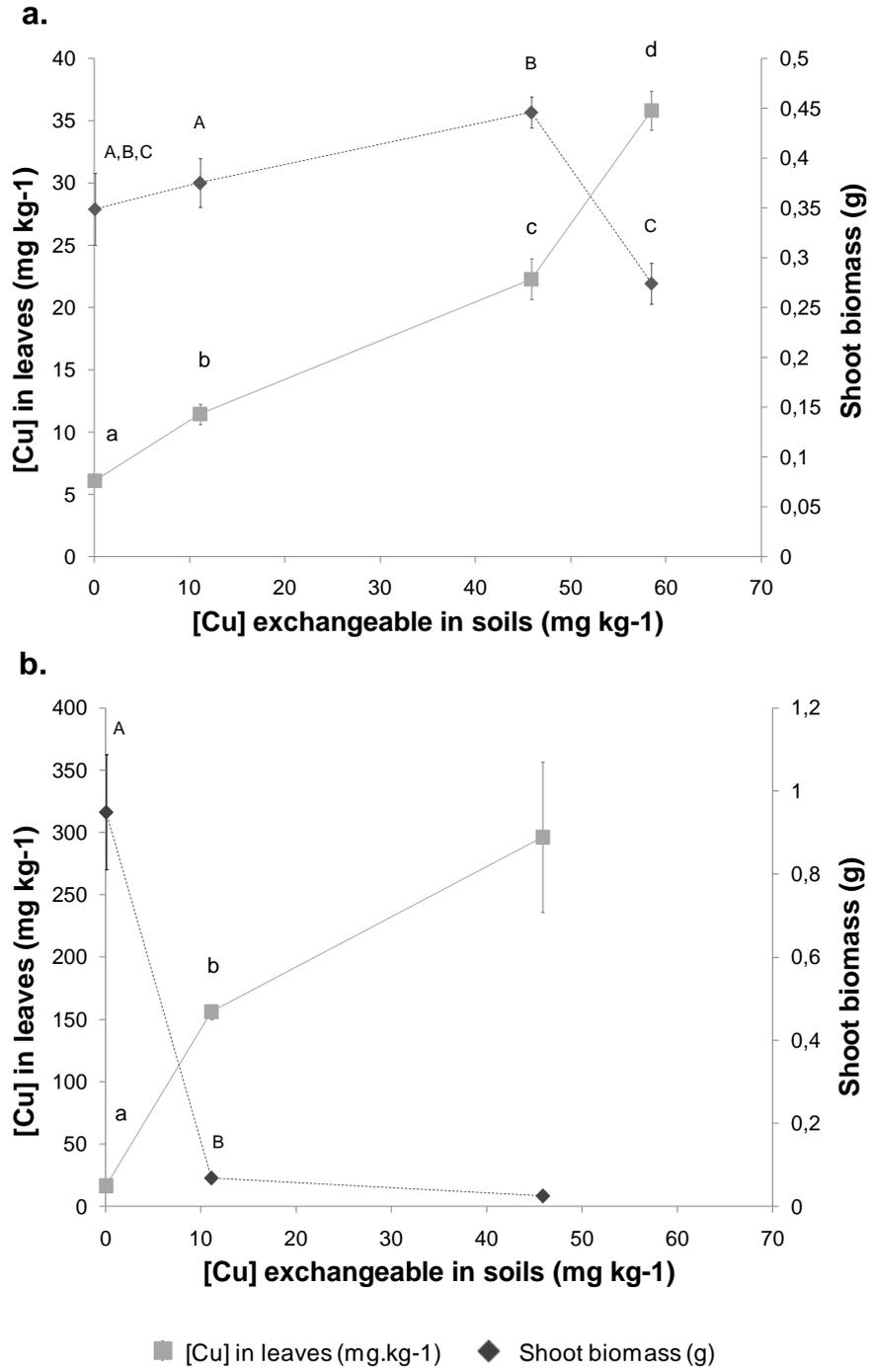
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469 Fig. 3



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471 Fig. 4



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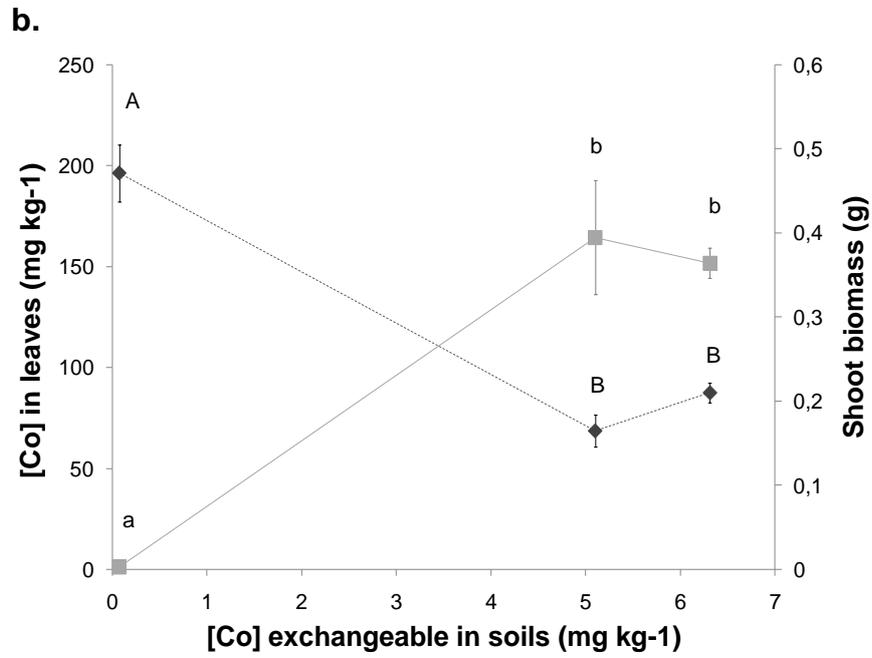
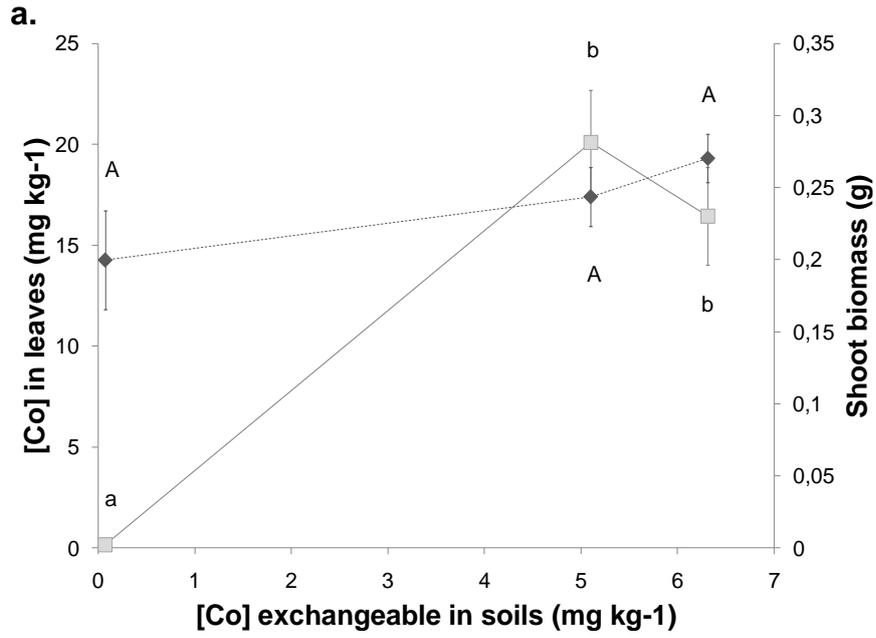
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479 Fig. 5



■ [Co] in leaves (mg.kg-1) ◆ Shoot biomass (g)

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