Variation in copper and cobalt tolerance and accumulation among six populations of the facultative metallophyte Anisopappus chinensis (Asteraceae)

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Variation in copper and cobalt tolerance and accumulation among six populations of the facultative metallophyte *Anisopappus chinensis* (Asteraceae)

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Highlights

- Population variation in Co tolerance and translocation is demonstrated in *Anisopappus chinensis*;
- Population variation in Co tolerance is correlated to Co concentrations in the native soil;
- Copper tolerance is weakly expressed in metallicolous populations grown in hydroponics;
- Cobalt tolerance may have evolved in response to directional selection in *Anisopappus chinensis*.

Abstract

Advances on the ecology and evolution of adaptation to metal toxicity are based on studying metallophytes that are not restricted to soils strongly enriched in trace elements. The evolution of Cu and Co tolerance and accumulation, which principally occurs among the Copperbelt of Central Africa, is poorly known. In this paper, we studied Cu and Co tolerance and accumulation in a facultative metallophyte occupying a very broad ecological niche in southeastern Democratic Republic of Congo: *Anisopappus chinensis* (Asteraceae). The population variation in Cu and Co tolerance and accumulation was experimentally investigated using four metallicolous and two non-metallicolous populations from contrasted habitats. Surprisingly, Cu tolerance was poorly expressed in metallicolous populations grown in hydroponics, suggesting that specific rhizosphere processes may account for the ability to grow without toxicity symptoms under high Cu concentration on metalliferous soils. Population variation in Co tolerance and accumulation was demonstrated, which positively correlated to the concentration of Co in the native soil. Increased tolerance seems to have evolved in populations originating from Co-enriched soils. Foliar Co accumulation above 300 mg kg^{-1} dry weight with increased translocation was observed in the most tolerant populations, possibly making such populations promising materials to test for Co-phytomining applications.

Keywords Hyperaccumulation; Intraspecific variation; Metallicolous; Non-metallicolous; Plant evolution

1. Introduction

Facultative metallophytes, *i.e.* metallophytes that are not restricted to soils strongly enriched in trace elements (TEs), represent powerful models for studying evolution of adaptation to metal toxicity (Baker, 1981; Pollard et al., 2014). Studies of the past decades have mostly focused on species comprising populations adapted to Zn-, Cd-, Ni- and Cu-enriched soils (Antonovics et al., 1971; Macnair et al., 1993; Bert et al., 2000; Escarré et al., 2000; Dechamps et al., 2006; Pauwels et al., 2006; Meyer et al., 2010; Faucon et al., 2012; Schvartzman et al., 2018). In rare facultative metallophytes, TEs tolerance is constitutive at the level of the species; such is the case for the Zn- and Cd-tolerant *Arabidopsis halleri* and *Noccaea caerulescens* (Bert et al., 2002). Most often, increased tolerance to one or several TEs has been found in metallicolous populations compared to their relatives originating from normal soils (Gonnelli et al., 2001; Assunção et al., 2003). However, the plant response when increasing soil TEs concentration can vary widely between species and populations, and Baker (1981) proposed three main conceptual strategies of tolerance, including so-called “excluder”, “indicator” and true “hyperaccumulator” plants (*i.e.* accumulation occurring from either low or high available concentration in the growth medium).

Most often, facultative metallophytes from Cu and/or Co-enriched soils behaved as Cu and/or Co excluders or indicators in culture, with limited metal translocation from the roots to the leaves (Lange et al., 2017a). Foliar Cu and/or Co concentrations above 300 mg kg^{-1} dry weight (DW) may be due to passive transport at phytotoxic concentrations in the growth medium (Clemens et al., 2002; Verbruggen et al., 2009). Plant populations established on naturally Cu- and Co-enriched soils are almost restricted to southeastern Democratic Republic of Congo (D.R. Congo) (Faucon et al., 2016; copperflora.org). These plants can thrive on soils extremely enriched with Cu and/or Co (1 to 10 g kg^{-1} inorganic Cu–Co) deriving from the natural Cu- and Co-outcrops of the so-called "Katangan Copperbelt". Some of the facultative metallophytes from that region have been reported to accumulate Cu and Co above 300 mg kg^{-1} DW in the leaves *in situ* (Lange et al., 2017a). Cobalt, with no physiological function established so far in higher plants, is known to be more highly phytotoxic compared to Cu (Palit et al., 1994; Morrissey et al., 2009; Pilon-Smits et al., 2009), which contrasts the rather accepted Irving Williams theory on metal toxicity (Stumm and Morgan 1996). It has been hypothesized that Co could be an important driver in the evolution of local adaptation in metallophytes (Duvigneaud, 1959; Malaisse et al., 1983). However, large variations in shoot biomass and foliar Cu and/or Co concentrations are observed between populations and plants (Faucon et al., 2009; Lange et al., 2014; Delhaye et al., 2016). These variations could be accounted for by variations in Cu and Co mobility in soil (varying widely among sites) (Pourret et al., 2016), but also by variation in the capacity to tolerate and accumulate Cu and/or Co (Lange et al., 2014). However, only few works have investigated variation in Cu and Co tolerance and accumulation within the flora of Cu and Co-enriched soils.

Baker et al. (1983) observed a variation in Cu and Co tolerance between three *Silene* taxa. Variation in Cu–Co tolerance was found in the *Silene burchelii* complex, correlated to the Cu–Co status of native soils. More recently, increased tolerance to Cu was found in metallicolous populations of *Crepidorhopalon tenuis* (Faucon et al., 2012) and *Haumaniastrum katangense* (Peng et al., 2012). Most often, increased tolerance is coupled to decreased Cu concentrations in shoots, as a result of specific tolerance mechanisms restricting Cu uptake and/or translocation (Ouzounidou et al., 1994; Gonnelli et al., 2001). The plant response to Cu treatment can vary widely depending on the concentration, the species and the growth medium including growth inhibition, no effect on growth to growth stimulation (Jiang et al., 2004; Lou et al., 2004; Lange et al. 2016). Increased requirements of Cu were found in *Crotalaria cobalticola* (Boisson et al., 2016) and *Crepidorhopalon perennis* (Faucon et al., 2012) two strict endemic species of Cu-enriched soils. In contrast to Cu, studies on intraspecific variation in Co tolerance and accumulation are very scarce. Paliouris and Hutchinson (1991) found increased tolerance to Co in a population of *Silene vulgaris* from mine tailings compared to a population from uncontaminated site. In another study, a genetic variation in Co tolerance was shown among clones of *Agrostis gigantea* (Hogan and Rauser, 1979). A more recent study has demonstrated that Co tolerance may be associated with the translocation of Co from the root to the shoot, and accumulation in *Haumaniastrum katangense* and *H. robertii*, two metallophytes from the Katangan Copperbelt (Kabeya et al., 2018).

Anisopappus chinensis (Asteraceae) is a widespread facultative metallophyte in tropical Africa. Compared to the few facultative metallophytes studied so far, *A. chinensis* appears to have a broader niche, being very widespread on non-contaminated soils in tropical Africa (Lange, 2016), and being particularly frequent on cobalt-rich soils in southeastern D.R. Congo (Duvigneaud 1959). The ecology and evolution of Cu and Co tolerance and accumulation in facultative metallophytes remains poorly studied, especially for Co and species that present a high occurrence on non-metalliferous soils. Our working hypothesis is that differentiation in Cu and Co tolerance and accumulation exists between populations of such species, and is related to variations in the level of Cu and Co in the native soil.

In this paper, we assessed Cu and Co tolerance and accumulation in four metallicolous and two non-metallicolous populations of the facultative metallophyte *A. chinensis*. We addressed the following questions: (i) Is *A. chinensis* constitutively tolerant to Cu and/or Co? (ii) Does intraspecific variation in Cu and Co tolerance, if any, related to Cu and Co concentrations in native soils? (iii) Does increased tolerance to Co, if any, rely on Co translocation and foliar accumulation?

2. Materials and methods

2.1. Plant material

Anisopappus chinensis L. Hook.f. & Arn. (Asteraceae, subfam. Asteroideae, tribe Anthemideae) is a perennial facultative metallophyte native of and widely distributed in tropical central, austral, western and eastern Africa (but also extended to China and southeastern Asia) (Wild, 1963). In south-eastern D.R. Congo, *A. chinensis* is widespread in grasslands of natural Cu–Co outcrops and the surrounding wooded savannas (Séleck et al., 2013; Lange et al., 2017b). Four metallicolous (M) and two non-metallicolous (NM) populations were sampled. At each site, the seeds from 20 plants were harvested and pooled. The sites of harvest were: Etoile (Et, M), Fungurume V (F5, M), Goma II (G2, M), Niamumenda (Nm, M), Mikembo (Mi, NM) and Kiswishi (Ki, NM) (Table 1). In nature, M populations exhibit broad variations in leaf Cu and Co concentrations: 3–1335 and 4–2820 mg kg^{-1} DW, respectively (Lange et al., 2014). *Leucanthemum superbum* D.H.Kent (Asteraceae, subfam. Asteroideae, tribe Anthemidae), an ornamental perennial herb which has not evolved mechanisms to tolerate Cu–Co, was chosen as control species (hereafter "Ctrl"). Seeds were commercially purchased (Vilmorin©).

Table 1 Populations of *Anisopappus chinensis*: location and habitat description

Sites	**Status**	**Coordinates** (GCSWGS84 DD)	**Habitat**	**Soil inorganic Cu** (mg kg^{-1})	**Soil inorganic Co** (mg kg^{-1})
Etoile (E)	M	11.38'S 27.35'E	Cu-Co hill disturbed by mining. Secondary vegetation on mine waste.	953 *(2836)*	1311 *(2541)*
Fungurume 5 (F5)	M	10.62'S 26.29'E	Natural Cu-Co hill not disturbed by mining. Grassland.	4774 *(9070)*	2558 *(2685)*

Goma 2 (G2)	M	10.60’S 26.14’E	Natural Cu-Co hill marginally disturbed by mining. Grassland.	8469 *(13 263)*	456 *(236)*
Niamumenda (Nm)	M	11,60’S 27,29’E	Natural Cu-Co hill marginally disturbed by mining. Grassland.	1950 *(4735)*	29 *(34)*
Mikembo (Mi)	NM	11.47’S 27.66’E	Miombo woodland with grassy herbaceous layer	0 *(5e-4)*	1 *(0.02)*
Kiswishi (Ki)	NM	11.53’S 27.46’E	Miombo woodland with grassy herbaceous layer	0 *(3e-4)*	44 *(1.2)*

M = metallicolous, NM = non-metallicolous. Inorganic concentrations of Cu and Co in the rooting zone (ionic forms) were obtained by Cu and Co speciation modelling (Lange et al., 2014; Pourret et al., 2016). Standard deviation is given in brackets.

2.2. Experimental design

A hydroponic experiment was conducted in a greenhouse from April to June 2015 (three months). Seeds were germinated in vermiculite watered with nutrient solution for three weeks before being transferred in hydroponic conditions. Three-week old seedlings were then grown for eight weeks in 5 L polyethylene pots filled with a nutrient solution composed of: 20 µM Fe-EDDHA (iron salt of ethylenediamine-di-o-hydroxyphenylactic acid), 0.88 mM K_2SO_4, 0.25 mM KH2PO4, 10 µM NaCl, 1000 µM $Ca(NO_3)_2$, 1000 µM $MgSO_4$, 10 µM H_3BO_3, 1 µM ZnSO4, 1 µM MnSO4, 0.5 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$ (adapted from Meyer *et al.*, 2010). The nutrient solution was adjusted to pH 5.8 with KOH (0.01 M) and changed each week. Five treatments were performed using Cu (II) sulfate ($CuSO_4 \bullet 5H_2O$) or Co (II) sulfate ($CoSO_4 \bullet 7H_2O$): Control (with no addition of Cu and Co), +10 µM Cu, +50 µM Cu, +5 µM Co, +20 µM Co. Plants of *A. chinensis* (one plant from each population) and one plant of *L. superbum* were grown for each treatment (one pot per treatment). Pots were replicated 10 times (*n* = 10) and randomized each week.

2.3. Measurements

After eight weeks in hydroponics, plants were harvested and separated into shoots and roots. Shoots were washed with Alconox® 1% in deionized water, dried at 65 °C for 48 h and weighed (Faucon et al., 2007). Roots were washed with 0.1 mM EDTA for 5 min, rinsed three times for 5 min in deionized water before being dried at 65 °C for 48 h and weighed (Chipeng et al., 2010). Leaf Cu–Co and root Cu–Co concentrations in *A. chinensis* and *L. superbum* were determined using Inductively Coupled Plasma–Mass Spectrometry (Thermo Scientific XSERIES2) after a dry ashing (Faucon et al., 2009). Briefly, 0.2 g (accurately weighed) of leaf or root powder were ashed at 500 °C for 12 h in a muffle furnace. Then, ashes were dissolved in a 32.5% HNO_3 solution (3 mL) at 80 °C for 5 min. Samples were then diluted to a 5% HNO_3 solution (50 mL) and stored at 4 °C until being analyzed. The precision and accuracy of analysis were determined using the SRM 1547 (peach leaves, NIST); bias of ± 5% were obtained in this case. At each treatment, average tolerance indices (TI) of populations were calculated: average biomass on metal divided by average biomass on control.

2.4. Statistical analysis

Plant biomass, leaf Cu and leaf Co were analyzed by partially nested ANOVAs on log-transformed data. The “edaphic group” (*i.e.* M *vs* NM) and “treatment” factors were fixed factors. The “population” factor was a random factor nested in the “edaphic group” factor. The “block” factor was tested as a random factor and did not have a significant effect on any variable. Data for Cu and Co were tested independently. The population variation in leaf Cu, root Cu, leaf Co, root Co and leaf/root Cu–Co ratio (Cu–Co translocation factor) were investigated by one-way ANOVAs on log-transformed data. The control species was not considered for the population variation analysis. Pearson’s correlations were used to test the relationship between shoot and root biomass and between leaf and root Cu–Co. The population variation in average TI was not tested statistically due to the lack of replication. Relationship between population TI and native soil concentration or translocation factor was tested for each treatment using Spearman's rank correlation (corrected by Monte Carlo permutation test based on 999 replications). The analyses were performed using R software 3.2.2 (www.R-project.org).

3. Results

3.1. *Response of shoot biomass and leaf copper and cobalt concentrations to Cu and Co in the growth medium*

3.1.1. *Response to Cu*

In all tested plants (including the Ctrl plants), except those from the M population F5, shoot biomass decreased dramatically with increasing Cu to +10 µM (Fig. 1). Growth was very poor at +50 µM Cu in all populations and plants from Mi (NM) did not survive at this concentration (Fig. 1). In contrast to other populations, leaf Cu of F5 plants did not increase significantly with an increase of Cu to +10 µM, whereas a steady increase was observed in all populations, up to 250–500 mg kg^{-1} DW in leaves, with increasing Cu from +10 to +50 µM.

Tolerance indices for Cu were consistently very low in NM populations at +10 and +50 µM Cu (TI ≤ 0.07), while they showed extensive variation among M populations (TI: 0.12–0.51 and 0.08–0.29 at +10 and +50 µM Cu, respectively). The ranking of population TI differed between +10 and +50 µM Cu and did not match the ranking in inorganic Cu concentration in the native soil (rank correlation not significant).

Shoot and root biomass were highly correlated at +10 and +50 µM Cu (r = 0.96 and r = 0.76; p<0.001, respectively). ANOVA on plant biomass did not show a significant effect of the edaphic group (*i.e.* M *vs* NM) (Table S1). Otherwise all factors and interactions were significant. Patterns of variation in plant biomass varied between +10 and +50 µM Cu (data not showed), in accordance with the highly significant interaction population within edaphic group*treatment.

3.1.2. Response to Co

The response of shoot biomass to the increase of the concentration of Co in the nutrient solution varied widely among populations. The growth of M populations Et, F5 and G2 was not significantly affected, and leaf Co concentrations increased progressively up to >300 mg kg^{-1} DW (Fig. 2). For the M population Nm, increasing Co to +5 µM had no significant effect on shoot biomass, and leaf Co increased only slightly (+25 mg kg^{-1}). However, increasing Co from +5 to +20 µM led to a significant decrease in shoot biomass and a steady leaf Co increase in these plants (from 25 to 250 mg kg^{-1}). In both NM populations Mi and Ki and the Ctrl species, increasing Co in the growth medium had a strong negative impact on shoot biomass, coupled to dramatic increase in the leaf Co (>400 mg kg^{-1}).

Tolerance indices were consistently lower in both NM populations and the Ctrl species at +5 and +20 µM Co compared to M populations. Large variations of TI were observed among populations at +20 µM Co (from 0.03 to 0.75). The ranking of populations for TI did not differ between +5 and +20 µM Co and matched remarkably the ranking of native soils for Co concentration: TI = 0.03 (Mi) < 0.08 (Ki) < 0.22 (Nm) < 0.66 (G2) < 0.74 (Et) < 0.75 (F5) (r_s = 0.89; p=0.015 and r_s = 0.94; p=0.005 at +5 and +20 µM Co, respectively).

Shoot and root biomass were highly correlated at +5 and +20 µM Co (r = 0.92 and r = 0.94; p<0.001, respectively). Similarly to Cu, ANOVA on plant biomass did not show a significant effect of the edaphic group, while all factor and interaction effects were significant (Table S2). The interaction population within edaphic group*treatment was slightly significant.

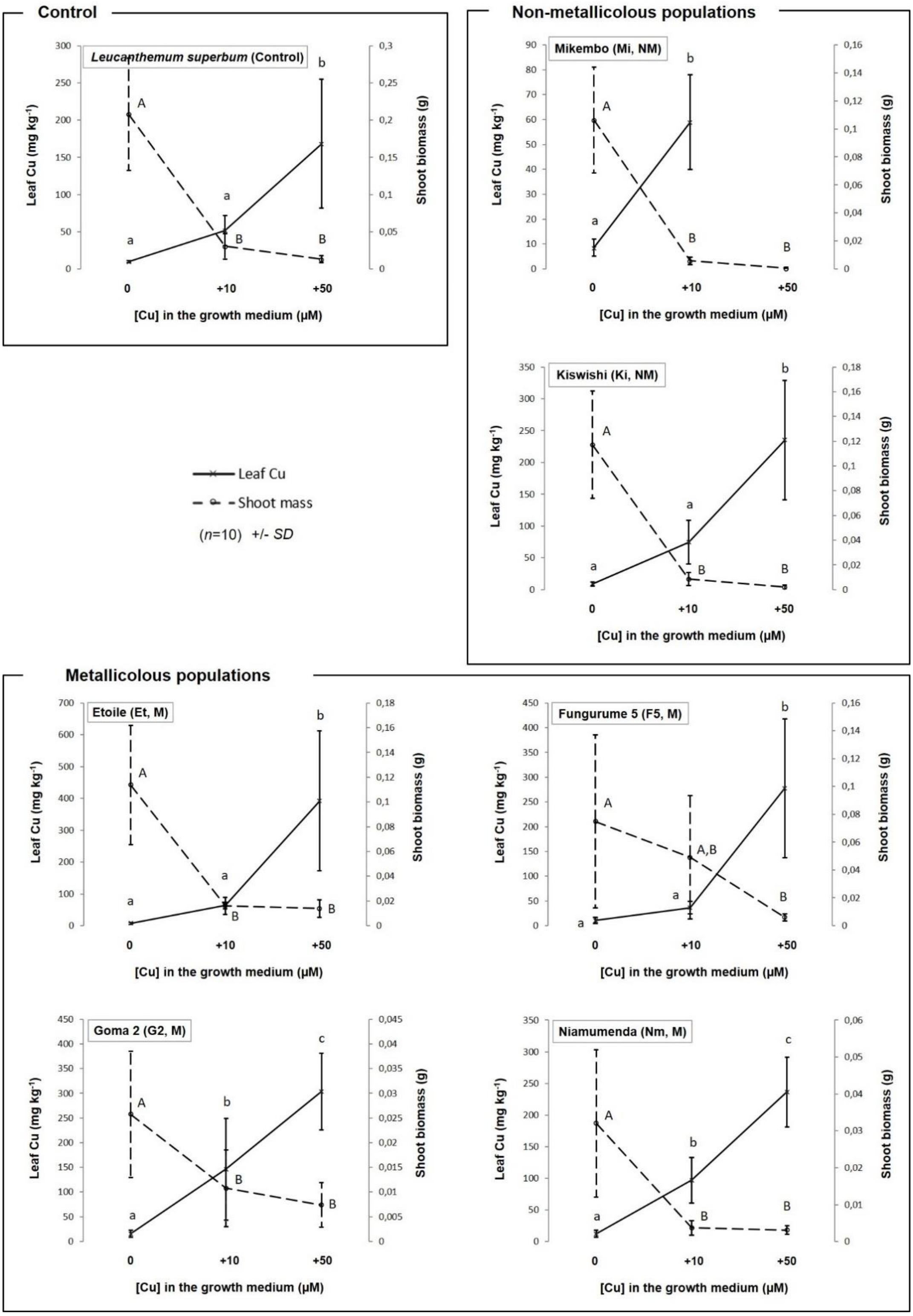

Figure 1 Shoot mass and leaf Cu as a function of increasing Cu in the nutrient solution in four metallicolous and two non-metallicolous populations of *Anisopappus chinensis*. *Leucanthemum superbum* was used as a control. Three Cu treatments: Control, +10 µM Cu, +50 µM Cu. Mean ± standard deviation (n = 10). Mean values with the same superscript are not significantly different (one-way ANOVAs on log-transformed data followed by post-hoc multiple comparison, Tukey HSD test).

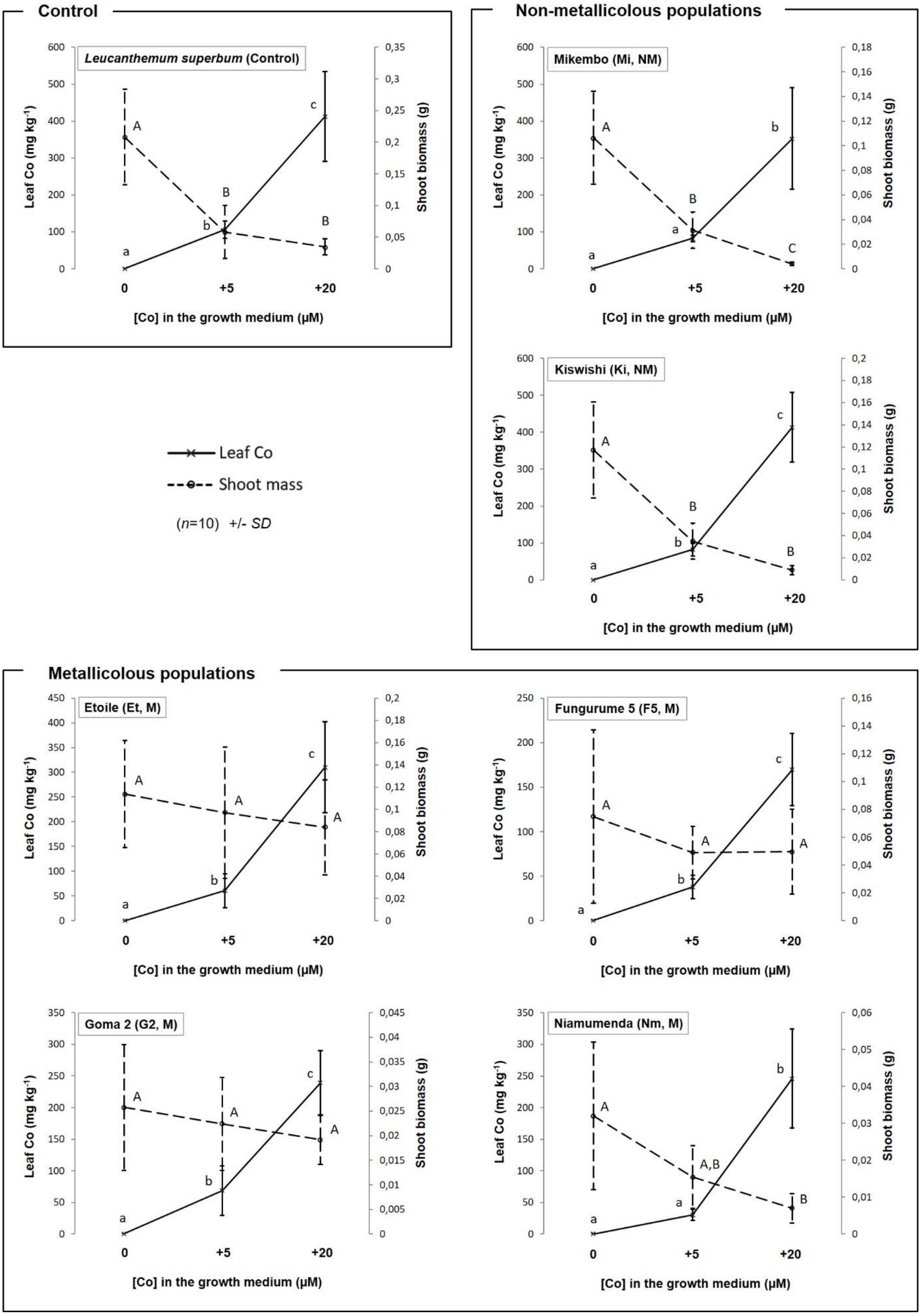

Figure 2 Shoot mass and leaf Co as a function of increasing Co in the nutrient solution in four metallicolous and two non-metallicolous populations of *Anisopappus chinensis*. *Leucanthemum superbum* was used as a control. Three Co treatments: Control, +5 µM Co, +20 µM Co. Mean ± standard deviation (n = 10). Mean values with the same superscript are not significantly different (one-way ANOVAs on log-transformed data followed by post-hoc multiple comparison, Tukey HSD test).

3.2. Population variation in leaf and root Cu and Co concentration

3.2.1. Leaf and root Cu concentration

Leaf Cu ranged from 16 to 338 mg kg^{-1} and from 76 to 761 mg kg^{-1} at +10 and +50 µM Cu, respectively. Root Cu ranged from 828 to 6813 mg kg^{-1} and from 4347 to 81 088 mg kg^{-1} at +10 and +50 µM Cu, respectively. Leaf Cu and root Cu were not significantly correlated.

ANOVAs on leaf and root concentrations showed a significant effect of all factors and interactions, except the interaction edaphic group*treatment interaction (Table S3). The few variations of leaf and root Cu observed at +10 µM Cu did not match TI (rank correlation not significant) (Fig. 3a). Leaf Cu did not vary significantly between populations at +50 µM Cu (Fig. 3b).

Leaf/root Cu ratio was very low in all populations and the Ctrl, and varied weakly at +10 µM (from 0.01 [Ctrl] to 0.05 [G2, M]) and at +50 µM (from 0.00 [Ctrl] to 0.04 [Ki]) (Fig. 3). Variations in leaf/root Cu ratio did not match TI (rank correlation not significant).

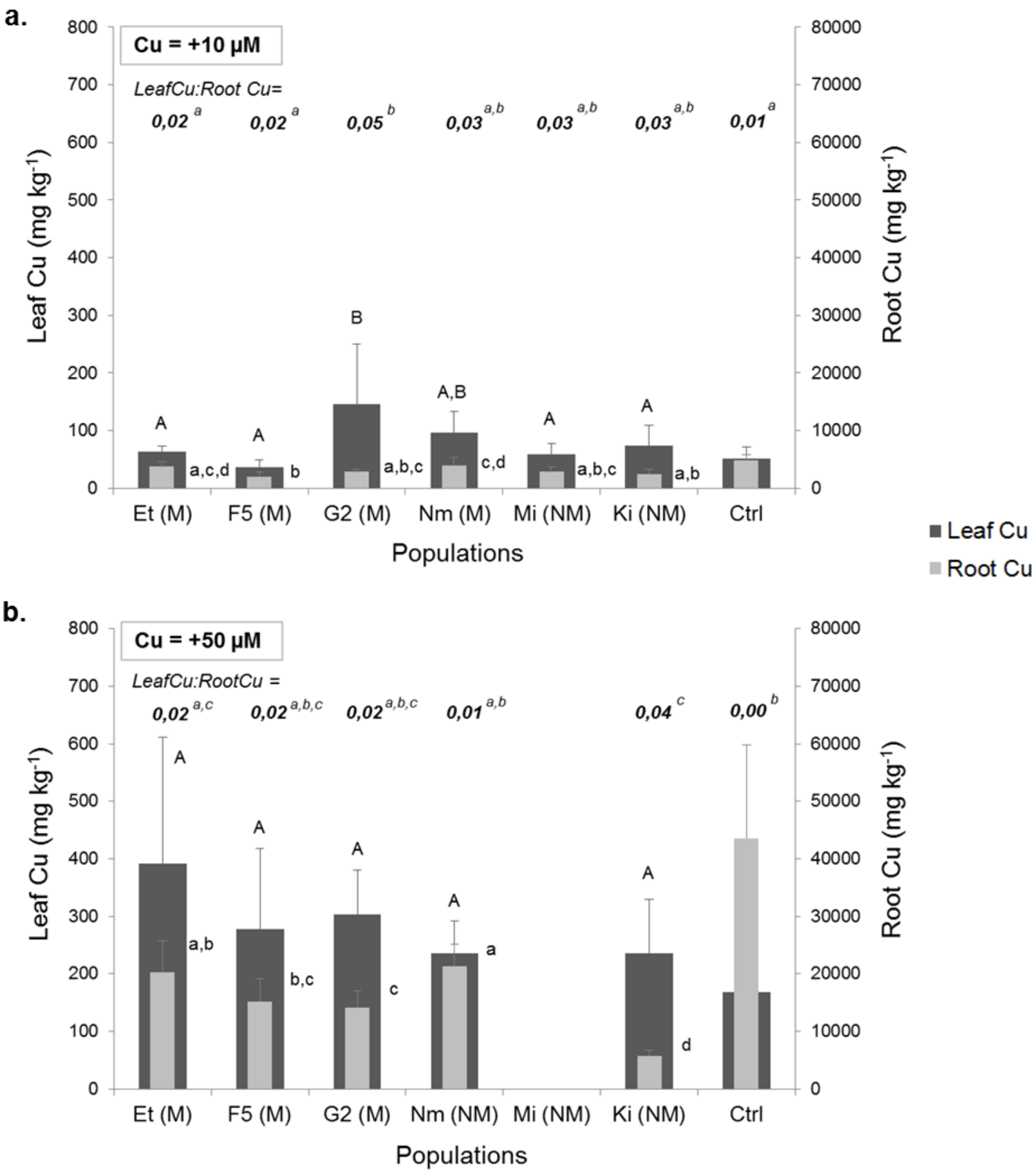

Figure 3 Leaf and root Cu concentrations in four metallicolous and two non-metallicolous populations of *Anisopappus chinensis* at two concentrations of Cu in the nutrient solution ***a.*** +10 µM Cu in the nutrient solution ***b.*** +50 µM Cu in the nutrient solution.
Et (Etoile), F5 (Fungurume V), G2 (Goma II) and Nm (Niamumenda) are metallicolous (M) populations. Mi (Mikembo) and Ki (Kiswishi) are non-metallicolous (NM) populations. Ctrl is the control species: *Leucanthemum superbum*. Shoot mass and root mass, as well as leaf Cu and root Cu were tested independently, without the Ctrl. Translocation factors (leaf/root Cu ratios), including the Ctrl, were also calculated. Mean ± standard deviation (n = 10) with the same superscript are not significantly different (results of one-way ANOVAs on log-transformed data followed by post-hoc multiple comparison, Tukey HSD test).

3.2.2. Leaf and root Co concentration

At +5 µM Co, leaf Co ranged from 19 to 149 mg kg^{-1} in M populations and from 49 to 116 mg kg^{-1} in NM populations; and root Co ranged from 204 to 1186 mg kg^{-1} in M populations and from 1729 to 6577 mg kg^{-1} in NM populations. At +20 µM Co, leaf Co ranged from 110 to 504 mg kg^{-1} in M populations and from 89 to 620 in NM populations; and root Co ranged from 485 to 2690 mg kg-1 in M populations and from 2995 to 7794 mg kg^{-1} in NM populations. Leaf Co and root Co concentration were positively correlated (r = 0.51, p<0.001).

ANOVA on leaf and root Co showed a significant effect of all factors and interactions, except the population effect for leaf Co (Table S4). In contrast to Cu, leaf and root Co varied among populations at the intermediate and highest treatments (Fig. 4a,b). At +5 µM Co, root Co was much lower in M populations (442 ± 120 mg kg^{-1}) compared to NM populations (3967 ± 1318 mg kg^{-1}) and the control species (2198 ± 575 mg kg^{-1}). A similar pattern was observed at +20 µM Co. Variations in root Co among populations matched variations in TI at +20 µM Co, with higher root Co concentrations in populations having lower TI (Mi and Ki) (r_s = -0.94; p<0.05).

Leaf/root Co ratio varied weakly at +5 µM Co (Fig. 4a). At +20 µM Co, leaf/root Co ratio was significantly higher in the M populations F5 (0.29) and G2 (0.24) compared to the NM populations Mi (0.06) and Ki (0.09) (Fig. 4b). A positive correlation was observed between leaf/root Co and TI at +20 µM Co (r_s = 0.94; p<0.01).

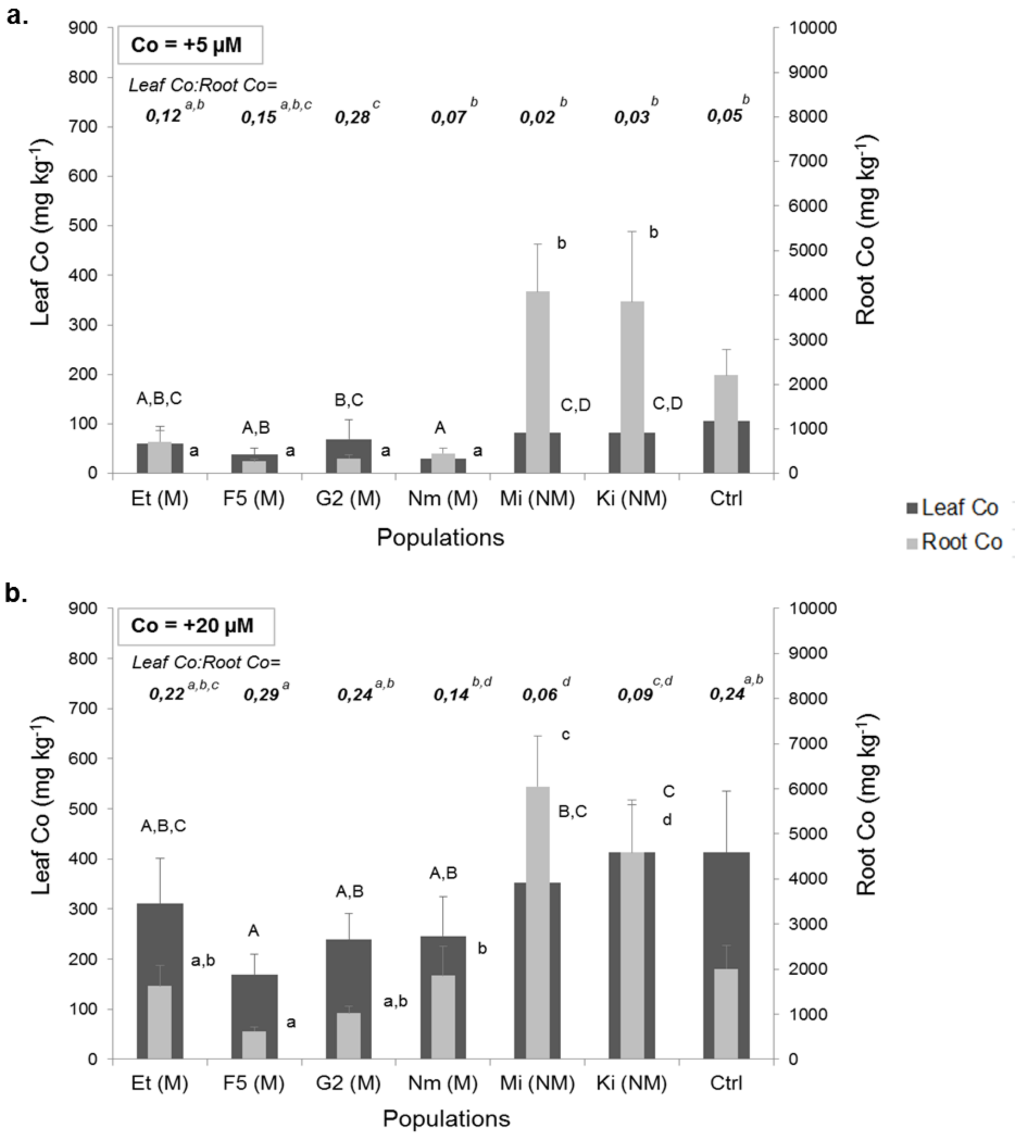

Figure 4 Leaf and root Co concentrations in four metallicolous and two non-metallicolous populations of *Anisopappus chinensis* at two concentrations of Co in the nutrient solution ***a.*** +5 µM Co in the nutrient solution ***b.*** +20 µM Co in the nutrient solution.

Et (Etoile), F5 (Fungurume V), G2 (Goma II) and Nm (Niamumenda) are metallicolous (M) populations. Mi (Mikembo) and Ki (Kiswishi) are non-metallicolous (NM) populations. Ctrl is the control species: *Leucanthemum superbum*. Shoot mass and root mass, as well as leaf Co and root Co were tested independently, without the Ctrl. Translocation factors (leaf/root Co ratios), including the Ctrl, were also calculated. Mean ± standard deviation (n = 10) with the same superscript are not significantly different (results of one-way ANOVAs on log-transformed data followed by post-hoc multiple comparison, Tukey HSD test).

4. Discussion

4.1. Low expression of Cu tolerance in metallicolous populations grown in hydroponics

Anisopappus chinensis is a facultative metallophyte occupying a broad niche including non-metalliferous habitats and soils enriched in Cu and Co in southeastern D.R. Congo. The latter species presents a high occurrence on non-metalliferous soils, as opposed to species from the Copperbelt studied so far (Lange, 2016). Results of growth under Cu stress for the control species and *A. chinensis* plants demonstrated that tolerance to Cu is not constitutive in the species. Surprisingly, a very poor expression of Cu tolerance was observed in hydroponics for metallicolous populations originating from soils extremely concentrated in Cu (Fig. 1, Table 1). We expected higher tolerance to Cu in metallicolous compared to non-metallicolous populations, with respect to the metallic status of their native soils. Despite somewhat higher tolerance indices in metallicolous populations, *A. chinensis* contrasts with other facultative metallophytes such as *C. tenuis* and *H. katangense.* The latter species consistently showed a strong increased tolerance to Cu in metallicolous compared to non-metallicolous populations (Faucon et al., 2012; Peng et al., 2012). For *C. tenuis*, increasing Cu in the growth medium even showed a growth stimulation. Contrast in the realized ecological niche between *A. chinensis* and species that present a higher occurrence on metalliferous soils may account for contrast in the degree of tolerance and response to Cu of M populations.

Elevated tolerance to Cu was demonstrated in metallicolous plants of *H. katangense* and *C. tenuis* growing in hydroponics or MS medium (Chipeng et al., 2010; Faucon et al., 2012). At an equivalent concentration, metallicolous plants of *A. chinensis* suffered from Cu toxicity. Metallicolous populations of *A. chinensis* may have evolved contrasting tolerance to Cu, which could be the result of specific soil-plant interactions not expressed in hydroponics. For instance, specific rhizosphere processes involving the microbial communities might account for the ability to grow under high Cu concentration on metalliferous soils. Among them, metal-tolerant arbuscular mycorrhizal fungi (AMF) and endophytic bacteria can have a beneficial effect on Cu tolerance by plants (Cornejo et al., 2008; Sun et al., 2010; Ma et al., 2011; Lenoir et al., 2016). Future work should test the presence of AMF and endophytic bacteria in M populations of *A. chinensis* and, if any, explore possible relationships with the ability to grow under high soil Cu levels.

4.2. Increased tolerance to Co in populations from Co-enriched soils as a result of local adaptation

Results showed that tolerance to Co is not constitutive in *A. chinensis*. Non-metallicolous populations of *A. chinensis* have not evolved mechanisms to tolerate concentrations of Co that are toxic for nontolerant plants such as *L. superbum*. In contrast to Cu, large intraspecific variation in the response to increasing Co in the growth medium was observed in *A. chinensis*. This study is only the second one to report intraspecific variation in tolerance to Co in a metallophyte. Paliouris and Hutchinson (1991) demonstrated an increased tolerance to Co in *Silene vulgaris* from a Co-contaminated soil compared to its relative on uncontaminated soil. Interestingly, these authors did not find any relationship between tolerance indices and Co concentrations in the native soils (water extract). Our work is the first to report population differentiation in Co tolerance related to the level of Co in the native soil. Tolerance indices at each Co treatment matched remarkably well the ranking of native soils for Co concentration. Selective pressure stemming from Co toxicity in highly contaminated soils may have induced local adaptation and enhanced tolerance to Co in the populations Et, F5 and G2. Therefore, the broad ecological niche of *A. chinensis* in southeastern D.R. Congo might be ascribed to within species genetic variation in Co tolerance. A similar conclusion was drawn by Faucon et al. (2012), for the facultative metallophyte *Crepidorhopalon tenuis* for Cu. The existence of such Co-tolerant ecotypes suggests that Co tolerance can explain the broad ecological niche of facultative metallophytes growing on Cu-enriched soils.

4.3. High accumulation of Co and increased translocation in populations adapted to extreme Co-levels

Relatively high foliar concentrations of Cu and Co have been reported from field growing plants of *A. chinensis* among M populations. *Anisopappus chinensis* from M populations was classified by Lange et al. (2014) as a Cu-Co hyperaccumulator based on operationally defined threshold values proposed by van der Ent et al. (2013) for foliar Cu and Co (>300 mg kg^{-1} DW). In our experimental conditions, the foliar concentration increase up to >300 mg kg^{-1} DW in all population with increasing Cu to +50 µM might be explained by passive absorption (Baker and Walker, 1989; Verkleij and Schat, 1989). This study is the first demonstration of a high degree of tolerance to Co associated with foliar Co accumulation up to >300 mg kg^{-1} DW in a facultative metallophyte largely distributed among non-metalliferous soils. Interestingly, Co tolerance was positively correlated to Co translocation to the leaves at elevated concentration, implying that the strategy of Co tolerance in *A. chinensis* does not rely on Co exclusion. The lower root Co concentration in the most tolerant compared to non-tolerant populations seems to confirm this hypothesis. Our results suggest that *A. chinensis* from soils highly enriched in available Co would behave as Co indicator rather than true hyperaccumulator *sensu* Baker (*i.e.* accumulation occurring from either low or high available concentration in the growth medium). The latter hypothesis should be verified by looking at the response of metallicolous plants grown on a natural metalliferous soil of reference with different levels of Co.

Intraspecific variation in leaf and root Co accumulation was shown for the first time in a facultative metallophyte. Interesting work could be to test the possible use of the most tolerant populations of *A. chinensis,* which translocate and accumulate Co above 300 mg kg^{-1} DW in leaves, in phytomining applications, for the production and valorization of a Co bio-ore (Faucon et al., 2018). Interestingly, the latter populations that also present the lowest root Co concentrations in our study, originate from soils with the highest level of inorganic Co. Selective pressure stemming from Co toxicity in the soil of origin may have induced a particular ability in (*i*) limiting Co storage in the root system and (*ii*) translocating Co to the leaves in plants originating from Co-enriched soils. The ecophysiology of Co tolerance and accumulation and interaction between is still poorly understood and particular adaptation mechanisms are expected.

5. Conclusion

Tolerance to Cu and Co are not constitutive in *Anisopappus chinensis*. The contrast between Cu toxicity in our study and field observations on Cu-enriched soils needs to be clarified. For instance, rhizospheric processes and/or biotic interactions which can have a beneficial effect on metal tolerance by plants but cannot be expressed in hydroponics, such as associations with mycorrhizal fungi and/or endophytic bacteria, should be investigated. Such processes might also account for the high concentrations of Cu in field growing plants. In contrast, populations form Co-enriched soils did express increased tolerance to Co. Variation in the degree of tolerance among these populations was related to the concentration of Co in the native soil, strongly suggesting that Co tolerance has evolved in response to directional selection. This study is the first to report population differentiation in Co tolerance in a facultative metallophyte depending on Co soil status. Interestingly, Co tolerance was positively correlated to Co translocation to the leaves up to >300 mg kg^{-1} dry weight at phytotoxic concentration in the nutritive solution (+20 µM Co). This suggests that *A. chinensis* from soils highly enriched in available Co would behave as Co indicator *sensu* Baker (1981) and that increased capacity to translocate Co may have evolved in the most tolerant populations.

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7. Author contributions

BL, GD, SB, NV, MPF and PM designed the experiment. BL conducted the experiment with the participation of GD. BL did the mineral and statistical analyses. BL wrote the manuscript with the participation of NV, MPF and PM. All authors critically revised the manuscript and gave their final approval.

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