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Highlights

-Organic matter supply decreased Cu mobility and accumulation in *Anisopappus chinensis* at 500 mg kg^{-1} Cu.

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

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

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

Abstract

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

Keywords

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

1. Introduction

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

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

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

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

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

Therefore, the availability of Cu and Co in soil can be indirectly driven by the complex interplay of many soil chemical parameters, including the mineralogical context, and ecological processes driving OM concentration in the soil. Variation in metal accumulation in plants may thus be strongly dependent on a broad range of factors, beyond the concentration of Cu and Co themselves. A previous study (Lange et al. 2014) explored the phenotypic 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}, respectively), collected *in natura* among strongly pedogeochemically contrasted sites. It was found that metal accumulation in plants was influenced not only by the free Cu and Co concentration in the soil solution. Copper bound to MnOx and Co bound to OM represented a significant pool of available Cu and Co for plants. The concentrations of Cu and Co bound to FeOx also had a positive influence upon the Cu and Co accumulation variations, respectively.

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

2. Materials and methods

2.1. Plants and soil origin

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

The soil for the pot experiment was obtained from the upper 20 cm of a Luvisol from homogeneous old forest area (Beauvais, France, coordinates: N 49°28'13.88'', E 2°4'0.45''). The soil was sieved (5 mm) and mixed with river sand to obtain 20% sand. The soil was analysed by Acme Analytical Laboratories Ltd. (Vancouver Canada), accredited under ISO 9002. Briefly, 0.25 g soil was heated in HNO_3–$HClO_4$–HF to fuming and taken to dryness; the residue was further dissolved in HCl solution and diluted with ultra-pure water before Inductively Coupled Plasma–Mass Spectrometry (ICP–MS) measurements. According to data quality result assessment, the measurement accuracy was estimated at ±5% for all the considered elements. The total concentrations of Cu, Co,

Fe_2O_3 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 content (loss on ignition, 500 °C for 8 h) was 6.0 ± 0.15%. The CaCl2 (0.01 M)-extractable Cu and Co 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), respectively.

2.2. Experimental Design

A glasshouse pot experiment was conducted from March to July 2013. Both species were cultivated in the same conditions and received the same treatments. The mass of soil in each pot was 500 g. Two soil concentrations of Cu (200 and 500 mg kg^{-1}), and two concentrations of Co (20 and 100 mg kg^{-1}), were prepared as follows. The Cu and Co supply was performed per pot using 30 mL of pH-adjusted solutions (pH = 4.5) of copper (II) sulfate ($CuSO_4{\bullet}5H_2O$, *Sigma-Aldrich*®) and/or cobalt (II) sulfate ($CoSO_4{\bullet}7H_2O$, *Sigma-Aldrich*®). Concentrations of solutions were 0.05 and 0.13 M $CuSO_4$ for 200 and 500 mg kg^{-1} Cu treatments, respectively; and 0.0056 and 0.028 M $CoSO_4$ for 20 and 100 mg kg^{-1} Co treatments, respectively. For each Cu and Co concentration, the concentrations of MnOx, FeOx and OM content were modified according to several treatments as follows: control, +OM, +MnOx, +FeOx, +OM+FeOx, +OM+MnOx, +FeOx+MnOx, +FeOx+MnOx+OM. For Cu, two additional treatments were tested: +Co and +MnOx+Co. A non-treated soil, hereafter referred to as "control-0" in the paper, was also included. Each treatment was carried out in six replicates, divided in three blocks, each block containing two replicates. For treatments with MnOx and FeOx supply, 130 mg kg^{-1} pyrolusite (MnO_2, *Sigma-Aldrich*®) (65 mg per pot) and 3,000 mg kg^{-1} hematite (Fe_2O_3, *Sigma-Aldrich*®) (1,500 mg per pot) were added in their commercial forms. These are the most widespread oxides (Pourret et al. 2015b) in the Cu- and Co-rich soils in Katanga. The concentrations were chosen to get an increase of up to more than 10% of the total oxide concentrations. For the study of the OM impact, 4% peat moss (*Sphagnum*) were added, to work with an acidic recalcitrant OM (pH = 4.5 ± 0.05) with the same pH as the study soil, and low decomposition kinetics (Moore and Basiliko 2006). A level of +4% OM was chosen to get the OM content close to that usually found in soils in Katanga (Lange et al. 2014). One week before sowing, the soil of each pot was carefully homogenized. During the experiment, all pots were watered with deionized water by filling up saucers every two days, and pots of each block were randomized weekly.

2.3. *Plant and soil analyses*

After harvesting, plants were carefully brushed (whole shoots), washed with Alconox® 1% in deionized water, dried at 65 °C for 48 h and weighed based on the procedure by Faucon et al. (2007). The Cu and Co concentrations in leaves of *A. chinensis* and *H. annuus* were determined using ICP–MS following digestion using a closed microwave system. Briefly, 0.2 g (accurately weighed) of leaf powder was mixed with 8 mL concentrated HNO_3 and 2 mL concentrated HCl (Avula et al. 2010) directly in a microwave Teflon vessel (Lavilla et al. 2009). After homogenization of the mixture, the vessels were placed in the microwave system (Mars 5, CEM Corporation, USA) according to the protocol of Avula et al. (2010). The digest was then diluted to approximately 30 g (accurately weighted) and then stored at 4 °C. The Cu and Co concentrations in digested samples were measured by ICP–MS (Thermo Scientific XSERIES2). Quality control was performed using the SRM1573a CRM (tomato leaves, Gills 1995). For Cu and Co in this case, the bias was below 5% (Lange et al. 2014). For the soil, pH (water) and OM content (loss on ignition, 500 °C for 8 h) were measured at the end of the experiment for each treatment. The OM content did not vary while the pH of soils enriched with OM were slightly lower (pH = 4.7 ± 0.05) than those of soils without added OM (pH = 4.9 ± 0.05). Then, the soil of each pot was dried at room temperature, sieved (2 mm), and then Cu and Co were extracted with $CaCl_2$ (0.01 M) (Van Ranst et al. 1999). Copper and Co concentrations were measured using Inductively Coupled Plasma–Optical Emission Spectrometry (ICP–OES; Varian Vista MPX). The precision and accuracy of analysis were determined using in-house standards for soils; biases of ± 5% were obtained in this case. In this study, variations of Cu and Co mobility were assessed by investigating variations of $CaCl_2$-extractable Cu and Co concentrations, which indicate variations of Cu and Co exchangeable concentration in soils (Van Ranst et al. 1999). Exchangeable Cu and Co in the control-0 soils at the 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 Co. Exchangeable Cu and Co in the control-0 soils did not vary from those of the soil of origin.

2.4. Statistical analysis

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

3. Results and discussion

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

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

For Co, the results showed a concentration effect on the exchangeable concentration (Table 1). 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. 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 not graphically represented). Contrary to expectations, MnOx supply did not influence Co mobility, while a reduction of mobility was expected due to the high affinity between Co and MnOx (Lange et al. 2014, Pourret et al. 2015). This negative result might be explained by the low amount of MnOx added in the +MnOx treatments (+ 65 mg, i.e. an increase of more than 10% of the total MnOx) compared with the total amount of soil in pots (500 g). The influence of MnOx supply on the Cu and Co mobility should be tested with higher MnOx enrichment. At

20 mg kg^{-1} Co, exchangeable Co was higher in +OM, +FeOx, +OM+FeOx, +OM+MnOx and +FeOx+MnOx+OM 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 explained by the fact that Co desorption is easier when bound to humic acids than to oxides (McLaren et al. 1986). 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 mobility (McLaren and Crawford 1973; Alloway 1995; Kabata-Pendias and Pendias 2001; Chaignon et al. 2002; Faucon et al. 2011). Surprisingly, FeOx addition increased the Co mobility (Fig. 2). The fraction of Co adsorbed onto oxides is not easily desorbed into the soil solution (McLaren et al. 1986; Collins and Kinsela 2010). However, in acidic conditions, iron oxides are able to uptake complexing ligands, and especially Co complexed by natural organic matter (Davis and Leckie 1978; Gu et al. 1994). These associations are known to occur as surface binding (Davis and Leckie 1978), which are hence readily desorbed into soil solution compared with oxide–ions chemisorption, i.e. more easily extracted by $CaCl_2$ extraction. In our case, iron oxides might have also adsorbed Co–OM ligands at their surfaces, explaining the increase of Co mobility in soils enriched with FeOx.

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

The two plant species showed contrasting patterns in terms of Cu and Co accumulation. Foliar Cu ranged 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 mg kg^{-1} in *A. chinensis* and from 1 to 377 mg kg^{-1} in *H. annuus*. *Helianthus annuus* exhibited symptoms of toxicity (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. Only a few plants of *A. chinensis* and no plants of *H. annuus* survived at 100 mg kg^{-1} Co. Consequently, statistical 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. chinensis*, a pseudometallophyte previously defined as facultative Cu and Co hyperaccumulator species because of the large variations of foliar concentrations observed in its natural habitat (Faucon et al. 2009; Lange et al. 2014), the plants did not accumulate Cu and Co up to the hyperaccumulation threshold of 300 mg kg^{-1} (Krämer 2010; van der Ent, et al. 2013). In the same way, the few studies regarding Cu and Co hyperaccumulation by plants under controlled conditions did not demonstrate the phenomenon (Morrison et al. 1979, 1981; Baker et al. 1983; Chipeng et al. 2010; Faucon et al. 2012). The Cu and Co hyperaccumulation may express in very specific *in natura* conditions, which are difficult to reproduce.

For foliar Cu in *A. chinensis*, there was an effect of the concentration, the treatment and the interaction concentration × treatment, while no treatment effect was found for *H. annuus* (Table 2). At 200 mg kg^{-1} Cu, foliar 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).

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

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

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

3.3. Response of growth and accumulation to metal mobility

Response of shoot biomass

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

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

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

Response of accumulation

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

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

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

4. Conclusion

This study attempted to test experimentally the influence of FeOx, MnOx and OM supply on the Cu and Co mobility in soil, and accumulation in a metallophyte as compared with a well-known non-tolerant control species from the same family. Variations in the amount of added Cu and Co had more influence than amendments on concentrations of mobile Cu and Co and on plant accumulation. Organic matter lowered Cu mobility and increased Co mobility. Future work is needed to understand better the factors governing Cu and Co mobility and accumulation in the naturally Cu- and Co-rich soils in Katanga, with a particular attention to biological interactions at the rhizosphere level.

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

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

Exchangeable Cu and Co concentrations were determined by $CaCl_2$ extraction (0.01 M). The 'Concentration' factor (two levels for each metal) corresponds to the soil metal concentration: 200 or 500 mg kg^{-1} for Cu and 20 or 100 mg kg^{-1} for Co. The 'Treatment' factor (10 levels for Cu and eight levels for Co) corresponds to soil treatments. For Cu and Co, the treatments were: control, +OM, +MnOx, +FeOx, +OM+FeOx, +OM+MnOx, +FeOx+MnOx, +FeOx+MnOx+OM, with two additional treatments for Cu: +Co and +Co+MnOx.
***, p<0.001; **, p<0.01; *, p<0.05; *ns*, not significant.

Table 2. ANOVAs on the Cu and Co leaf concentrations of *Anisopappus chinensis* and *Helianthus annuus*
The ‘Concentration’ factor (two levels for Cu and one level for Co) corresponds to the soil metal concentration: 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 Co) corresponds to soil treatments. For Cu and Co, the treatments were: control, +OM, +MnOx, +FeOx, +OM+FeOx, +OM+MnOx, +FeOx+MnOx, +FeOx+MnOx+OM, with two additional treatments for Cu: +Co and +Co+MnOx. For *H. annuus*, no results were obtained for plants growing on the 500 mg.kg^{-1} Cu soils.
***, p<0.001; **, p<0.01; *, p<0.05; *ns*, not significant.

Fig. 1. Copper exchangeable in soils ($CaCl_2$ 0.01 M) and accumulated in *Anisopappus chinensis* according to soil treatments
a. Total Cu added: 200 mg kg^{-1} **b.** Total Cu added: 500 mg kg^{-1}
Each treatment (n = 6) was compared with the control (n = 6) using Student’s t-test on log-transformed data. [Cu] exchangeable and [Cu] accumulated were tested separately. Treatments with symbols were significantly different from the control. Error bars are standard errors.

Fig. 2. Cobalt exchangeable in soils ($CaCl_2$ 0.01 M) and accumulated in *Anisopappus chinensis* according to soil treatments
Each treatment (n = 6) was compared with the control (n = 6) using Student’s t-test on log-transformed data. [Co] exchangeable and [Co] accumulated were tested separately. Treatments with symbols were significantly different from the control. Error bars are standard errors.

Fig. 3. Cobalt exchangeable in soils ($CaCl_2$ 0.01 M) and accumulated in *Helianthus annuus* according to Co treatments
Each treatment (n = 6) was compared with the control (n = 6) using Student’s t-test on log-transformed data. [Co] exchangeable and [Co] accumulated were tested separately. Treatments with symbols were significantly different from the control. Error bars are standard errors.

Fig. 4. Shoot biomass and foliar concentrations of Cu as a function of exchangeable Cu in soil
a. *Anisopappus chinensis* b. *Helianthus annuus*
Mean exchangeable Cu (n = 6) deriving from the 20 treatments were associated according to four exchangeable 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 concentrations, per range are graphically represented. Error bars are standard errors. Mean values with the same letter are not significantly different (Mann–Whitney pairwise comparisons). Shoot biomass and foliar concentration were tested independently. For *H. annuus*, plants growing on 40–50 mg kg^{-1} exchangeable Cu in soil were not considered for statistics due to less than three replications. No plants of *H. annuus* survived at 50–60 mg kg^{-1} exchangeable Cu.

Fig. 5. Shoot biomass and foliar concentrations of Co as a function of exchangeable Co in soil
a. *Anisopappus chinensis* b. *Helianthus annuus*
Mean exchangeable Co (n = 6) deriving from 20 mg kg^{-1} Co treatments (n = 8) were associated according to three exchangeable ranges: 0–1 (control-0); 4–6 (n = 4) and 6–7 (n = 4). Mean shoot biomass, or foliar concentrations, per range are graphically represented. Error bars are standard errors. Mean values with the same letter are not significantly different (Mann–Whitney pairwise comparisons). Shoot biomass and foliar concentration were tested independently.

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		

Table 2.

Sources	*Anisopappus chinensis*				*Helianthus annuus*			
	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		

Fig. 1

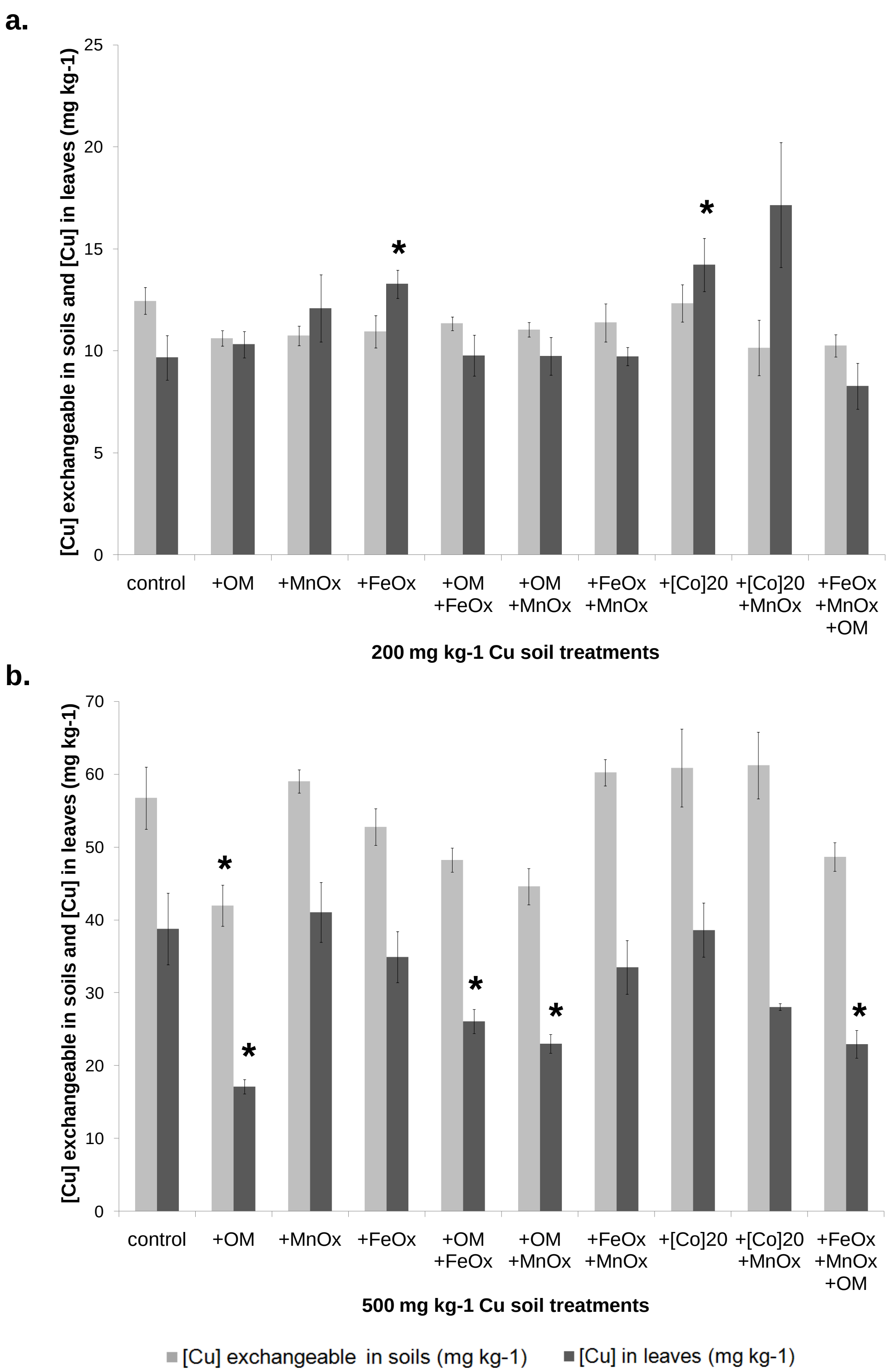

a.
[Cu] exchangeable in soils and [Cu] in leaves (mg kg-1)
0
5
10
15
20
25
*
*
control
+OM
+MnOx
+FeOx
+OM +FeOx
+OM +MnOx
+FeOx +MnOx
+[Co]20
+[Co]20 +MnOx
+FeOx +MnOx +OM
200 mg kg-1 Cu soil treatments
b.
[Cu] exchangeable in soils and [Cu] in leaves (mg kg-1)
0
10
20
30
40
50
60
70
*
*
*
*
*
control
+OM
+MnOx
+FeOx
+OM +FeOx
+OM +MnOx
+FeOx +MnOx
+[Co]20
+[Co]20 +MnOx
+FeOx +MnOx +OM
500 mg kg-1 Cu soil treatments
[Cu] exchangeable in soils (mg kg-1)
[Cu] in leaves (mg kg-1)

Fig. 2

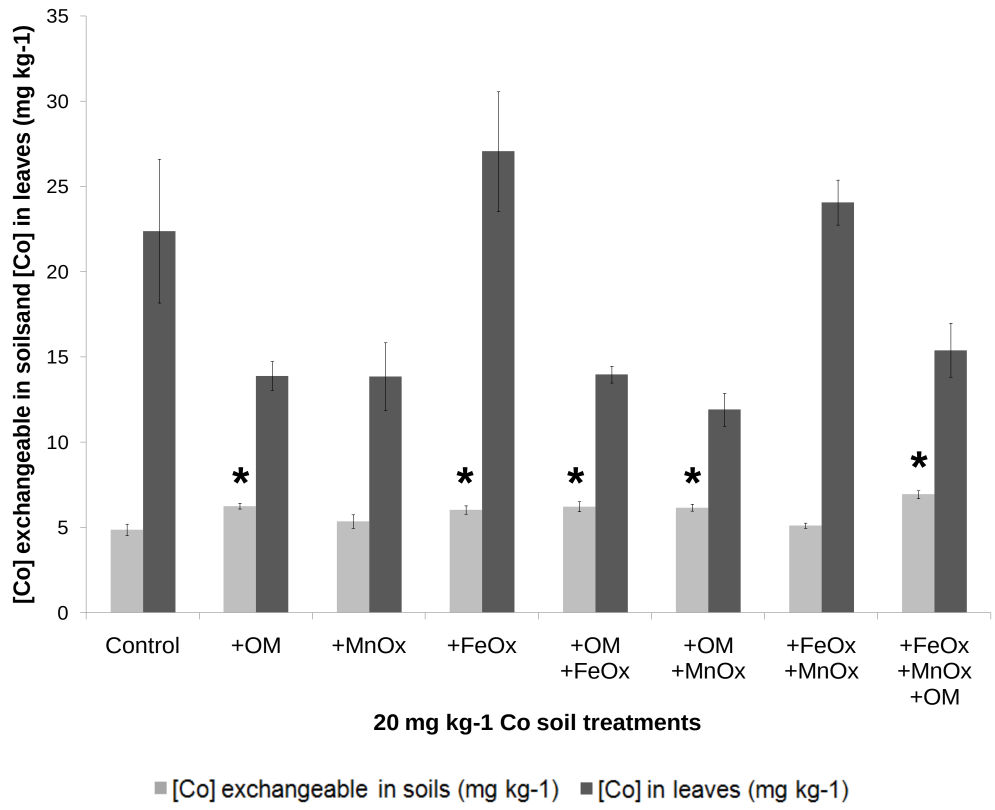

[Co] exchangeable in soilsand [Co] in leaves (mg kg-1)
35
30
25
20
15
10
5
0
*
*
*
*
*
Control
+OM
+MnOx
+FeOx
+OM +FeOx
+OM +MnOx
+FeOx +MnOx
+FeOx +MnOx +OM
20 mg kg-1 Co soil treatments
[Co] exchangeable in soils (mg kg-1)
[Co] in leaves (mg kg-1)

Fig. 3

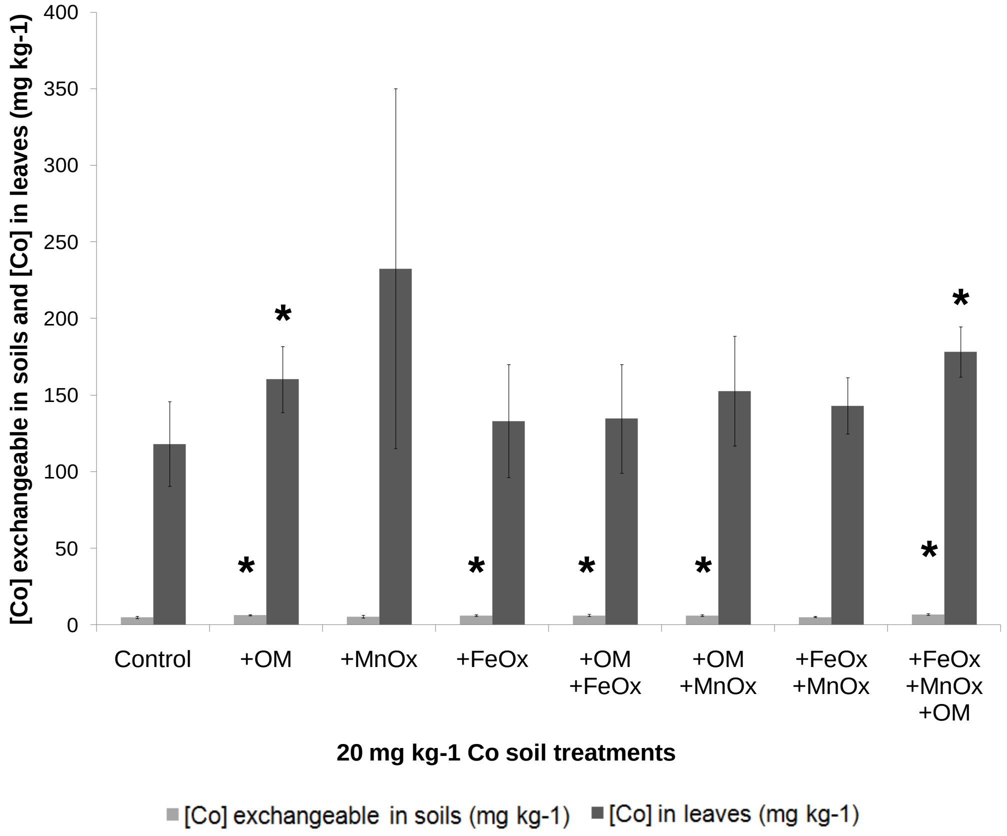
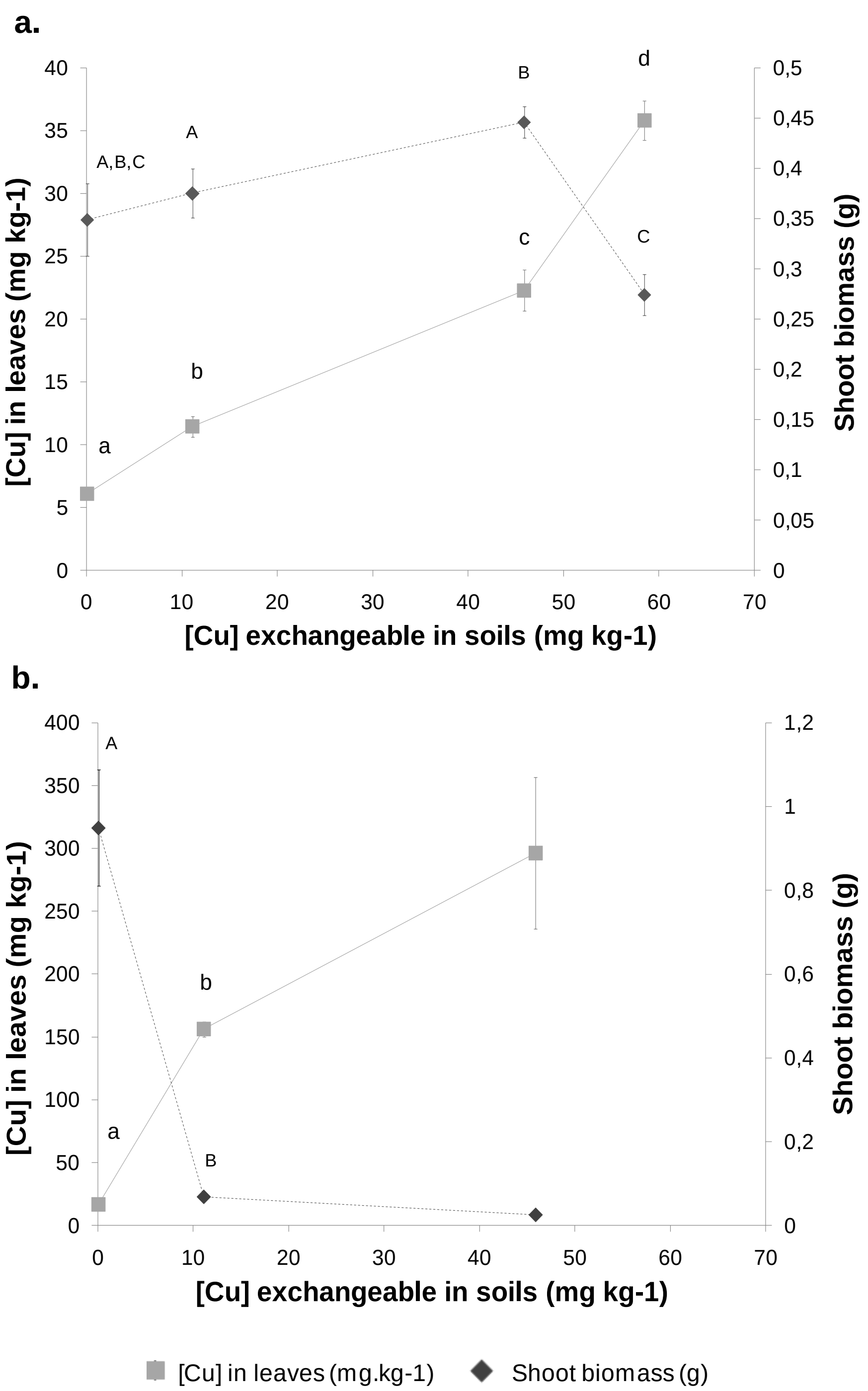
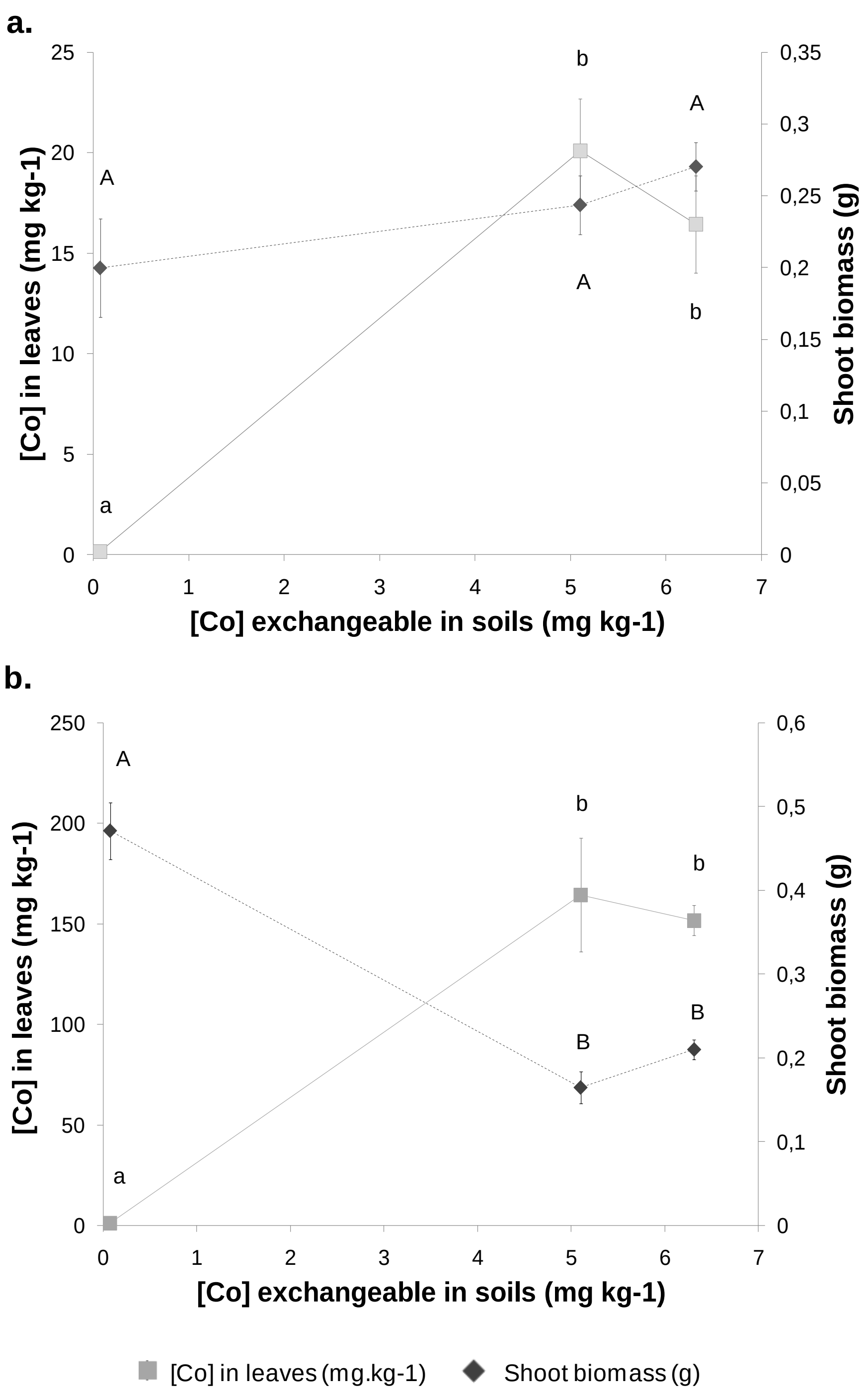

[Co] exchangeable in soils and [Co] in leaves (mg kg-1)
400
350
300
250
200
150
100
50
0
*
*
*
*
*
*
*
*
Control
+OM
+MnOx
+FeOx
+OM +FeOx
+OM +MnOx
+FeOx +MnOx
+FeOx +MnOx +OM
20 mg kg-1 Co soil treatments
[Co] exchangeable in soils (mg kg-1)
[Co] in leaves (mg kg-1)

Fig. 4



a.
[Cu] in leaves (mg kg-1)
Shoot biomass (g)
[Cu] exchangeable in soils (mg kg-1)
A,B,C
A
B
C
a
b
c
d
b.
[Cu] in leaves (mg kg-1)
Shoot biomass (g)
[Cu] exchangeable in soils (mg kg-1)
A
B
a
b
[Cu] in leaves (mg.kg-1)
Shoot biomass (g)

Fig. 5


a.
[Co] in leaves (mg kg-1)
Shoot biomass (g)
[Co] exchangeable in soils (mg kg-1)
b.
[Co] in leaves (mg kg-1)
Shoot biomass (g)
[Co] exchangeable in soils (mg kg-1)
[Co] in leaves (mg.kg-1)
Shoot biomass (g)

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