

Copper tolerance of three *Crotalaria* species from southeastern D.R. Congo at the early development stage

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Description of the subject. Microevolutionary processes in metallophytes established on copper enriched soils can lead to a diversity of plant species showing distinct tolerance capacities among genus. Researches about the relationship between these endangered plants and soil copper concentrations are critical in order to improve *ex situ* conservation methods in southeastern Democratic Republic of Congo (Katangan Copperbelt).

Objectives. The aim of the study was to test the effect of copper on the germination and root elongation of three *Crotalaria* species naturally occurring along a natural copper gradient. The hypothesis is that copper concentrations have different effects on germination and root elongation according to the species of *Crotalaria* genus.

Method. Three species were selected: *Crotalaria cobalticola*, *Crotalaria peschiana* and *Crotalaria cornetii*, occurring on soils with the highest to the lowest copper concentrations respectively. Germination and root elongation tests were performed *in vitro* (MS vitamin-enriched medium) in six copper mediums ranging from 0 to 125 μM Cu^{2+} .

Results. No significant differences in germination percentage were observed according to the copper concentrations. *Crotalaria cornetii* had the lowest germination percentage. Root elongation of *C. peschiana* did not differ with copper concentration, but root elongation of *C. cobalticola* was higher at the greatest copper concentration (125 μM Cu^{2+}).

Conclusions. Even if *C. cobalticola* presented better growth at highest Cu concentrations, it appeared that *C. cobalticola* and *C. peschiana* do not require copper for their early stages of development and could thus be conserved in non-contaminated substrate. *Crotalaria cornetii* seemed to present a physical seed dormancy.

Keywords. *Crotalaria*, biodiversity conservation, indigenous organisms, seed germination, root length, *in vitro* experimentation, copper, soil chemico-physical properties, D.R. Congo.

Tolérance au cuivre de trois espèces de *Crotalaria* de l'Arc Cuprifère Katangais dans les premiers stades de croissance

Description du sujet. Les processus microévolutifs des métalphytes établies sur des sols naturellement enrichis en cuivre peuvent conduire à une diversité d'espèces végétales présentant des capacités de tolérance distinctes au sein d'un genre. Dans l'Arc Cuprifère katangais (sud-est de la R.D. Congo), l'étude des relations entre les espèces menacées et leurs conditions édaphiques est essentielle pour améliorer les méthodes de conservation *ex situ*.

Objectifs. L'objectif de l'étude est de tester l'effet de la concentration en cuivre sur la germination et la croissance racinaire de trois espèces de *Crotalaria*, présentes le long d'un gradient de cuivre. L'hypothèse est que les concentrations en cuivre ont un impact différent sur la germination et l'élongation des racines selon l'espèce du genre *Crotalaria*.

Méthode. Trois espèces du genre *Crotalaria* ont été sélectionnées : *Crotalaria cobalticola*, *Crotalaria peschiana* et *Crotalaria cornetii*, présentes sur des sols avec des concentrations en cuivre respectivement des plus élevées aux plus faibles. Des tests de germination et d'élongation racinaire ont été effectués pour six concentrations en cuivre échelonnées de 0 à 125 μM Cu^{2+} .

Résultats. Les résultats n'ont mis en évidence aucun effet des concentrations en cuivre sur le pourcentage de germination. *Crotalaria cornetii* a présenté les plus faibles pourcentages de germination. L'élongation racinaire de *C. peschiana* n'a pas différé en fonction des concentrations en cuivre, cependant l'élongation racinaire de *C. cobalticola* était plus grande à la concentration en cuivre la plus élevée (125 μM Cu^{2+}).

Conclusions. Même si *C. cobalticola* présente une meilleure croissance à des concentrations en cuivre plus élevées, il apparaît que *C. cobalticola* et *C. peschiana* ne nécessitent pas de cuivre dans leurs premiers stades de développement et peuvent donc être conservées dans un milieu non enrichi en cuivre. Une probable dormance physique des graines pourrait affecter la germination de *C. cornetii*.

Mots-clés. *Crotalaria*, conservation de la diversité biologique, organisme indigène, germination des graines, longueur des racines, expérimentation *in vitro*, cuivre, propriété physique du sol, R.D. Congo.

1. INTRODUCTION

Metalliferous areas present high concentrations of trace metals associated with other environmental constraints including xericity and poor depauperate nutrient content (Ernst, 1974; Kruckeberg, 1985). These harsh abiotic conditions are strong drivers for local adaptation of plant species (Antonovics et al., 1971; Brady et al., 2005) that results in the formation of highly distinctive plant communities (Duvigneaud et al., 1963; Ernst, 1974; Rajakaruna, 2004; Saad et al., 2012). Such ecosystems are found in many places around the world (*e.g.* Arizona, Peru, Chili, Australia or Democratic Republic of Congo). They are usually recognized as major hotspots of biodiversity (Whiting et al., 2004; Veldman et al., 2015), offering outstanding examples of microevolution and speciation processes (Antonovics et al., 1971; Faucon et al., 2010; Harrison et al., 2011).

Ecosystems established on soils naturally enriched with copper are rare, except in South Central Africa where occurs a large number of copper-cobalt mineralized areas (Duvigneaud et al., 1963; Baker et al., 2010; Chipeng et al., 2010). The Katangan Copperbelt, in the Katanga province (South East of Democratic Republic of Congo), extends over 300 km long between Kolwezi and Lubumbashi with more than 150 copper- and cobalt-rich outcrops (François, 1988; Cailteux et al., 2005). These outcrops appear as isolated hills scattered in the Miombo woodland matrix, and available copper concentrations often exceed 10,000 mg Cu·kg⁻¹ at the outcrop summit. Those copper hills support more than 550 metallophyte taxa (Leteinturier, 2002). Recent studies show that trace metals and related nutrient concentrations play a significant role to structure plant communities along the metal gradient (Saad et al., 2012; Ilunga wa Ilunga et al., 2013; Séleck et al., 2013).

In Katanga, copper hills represent a biodiversity hotspot (Malaisse, 1983; Brooks et al., 1985; Leteinturier et al., 2002; Faucon et al., 2010), including 56 endemic species in which 32 are exclusively restricted to copper enriched soils (absolute metallophytes) (Faucon et al., 2010). Some authors have shown that the edaphic endemism could result of both abiotic and biotic interactions (Meyer, 1986; Palacio et al., 2007). First, taxa may be restricted to a limited area because of their specialization to particular physicochemical soil conditions making them more competitive than in regular soils (Meyer, 1986). Second, taxa can be stress-tolerant, but not specifically adapted, to adverse physicochemical conditions, and take refuge in the

stressful area where competition with other plant species is lowest (Gankin et al., 1964). Plants of the Katanga (D.R. Congo) occurring on copper-rich soils remained poorly studied (Faucon et al., 2012). To focus on endemic and non-endemic species within a genus appears interesting in order to understand the relationship between plant and copper concentrations in soil.

The copper hills have been severely disturbed for more than one century through the mining of natural resources leading in some cases to their degradation or total destructions (Brooks et al., 1992; Faucon et al., 2010). The determination of the *in vitro* multiplication ability of some endemics appears useful in a purpose of *ex situ* conservation. In this paper, we aimed to test the effect of copper on the germination and the root elongation of *Crotalaria cobalticola*, *Crotalaria peschiana* and *Crotalaria cornetii*. The results also contribute to discuss the relationship between the endemic status of the three species and their affinity for copper.

2. MATERIALS AND METHODS

We investigated both the germination and the root elongation in the first weeks according to different copper concentrations for three species of the genus *Crotalaria* (Fabaceae) from the Katangan Copperbelt. These species, *Crotalaria cobalticola*, *Crotalaria peschiana* and *Crotalaria cornetii*, were chosen according to their supposed copper tolerances (*i.e.* present in their habitat) and their endemism status in natural ecosystem; *C. cobalticola*, being the most tolerant species to strong copper concentrations and *C. cornetii* the less tolerant species.

2.1. Plant species

Crotalaria is a genus of the Fabaceae family. The genus *Crotalaria* appears to be mainly adapted to the seed dispersal by wind, water and gravity (Le Roux et al., 2011). *Crotalaria cobalticola* P.A. Duvign. & Plancke is a strict endemic and a threatened species (proposed IUCN status: EN) present in the natural highest Cu-enriched steppes of the copper hills or in disturbed copper and cobalt soils in the Katangan Copperbelt (**Figure 1**, **Table 1**). This annual forb has a natural density of about 5 to 10 individuals·m⁻² and was identified as an hyper-accumulator of cobalt in concentrations higher than 3,000 mg·kg⁻¹ (Duvigneaud et al., 1959; Brooks et al., 1981). *Crotalaria cornetii*

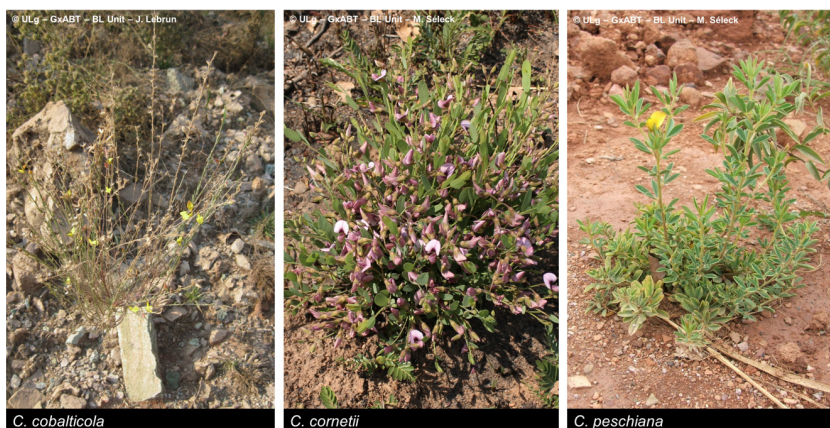


Figure 1. Pictures of adult individuals of the three tested *Crotalaria* species — Photographies des individus adultes des trois espèces testées de *Crotalaria* (<http://copperflora.org/eflora>).

Taub. & Dewevre is a non-endemic species presenting a large distribution in the southeastern D.R. Congo with a density of about 0.5 to 1 individual per m². It occurs on the low-enriched substrate (**Figure 1**, Duvigneaud et al., 1963), located in the steppic savannahs of the copper hills (**Table 1**, Duvigneaud et al., 1959). To date, its IUCN status has not been assessed. *Crotalaria peschiana* P.A. Duvign. & Timp. is a strict endemic and a critically endangered species (proposed IUCN status: CR) occurring in the slopes of steppic savannah of the natural copper hills in the Katangan Copperbelt (Duvigneaud et al., 1959; Faucon et al., 2010). Its natural density is about 1 to 2 individuals per m² (**Figure 1**, **Table 1**).

season minima can reach 5 °C at night (Saad et al., 2012).

Seeds were collected on 72 individuals scattered in one population of *C. cobalticola* (ca. 100-500 individuals), on 75 individuals in one population of *C. cornetii* (ca. 100-500 individuals) and on 75 individuals in one population of *C. peschiana* (ca. 500-1,000 individuals). Among population, mature and well-developed individuals were targeted in order to obtain mature seeds. For the three species, fruits are dehiscent pods and seed sizes comprised between 3 and 5 mm. *Crotalaria cornetii* has largest seeds (4-5 mm) (**Table 1**). For each species, seeds from the different individuals were pooled and seed samples were then placed in conserva-

2.2. Seed collection

Seeds were harvested in populations occurring in the southeastern D.R. Congo, in the Copperbelt, between the cities of Tenke (10.61°S; 26.12°E) and Fungurume (10.62°S; 26.32°E) during the year 2013. This region presents 40 isolated copper hills between 1,300 m to 1,400 m. The climate is subtropical with a rainy season from November to the end of March and a dry season from May to September. October and April are the transition months between the two seasons. Annual rainfalls are ca. 1,300 mm, most of which occur during the rainy season. Temperatures range from 15 °C to 17 °C at the beginning of the dry season and from 31 °C to 33 °C between September and October. Dry

Table 1. General characteristics of the studied species — *Caractéristiques générales des espèces étudiées.*

Taxon	Life form	Habitat in copper hills	Fruiting time	Fruit type	Seed size	Proposed IUCN status	Conservation status
<i>Crotalaria cobalticola</i>	Annual	Highly Cu/Co-enriched steppe	April-May	Dehiscent pod	3-4 mm	EN	Strict endemic
<i>Crotalaria cornetii</i>	Perennial	Low Cu/Co-enriched steppic savannah	September-October	Dehiscent pod	4-5 mm	NA	Non-endemic
<i>Crotalaria peschiana</i>	Perennial	Cu/Co-enriched slope	August-September (April-May)	Dehiscent pod	3-4 mm	CR	Strict endemic

Habitat descriptions and life forms are from Duvigneaud et al. (1959), Duvigneaud et al. (1963) and Saad et al. (2012). Conservation status and proposed IUCN status are from Faucon et al. (2010). Fruiting time, fruit type, seed sizes are from field observations — *La description des habitats et les formes de vies proviennent des travaux de Duvigneaud et al. (1959), Duvigneaud et al. (1963) et Saad et al. (2012). Les statuts de conservation et les statuts UICN proviennent de Faucon et al. (2010). Les périodes de fructification, les types de fruits et les dimensions des graines proviennent d'observations de terrain*; CR: Critically endangered — *fort en danger*; EN: Endangered — *en danger*; NA: Not assessed — *non évalué*.

tion. Considering that the three species have different conservation purposes (*i.e.* short-term conservation for *C. cornetii* and *C. peschiana* and long-term conservation for *C. cobalticola*), they were stored in different sites during a maximum two years. *Crotalaria cobalticola* was stored in the Botanic Garden Meise (Belgium) at -20 °C and 15% RH in order to apply long term conservation strategy (Godefroid et al., 2013). The two other species (*C. cornetii* and *C. peschiana*) were conserved at 5 °C in the nursery of Tenke Fungurume Mining Sarl (D.R. Congo) in order to use their seeds in propagation programs (Table 2).

2.3. Germination tests

Germination tests were performed in controlled conditions between October and December 2014. Mediums were prepared with agar (8 g·l⁻¹), Murashige and Skoog (MS)-vitamins (2.2 g·l⁻¹), sucrose (10 g·l⁻¹), MES-buffer (0.5 g·l⁻¹) and copper sulfate solution (CuSO₄·5H₂O 0.1M). Six copper mediums were prepared: 0 (the control), 25, 50, 75, 100 and 125 μM (*i.e.* μmol·l⁻¹) copper added, based on the results of Chipeng et al. (2010) and two other studies about non-tolerant legumes (Di Salvatore et al., 2008; Lin, 2011). The pH was adjusted at 5.7 with HCl 0.5 M or NaOH 0.1 M. Fifty ml of medium were put in each Petri dish; each medium was previously sterilized in autoclave during 90 min.

In order to limit the pathogen development, seeds were first treated with a bleach water solution (5%) during 5 min and rinsed three times with distilled water followed by a 5 min soak in an ethanol solution (70%) (Boisson et al., 2016). Seeds were then rinsed again three times in distilled water. Then, for each species and for each copper concentration, 90 seeds were placed in three Petri dishes (*i.e.* 30 seeds by Petri dish according to a systematic grid following six columns and five rows).

Petri dishes were placed horizontally in a tropical growth chamber with photoperiod 12/12 at 25 °C and 70% relative humidity at 450 μE light and 15 °C and 50% relative humidity in the dark during 38 days

(23 October – 01 December). Germination was recorded when the radicle was at least 2 mm long. Germination was monitored twice a week during three weeks and once a week as soon as one germinated seed maximum was recorded by Petri dish.

2.4. Root elongation tests

In vitro elongation tests were performed for *C. cobalticola* and *C. peschiana*; the number of *C. cornetii* seedlings was too low in order to test the effect of copper on root elongation. The root elongation test mediums were the same than the germination mediums; 0 (the control), 25, 50, 75, 100 and 125 μM (*i.e.* μmol·l⁻¹) copper added. Seedlings were germinated on MS, and transplanted when cotyledons appeared after a treatment with bleach water (10%) during 5 min followed by five rinsing baths in distilled water in order to keep it in sterile conditions. For each species and for each copper concentration, ten seedlings were transplanted in two Petri dishes (*i.e.* five seedlings by Petri dish) and dishes were installed vertically in the same conditions than for the germination tests during four weeks. The distance (mm) between the crown and the apical point of the roots was measured at the end of the test.

A first test demonstrated the independence of seedlings root elongation among the Petri dishes. For this purpose, seedlings were germinated in the non-enriched MS. Five seedlings were transplanted respectively in five Petri dishes while five seedlings were transplanted in one Petri dish. The final distance (mm) between the crown and the apical point of the roots was also measured.

2.5. Data analysis

Germination percentages were calculated as the ratio between the number of germinated seeds and total number of seeds in a Petri dish. Cumulative germination curves were created in order to evaluate the global dynamic of the germination process. The

Table 2. Location of harvested sites with collecting and conservation data of the seeds of the three studied species — *Localisation des sites de récoltes des espèces et des informations sur la collecte et la conservation des graines des trois espèces.*

Taxon	Collecting date			Conservation data		
	Date	Site	Sampled ind.	Date	Method	Site
<i>Crotalaria cobalticola</i>	07/05/2013	Fungurume II	72	28/05/2013	-18 °C - 15% HR	BGM
<i>Crotalaria cornetii</i>	03/01/2013	Kavifwafwaulu I	75	02/01/2013	5 °C - ambient HR	TFM
<i>Crotalaria peschiana</i>	24/09/2013	Kazinyanga	75	24/09/2013	5 °C - ambient HR	TFM

BGM: Botanic Garden Meise; TFM: Tenke Fungurume Mining sarl.

mean germination time (MGT) was calculated as a proxy of the germination speed:

$$\sum_{t_0}^{t_f} \frac{g_{ii} - p_{ii}}{N_g}$$

where t_0 was the beginning of the test (in days); t_f was the time at test closure (in days); g_{ii} was the number of germinated seeds at the time t_i ; p_{ii} was the period (in days) between t_i and t_0 ; N_g was the total number of germinated seeds (Ellis et al., 1981). The effect of copper concentration on both the final germination percentages and on the mean germination time (MGT) was analyzed by a one-way ANOVA.

Effect of copper concentration on root elongation was assessed using one-way ANOVA. The independence test had confirmed the absence of effect of the Petri dishes on the root elongation (one-way ANOVA *C. cobalticola*: $F = 1.03$, p -value > 0.05 ; one-way ANOVA *C. peschiana*: $F = 3.59$, p -value > 0.05). So, ten replications were considered for the analysis. However, some seedlings died during the test. The number of replications was lower than 10 in two treatments for *C. cobalticola* (25 μM , $n = 9$; 75 μM ,

$n = 5$) and for one treatment for *C. peschiana* (50 μM , $n = 9$).

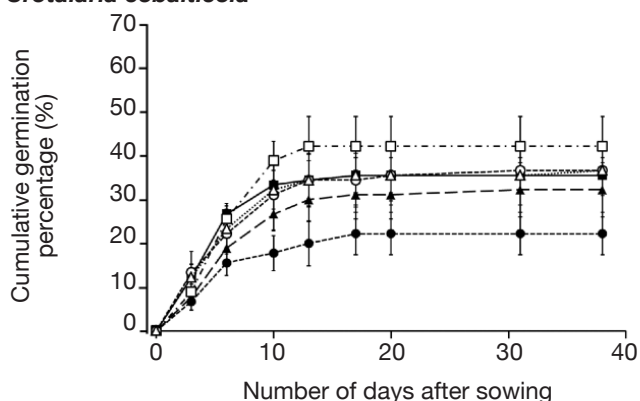
For all analyses, the normality and heteroscedasticity were verified. Root elongation data for *C. cobalticola* were square-root transformed. All significant differences in analyses were followed by Tukey's range test (HSD) to structure the means. Statistical analyses were performed using the R statistical software (R Development Core Team, 2010).

3. RESULTS

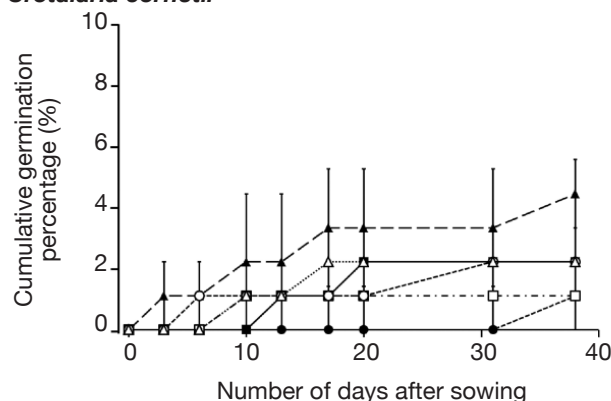
3.1. Effect of copper on germination

The final germination percentages were $34.3 \pm 2.4\%$ for *C. cobalticola*, $46.0 \pm 2.8\%$ for *C. peschiana* and $3.0 \pm 0.5\%$ for *C. cornetii*. Although no significant differences of the final germination percentage were highlighted between copper concentrations (Figures 2 and 3), some trends were observed on the cumulative germination curves. The cumulative germination percentage of *C. cobalticola* at 25 μM Cu^{2+} stayed the lowest ($22.0 \pm 4.8\%$ at the end of the experiment), while the cumulative germination percentage at 75 μM

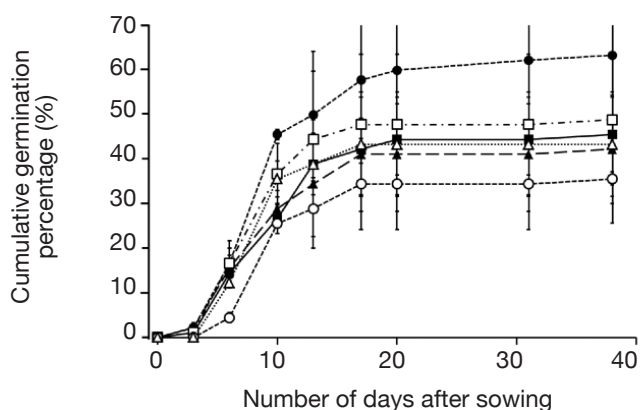
Crotalaria cobalticola



Crotalaria cornetii



Crotalaria peschiana



Copper concentrations (μM)

■ 0 ● 25 ▲ 50 □ 75 ○ 100 △ 125

Figure 2. Cumulative germination curves of the percentage of germination (%; $n = 3$) of *Crotalaria cobalticola*, *Crotalaria cornetii* and *Crotalaria peschiana* after sowing of seeds in Petri dishes containing six concentrations of copper. Error bars correspond to standard error (SE) — *Courbes d'accumulation du pourcentage de germination (%; $n = 3$) de Crotalaria cobalticola, Crotalaria cornetii et de Crotalaria peschiana après leur semis en boîtes de Petri dans six concentrations en cuivre. Les barres d'erreur correspondent à l'erreur standard (SE).*

Cu^{2+} was the highest from the day 6 ($42.0 \pm 6.8\%$ at the end of the experiment). *Crotalaria peschiana* had the highest cumulative germination percentage at $25 \mu\text{M Cu}^{2+}$ ($63.3 \pm 6.7\%$ at the end of the experiment). *Crotalaria cornetii*, which had overall low final germination percentages, had a highest final germination percentage ($4.4 \pm 1.1\%$) at low copper concentrations (0, 25 and $50 \mu\text{M Cu}^{2+}$). However, no well-defined trend on the cumulative germination curves was observed (Figure 2).

No significant differences of the MGT were highlighted between copper concentrations for *C. cobalticola*, with a MGT of 7.3 ± 0.3 days (Figure 3) and for *C. peschiana* with a MGT equal to 10.8 ± 0.3 days (Figure 3). For *C. cornetii*, the MGT differed according to copper concentrations ($F = 5.36$, p -value < 0.01), seeds tended to germinate more rapidly when copper concentrations increased. The highest MGT was observed for $25 \mu\text{M}$ of copper (35.6 ± 2.3 days), although it was not significantly different from that at 0 and $50 \mu\text{M Cu}^{2+}$ (27.8 ± 5.3 days). Its lowest MGT was at $75 \mu\text{M Cu}^{2+}$ (3.3 ± 3.3 days) that was significantly different than the MGT at $25 \mu\text{M Cu}^{2+}$ (Figure 3).

3.2. Effect of copper on root elongation

Crotalaria cobalticola had a higher root elongation at $125 \mu\text{M Cu}^{2+}$ (150 ± 41 mm) compared to the other copper concentrations ($F = 6.31$, p -value < 0.001 , Figure 4). *Crotalaria peschiana* presented an equal elongation root uneven the copper concentrations (25 ± 1 mm, Figure 4).

4. DISCUSSION

4.1. Germination and early establishment

The three tropical metallophyte species, *C. cobalticola*, *C. peschiana* and *C. cornetii*, germinated under all copper concentrations, without a clear effect of copper concentrations on the germination (*i.e.*, germination percentage and mean germination time). This pattern was already pointed out for another copper endemic species *Diplolophium marthozianum* (Apiaceae) (Boisson et al., 2016). Chipeng et al. (2010) also showed that copper addition did not significantly

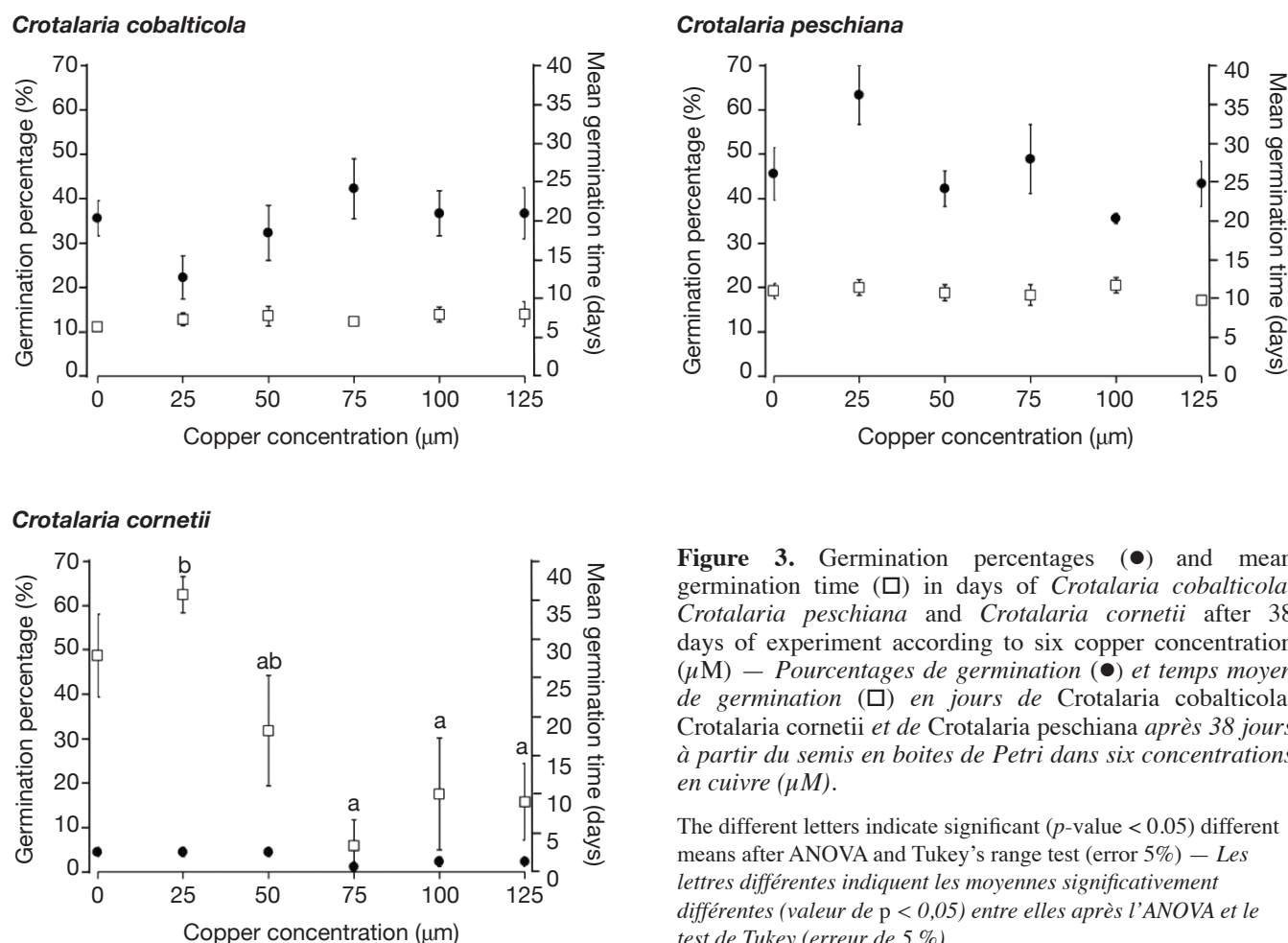


Figure 3. Germination percentages (●) and mean germination time (□) in days of *Crotalaria cobalticola*, *Crotalaria peschiana* and *Crotalaria cornetii* after 38 days of experiment according to six copper concentration (μM) — Pourcentages de germination (●) et temps moyen de germination (□) en jours de *Crotalaria cobalticola*, *Crotalaria cornetii* et de *Crotalaria peschiana* après 38 jours à partir du semis en boîtes de Petri dans six concentrations en cuivre (μM).

The different letters indicate significant (p -value < 0.05) different means after ANOVA and Tukey's range test (error 5%) — Les lettres différentes indiquent les moyennes significativement différentes (valeur de $p < 0,05$) entre elles après l'ANOVA et le test de Tukey (erreur de 5 %).

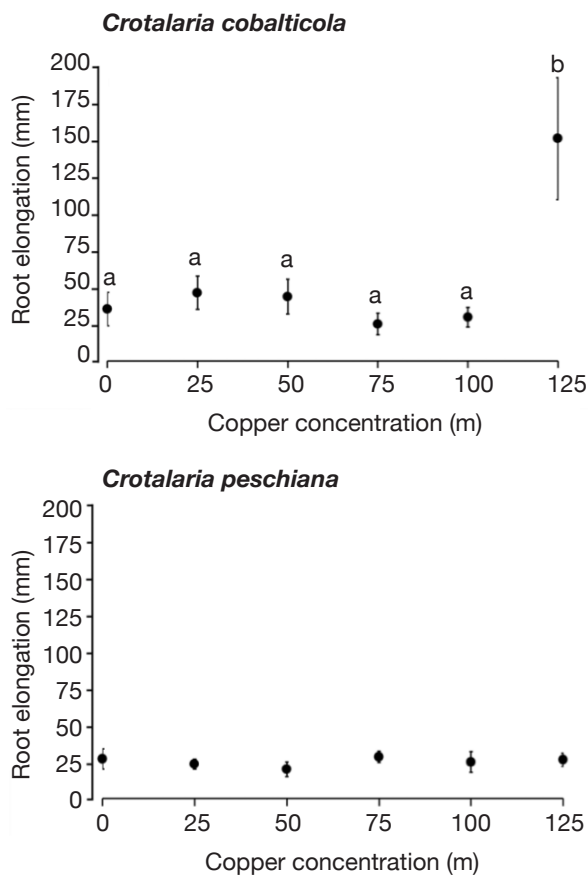


Figure 4. Copper concentrations effects on the root elongation of *Crotalaria cobalticola* and *Crotalaria peschiana* after 28 days of exposition to solutions containing 0, 25, 50, 75, 100 and 125 μM Cu^{2+} — *Effet des concentrations en cuivre sur l'élongation racinaire de Crotalaria cobalticola et Crotalaria peschiana après 28 jours d'exposition à des solutions contenant 0, 25, 50, 75, 100 et 125 μM Cu^{2+} .*

Data are presented as the mean root elongation (mm \pm SE) of 10 seedlings for each concentration but 25 μM (n = 9) and 75 μM (n = 5) for *C. cobalticola* and 50 μM (n = 9) for *C. peschiana* — *les données sont exprimées en moyennes d'élongations racinaires (mm \pm SE) de 10 plantules, excepté pour les concentrations 25 μM (n = 9) et 75 μM (n = 5) de *C. cobalticola* et 50 μM (n = 9) de *C. peschiana*; dead seedlings were removed from the analysis — *les plantules mortes ont été retirées des analyses*; error bars correspond to standard error (SE) — *les barres d'erreur correspondent à l'erreur standard (SE)*; letters above dots indicate significant (p -value < 0.05) differences after ANOVA and Tukey's range test — *les lettres indiquent les différences significatives (valeur de p < 0,05) à la suite d'une ANOVA et d'un test de Tukey*; means sharing the same letter are not significantly different — *les moyennes partageant les mêmes lettres ne sont pas significativement différentes.**

improve the germination of the high copper tolerant *Haumaniastrum katangense* (Lamiaceae). On the other hand, *Crotalaria cornetii* seems more sensitive to higher copper concentration, since this species showed lower mean germination time at the highest copper

concentrations. Seed coats can constitute a barrier between the embryo and its direct environment or adsorb metals as reported by previous studies (Mayer et al., 1989; Araújo et al., 2005; Di Salvatore et al., 2008). Seeds of Fabaceae are known for their physical dormancy, where an impermeable seed-coat prevents the entry of water to the embryo (Bewley et al., 1994). The low germination percentages recorded for *C. cornetii* (< 5%) may indicate a dormancy of seeds; scarification with sandpaper or soaking in hot water permitted to increase the percentage of germination at 56% (Le Stradic, personal communication).

The genus *Crotalaria* permitted to study two endemics that have distinct life histories. *Crotalaria cobalticola* is an annual species while *C. peschiana* is perennial. Due to the shorter life cycle, annual species have to grow rapidly in order to reproduce (Garnier, 1992). While the percentages of germination were higher for the perennial *C. peschiana*, the MGT was also longer and could be partially the consequence of the perennial behavior of the species. *Crotalaria cobalticola* germinates in November and has to produce seeds before the dry season (May). This could explain that the root elongation was higher for *C. cobalticola*. These two endemic species from the Katangan Copperbelt are classified in the same clade Dispermae contrary to *C. cornetii* which is in Glaucace (Le Roux, personal communication). This phylogenetic proximity supports supposed higher copper tolerance of *C. cobalticola* and *C. peschiana* compared to *C. cornetii*.

As expected, *C. cobalticola*, which is a strict endemic of copper hills, was the most copper tolerant species at the early stages of growth (Duvigneaud et al., 1959). Root growth for *C. cobalticola* was stimulated by highest copper concentration, favoring a rapid development of the individuals, which was not the case for *C. peschiana* at least for the copper concentrations used. Root growth was already reported to be more sensitive than germination to metal toxicity (Jeliazkova et al., 1998; Araújo et al., 2005). Growth stimulation by elevated concentrations of copper has rarely been reported (Faucon et al., 2012). Rapid development of individuals confers an advantage in terms of competition in the highest copper-enriched areas. This high tolerance capacity could also lead to a physiological cost, reducing the fitness and competitive ability of individuals (Cox et al., 1981; Bradshaw, 1984). These assumptions support the hypothesis that this species could not grow in regular soils of the savannahs surrounding the hills where copper content is low ($\leq 100 \text{ mg}\cdot\text{kg}^{-1}$ Cu) (Duvigneaud et al., 1959; Saad et al., 2012). Additional studies should focus on a broader range of metal concentrations in order to obtain the total copper response curve of the species. Considering that Duvigneaud et al. (1959) has

described it as a species highly dependent on cobalt in soils, the identification of its response to cobalt would also be interesting in terms of comparison with copper response.

Crotalaria peschiana was able to grow in all copper concentrations without expressing any quantified stress. Contrary to *C. cobalticola*, copper seems not to be associated with a physiological need for its growth in the early growth stage; it did not limit its growth either. *Crotalaria peschiana* is not recorded in non-metaliferous substrate in the Katangan province (Faucon et al., 2010), which implies that this stress-tolerant endemic species might not be limited by edaphic conditions, but is likely excluded from the dominant vegetation by biotic interactions as competition (Gankin et al., 1964) or herbivory. The less productive ability in regular soils leads this species in "refuge areas" (*i.e.* metalliferous substrates) in which competition is lower (McNeilly et al., 1968). Copper is also known as a broad-spectrum fungicide. Copper-rich soils may have lower pathogen content in comparison to regular soils, conducting to a loss of pathogens defense (Tadros, 1957; Chipeng et al., 2010). Some studies have pointed out the loss of resistance of some copper tolerant species to pathogens (Malaisse et al., 1982; Paton et al., 1996; Chipeng et al., 2010; Faucon et al., 2012). According to the elemental defense hypothesis, metal accumulation in the apoplast can inhibit growth of some bacterial pathogens (Fones et al., 2010). On the copper hills, *C. peschiana* are absent in the steppe where copper concentrations exceed 4,000 mg Cu·kg soil⁻¹, suggesting that this species tolerates lower copper concentration in soil than *C. cobalticola* (Séleck et al., 2013). Additional studies are required to evaluate the growth response of *C. peschiana* and *C. cobalticola* in higher copper concentrations than 125 μM in order to highlight their upper tolerance limits.

4.2. Conservation of copper endemics and revegetation

From a conservation point of view, the two endemic and threatened species *C. cobalticola* and *C. peschiana* could be put in germination in non-contaminated mediums, like *C. cornetii*. The *ex situ* conservation should allow to export and preserve the plant material outside its natural habitat. It is interesting to germinate seeds *in vitro* before transplanting them in the nursery to keep a maximum of individuals. Then, a non-copper enriched medium would be sufficient to grow these two species during their installation stages, which should facilitate the multiplication of such species. However, more studies should be performed to determine the other factors (*i.e.* origin of population, biotic interactions or dormancy) which might influence their growth. As the response could be different between populations due

to local adaptations, conservation actions should focus on several sites (Faucon et al., 2012; Godefroid et al., 2013; Ilunga wa Ilunga et al., 2013).

In addition, physiological plant response to available metals is related to other physico-chemical conditions in soil with all chemical edaphic conditions (Austin, 2007; Soberón et al., 2009), which are not reproduced in our experiments. Similarly, bioavailability of copper and cobalt depends on the total edaphic metal content, pH, clay and hydrous oxide content, organic matter and redox conditions (Reichman, 2002; Saad et al., 2012; Séleck et al., 2013). So, to define a clear relationship between the availability of copper in agar and soils is still difficult (McLean et al., 1992). The determination of the copper and cobalt bioavailability by mathematical models is currently performed in order to study the ecological response to metals of some endemic species and is necessary to improve conservation strategies (Lange et al., 2014).

These results could also contribute to the implementation of other strategies, as revegetation or phytoremediation. The knowledge about ecology of metallophyte endemics is the basis to associate phytotechnologies and *ex situ* conservation strategies. Secondary habitats created by the mining exploitation are opportunities to preserve part of the threatened metallophyte and endemic species (Whiting et al., 2004; Faucon et al., 2011). These secondary metalliferous habitats present often high physico-chemical heterogeneity in metal concentrations and species having a broad response to metals should be preferred. While restoration of mining sites has to be performed after exploitation, *ex situ* conservation of these metallophyte and endemic species in favorable sites should be a priority, and these secondary habitats created by the mining can be an opportunity to preserve this biological resource.

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