Germination capacity and seed storage behaviour of threatened metallophytes from the Katanga copper belt (D.R.Congo): implications for *ex situ* conservation

Sandrine Godefroid[1,*], Ann Van de Vyver[1], Julie Lebrun[2], Wilfried Masengo Kalenga[3], Guylain Handjila Minengo[4], Charles Rose[4], Michel Ngongo Luhembwe[3], Thierry Vanderborght[1] & Grégory Mahy[2]

[1]National Botanic Garden of Belgium, Domein van Bouchout, BE-1860 Meise, Belgium
[2]Biodiversity and Landscape Unit, Gembloux Agro-Bio Tech, University of Liège, Passage des Déportés 2, BE-5030 Gembloux, Belgium
[3]Faculté d'Agronomie, Université de Lubumbashi, Campus de la Kasapa, Lubumbashi, D.R.Congo
[4]Tenke Fungurume Mining SARL, Avenue Panda n° 790, Lubumbashi, Katanga, D.R.Congo
*Author for correspondence: sandrine.godefroid@br.fgov.be

Background and aims – Plant species adapted to metalliferous soil are of high conservation value, and actions for preserving these species (some of them are endemics) are urgent given the threat of mining activities. In the framework of an integrated conservation programme of cuprophytes (plants that tolerate a soil with a high level of copper) in Katanga (D.R.Congo), this study aims at: (1) providing new data on species whose germination has never been studied so far; (2) gaining new insight into the storage behaviour of these species; (3) discussing implications for *ex situ* conservation of these highly threatened species.

Methods – Germination tests were conducted on fresh seeds of nineteen species. These tests were repeated after 6, 12 and 24 months of storage in dry-cold conditions.

Key results – Most species kept or increased their germination capacity after 2 years storage in dry-cold conditions. Nine species showed a slight decrease in their viability (from 100% to > 80%) after 2 years storage in dry-cold conditions. The present study gives evidence that at least six of the 19 studied species are desiccation-tolerant (orthodox). Among these, two are strict endemics, *Haumaniastrum robertii* and *Faroa malaissei*, and two are broad endemics, *Diplolophium marthozianum* and *Gladiolus robiliartianus*. This means that *ex situ* seed banking of these species could form a useful part of a more comprehensive conservation strategy. Only two species have been identified as desiccation-sensitive (recalcitrant), i.e. inappropriate for conservation in standard seed bank conditions. An orthodox behaviour has not been ruled out for the other species tested, but their response was less clear and needs further investigation .

Key words – Endangered species, endemic, heavy metals, metallophyte, seed banking, seed viability.

INTRODUCTION

Heavy metal-rich substrates have driven the evolution of some of the world's most remarkable and rare plants (Whiting et al. 2002). Thanks to their remarkable physiological adaptations to substrates enriched in heavy metals, metallophytes represent valuable phytogenetic resources for revegetation and restoration programs, and for the remediation of heavy metal pollutions (Leteinturier et al. 1999, Baker et al. 2000, Whiting et al. 2002, Shutcha et al. 2010). The flora of metalliferous sites is therefore of high conservation value, and actions for preserving these species are imperative given the threat of mining activities (Whiting et al. 2004). The most common natural outcrops of metalliferous rock are ser-

pentines rich in nickel, iron and magnesium (Brooks 1987). Ecosystems established on soils naturally enriched with copper are rare on world scale (Faucon et al. 2010). South Central Africa is however exceptional in the occurrence of Cu-enriched substrates (Duvigneaud & Denaeyer-De Smet 1963), and the Katanga copper belt (D.R.Congo) has been recognized as a hotspot for metallophytes (Wild & Bradshaw 1977, Malaisse et al. 1983). Plant populations established on these substrates form unique, spatially limited communities that have received attention from the late fifties onwards (Duvigneaud 1958, 1959, Duvigneaud & Denaeyer-De Smet 1960, 1963). Since this pioneering work, cuprophytes (plants that tolerate a soil with a high level of copper) have been

studied from various points of view, e.g. their taxonomy (Macnair 1988), evolution (Brooks & Malaisse 1990), phytogeography (Malaisse 1983, Leteinturier & Malaisse 1999) and physiology (Baker et al. 1983). It is only recently that scientists began to focus on the conservation of these highly endangered species (Bizoux et al. 2004, Faucon et al. 2010, Saad et al. 2012). Recently, Faucon et al. (2010) have established an inventory of endemics of Cu-enriched soils in Katanga. They also provided a first assessment of their conservation status using the IUCN criteria. More than 600 taxa are currently part of the Katanga copper flora from which 32 are strict endemics (Faucon et al. 2010). In this area, a conservation project has been launched by scientists supported by the mining company Tenke Fungurume Mining (TFM). The project called "Biodiversity Action Plan" (Saad et al. 2012) results from an Environmental and Social impact Assessment (ESIA) in accordance with Equator principles, IFC Performance Standards (www.equator-principles.com) and the Congolese mining code. The aim is to elaborate a programme that allows copper-cobalt flora conservation while being compatible with mining activities. The project combines various conservation strategies including ecosystem reconstruction, species translocations, protected areas designation and the development of *ex situ* collections in Belgium and in D.R.Congo (University of Lubumbashi). The D.R.Congo is one of the countries recently identified as in greatest need of conservation assistance (Giam et al. 2010). Meersseman (2008) estimates that by 2040 the number of endemic species from the Katanga copper belt will have decreased by over 40 percent. Prompt action is therefore necessary to safeguard this diversity before it disappears.

Seed storage is the preferred method of *ex situ* conservation due to the relative low cost of maintaining collections and its superior capability in representing the species' genetic variability (Weisenberger et al. 2010). Some *ex situ* seed banks of metal hyperaccumulator plants exist, e.g. at the Universities of Oxford and Melbourne (Whiting et al. 2004), but to our knowledge no such structure exists for the cuprophytes of Central Africa. *Ex situ* conservation requires knowledge of seed germination requirements and storage behaviour. There has been no previous research on germination, dormancy or storage behaviour of metallophyte species from the Katanga copper belt. Yet, it is important to be able to germinate the collected seeds if they are to be used in species recovery and vegetation restoration projects. Identifying their storage behaviour (orthodox vs. recalcitrant seeds) is also essential in order to know whether these species can be stored in standard seed bank conditions. Recalcitrant seeds (i.e. damaged by dryness and subzero temperature) are most common in tropical ecosystems (Tweddle et al. 2003). However, contrary to what is often believed, many tropical species can be banked (Linington et al. 2003).

This paper analyses seed collections of nineteen cuprophytes (table 1) from copper hills in Katanga (D.R.Congo) by determining their germination capacity, viability, dormancy and vigour on fresh seeds and after 6, 12 and 24 months of storage in dry-cold conditions (15%RH; -20°C). These species were the first to be banked at the National Botanic Garden of Belgium and are therefore the only ones for which we have results over two years. Our study aims at: (1) providing new data on species whose germination has never been studied so far; (2) gathering new insight in the storage behaviour of these species; (3) discussing opportunities for *ex situ* conservation of these highly threatened species.

MATERIALS AND METHODS

Study area

The TFM mining concession is located in the phytogeographical district of Eastern Upper-Katanga (Zambezian domain of the Sudano-Zambezian region, Duvigneaud 1958), South D.R.Congo. This region includes approximately 300 metalliferous sites scattered over more than 400 km (W-E) and forms the so-called "Katangan Copper Belt" (Leteinturier 2002, Baker et al. 2010).

Climate of Upper-Katanga is humid subtropical (Köppen climate classification Cwa) with one dry season (May to September) and one wet season (November to April). Annual mean rainfall is about 1300 mm, the majority falling during the wet season. Temperature fluctuates from 15–17°C (early dry season) to 31–33°C (late dry season). Mean annual temperature is about 20°C (Saad et al. 2012).

The study area is part of the Zambezian regional centre of endemism with more than 50 percent of the species endemic and more than 1000 species recorded (White 1983). The climax vegetation was a semi-deciduous dry forest ("Muhulu") gradually replaced by the "Miombo" open forest arising from fires and deforestation (Schmitz 1971). For a detailed description of these forest types, we refer to White (1983).

Metalliferous sites of Katanga are expressed in the form of hills emerging from the Miombo forest and often topped by siliceous cellular rocks. Total copper concentration in the topsoil is highly variable, ranging from 100 to 100000 mg kg^{-1} (Duvigneaud & Denaeyer-De Smet 1963). Heavy metal concentrations prevented the development of trees leading to the formation of highly distinctive types of vegetation (swards, steppes and steppic savanna).

Seed collection

In the framework of the Biodiversity Action Plan, 205 seed samples from 65 taxa have been collected from the Katanga copper belt between 2007 and 2010. Most of these seed samples come from the Fungurume area and were first collected on copper hills planned to be mined in a near future.

Within the target populations, seeds or fruits were collected when fully mature (when they can easily be detached from the parent plant), from at least fifty individuals. In case of very small populations including less than fifty individuals and highly threatened in short term, all individuals were sampled. The strategy for seed collection followed the recommendations from Way (2003) and the Millennium Seed Bank Project (Royal Botanic Gardens Kew 2006). The identity of target taxa from all populations sampled was verified by vouchering specimens at the herbarium of the National Botanic Garden of Belgium. Identifications were based on the Flore d'Afrique Centrale (Bamps 1973–1993), Flora Zambesiaca (Board of trustees Kew Royal Botanic Gardens

Table 1 – Taxa from the Katanga copper belt included in this study (ranked by alphabetical order of family name).
IUCN equivalent status is provided for strict and broad endemics, according to Faucon et al. (2010). [a] "Strict endemics" are species with 100 percent of the records on Katanga copper soil; "Broad endemics" are species with more than 75 percent of the occurrences on Katanga copper soil. Hyperaccumulators are species that accumulate unusual amounts of trace elements in their biomass which, for copper, exceed 1000 mg kg^{-1} (Boyd 2004). [b] IUCN equivalent status: CR: Critically endangered, EN: Endangered, VU: Vulnerable, NT: Near Threatened, LC: Least Concern, DD: Data Deficient.

Family	Taxa	Concern[a]	IUCN equivalent status[b]
Apiaceae	*Diplolophium marthozianum* P.A.Duvign.	Broad endemic	EN
Apiaceae	*Diplolophium zambesianum* Hiern		
Apiaceae	*Peucedanum nyassicum* H.Wolff		
Asteraceae	*Anisopappus davyi* S.Moore	Hyperaccumulator	
Asteraceae	*Dicoma anomala* Sond.		
Asteraceae	*Pleiotaxis rogersii* S.Moore		
Campanulaceae	*Wahlenbergia collomioides* (A. DC.) Thulin		
Fabaceae	*Crotalaria cobalticola* P.A.Duvign. & Plancke	Strict endemic	EN
Gentianaceae	*Faroa malaissei* Bamps	Strict endemic	CR
Iridaceae	*Gladiolus gregarius* Welw. ex Baker		
Iridaceae	*Gladiolus robiliartianus* P.A.Duvign.	Broad endemic	EN
Lamiaceae	*Haumaniastrum robertii* (Robyns) P.A.Duvign. & Plancke	Strict endemic & hyperaccumulator	VU
Lamiaceae	*Haumaniastrum rosulatum* (De Wild.) P.A.Duvign. & Plancke	Hyperaccumulator	
Malvaceae	*Hibiscus rhodanthus* Gürke ex Schinz		
Melastomataceae	*Antherotoma naudinii* Hook. f.		
Orobanchaceae	*Buchnera inflata* Skan		
Orobanchaceae	*Buchnera quadrifaria* Baker		
Orobanchaceae	*Buchnera trilobata* Skan		
Orobanchaceae	*Sopubia neptunii* P.A.Duvign. & van Bockstal	Broad endemic	VU

1960–2010) and Flora of Tropical East Africa (Royal Botanic Gardens Kew 1952–2008).

Small populations not threatened in the short term were not sampled since the priority was to first collect those populations that are in danger of destruction (according to the schedule of the mining company). Considering the high risk of habitat destruction due to mining activities, as many seeds as possible were collected, depending on time and people availability.

Seeds harvested within the population were bulked into a labelled paper bag. A passport data sheet was filled in for each population including GPS coordinates, voucher reference, sampling area, population size, proportion of mature individuals, number of sampled individuals and habitat description (aspect, slope, percent of rocks, associated species).

After collection, seeds and fruits were air dried and stored at ambient laboratory conditions (c. 21°C, 50%RH) for approximately three months until germination testing started.

Germination test

For the purpose of this study, we selected a subsample of the collected species to be tested for germination, i.e. 42 seed samples belonging to nineteen taxa collected from six different copper hills. This choice was made so as to have a mixture of various metallophytes (e.g. hyperaccumulators, strict and broad endemics) belonging to ten different families. The tested taxa are listed in table 1.

Seed viability was first estimated by performing germination tests. Since no information on our study species could be retrieved from the Seed Information Database (Royal Botanic Gardens Kew 2008) and existing literature, we applied a treatment that is close to climate conditions experienced by the species at the time of germination on the field. Some families are known to have a physical dormancy and therefore require scarification (Baskin & Baskin 1998). These seeds had their seed coat cut with a scalpel. A total of 174 germination trials were conducted on nineteen species un-

der controlled environmental conditions in incubators set with a constant temperature of 22°C, with a diurnal period of 12 hours light and 12 hours dark. Experiments were undertaken on 'fresh' seeds (stored at ambient conditions: 21°C and 50%RH for ~ 3 months), and after 6, 12 and 24 months in seed bank conditions: seeds were first dried in a drying chamber during 3 months at 15°C and 15%RH, and subsequently stored at -20°C with 5% moisture content, according to international standards (FAO/IPGRI 1994).

Seeds were placed on 1 percent agar (10 g/l) in plastic Petri dishes. Testing was applied to a total of 50 or 100 seeds per accession (depending of the availability of seeds), divided into two or four Petri dishes of 25 seeds. For each taxon, one to five accessions were collected, which means that 2 to 20 replicates per taxa (Petri dishes × accessions) were available for analysis. Germination percentage of each accession was obtained by pooling the results of the two or four Petri dishes. Germination was recorded when the radicle was at least 2 mm long. Germination was monitored until one month after the last seed germinated, and many germination tests were left to run for long periods, the longest being 187 days. Seeds that did not germinate were subjected to a cut test in order to identify the number of fresh, mouldy, empty and infested seeds.

Data analysis

Germination percentage is calculated as follows:
Germination % = number of germinated seeds / (total number of tested seeds - empty ones). The results of germination tests were indeed corrected to eliminate the proportion of empty seeds. Since empty seeds are never viable, it is not appropriate to consider them as part of the seed population (Gosling 2003).

Seed viability was calculated as the number of germinated seeds plus the number judged viable from the cut test expressed in percentage of the total (Offord et al. 2004, Crawford et al. 2007).

An indication of dormancy status was calculated using Offord's et al. (2004) equation:
Dormancy index = 1-(seed germinated % / viability %)

An index > 0.4 was used as the threshold value to indicate dormancy (Offord et al. 2004).

As a seed vigour trait, we used the mean time to germination (MTG), allowing to calculate the speed of germination. For each species, the mean time to germination (MTG) was calculated using the following equation (e.g. Tompsett & Pritchard 1998, Daws et al. 2005):
$$MTG = \sum (n_i \cdot d_i / N)$$
Where, n_i = number of germinated seeds at d_i days; d_i = incubation period in days at n_i ; N = total number of seeds germinated in the treatment.

RESULTS

Germination data at six months interval for up to two years were available for nineteen species, representing fourteen genera. Considering all species together (fig. 1), germination and viability of fresh seeds reached on average 71 percent
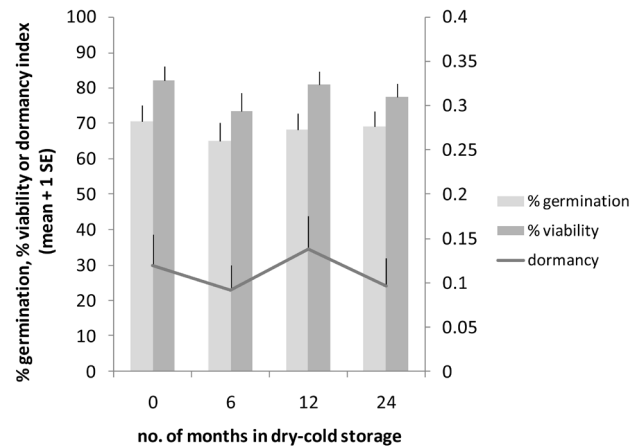

Figure 1 – Temporal evolution of the germination capacity, viability and dormancy (mean + 1 S.E.) of the nineteen tested species (42 replicates) after storage in seed bank conditions (15%RH, -20°C).

and 82 percent, respectively. Globally, there was no significant decrease in germination, viability or dormancy with the duration of storage in dry-cold conditions (P = 0.89, 0.10 and 0.47, respectively; Kruskal-Wallis tests).

Out of nineteen species, eleven showed a germination percentage of fresh seeds above 75 percent, whereas eight had less than 50 percent fresh seeds that germinated (fig. 2). Most species kept or increased their germination after 2 years storage in dry-cold conditions. Nine species showed a slight decrease in their viability (from 100% to > 80%) after 2 years storage in dry-cold conditions: *Anisopappus davyi, Buchnera quadrifaria, Crotalaria cobalticola, Faroa malaissei, Haumaniastrum robertii, H. rosulatum, Hibiscus rhodanthus, Sopubia neptunii* and *Wahlenbergia collomioides*. Two species had a dramatic decline in their viability after storage in the seed bank: *Dicoma anomala* and *Pleiotaxis rogersii*. Conversely, four species showed an increased viability after storage: *Gladiolus gregarius, Diplolophium marthozianum, Peucedanum nyassicum* and *Buchnera trilobata*. The contrasted behaviour of some species is detailed in figure 4.

Dormancy index was found to be particularly low for the vast majority of the studied species (fig. 5). It was zero for half of the tested taxa. Only two species exhibit some kind of dormancy with an index higher than 0.4: *Buchnera quadrifaria* and *Wahlenbergia collomioides*. For the remainders, dormancy index was lower than 0.2, and for most of them it was further reduced after storage in the seed bank.

Mean time to germination (fig. 6) ranged between six and 67 days for fresh seeds (mean: 26; median: 23) and between eight and 42 days for 2 years-banked seeds (mean = median: 13). Those species that germinated very fast (< 10 days) were *Haumaniastrum robertii, Hibiscus rhodanthus* and *Crotalaria cobalticola*. Conversely, the slowest germinator was *Peucedanum nyassicum. Dicoma anomala* and *Antherotoma naudinii* exhibited a slower germination after storage in dry-cold conditions, whereas *Peucedanum nyassicum, Gladiolus gregarius* and *Diplolophium zambesianum* are examples of species showing a faster germination after dry-cold storage.
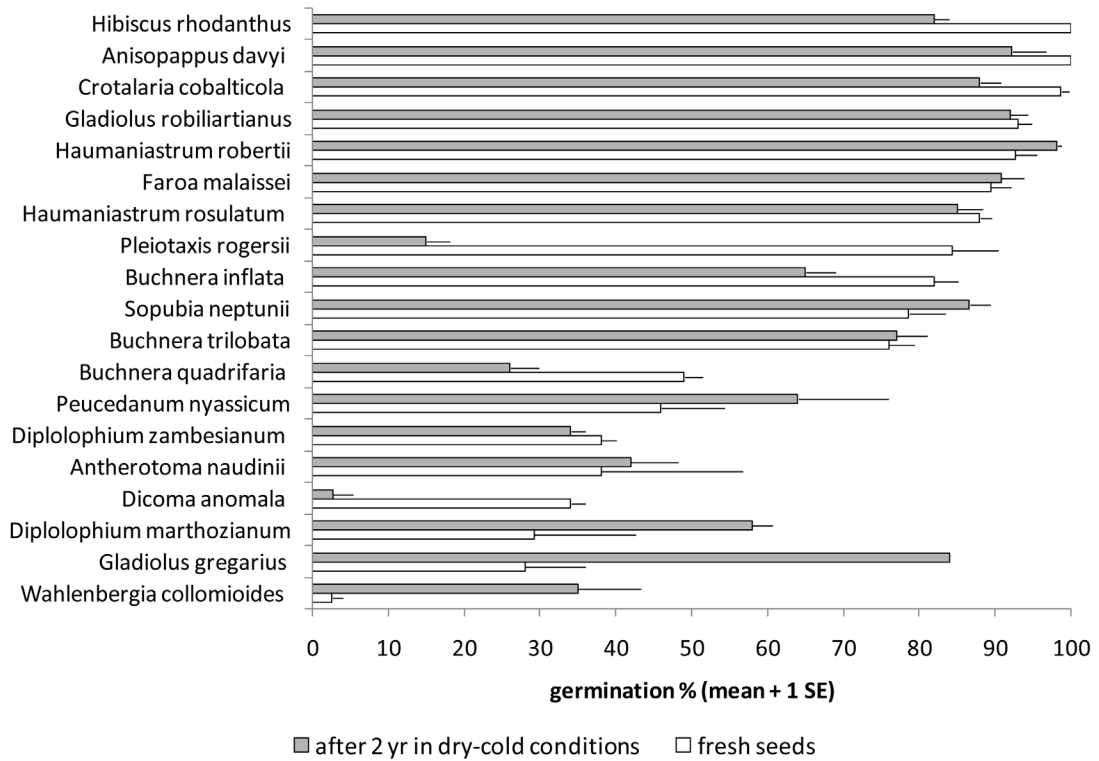
Figure 2 – Germination capacity of the tested species (fresh seeds vs. seeds stored for 2 years at 15%RH, -20°C). Species are ranked by increasing order of germination percentage of fresh seeds.
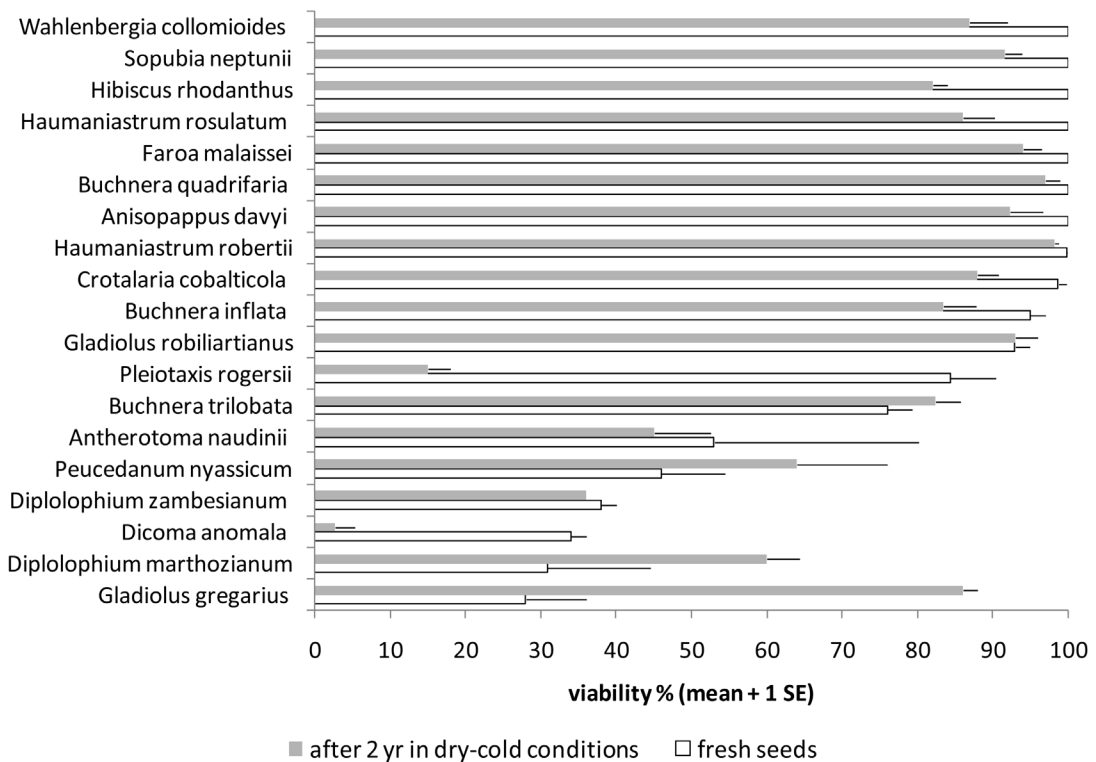
Figure 3 – Viability of the tested species (fresh seeds vs. seeds stored for 2 years at 15%RH, -20°C). Seed viability was calculated as the number of germinated seeds plus the number judged viable from the cut test expressed in percentage of the total. Species are ranked by increasing order of viability of fresh seeds.
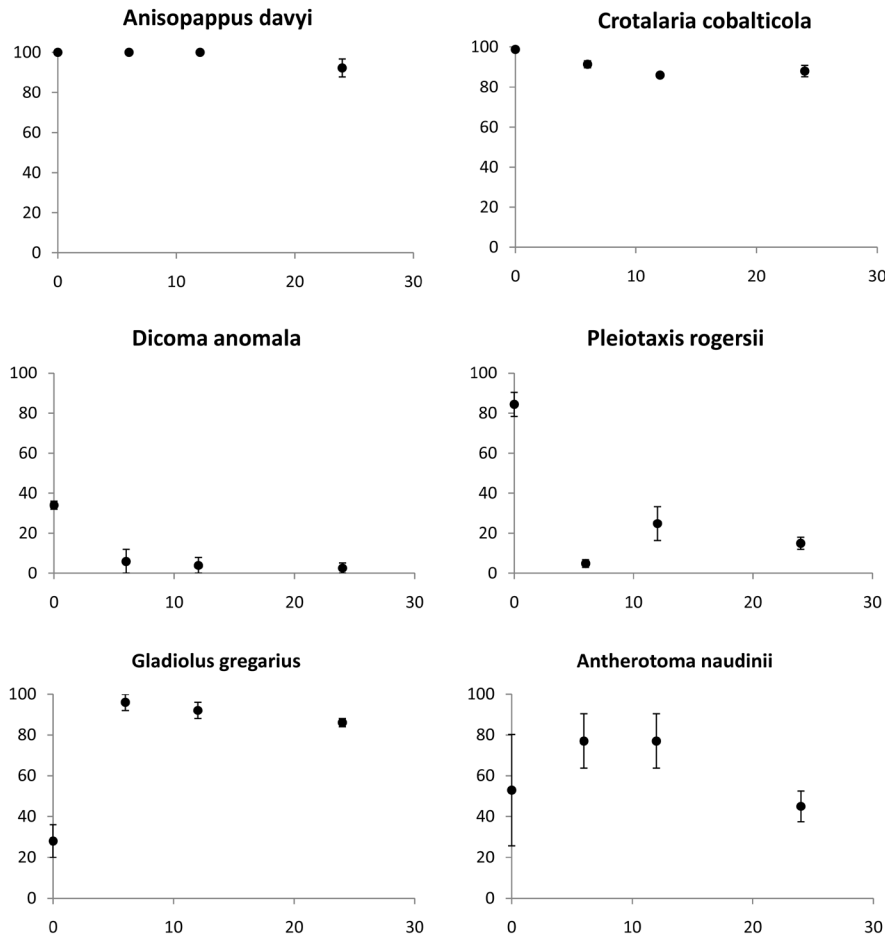
Figure 4 – Temporal evolution of seed viability (after storage at 15%RH, -20°C) of some species showing contrasting behaviours. X-axis: number of months in dry-cold conditions; Y-axis: seed viability (%).

DISCUSSION

Seed viability of cuprophytes from Katanga

According to our results, the germination percentage of the nineteen tested species reached on average 71 percent for fresh seeds, and 65, 68 and 69 percent after 6, 12 and 24 months dry-cold storage, respectively. This is higher than most figures mentioned for seed collections from other floras in the world, e.g. 58 percent for 276 species within the USDA National Plant Germplams System (Walters et al. 2005), 59 percent for 250 species from the Belgian flora stored at the National Botanic Garden of Belgium (Godefroid et al. 2010), 62 percent for 229 species stored in the New South Wales Seedbank (Offord et al. 2004), 71 percent for 15 species from the Irish Threatened Plant Genebank (Walsh et al. 2003). This shows that for these taxa from the Katanga copper belt, whose germination behaviour was still unexplored, an adequate protocol has generally been found.

Seed maturity is one of the factors that can influence seed longevity (Hay et al. 1997). Seeds collected too early may have poor storage potential as a result (Hay & Smith 2003, Crawford et al. 2007). Knowledge of the right state and time of maturity is therefore essential for collection of healthy and

vigorous seeds (Phartyal et al. 2002). Since we are dealing with poorly known tropical species, time of maturity may not be optimal. As a result, one possible explanation for the low germination of some species is that their collections were made of immature seeds. Nevertheless, we have minimized this risk since four major collecting trips were organized each year and a team is permanently on site. As immature seeds quickly become covered with fungi (Baskin & Baskin 1998), we can however not exclude this hypothesis if we consider the number of mouldy seeds. During germination, 26 percent of all tested accessions showed a high degree of mouldiness (> 30%), especially *Antherotoma naudinii*, *Dicoma anomala*, *Diplolophium marthozianum*, *D. zambesianum*, *Gladiolus gregarius* and *Peucedanum nyassicum*. Discounting mouldy seeds when calculating the germination percentage would give a figure of 85 percent on average.

Dormancy may also account for the low germinability of some tested species. In Neotropical savannas, it has been shown that many seeds dispersed in the dry season are dormant (Salazar et al. 2011). In our study area, most of the studied species disperse their seeds in the dry season. However, only *Buchnera quadrifaria* and *Wahlenbergia collomioides* had a dormancy index higher than 0.4. Since *Buchnera quadrifaria* is an Orobanchaceae, its parasitic character may
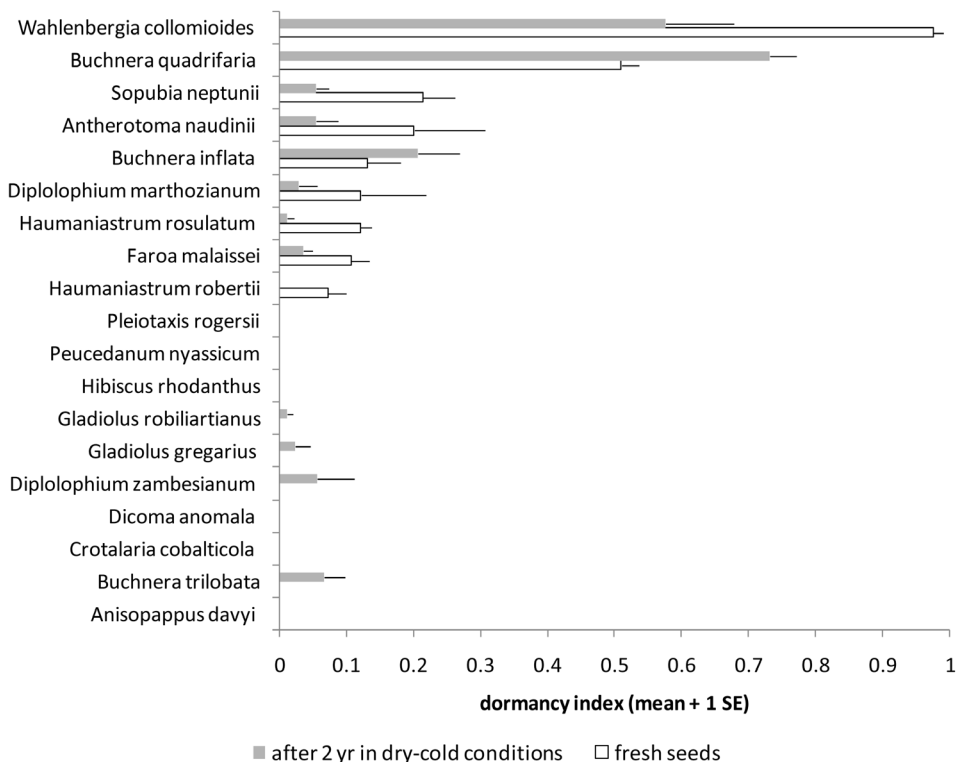
Figure 5 – Dormancy index of the tested species (fresh seeds vs. seeds stored for 2 years at 15%RH, -20°C). Dormancy index = 1-(seed germinated % / viability %). Species are ranked by increasing order of dormancy of fresh seeds.
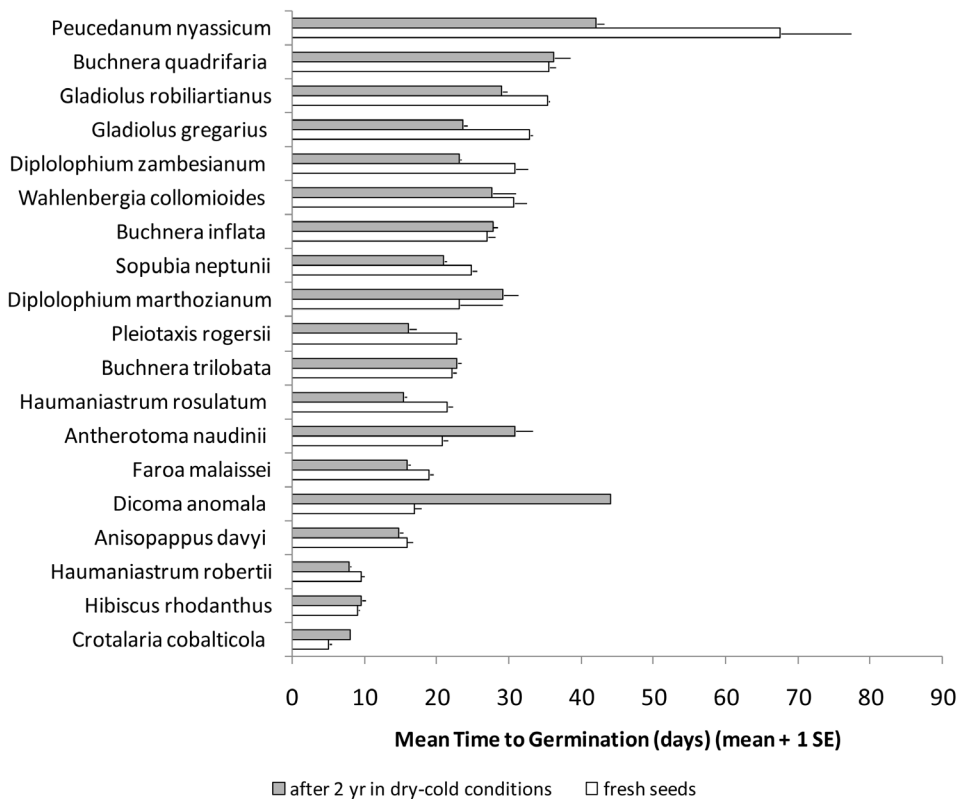
Figure 6 – Mean time to germination (days) of the tested species (fresh seeds vs. seeds stored for 2 years at 15%RH, -20°C). Species are ranked by increasing order of mean time to germination of fresh seeds.

also cause a poor germination in the absence of the host plant (Scott 2008).

Storage behaviour of cuprophytes from Katanga

Desiccation-tolerant (orthodox) seeds maintain a high vigour and viability for many decades when dehydrated to around 5% moisture content and stored at -18°C (FAO/IPGRI 1994). On the contrary, desiccation-sensitive seeds show germination-associated changes in storage and this property underlies the phenomenon of recalcitrance (Berjak & Pammenter 2002). Recalcitrant seeds are normally killed if the moisture content is reduced below some relatively high critical value of 12–31% (Roberts 1973). Using our data we therefore expect to be able to identify the storage behavior of the studied species. Since recalcitrant seeds are most common in tropical environments (Tweddle et al. 2003) we cannot exclude a recalcitrant character as a possible cause of the low germinability of some species. Comparing the results obtained for germination, viability and vigour (mean time to germination) between fresh seeds and after 2 years storage in dry-cold conditions makes us believe that following species are orthodox: *Haumaniastrum robertii*, *Gladiolus robiliartianus*, *Faroa malaissei*, *Peucedanum nyassicum*, *Diplolophium marthozianum* and *Gladiolus gregarius*. These species had both a faster and a more abundant germination after dry-cold storage, suggesting some tolerance to desiccation. On the contrary, for *Dicoma anomala* and *Pleiotaxis rogersii*, the steady decrease in their germination and viability after storage leaves no doubt as to their recalcitrance. The remaining species may have either an orthodox or intermediate behaviour and in any case require further investigation to ascertain their ability to be stored in seed bank.

Implications for biodiversity conservation

Seed banks are a good way of conserving biodiversity, providing that seeds are of high quality and at maximum viability. For the nineteen species tested, seed viability after two years storage was 77 percent on average, while at least 30 percent of them were identified as desiccation-tolerant (orthodox), which is an encouraging result. Among species identified as orthodox, two are strict endemics [*H. robertii* (VU) and *F. malaissei* (CR)] and two are broad endemics [*D. marthozianum* (EN) and *Gladiolus robiliartianus* (EN)]. This means that *ex situ* seed banking of these species can be included in a more comprehensive conservation strategy. These results also open new perspectives on revegetation and restoration programmes. Storage behaviour of other cuprophytes remains however unknown, and further studies are necessary in order to define the optimal conditions for the *ex situ* conservation of these species. Although recalcitrance is more typical in woody species, we have identified two species being desiccation-sensitive. This kind of behaviour limits storage to a few months at ambient temperatures and moist conditions. Because of the difficulty of storing recalcitrant seeds, their use in revegetation of mines and metal-contaminated sites and *ex situ* conservation programs is problematic (Daws et al. 2005). The need to develop appropriate methods to support the conservation of species with limited desiccation tolerance has been highlighted in Target 8 of the Global Strategy for Plant Conservation (GSPC), stating that the number of threatened species maintained in seed banks could be increased "with additional resources, technology development and transfer, especially for species with recalcitrant seeds" (http://www.cbd.int/gspc/). By studying seed quality of relatively unknown tropical species, this paper contributes to achieve target 8 of the GSPC.

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