STUDIES ON PLANTS THAT HYPERACCUMULATE COPPER, COBALT AND NICKEL: THEIR POTENTIAL FOR USE IN PHYTOMINING AND PHYTOREMEDIATION.

A thesis presented in partial fulfillment of the requirements for the degree of Masterate of Science in Soil Science at Massey University.

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ABSTRACT

This thesis reviews three lines of research on hyperaccumulators to examine their potential for phytoremediation and phytomining. The first line of research was to test the affect of nutrient addition on biomass and nickel uptake by two nickel hyperaccumulators, *Alyssum bertolonii* and *Streptanthus polygaloides*. Addition of fertiliser increased the biomass although the maximum amount added was found to be suboptimal. Nutrient addition did not affect the rate of nickel uptake. Larger plants contained a more dilute nickel content but still had an overall larger amount than smaller plants.

The second line of research was to test the affect of chelates on metal uptake by copper and cobalt flora of the Democratic Republic of Congo (formally Zaïre) and a copper tolerant plant from Spain, *Erica andevalensis*. EDTA and Citric Acid increased uptake of copper in these plants but had no effect on the uptake of cobalt and nickel. EDTA increased the uptake of lead by *Alyssum bertolonii* but did not affect the uptake of zinc and cadmium.

The third line of research was to examine the reality of hyperaccumulators of copper and cobalt. Copper and cobalt hyperaccumulation does in fact exist but not to the extent reported previously. There is a good possibility that the previously reported values for copper and cobalt hyperaccumulation are in some cases erroneous due to high iron levels indicating contamination of plant samples by soil.

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INTRODUCTION

HYPERACCUMULATORS

Natural mineral deposits containing particularly large quantities of heavy metals are present in many regions of the globe. These areas often support very characteristic plant assemblages and species that thrive in these metal-enriched environments. Examples of such distinct plant communities include the serpentine flora (growing on Ni, Cr, Mn, Mg and Co rich soils), the seleniferous flora (growing on Se rich soil), the uraniferous flora (growing on U rich soils), the calamine flora (growing on Zn and Cd rich soils), and Copper/Cobalt floras. As a result of their association with specific ore deposits, many metallophyte plants are used as so-called indicator species in prospecting for mineral deposits. For example, the copper flower (*Haumaniastrum katangense*) is a classical indicator of Cu and Co ores in central Africa.

There are three basic categories of plant that grow on metalliferous soils. Metal excluders effectively prevent metal from entering their aerial parts when growing in a broad range of metal concentrations in the soil. Metal non-excluders actively accumulate metals in their above-ground tissues and can be roughly divided into two groups: indicators and hyperaccumulators. Metal levels in the tissues of indicator species generally reflect metal levels in the soil. Hyperaccumulators can concentrate metals in their above-ground tissues to levels far exceeding those present in the soil.

Baumann (1885) recorded the first hyperaccumulator of any metal when he reported over 1% zinc in the dried leaves of *Thlaspi calaminare* and *Viola calaminaria* growing over zinc deposits near Aachen, Germany. Minguzzi and Vergnano (1948) were the first to discover high nickel levels in plants. They reported over 1% nickel in leaves of *Alyssum bertolonii* growing over ultramafic rocks in Tuscany.

By far the greatest volume of work and general interest in hyperaccumulators has been centered on the genus *Alyssum*. This followed the original discovery of over 1% Ni in *Alyssum bertolonii* by Minguzzi and Vergnano in 1948. Doksopulo (1961) reported the discovery of a second hyperaccumulator of nickel, *Alyssum murale*, in 1961. In the early

1970's, Severne and Brooks (1972) discovered a third nickel plant, *Hybanthus floribundus*, in Western Australia. Following this early work, over 200 hyperaccumulators of Ni have now been reported, largely as the result of chemical tests on herbarium specimens.

The term *hyperaccumulator* was first used by Brooks *et al.* (1977a) and was originally used to define plants containing more than 1000 μg/g nickel in dry tissue. This threshold was based on histograms showing discrete plots of two different populations of the same plant genus with no overlap of hyperaccumulators and non-accumulators at the 1000 μg/g Ni boundary (see Figure 1). By 1983, Brooks using mainly herbarium specimens had identified 44 species of the *Alyssum* genus as hyperaccumulators of Ni.

The threshold of hyperaccumulation will vary considerably for different trace elements. Reeves *et al* (1995) have suggested the following concentrations (in the plant dry matter) as thresholds to define accumulation: 10000 μg/g for Zn and Mn; 1000 μg/g for Ni, Co, Cu, Cr and Pb; and 100 μg/g for Cd and Se. These values represent a concentration about 100 times greater than the normal elemental concentration ranges found in plants.

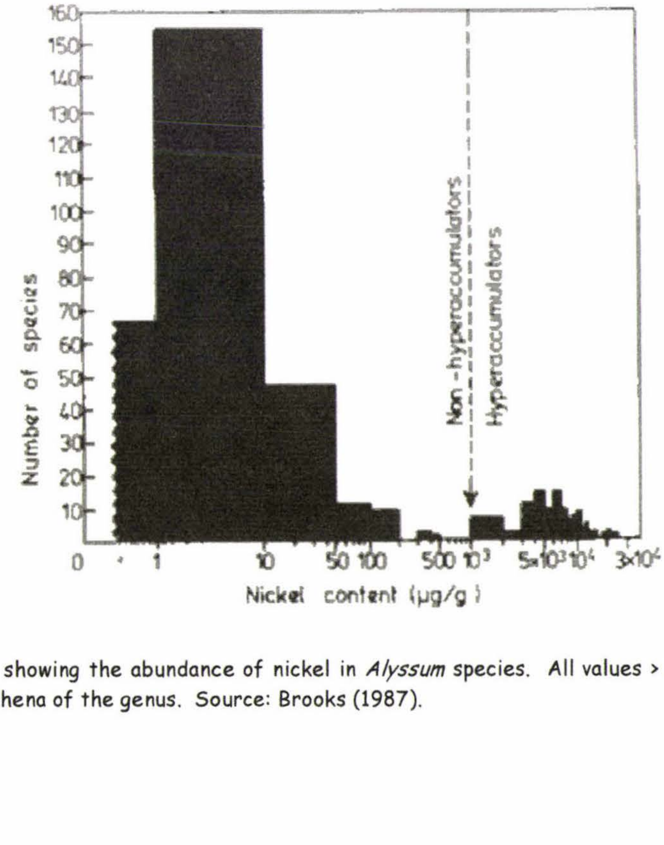


Figure 1: Histograms showing the abundance of nickel in *Alyssum* species. All values > 1000 μg/g (0.1%) are from section Odontarrhena of the genus. Source: Brooks (1987).

Almost all metal-hyperaccumulating species known today were discovered on metalliferous soils, either natural or man-made, and almost all hyperaccumulators are endemic to these soils. This suggests that hyperaccumulation is an important ecophysiological adaptation to heavy-metal stress and one of the manifestations of heavy-metal resistance.

For a long time the ability of plants to accumulate metals was considered a detrimental trait. Being at the bottom of many natural food chains, metal-accumulating plants are directly or indirectly responsible for a large proportion of the dietary uptake of toxic heavy metals by humans and other animals. Although some heavy metals are required for life, their excessive accumulation in living organisms is always toxic.

There are three fields in which hyperaccumulators have made significant impacts: mineral prospecting, phytoarchaeology, and 'green remediation' of toxic soils.

Use of Hyperaccumulators in Mineral Prospecting

Hyperaccumulators can be used for geobotanical exploration whereby the type of vegetation can be used to determine and map areas of mineralisation (Nicks *et al*, 1995). Biological methods of prospecting involve mapping the distribution of plant species confined to certain types of mineralisation. Although there are over 400 hyperaccumulators of nickel, there are only about 20 species that can tolerate and accumulate copper and cobalt. Until recently, these plants were believed to be confined solely to the Shaban Copper Arc in Shaba Province, Democratic Republic of Congo (Zaïre) which has the world's richest and most diverse copper flora. However, there have been reports that the Chinese plant, *Elsholtzia haichowensis*, a member of the Lamiaceae (mint family) and confined to copper mines in the Daye region of Hubei province, Central China, is also a hyperaccumulator of copper.

Two species of *Haumaniastrum*, *H. katangense* and *H. robertii* were once known as copper flowers and were used by early prospectors to delineate mineralised ground since they will not grow in soils containing less than 100 μg/g copper and/or cobalt. Brooks *et al.* (1980) have now identified 26 hyperaccumulators of cobalt and 24 of copper. A further 9 taxa were found to be able to hyperaccumulate both metals. All of these 38 plant species can be used, or have been used, to prospect for copper and/or cobalt in Democratic Republic of Congo (Zaïre).

Many hyperaccumulators of nickel have been, or could be used for mineral prospecting. For example, all of the 48 *Alyssum* species with this characteristic indicate the presence of ultramafic substrates and by inference the presence of Ni, Cr and Co that are associated with this rock type. Most hyperaccumulators of nickel are endemic to nickel-rich ultramafic substrates and as such can serve as indicators of this rock type.

The association of the hyperaccumulators *Viola calaminaria* and *Thlaspi calaminare* with calamine zinc deposits in Western Europe has been known for over 100 years. As in the case of nickel, virtually all of the "zinc plants" are geobotanical indicators of zinc and/or other sulphide deposits.

There is only one clearly documented case of a lead hyperaccumulator. *Thlaspi rotundifolium subsp. cepaeifolium* from Raibl on the Austrian/Italian border has been found to contain 7000 μg/g of this element in dried tissue (Reeves *et al.*, 1983). This species has not yet been used for mineral exploration but does have this potential.

Cannon (1959, 1960, 1964) has reported the use of species of *Astragalus* for geobotanical prospecting for uranium in the western United States. Some *Astragalus* species such as *A. pattersoni* and *A. preussi* are confined to selenium-rich soils and indirectly indicate the presence of uranium in the substrate (selenium and uranium are associated geochemically in the mineral carnotite).

Phytoarchaeology and Hyperaccumulators

The hyperaccumulator *Alyssum corsicum* was first discovered growing over a few hectares of ultramafic rock in the suburbs of Bastia in Corsica. Later however it was found to have its origin in Anatolia (Turkey) on the eastern shores of the Mediterranean. It was concluded that the seed had been brought to Bastia by the Venetians as weed seed in shipments of corn and had colonised this small part of Bastia since it would only grow over ultramafic soils. This small colony of hyperaccumulating *Alyssum* traces an ancient trade route.

The Zaïrean copper flower *Haumaniastrum katangense* can also be used to indicate the presence of archaeological remains beneath the soil. Artisans of the 14th Century Kabambian culture of what is now Shaba Province, Democratic Republic of Congo (Zaïre) used to smelt copper to produce copper crosses that were used for currency. The smelters were fashioned from abandoned termite mounds and copper ores were brought to the site from elsewhere. After several years, the termite hills weathered to ground level and the residual copper ore formed a toxic ring around the original smelters. This toxic soil became colonised by *H. katangense* and the sites were later recognisable as bare open areas in the savanna. Archaeologists digging below the carpet of this hyperaccumulator of copper have been able to uncover numerous artifacts from the Kabambian and later cultures (Figure 2).

Figure 2: Abandoned native copper smelter fashioned from a termite hill at Luisha colonised by *Haumaniastrum katangense* (not in flower) and the fern *Nephrolepis undulata*. Source: Brooks *et al.* (1992).

Hyperaccumulators and Green Remediation of Soils

Only recently has the value of metal-accumulating plants for environmental remediation been fully realised. At present, remediation technologies consist primarily of removal and replacement, or simply isolation of contaminated sites. Water treatment facilities also do a relatively poor job of removing toxic metals from residential and industrial effluents. Hyperaccumulators can also be used to colonise polluted areas, such as old mine dumps and further to remove pollutants from the soil (Baker *et al*, 1995). This is termed phytoremediation (removal of pollutants from the soil by use of plants) and is based on the principle that a crop of hyperaccumulating plants may be grown on the heavy metal enriched soil and harvesting of these plants would lower the heavy metal concentrations. Phytomining is a process where these plants may be used to extract heavy metals from low grade ores that would otherwise not be economic (Chaney, 1983; Robinson *et al.*, 1997). Burning the crop produces a 'bio-ore' which can then be sold to small smelting companies as a sulphur-free, non-polluting raw material (Nicks and Chambers, 1994; 1995).

The application of plants in environmental cleanup is an emerging technology that has been called phytoremediation (I Raskin, Grant Proposal #R81869, 1991). Three subsets of this technology are being developed. First, phytoextraction (Salt *et al.*, 1995), in which metal-accumulating plants are used to transport and concentrate metals from the soil into the harvestable parts of roots and aboveground shoots. Second, rhizofiltration (Dushenkov *et al.*, 1995), which uses plant roots to absorb, concentrate and precipitate toxic metal from polluted effluents. Finally, phytostabilisation, which involves the use of plants to eliminate the bioavailability of toxic metals in soils.

REVIEW OF THE LITERATURE

PHYTOREMEDIATION

The extraction of heavy metals from their ores is a process involving a large amount of energy. Hyperaccumulators are able to achieve this by a low energy process (via sunlight) and if this could be emulated technologically, the benefits are obvious (Morrison *et al*, 1979). The use of such hyperaccumulator plants to extract metals from surface contaminated land could represent a low technology, natural means of *in situ* soil remediation (phytoremediation).

An alternative soil remediation technology to removal and replacement of contaminated soils has been proposed that would use heavy-metal-tolerant plant species that are able to hyperaccumulate metals in plant shoots.

Phytoextraction requires the translocation of heavy metals to the easily harvestable shoots. Several sequential crops of hyperaccumulators may be used to reduce soil concentrations of heavy metals to environmentally acceptable levels. Dried, ashed, or composted plant residues highly enriched in heavy metals may be isolated as hazardous waste or recycled as metal ore (Kumar, 1995).

Brown (1995) compared two metallophytes, *Thlaspi caerulescens*, a Zn and Cd hyperaccumulator from Prayon, Belgium, and a Zn-tolerant ecotype of bladder campion (*Silene vulgaris*) from Palmerton, PA, with tomato (*Lycopersicon lycopersicum*) in nutrient solution to characterise Zn and Cd uptake and tolerance.

Thlaspi caerulescens showed much greater tolerance to Zn/Cd treatments than the other species, with toxicity stress only apparent at the 10000 μg Zn/200 μg Cd treatment. Shoot concentrations of Zn and Cd were 33600 and 1140 μg/g, respectively. Zinc concentrations in shoots of *Thlaspi caerulescens* were higher than the other species in all Zn/Cd treatments. Extreme Zn and Cd uptake and tolerance is evident in *Thlaspi caerulescens*, with >25000 μg Zn/g and 1000 μg Cd/g before yield is reduced.

These tolerance mechanisms could be exploited to remove heavy metal pollutants from soil (Chaney, 1983).

For phytoremediation to prove effective, it is necessary to delineate patterns of uptake and limits of tolerance of potential phytoremediation plant species. It is also important to define accumulation patterns at lower concentrations of soil metals to determine if phytoremediation efficiency will decline as soils approach natural metal concentrations.

Except for low biomass, the metal-accumulating properties of *Thlaspi caerulescens* are those necessary for plants that could be used for phytoremediation of metal-contaminated soils. Concentrations of Zn in harvestable shoots are high enough that plant tissue could be treated as a low-grade ore (Chaney, 1983). Smelting dried *T. caerulescens* shoots could recycle Zn and Cd. Low yield and slow growth rate are the two major factors that limit the potential for the phytoremediation of Zn/Cd-contaminated soils by successive croppings of *T. caerulescens*.

Successive cropping with metal accumulating plants may prove an effective and practical means of removing metals from superficially contaminated soils. McGrath *et al* (1993) found that thirteen croppings with *Thlaspi caerulescens* would be required to extract the excess loading of Zn (assuming subsequent crops removed metals at the same rates as the first crop) compared with over 800 croppings with *Brassica napus* and more than 2000 croppings with *Raphanus sativus*, both non-accumulating crop plants.

For Cd, McGrath *et al* (1993) found that *Thlaspi caerulescens* could remove 95% of the European Directive Mandatory Limit for the annual application of Cd to agricultural land in a single cropping which was more than 10 times the rate of extraction by a non-accumulating plant. A single cropping with *Alyssum tenium* could remove 45% of the permitted addition of Ni to agricultural land (with ~2 croppings needed to remove the total amount).

Most of the metal accumulating plants identified so far are slow growing, small and/or weedy plants that produce low biomass and have unidentified growth requirements and characteristics. Indian mustard (*Brassica juncea*), in contrast, is a high biomass crop plant that efficiently accumulates Pb and other heavy metals. In addition to being a Pb accumulator, *B. juncea* produces 18 t/ha of biomass and can be easily adapted to cultivation in various climatic conditions using existing agricultural practices.

Kumar (1995) found that the rate of Pb uptake into the roots of *Brassica juncea* slowed as a function of the exposure time while the rate of translocation to the shoots showed an increase with time. Formation of insoluble inorganic complexes in soil and inside the plant can significantly reduce the phytoextraction efficiency of *B. juncea*.

EDTA

Soil amendments such as acidifiers and chelators will make such tightly bound and relatively immobile heavy metals such as Cu, Pb and Cr^{3+} more available for plant uptake and translocation. The most common chelating agent, used to enhance the water solubility of iron in hydroponic systems, is EDTA (ethylenediaminetetraacetic acid). The formation of $[Fe(EDTA)]^-$ is very pH dependent and other trace metals, such as Cu and Zn can compete with Fe for the EDTA. Laurie *et al* (1991) found that EDTA at a concentration of 4.0×10^{-5}(treatment 5) caused chlorosis in all the barley (*Hordeum vulgare)* plants. This was attributed to a deficiency in zinc. The plants grown in lower concentrations (2.0×10^{-5}(treatment 1), 2.1×10^{-5}(treatment 2), 2.2×10^{-5}(treatment 3) and 2.4×10^{-5}(treatment 4)) all had green leaves and appeared healthy. Mn, Cu and Zn shows increasing concentration with increasing EDTA levels for treatments 1-4 but significant decreases in the chlorotic plants of treatment 5. Increased complexation led to increases in concentrations in the plants.

Huang and Cunningham (1996) found that addition of HEDTA (2-hydroxyethylethylenediaminetetraacetic acid) to a Pb-contaminated soil resulted in a surge of Pb accumulation in corn. The shoot Pb concentration was increased from 40 μg/g for the control to 10600 μg/g (1.06%) for the HEDTA treated soil.

FERTILISATION OF HYPERACCUMULATORS

Bennett *et al* (1998) found that *Alyssum bertolonii* and *Thlaspi caerulescens* showed a slight reduction in concentration of nickel and zinc respectively when the biomass of each plant was increased by the addition of nitrogen fertilisers. The trade-off of biomass against metal content was however slight. Some of this work is part of the present thesis.

Nicks and Chambers (1994) found there was a 5-fold increase of biomass of *Streptanthus polygaloides* when N, P, K fertiliser was added.

Robinson *et al* (1996) found that adding fertiliser to *Alyssum bertolonii* plants can result in a dramatic increase of biomass without corresponding loss of nickel concentration in the tissue. Their results showed that the best fertiliser treatment (N+P+K) gave a 3-fold increase of the biomass of the reproductive matter without dilution of the unfertilised nickel content.

Chaney proposed the concept of using plant hyperaccumulators for phytoremediation in 1983 and the first thorough scientific research under field conditions was done by McGrath *et al.* (1993) who showed that hyperaccumulator plants might be used for removal of pollutants from soil.

The idea of using these plants to grow a crop of a given heavy metal (phytomining) appeared in 1995 (Nicks and Chambers, 1995, 1998). The potential for using hyperaccumulators as a means of "growing a commercial crop of nickel" is due to the pioneering work of L. Nicks and M.F. Chambers at the US Bureau of Mines, Reno, Nevada (Nicks and Chambers, 1994, 1995, 1998). Their work was based on the principle that it might be possible to use plants to extract Ni from low-grade ores that would otherwise not be economic.

THE GEOLOGICAL AND CLIMATOLOGICAL ENVIRONMENT OF THE HYPERACCUMULATORS STUDIED

THE SHABAN COPPER ARC

The dominant physiographic feature of the Democratic Republic of Congo (formally Zaïre) is the basin of the Congo River, also known as the Zaïre River. This region, constituting the entire central area, is a vast depression that slopes upwards on all sides into plateaus and mountain ranges. In the southeast, the basin is fringed by rugged mountain country called the Katanga, or Shaba, Plateau. This region, about 1220m above sea level contains rich copper and cobalt fields, uranium, and other mineral deposits. Such mineralised areas are highly toxic to 'normal' plants but tolerant populations have evolved on them. Several of these such as *Haumaniastrum* and *Aeollanthus* have been studied in this present thesis.

The copper-cobalt deposits of southcentral Africa in the Democratic Republic of Congo (Zaïre) and Zambia are found in an area of about 35000 km^2, of which the greater part is in the Shaba Province in the southeast of the Democratic Republic of Congo (Zaïre). These deposits comprise about 100 separate ore occurrences with a significant surface expression. The Shaban Copper Arc in the Democratic Republic of Congo (Zaïre) extends from Kolwezi in the west to Lubumbashi in the southeast of the Shaba province and has a length of about 300 km with a width of about 50 km (see figure 3).

The Shaban Copper Arc is situated along an 800 km structural trend (the Lufilian fold belt) of folded Katangan sediments extending from Namibia in the west through Zambia and Angola and into the southern part (Shaba) of the Democratic Republic of Congo (Zaïre) and then back into Zambia at Konkola. This structural setting is shown in figure 4.

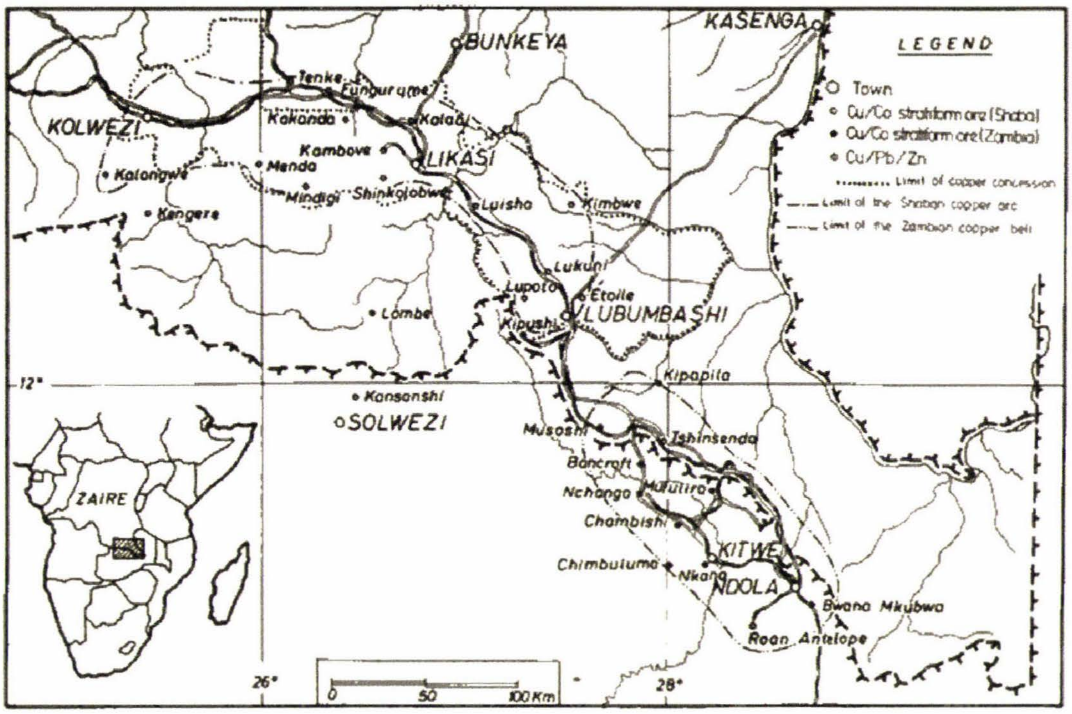


Figure 3: The Shaban Copper Arc and Zambian Copperbelt. Source: François (1973).

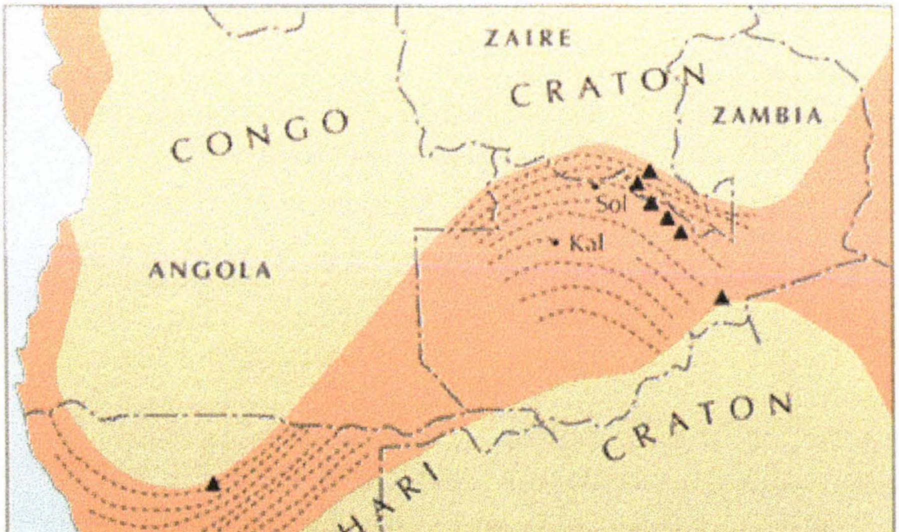


Figure 4: Structural setting of the Shaban Copper Arc. After Bowen and Gunatilaka (1976).

The younger parts of the Lufilian orogenic belt, the Damaran-Katangan system, are about 550 million years old. The copper ores of the Katangan system consist of mainly sulphides (pyrite, chalcopyrite, bornite etc) with important secondary oxide ores overlying schists and granite of the Basement Complex. The formations hosting the ores are mainly argillites and micaceous dolomites which are only locally mineralised to ore grade. They are mostly 5-15 m thick with an ore content of around three to four percent. (Huywel-Davies, 1972)

The Katangan sequence comprises marine sediments formed in a series of marine transgressions. The initial transgression left a layer of sandstones and conglomerates on the flanks of the paleoridges. Above this, the Lower Roan clastic sediments form a layer of 800-2000 m thick covering the paleoridges. The Lower Roan siliclastic deposits gradually change to dolomite-rich carbonate deposits of the Upper Roan period. These, in turn, grade into carbonaceous shales of the Mwashia group. The Mwashia group is topped by an erosional contact with the Kundelungu group.

The Kundelungu group consists of tillite and marine sedimentary horizons laid in Upper Pre-Cambrian times. At least two major tillite horizons exist as a testimony to fluctuations in the paleoclimate of Central Africa at that time. Following the Kundelungu sedimentation, the Kundelunguan orogeny rapidly raised the area. This uplift was stronger in the north and in Shaba it formed a large arc with a succession of anticlines.

François (1973) divides the Precambrian Katanga system into three series: the Roan (R), the Lower Kundelungu (K_i) and the Upper Kundelungu (K_s). Each series is then subdivided into groups such as R1-R4 in the case of the Roan. Most of the important copper-cobalt mineralisation is in the R2 group of the Shaban Katanga system. The R2 is easily recognisable as a line of similar facies along the whole of the Shaban Copper Arc, even though it undergoes changes of thickness and facies. The minerals are believed to have been deposited as sulphides from a reducing, shallow marine environment. This would, however, give only a low grade ore. Further enrichment by diagenesis appears to have occurred but the exact nature of the process remains unknown.

In the Democratic Republic of Congo (Zaïre) some 100 copper-cobalt occurrences with a total expression of about 20 km^2 are scattered in a 20000 km^2 area of the Shaban Copper Arc from Kolwezi in the west to Lubumbashi in the southeast. The Shaban ore bodies tend to occur in mineralised hills, particularly in the west of the Shaban Copper Arc.

The climate of the Democratic Republic of Congo (Zaïre) is extremely hot and humid except in the upland regions where the climate is tempered by the high elevation (around 1220 m in the Shaba Province). The mean annual temperature is around 18.9°C in high altitude areas. The average rainfall is about 1200 mm. The year is divided into wet and dry seasons. Frequent heavy rains occur from early November to the end of March. Dry, warm conditions prevail from September to November with maximum temperatures in the upper 20s and lower 30s (°C) see Table 1.

Frosts are absent or rare in North Shaba but may occur, infrequently, in the south around and below Lubumbashi. The dryness of the dry season makes fire a major threat to plant life.

Table 1: Climatological data for stations in the Democratic Republic of Congo (Zaïre), Zambia and Zimbabwe. Sources: Leblanc and Malaisse (1978), Brelsford (1960).

		January			July			October			
	Altitude	Temperature °C		Rainfall	Temperature °C		Rainfall	Temperature °C		Rainfall	Total Rainfall
Location	m	max	min	mm	max	min	mm	max	min	mm	mm
Lubumbashi	1230	27	16	250	26	6	0	32	14	25	1231
Ndola	1272	26	17	312	24	8	0	31	17	20	1190
Sinoia	1156	28	18	208	24	13	1	32	15	23	818

The metalliferous soils of Shaba occur, generally, on low hills or crests. The tops of these structures have skeletal, gravelly soils with outcrops of the underlying rocks. The slopes are frequently composed of colluvial deposits of the crest soil. Where the slopes are of non-mineralised mother rock the heavy metal content of the colluvium is diluted. Illuvial deposits may, however, enrich such non-mineralised areas in metals, particularly during the rainy season. A contamination halo (or 'poisoned dambo'), formed by illuvial outwash, generally surrounds the crest or hill. The halo is surrounded by a corona until 'normal' heavy metal content is reached in the forest soils. The presence in the soil of large quantities of heavy metals severely restricts the growth of many plant species. In particular woody species are inhibited. The vegetation of these regions is thus herbaceous or, at most, weakly bushy.

The ultramafic outcrops of Southern Europe

The mafic and ultramafic complexes of the Apennines (Figure 5) where *Alyssum bertolonii* is found, consist of Mesozoic rocks (including Jurassic peridotite-serpentinite associations (ophiolites)) and pre-Mesozoic rocks. The ophiolites comprise serpentinites, gabbros and basalts that are associated with radiolarian cherts, limestones and shales ranging from late Jurassic to early Cretaceous and seem to represent sections of oceanic crust.

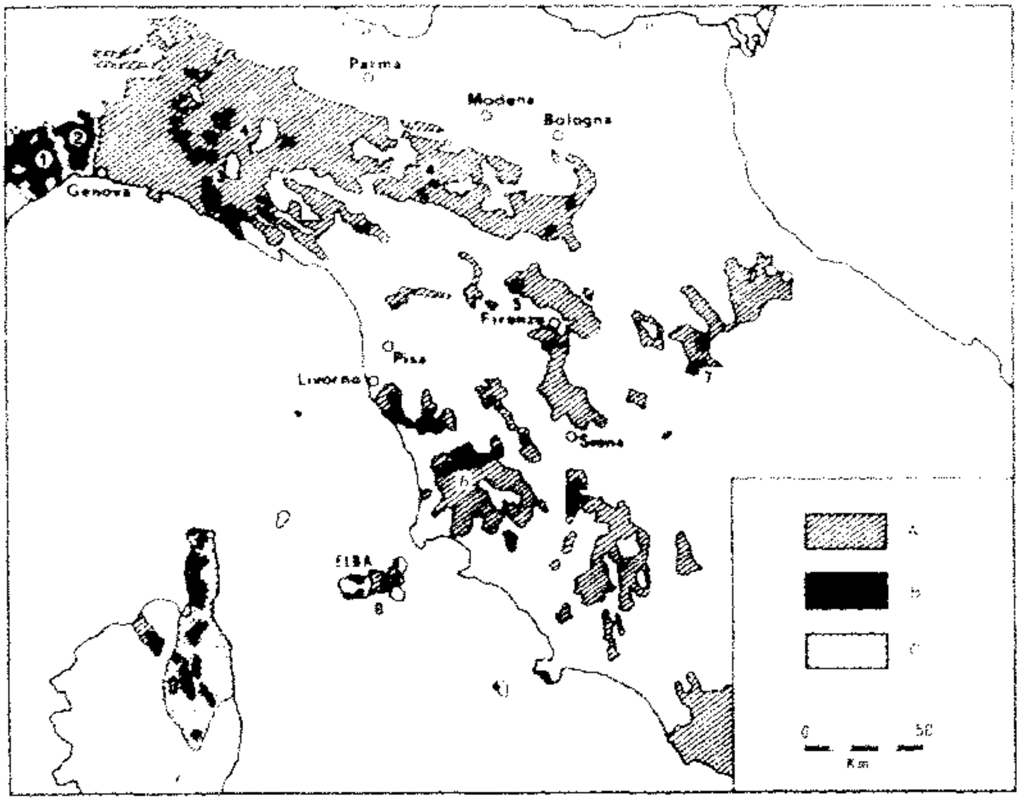


Figure 5: Distribution of ophiolites in the Apennines. A. Ligurids; B.Ophiolites; C. Schists Lustrès. Location of ophiolitic outcrops: 1. Voltri, 2. Sestri-Voltaggio, 3/4. Eastern Ligurian-Emilian Apennine, 5. Florence basin, 6. Southern Tuscany, 7. Tuscan Apennine, 8. Elba Island. (After Abbate, Bortolotti and Principi, 1980).

According to their geological occurrence, the ophiolites may be divided into three groups. To the west occur the high pressure, metamorphic ophiolites of the Gorgona and Giglio islands and of the Argentario promontory. In an intermediate belt, which is mainly developed in eastern Liguria between the coast and the Vara Valley, ophiolites and their sedimentary cover occur in a more or less coherent nappe structure.

In an eastern belt, ophiolites are dispersed in a large number of small masses and ophiolitic breccias in the external Ligurid nappes, an incompetent mass of clays, marls, marly limestones and sandstones which moved North-east as successive landslides, over-riding Tertiary deep sea formations.

Small masses of granite are occasionally associated with these ophiolites of the eastern Ligurids. The basin in which the oceanic crust was extruded was presumably of limited extent (about 100 km) and formed the southwest extension of the western Alpine basin. Ophiolites of both the internal and external Ligurids show low-grade metamorphism and their original structures are well preserved.

The ultramafic endemics are located on rocks, screes and pebbles. Because of the scarce tree cover, the dryness of the substrate and the dark colour of the rocks, the local climatic conditions are what is generally termed continental (temperature ranges are relatively low and rainfall is relatively high and uniformly distributed).

ULTRAMAFIC OUTCROPS OF THE SIERRA NEVADA RANGE, CALIFORNIA

The Sierra Nevada where *Streptanthus polygaloides* is found, is a prominent mountain range in eastern U.S. It is about 80 km wide and extends for some 400 km, in a generally north-south direction, and reaches elevations of 4400 m. The major rock type of the Sierra Nevada is granodiorite which is capped by volcanics and flanked on the western side by older ultramafic rocks. Exposures of these rocks, primarily serpentine and peridotite, in California and Oregon total over 400000 ha (see figure 6).

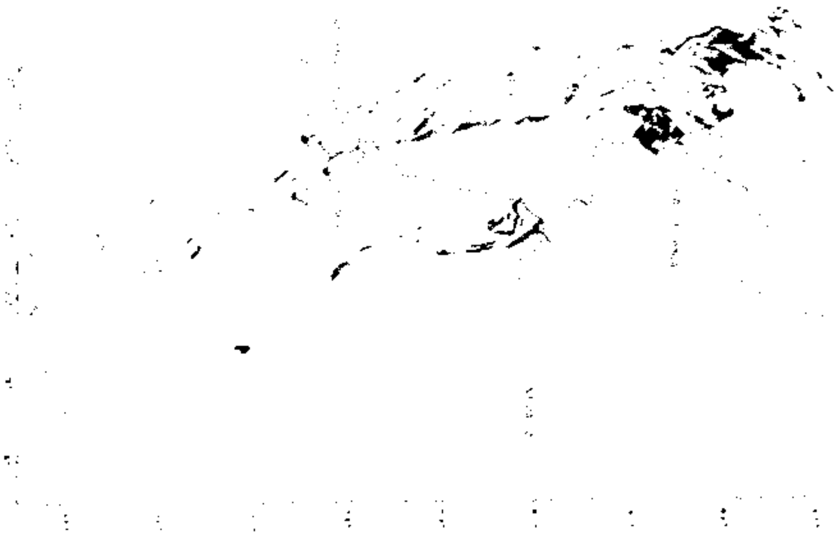


Figure 6: Ultramafic outcrops in California and Oregon. Source: Nicks and Chambers (1995).

According to plate tectonic models, ultramafic rock outcrops represent the accretion of the oceanic plate onto the continental North American plate as a result of their collision. Weathering of the ultramafics has produced what are generically termed serpentine soils which are characterised by a pH of 6-8, high ratios of Mg/Ca, low levels of N, P and K and potentially phytotoxic amounts of Ni and Cr.

Being derived from rocks foreign to the continental crust, serpentine soils are inhospitable to normal terrestrial plants. A few opportunistic plants have, however, evolved mechanisms for tolerating the extreme soil chemistry. Hyperaccumulation of nickel is apparently involved in one such mechanism.

THE PYRITIC OREBODIES OF HUELVA, SOUTHWEST SPAIN

Erica andevalensis, a new species of heather first described by Cabezudo and Rivera (1980), is found in the province of Huelva in the southwest of Spain. This heather grows on spoil heaps and outskirts of mines as well as along the banks of rivers where the acidic (H_2SO4) water is a dark red colour due to contamination by iron, copper and zinc (López Archilla *et al*, 1994). These wet habitats are claimed to be the natural habitat of *Erica andevalensis* (Nelson *et al*, 1985).

The surface rocks of the Huelva province in pre-Devonian times were quartzites which were folded and covered by pyroclastics laid down in shallow seas. The seawater contained iron, which reacted with the sulphur in the pyroclastics to form pyrite. The pyroclastic rocks were then also folded and thermal solutions from depth, carrying other metals such as gold, silver, copper, lead, manganese and zinc, reacted with the pyrite and more sulphides were precipitated. These rocks were overlain in turn by Tertiary deposits which were eroded in recent times to reveal the pyritic orebodies. The mine tips are composed predominantly of overburden from open-cast workings and pyroclastic rocks of non-commercial quality; both contain traces of many heavy metals, including copper, lead, manganese and zinc.

The climate of Spain is marked by extremes of temperature and generally insufficient rainfall, and the variegated physical features of the country ensure pronounced climatic differences. The climate of the Huelva province is Mediterranean with a mean annual temperature of 18.5°C and a mean annual rainfall which ranges from 600 to 800 mm.

4 NICKEL HYPERACCUMULATORS

INTRODUCTION

Serpentine protoliths are essentially ultramafic rocks. The most commonly recognised are ophiolite complexes and occur in most fold-mountain belts of the world. Serpentinised ultramafic rocks have many distinctive chemical and physical features which create an unusual environment for the plants which grow in the soils derived from them.

Serpentinised ultramafic rocks outcrop in many parts of the world and very often they are associated with an unusual flora with rare, endemic and different races of plants. Serpentines have a relatively high content of iron and magnesium and a relatively low content of silicon. Serpentines very commonly have other features of their chemical composition which may exert unusual influences on the soils and plants associated with them. Many have low calcium, potassium, phosphorus and molybdenum concentrations and also relatively high chromium, cobalt and nickel concentrations.

Nickel is toxic to most types of vegetation at relatively low concentrations and serpentine rocks are unfavourable substrates for plant growth for a variety of reasons (such as their characteristic enrichment in heavy metals and low levels of essential nutrients). Normal plant species tend to suffer severe phytotoxicity when their leaves contain 100 μg/g of nickel (dry weight) (Reeves *et al*, 1995). The high levels of nickel and chromium have a direct toxic effect on the plant and the very high magnesium content (~20%) can restrict calcium uptake by plants (Kruckeberg, 1954). These factors are responsible for the relative infertility of serpentine soils and dictate the nature of the vegetative cover.

The nickel content of plants does not usually exceed 1 μg/g (dry mass basis) except for certain species growing over serpentinites and other ultramafic rocks. These plants usually contain up to 100 μg/g. There are a small number of plants whose nickel content can, under certain circumstances, exceed by a factor of 10 even the highest values achieved by 'normal' plants growing over ultramafic, nickeliferous substrates. Plants with the ability accumulate high concentrations of toxic elements such as nickel have been known (Minguzzi and Vergnano, 1948) for many years.

Hyperaccumulation of nickel appears to be a strategy where genera such as *Alyssum* and *Streptanthus* have been able to evolve a physiological tolerance to phytotoxic nickel-rich soils and avoid competition from other species by flourishing in hostile environments.

PURPOSE AND GENERAL HYPOTHESES OF THIS RESEARCH

Hyperaccumulator species in their natural habitats tend to be small and insignificant plants. For such plants to be useful in any major way in phytoremediation a large biomass needs to be achieved; they would have to be grown quickly and in bulk. The efficiency of their hyperaccumulatory activity and its limiting factors need to be determined and, if possible, that efficiency increased.

In their natural environment hyperaccumulators enjoy a specialised niche denied to other plants. The primary advantage of their peculiar adaptation has to be this freedom from competition, but other advantages have been postulated. The high concentration of metals in their tissues could make them immune to attack by pathogens, particularly fungi, and could make them unpalatable and toxic to herbivores.

If hyperaccumulators are neither attacked by pathogens nor browsed by herbivores, then something else must account for their unthrifty appearance. They are characteristically herbaceous or at the most weakly bushy. The limiting factor on their growth could be the fact that they are growing in such an extreme environment or it could relate to the low fertility of the niche they occupy.

Serpentine soils are considerably different to 'normal' soils in that they are very rich in Cr, Co, Fe, Mg and Ni as well as being deficient in the nutrient chemicals Ca, Mo, N, P and K (Brooks, 1987). Unless the hyperaccumulator plants themselves were nitrogen fixers, their substrate soils could not be enriched by rhizobium-harbouring leguminous plants as these are not believed to be able to tolerate the large amounts of toxic metals in these soils.

As hyperaccumulators are potentially toxic to browsing animals, this does not provide a good scenario for the natural replenishment of nutrient via animal dung. Decomposition cycles are also unlikely to be efficient in such soils as they are not favorable to the necessary micro flora and fauna. Furthermore, it is suspected that the metals in such soils have a tendency to bind to any organic matter present, rendering both the metals and the nutrients in the organic matter insoluble and unavailable for absorption by the plants (Blaylock *et al.*, 1997). Thus, the manifest disadvantage of the adaptive specialisation of hyperaccumulation is that the plants are condemned to grow in a nutrient-poor environment.

If the nutrients available to hyperaccumulators growing in their prerequisite metal-rich medium were increased, a variety of possible results could ensue.

- The growth of the plant could in fact be genetically determined and largely unaffected by nutrient levels above the basic minimum.
- The growth of the plants could be limited by the 'metal stress' they suffer through growing in such an extreme environment - the cost-benefit of their adaptation.
- The growth of the plants could be considerably improved by an amelioration in the nutrient levels available to them.
- The addition of extra nutrients to the soil could theoretically render less metals available for uptake by the plants and thus leave them susceptible to attack by pathogens.
- The biomass of the plants could be considerably increased by the addition of fertiliser, but the rate of metal uptake could diminish, remain constant or increase.
- The further addition of chelating agents to the soil could possibly increase the absorption of a) the metals, b) the nutrients, c) both or d) neither.

This research is aimed at answering the above questions and in doing so will hopefully aid in the identification and propagation of species and cultivars of hyperaccumulators with a significant role in phytoremediation.

ALYSSUM BERTOLONII - TUSCANY, ITALY

Minguzzi and Vergnano (1948) were the first to discover high nickel levels in plants. They found up to 12200 μg/g (12.2%) nickel in the leaves of *Alyssum bertolonii*. The genus *Alyssum* contains about 150 species of which 48 species have hyperaccumulating ability. These plants are confined to a belt of ultramafic (serpentine) rocks stretching along Southern Europe from Portugal to Eastern Turkey. *Alyssum bertolonii* is a member of the *Brassicaceae* Family and is an endemic mainly localised to the ultramafics of Tuscany, Italy.

Figure 7: *Alyssum bertolonii*.

STREPTANTHUS POLYGALOIDES – CALIFORNIA

In 1981 it was reported by Reeves *et al* that *Streptanthus polygaloides*, a member of the Brassicaceae family endemic to serpentine soils in California, was capable of accumulating nickel to concentrations as high as 14800 μg/g (1.48%) in dry plant tissue. The plant samples used for the study were from herbarium specimens originally collected from soils overlying ultramafic rocks in the foothills of the Sierra Nevada.

Streptanthus polygaloides is the first hyperaccumulator of nickel found in the Americas. It is a member of the *Brassicaceae* Family and is endemic to serpentine soils in California.

Figure 8: *Streptanthus polygaloides*.

POT TRIALS

Alyssum bertolonii and *Streptanthus polygaloides* were grown in a mixture of 3 parts bark to 1 part crushed serpentine rock (v/v) (containing approximately 2000 μg/g nickel) with the addition of phosphate and nitrogen treatments. The aim of these experiments was to ascertain whether different concentrations of these nutrients would have an effect on the biomass of the plants and their uptake of nickel.

Sixteen different fertiliser treatments (Table 2) were used with 10 replicates of each treatment for each plant species. Treatments for nitrogen are N_0, N_1, N_2 and N_3 where N_0 is the control, N_2 is the usual recommended loading, N_1 is half this loading and N_3 double the recommended loading. There is a similar series for phosphorus.

Based on recommendations for nursery stock grown in peat or bark the optimum levels of nitrogen would be 50 μg/g and phosphorus 15 μg/g. Based on top dressing levels employed by farmers the usual recommended loading is 150 kg/ha calcium ammonium nitrate (28 μg/g nitrogen) and 300 kg/ha superphosphate (21 μg/g phosphorus) (assuming a soil bulk density of 1 g/cc in the upper 10 cms). The optimum levels of nitrogen and phosphorus used in this experiment were set at 50 μg/g nitrogen (185 μg/g calcium ammonium nitrate) and 20 μg/g phosphorus (220 μg/g superphosphate).

Treatment	Nitrogen (μg/g)	Phosphorus (μg/g)
N_0P_0	0	0
N_0P_1	0	10
N_0P_2	0	20
N_0P_3	0	40
N_1P_0	25	0
N_1P_1	25	10
N_1P_2	25	20
N_1P_3	25	40
N_2P_0	50	0
N_2P_1	50	10
N_2P_2	50	20
N_2P_3	50	40
N_3P_0	100	0
N_3P_1	100	10
N_3P_2	100	20
N_3P_3	100	40

Table 2: Sixteen different fertiliser treatments used in pot trials with *Alyssum bertolonii* and *Streptanthus polygaloides*.

To give the required fertiliser loadings a mixture of sand was prepared containing 1.85% (18 500 μg/g w/w) calcium ammonium nitrate (CAN) and a second mixture was prepared containing 2.2% (22000 μg/g w/w) superphosphate (SP). These mixtures were added to the test soils at loadings of 0%, 0.5%, 1% and 2% (w/w) to give media containing 0 μg/g, 25 μg/g, 50 μg/g, and 100 μg/g nitrogen and 0 μg/g, 10 μg/g, 20 μg/g and 40 μg/g phosphorus.

1.85% CAN = 18.5g CAN in 1kg of sand = 5g N in 1kg of sand = 5000 μg/g N
2.2% SP = 22g SP in 1kg of sand = 2g P in 1kg of sand = 2000 μg/g P
Addition of 0%, 0.5%, 1% and 2% of each mixture to the test soils gives:

0%	0 μg/g N	0 μg/g P
0.5%	25 μg/g N	10 μg/g P
1%	50 μg/g N	20 μg/g P
2%	100 μg/g N	40 μg/g P

Alyssum bertolonii was also grown in a 1:1 mixture of Tui tailings and sieved bark. These tailings are from the Tui mine which is located about 3 km northeast of the township of Te Aroha, in the Coromandel. About 100000 m^3 of sulphide-bearing tailings derived from the mining operation were deposited on the steep flanks of Mt Te Aroha at about 350 m altitude. The tailings are invariably finely ground sand or silt-sized particles comprised primarily of quartz but also contain significant amounts of As, Cd, Cu, Fe, Pb and Zn primarily in the from of sulphides. Lime was added to the tailings/bark mixture as the tailings are quite acidic (pH of around 3). Addition of 2% lime brought the pH up to 6.25 which is suitable for plant growth. A 0.5g sample of the tailings was dried overnight at 105°C. The dried sample was then dissolved in hydrofluoric acid and boiled dry over a water bath, redissolved in 15ml of 2M hydrochloric acid and analysed for cadmium, copper, zinc and lead by flame atomic absorption spectrophotometry. The tailings were found to contain 80 μg/g copper, 180 μg/g zinc, 3 μg/g cadmium and 12400 μg/g (1.2%) lead.

The *Alyssum* plants were grown in this substrate for 4 weeks after which a leaf sample from each plant was analysed. EDTA was then added to each cell at the rate of 0 (control), 0.7, 1.3 and 2.0g/kg (5 replicates of each treatment) and at weekly intervals the plants were re-analysed to determine if the EDTA had any affect on the uptake of cadmium, zinc and lead.

Seeds were germinated and grown for around 2 weeks in potting mix and then the seedlings were planted singly into cell trays with 150ml capacity. Over the next 3 to 4 weeks plants that died during this period were replaced by similar sized plants from the seed trays. The plants were watered 3 times a week for the next 17 weeks. At the end of the 20 week test period the plants were harvested, taking the entire plant from the growing medium upwards, and analysed.

ANALYTICAL METHODS

All implements and glassware were cleaned with a detergent and rinsed in deionised water prior to use to avoid any contamination by the elements under study, which would produce erroneous results. Plant samples were air-dried and the dry weight of each plant recorded. The dried samples were placed in 5cm^3 borosilicate test-tubes and ashed overnight at 500°C in a muffle furnace. The resulting ash was dissolved in 5ml of 2M HCl, shaken and then analysed for nickel by atomic absorption spectrophotometry. The absorption line at 232 nm was used.

RESULTS

STREPTANTHUS POLYGALOIDES

My experiments showed that the *S. polygaloides* plants need a moderate level of nitrogen (50 μg/g or greater) to grow and seedlings died after a few days when planted in media containing less than 50 μg/g nitrogen, even after successive replanting. Only 24 out of the 160 plants survived the 20-week test period. Future experiments should therefore use a higher range of fertiliser loading i.e. for *S. polygaloides* 50 μg/g nitrogen should be used as a minimum (rather than optimum) fertiliser loading.

MEAN BIOMASS AND MEAN NICKEL CONCENTRATION FOR *STREPTANTHUS POLYGALOIDES*

TREATMENT	MEAN BIOMASS (mg)	MEAN NICKEL CONC (μg/g)
N_2P_0	22	4100
N_2P_1	15	6300
N_2P_3	15	3000
N_3P_0	24	3700
N_3P_1	26	4200
N_3P_2	38	4200
N_3P_3	16	4000

Table 3: *Mean* biomass and Nickel concentration of *Streptanthus polygaloides* in relation to the different fertiliser treatments used

Table 3 shows that there was no significant increase in either the mean biomass or the mean nickel concentration with the different fertiliser treatments, although there was a slight increase in biomass with increasing nitrogen (see figure 9). The correlation coefficient of the data for the biomass and nickel concentration is -0.280. The probability is greater than 0.5 which indicates that there is no significant relationship. In other words increasing the biomass does not increase the nickel concentration.

Reeves *et al* (1981) analysed herbarium samples of *Streptanthus polygaloides* and obtained a mean value of 9750 μg/g with a range of 3300 to 14800 μg/g (the nickel content of the soil ranged between 2350 and 3840 μg/g). The results obtained for *Streptanthus polygaloides* in this experiment were a mean of 4500 μg/g with a range between 1500 and 7100 μg/g (nickel content of the growing medium is 550 μg/g).

These results show that *Streptanthus polygaloides* is clearly a hyperaccumulator of nickel but did not take up as much nickel as in the experiment conducted by Reeves *et al.*

This could be due to the lower nickel content in the growing medium (or lower available nickel due to the nickel being locked up in silicate lattices) or could be a lack of adequate nutrients resulting in decreased growth.

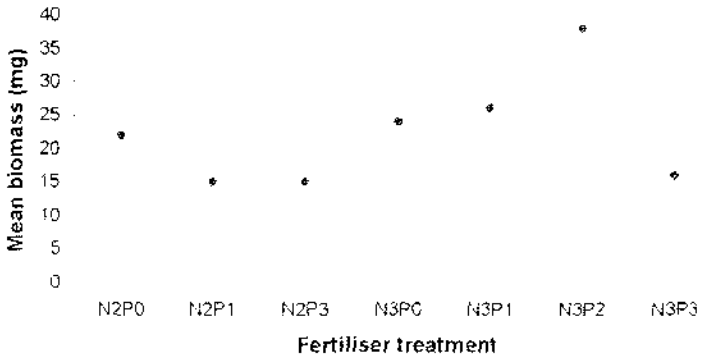


Figure 9: Graph showing mean biomass vs. fertiliser treatment for *Streptanthus polygaloides*.

Baker *et al* (1995) found that the concentration of nickel accumulated in plants previously identified as nickel hyperaccumulators increased in accordance with increasing total nickel concentration in the soil. These plants were found to respond to nutrient additions to the soils and so their growth potential can be enhanced by soil fertilisation.

ALYSSUM BERTOLONII

The results for *Alyssum bertolonii* showed that the biomass significantly increased with increasing nitrogen levels and the results also show some evidence for increase of biomass with increasing phosphorus levels but this was not as obvious as the nitrogen trend (see figure 10 and table 4).

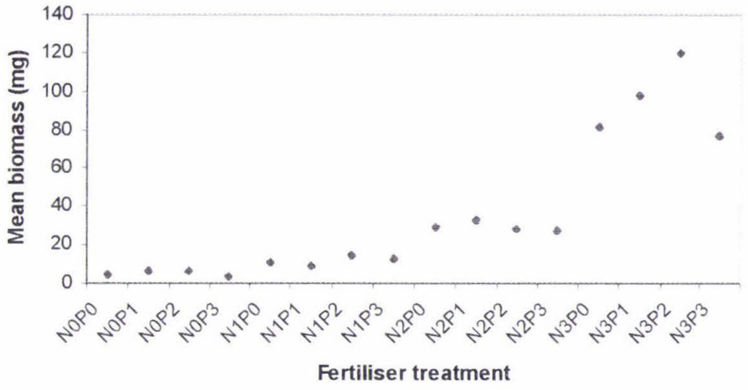


Figure 10: Graph showing mean biomass vs fertiliser treatment for *Alyssum bertolonii*.

MEAN BIOMASS AND MEAN NICKEL CONCENTRATION FOR *ALYSSUM BERTOLONII*

TREATMENT	MEAN BIOMASS (mg)	MEAN NICKEL CONC. (μg/g)
N_0P_0	5	4200
N_0P_1	6	3700
N_0P_2	6	5000
N_0P_3	4	5400
N_1P_0	11	4800
N_1P_1	9	5600
N_1P_2	15	5500
N_1P_3	13	5700
N_2P_0	29	5200
N_2P_1	33	5300
N_2P_2	28	5000
N_2P_3	27	4000
N_3P_0	81	3600
N_3P_1	98	4100
N_3P_2	120	3400
N_3P_3	77	3300

Table 4: Mean biomass and Nickel concentration of *Alyssum bertolonii* in relation to the different fertiliser treatments used.

Table 4 and figure 11 show that the phosphorus and nitrogen treatments do not make a significant difference to the nickel concentration in the plants. Nickel concentration stays relatively constant through the different fertiliser treatments. Although nickel concentration shows a slight decrease with increasing biomass (the nickel concentration is diluted by the larger biomass), for example an *Alyssum bertolonii* plant with a biomass of around 10 mg accumulates approximately 5000 μg/g nickel whereas a plant with a biomass of 85 mg accumulates around 3500 μg/g, it can be concluded that fertiliser does not significantly affect the uptake of nickel.

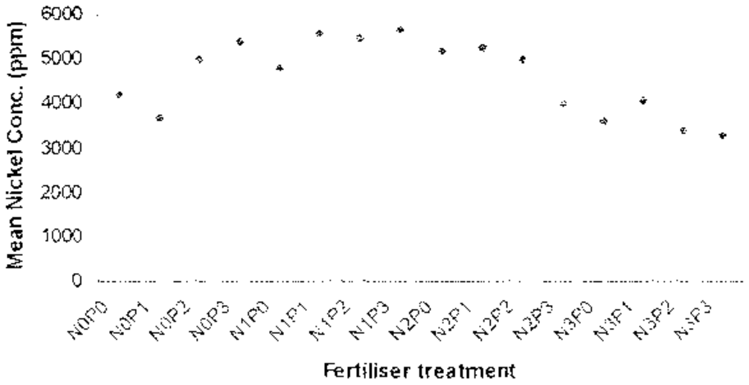


Figure 11: Graph showing mean nickel concentration vs. fertiliser treatment for *Alyssum bertolonii.*

Plants respond to nitrogen additions but it can be seen by visual inspection of the plants that additions of phosphorus do not greatly increase the biomass. There is no apparent relationship for phosphorus and biomass increase therefore only the means for the nitrogen data are used for the statistics.

MEAN BIOMASS AND MEAN NICKEL CONCENTRATION OF NITROGEN TREATMENTS FOR *ALYSSUM BERTOLONII*

TREATMENT	NITROGEN CONC. (μg/g)	MEAN BIOMASS (mg)	MEAN NICKEL CONC. (μg/g)
N_0	0	5	4600
N_1	25	12	5400
N_2	50	29	4900
N_3	100	94	3600

Table 5: Mean biomass and Nickel concentration of *Alyssum bertolonii* in relation to the different nitrogen fertiliser treatments used.

Table 5 shows that biomass significantly increases with increasing amounts of nitrogen fertiliser. There is a 5-fold increase of biomass with 50 μg/g nitrogen. Additional experimentation is needed to determine whether biomass would further increase with greater additions of nitrogen.

The data have a log-normal distribution which is shown by the median of the data corresponding to the geometric mean (see table 5 below).

MEDIANS AND GEOMETRIC MEANS FOR BIOMASS AND NICKEL CONCENTRATION FOR *ALYSSUM BERTOLONII*

TREATMENT	BIOMASS (mg)		NICKEL CONC. (μg/g)	
	MEDIAN	GEOMETRIC MEAN	MEDIAN	GEOMETRIC MEAN
N_0	5	4	3715	4182
N_1	11	10	5129	4231
N_2	27	28	4786	4728
N_3	85	86	3631	3521

Table 6: *Medians and geometric means for biomass and Nickel concentration of *Alyssum bertolonii* in relation to the different nitrogen fertiliser treatments used.*

STATISTICS FOR BIOMASS AND NICKEL CONCENTRATION OF *ALYSSUM BERTOLONII*.

BIOMASS (mg)							
TREATMENT	NUMBER	MEAN	MEDIAN	STD DEV	GEO MEAN	STD DEV RANGE	
N_0	33	0.63	0.70	0.28	4.26	2.24	8.11
N_1	41	1.02	1.04	0.25	10.45	5.91	18.48
N_2	42	1.44	1.43	0.16	27.72	19.27	39.87
N_3	39	1.94	1.93	0.18	86.11	56.88	130.35
NICKEL CONCENTRATION (μg/g)							
TREATMENT	NUMBER	MEAN	MEDIAN	STD DEV	GEO MEAN	STD DEV RANGE	
N_0	33	3.62	3.57	0.19	4182.07	2728.27	6410.56
N_1	41	3.63	3.71	0.59	4230.59	1095.23	16341.68
N_2	42	3.67	3.68	0.11	4727.64	3641.69	6137.42
N_3	39	3.55	3.56	0.10	3520.57	2799.92	4426.72

Table 7: Statistical treatment of biomass and Nickel concentration data for *Alyssum bertolonii*.

Log values are used for the statistical treatment of the data (Table 7) as this is a log-normal distribution. The standard deviation pooled and the students t-test was determined for the data to work out the probability that there is not a relationship between two sets of data for mean nickel concentration.

	Variables	t	ϕ	P	Significance
1	N_0 vs. N_1	0.094	72	0.5 to 0.2	NS
2.	N_0 vs. N_2	-0.746	73	0.5 to 0.2	NS
3.	N_0 vs. N_3	2.010	70	0.05 to 0.02	S
4.	N_1 vs. N_2	-0.434	81	>0.5	NS
5.	N_1 vs. N_3	0.839	78	0.5 to 0.2	NS
6.	N_2 vs. N_3	5.129	79	<0.001	S**

Table 8: Students t-test for data for *Alyssum bertolonii*. Where t=students t-test, ϕ=degrees of freedom, P=probability (NS=not significant, PS=possibly significant, S=significant, S*=highly significant, S**=very highly significant).

Only calculation 6. in Table 8 (N_2 vs N_3) shows any real relationship. The probability is less than 0.001 which is very highly significant. It can therefore be concluded that there is a relationship between these two sets of data. This may indicate that a threshold has been reached where the addition of nitrogen is causing a significant increase in biomass.

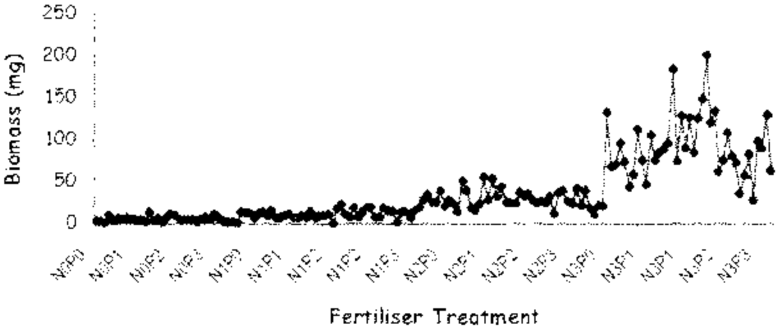


Figure 12: Effect of fertiliser treatment on the biomass of *Alyssum bertolonii*

There was a highly significant increase in biomass of *Alyssum bertolonii* with increasing addition of nitrogen (Figure 12). It is probable that there would have been a continuing increase of biomass if higher nitrogen concentrations had been used. From studies in the field by Robinson *et al.* (1997) it seems that this species is particularly susceptible to increasing its biomass with nitrogen fertilisers. These authors achieved an increase of 300% during a 12-month period. The results obtained for this experiment only showed a 1.5-fold increase of biomass from 50 µg/g nitrogen to 100 µg/g nitrogen which suggests that a greater increase of biomass could be obtained with increased fertilisation.

The relationship between biomass and nickel concentration is an inverse relationship. The correlation coefficient of the data for biomass and nickel concentration was -0.342. The probability was <0.001 which indicates that there is a very highly significant inverse relationship.

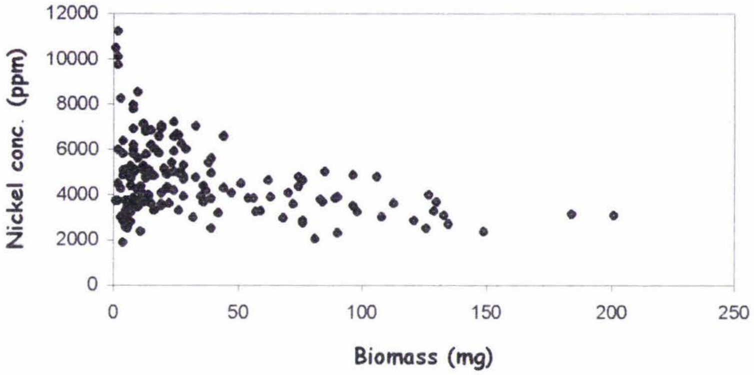


Figure 13: Graph showing mean nickel concentration vs. biomass for *Alyssum bertolonii*.

Figure 13 shows that as biomass increases nickel uptake decreases. The nickel concentration is inversely proportional to biomass but as the slope of the graph is very slight there is not a great disadvantage. In other words, although there is a slight tendancy for nickel concentration to reduce when biomass increases (diluted by the greater biomass), there is still an enormous advantage with greater biomass (see table 9). Similar findings were made by Robinson *et al.* (1997) in field trials with this species.

BIOMASS, NICKEL CONCENTRATION AND TOTAL NICKEL UPTAKE OF *ALYSSUM BERTOLONII*.

TREATMENT	BIOMASS (mg)	X	NICKEL CONC. (µg/g)	=	TOTAL NICKEL UPTAKE
N_0	4	X	4 182	=	16 728
N_1	11	X	4 231	=	46 541
N_2	28	X	4 728	=	132 384
N_3	86	X	3 521	=	302 806

Table 9: Statistics for biomass, nickel concentration and total nickel content for *Alyssum bertolonii*.

Table 9 shows that, although nickel concentrations decrease slightly as biomass increases, overall larger plants will take up much more nickel than smaller plants.

CADMIUM, LEAD AND ZINC UPTAKE BY *ALYSSUM BERTOLONII*

TREATMENT	CADMIUM (µg/g)	LEAD (µg/g)	ZINC (µg/g)
Control	1.9	31	49
Control	1.8	15	12
Control	1.2	23	19
Control	1.2	22	14
Control	3.7	14	11
0.7g/kg EDTA	0.3	304	32
0.7g/kg EDTA	0.1	259	17
0.7g/kg EDTA	1.2	67	57
0.7g/kg EDTA	0.2	91	22
0.7g/kg EDTA	0.4	185	19
1.3g/kg EDTA	0.5	677	18
1.3g/kg EDTA	0.3	604	17
1.3g/kg EDTA	3.9	613	20
1.3g/kg EDTA	0.2	196	14
1.3g/kg EDTA	2.2	740	29
2.0g/kg EDTA	0.2	521	38
2.0g/kg EDTA	0.1	604	18
2.0g/kg EDTA	2.1	130	39
2.0g/kg EDTA	0.2	308	40

Table 10: Analyses for cadmium, lead and zinc uptake by *Alyssum bertolonii* grown in 1:1 Tui tailings: bark

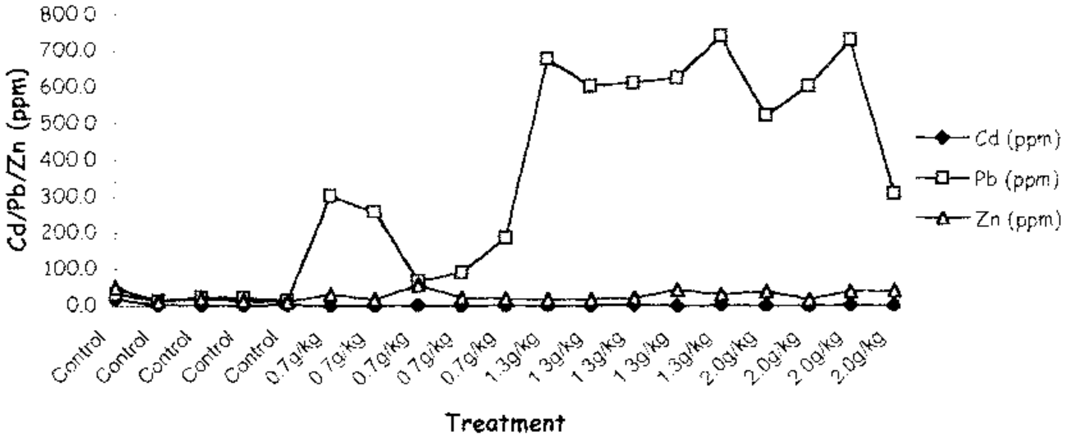


Figure 14: *Graph showing the effect of EDTA (after 3 weeks) on the uptake of cadmium, lead and zinc by Alyssum bertolonii.*

Table 10 and figure 14 show that the addition of EDTA does not affect the uptake of cadmium and zinc by *Alyssum bertolonii*. EDTA does have a significant affect on the uptake of lead by *Alyssum bertolonii*. Adding EDTA at a rate of 0.7 g/kg increased Pb uptake by a factor of 8 while EDTA rates of 1.3 and 2.0 g/kg increased uptake by a factor of 20.

GENERAL DISCUSSION

The experiments showed that *Streptanthus polygaloides* requires a moderate level of nitrogen and plants growing in the N_0 and N_1 series of pots (0 and 25 μg/g N) did not survive the 20 week period. For those that remained, there was no apparent relationship between fertiliser treatment and biomass and nickel content.

The addition of nitrogen and phosphorus fertiliser treatments increases biomass in both *Alyssum bertolonii* and *Streptanthus polygaloides* although optimum levels of fertiliser cannot be determined as the loadings used were too low. Further experimentation using increased fertiliser loadings would be necessary to ascertain the optimum fertiliser treatment that produces the greatest biomass under glasshouse conditions.

The mean nickel content of the *Streptanthus polygaloides* plants was well below the nickel content of 9750 μg/g dry weight reported by Reeves *et al.* (1981). In these experiments, the mean nickel content of individual plants was 4500 μg/g with a range of 1500 to 7100 μg/g.

Both *Alyssum bertolonii* and *Streptanthus polygaloides* have greatly exceeded the 1000 μg/g Ni threshold, showing clear hyperaccumulator status, and accumulate nickel even when biomass is low. Increasing the biomass has the effect of slightly decreasing the uptake of nickel in the plant tissues. The rate of uptake is possibly the same, but the rate of growth of the plant is increased. The product of biomass and nickel content is of greater importance in assessing the suitability of a species for phytoremediation or phytomining. Plants that are only moderate accumulators but have a high biomass may provide a higher overall metal yield than a hyperaccumulator with a low biomass.

There is a great potential for increasing biomass by addition of fertilisers although the optimum fertiliser treatment could not be determined in this experiment. This experiment does, however, indicate that although an increase in biomass decreases the nickel concentration, this effect is only slight and in general, larger plants will take up much more nickel than smaller plants. Increasing biomass (without subsequent decrease in nickel uptake) would increase the suitability of hyperaccumulators for bioremediation and biomining.

Plants do not take up lead because it is strongly held by soil organic matter and soil minerals, as well as being immobilised as insoluble $PbSO_4$ (anglesite) if any sulphate is present. The addition of EDTA mobilises the lead from the soil particles and makes it available for plant uptake. One problem with the addition of chelating agents to the soil is that plant growth is severely curtailed after the lead availability is enhanced. Once the plant has achieved accumulation, the EDTA weakens the plant so it accumulates less.

The addition of EDTA did not greatly enhance the uptake of cadmium and zinc which implies that lead was more vulnerable to leaching than Cd or Zn with added EDTA in soils. The Cd and Zn is either more tightly chelated in the soil than with EDTA or the Cd/Zn EDTA complex does not facilitate EDTA uptake. Further tests which examine whether the solution Zn and Cd increased when EDTA is added would determine which of these two situations is occurring. Added EDTA may increase the extractability of these metals but whether EDTA enhances metal plant availability may depend on soil conditions and metal concentrations.

COBALT AND COPPER HYPERACCUMULATORS

INTRODUCTION

Copper and cobalt are generally found together in mineralised soils. Copper concentrations in vegetation tend to be fairly constant irrespective of the amount of copper in the soil, because copper is an essential element in plant nutrition. Normal plant levels for cobalt are between 0.05 and 5 μg/g. Copper levels above 60 μg/g cannot be tolerated by most plants. Normal plant levels for copper are from 1 to 25 μg/g. Poisoning of plants by cobalt and copper results in chlorosis, stunting, reduced root growth and possibly necrosis. On mineralised soils most plants contain between 5 to 50 μg/g cobalt and 25 to 100 μg/g copper. Concentrations in hyperaccumulators can be more than 1000 times higher than for plants growing on normal soils, and can be 20 to 200 times higher than for most of the other plants that tolerate mineralised soil.

Element	Low	Normal	High	Hyperaccumulators
Fe	10 - 60	600	2 500 - 35 000	> 35 000
Mn	5 - 20	400	2 000	10 000 - 50 000
Zn	5 - 20	400	2 000	10 000 - 50 000
Cd	0.03 - 0.10	3	20	100 - 3 000
Pb	0.01 - 0.10	5	100	1 000 - 8 000
Ni	0.20 - 1.0	10	100	1 000 - 40 000
Co	0.05 - 0.20	5	50	1 000 - 10 000
Cr	0.05 - 0.20	5	50	1 000 - 2 500
Cu	1.00 - 5.00	25	100	1 000 - 12 500
Se	0.01 - 0.10	1	10	100 - 6 000

Table 11: Normal and abnormal concentrations of elements in plant leaves (μg/g). Source: Reeves *et al.* (1995).

The copper and cobalt content of mineralised soils can be very high. In Shaba Province, Democratic Republic of Congo (Zaïre), extensive copper/cobalt mineralisation exists in the 'Shaban Copper Belt'. Copper values for metalliferous soils in Shaba range from 900 to 2000 μg/g to more than 90000 μg/g and cobalt values range from 600 to 900 μg/g to over 40000 μg/g. The genus *Haumaniastrum* contains many plants which grow on these soils and some (e.g. *Haumaniastrum robertii* and *Haumaniastrum katangense*) are said to be 'copper flowers' which grow only over cobalt and copper deposits in the Shaban Copper Belt.

The concentration of any element in dried plant material will usually reflect to some extent the content of this same element in the underlying substrate. The term 'hyperaccumulator' was first used by Brooks *et al.* (1977a) to define plants containing more than 1000 μg/g nickel in dry tissue. Brooks *et al.* (1980) extended the concept of hyperaccumulation to include plants with that same defined level of copper and cobalt. Hyperaccumulation of zinc has been reported (Reeves *et al.* (1983)) but as the natural levels of zinc in plants can often reach 1000 μg/g, a hyperaccumulation threshold of 10000 μg/g (1%) zinc in dry material has been set for this element.

The flora of the 'Copper Belt' of Central Africa is characterised by a number of taxa adapted to high copper and cobalt concentrations in the soil overlying this region. The literature at present records 24 hyperaccumulators of copper and 26 of cobalt from the Shaban Copper Belt (Table 12), including 9 species that can be found with more than 1000 μg/g of both elements in their leaf tissue (Brooks, 1980).

The number of known hyperaccumulators may not present a picture of the total number that exist for any one element. The following table largely reflects the amount of effort that has been expended in identifying plants that hyperaccumulate a given element. This is particularly true for nickel. The number of 'manganese' plants may well exceed that for nickel, but there has been little interest in the former.

ELEMENT	NUMBER	FAMILIES
Cadmium	1	*Brassicaceae*
Cobalt	26	*Lamiaceae, Scrophulariaceae*
Copper	24	*Cyperaceae, Lamiaceae, Poaceae, Scrophulariaceae*
Manganese	11	*Apocynaceae, Cunoniaceae, Proteaceae*
Nickel	290	*Brassicaceae, Cunoniaceae, Euphorbiaceae, Violaceae, Flacourtiaceae*
Selenium	19	*Fabaceae*
Thallium*	1	*Brassicaceae*
Zinc	16	*Brassicaceae, Violaceae*

*Leblanc *et al.* (1998).

Table 12: Numbers of known hyperaccumulators for eight heavy metals and the families in which they are most often found

Haumaniastrum robertii and *Haumaniastrum katangense* will accumulate both copper and cobalt although *H. katangense* shows a much lower tolerance to both elements (Morrison *et al.*, 1979). Among the hyperaccumulators of copper, *Aeollanthus biformifolius* has the greatest concentrating ability. Malaisse *et al.*, (1979) reported as much as 13500 μg/g Cu in the whole plant (dry weight).

All three species will grow on soils containing low concentrations of copper or cobalt but in non-mineralised soils losses are particularly severe (especially in the case of *H. robertii*). These losses are mainly due to fungal attack and it is probably this factor in addition to an inability to withstand competition from other species that restricts these plants to mineralised soils. The normal substrates of all three species have copper and/or cobalt levels sufficiently high as to inhibit all fungal growth.

The genus *Haumaniastrum* was surveyed by Brooks (1977) and led to the first identification of a hyperaccumulator of cobalt, with a concentration of 10220 μg/g (1.02%) in *H. robertii.* This value is an order of magnitude higher than the previously recorded cobalt levels of the cobaltophyte *Crotalaria cobalticola* of 354 μg/g reported by Duvigneaud (1959) and 970 - 3010 μg/g reported by Brooks *et al.* (1980).

Hyperaccumulation of copper and cobalt appears to be much rarer than hyperaccumulation of nickel or zinc. Only *Haumaniastrum robertii* (1.02%) has surpassed the 1% cobalt level and *Aeollanthus biformifolius* (1.35%) the 1% copper level. The most important group of species in which copper and cobalt hyperaccumulation occurs is the flora of the Shaban Copper Arc in southeast Democratic Republic of Congo (Zaïre) and the Copper Belt of northwest Zambia. Some of these plants are extremely local in their distribution. For example, at the Mine de l'Etoile near Lubumbashi, the copper hyperaccumulators *Vigna dolomitica* and *Lindernia perennis* are confined to a few hectares around the abandoned copper mine and are found nowhere else in the world.

Most of the hyperaccumulators seem to have evolved from widespread species that surround, or once surrounded, the metal-rich patches without actually colonising them. Isolation has led to speciation and local adaptation in response to soil conditions. Most metal accumulating plants are rare endemics (Brooks *et al.*, 1985).

HAUMANIASTRUM ROBERTII

Haumaniastrum robertii (Figure 15) is a member of the Lamiaceae family and is one of the best known of the copper flowers of The Democratic Republic of Congo (Zaïre). It is found primarily in the region between Kolwezi and Likasi and is replaced in the east

(Lubumbashi) by *Haumaniastrum katangense* (Figure 16). *H. robertii* is found exclusively over copper/cobalt deposits either of ancient or recent origin. In such localities it is said to identify areas where there are up to 100000 μg/g (10%) copper in the soil. There is evidence that *H. robertii* is in fact an indicator of cobalt rather than copper (Brooks, 1977). It can contain up to 1.02% cobalt on a dry weight basis whereas uptake of copper does not exceed 0.20%. Even if *H robertii* indicates cobalt rather than copper, the occurrence of this plant can nevertheless be taken as an indicator of the presence of copper as copper and cobalt are almost always found together in the ores of The Democratic Republic of Congo (Zaïre).

Figure 15: The cobalt hyperaccumulator and indicator plant *Haumaniastrum robertii* at Mindingi, Democratic Republic of Congo (Zaïre). Source: Brooks *et al.* (1985).

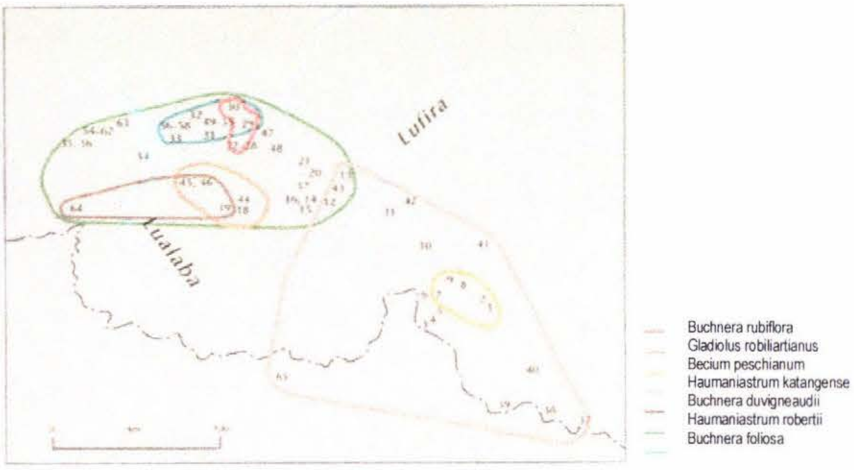


Figure 16: Distribution of some Shaban plants endemic to copper/cobalt deposits. Source: Brooks *et al.* (1992).

HAUMANIASTRUM KATANGENSE

Haumaniastrum katangense can also accumulate both copper and cobalt. It is confined to eastern Shaba province, Democratic Republic of Congo (Zaïre) and to the northern part of Zambia (Figure 16). Like its true relative *H. robertii*, it grows exclusively on copper-rich soils but does not accumulate as much cobalt as the latter. A maximum of 0.22% cobalt and 0.84% copper has been reported (Brooks *et al.*, 1980) for dried leaves of *H. katangense.*

H. katangense is not only an indicator of mineralisation but also a phytoarchaeological indicator. Brooks *et al.* (1985) observed its presence at numerous localities throughout Shaba. The plant seems to have escaped from its original habitat and is now found on man-made substrates which are lightly or heavily mineralised in copper. Such substrates include sites of furnaces traditionally used in precolonial days for the production of small copper crosses, sites of exploitation of copper minerals during the early decades of colonisation, and at the sides of roads passing through non-mineralised terrain. This latter occurrence is due to the local custom of using sterile waste from smelting activities (gangue) to dress the dirt roads after the rainy season has ended (the copper content of this gangue can sometimes approach 3%). Passing traffic deposits a narrow band of lightly mineralised dirt along the roadside during the dry season, and this gives a substrate suitable for the establishment of a community of metal tolerant plants.

The inadvertent presence of metallophytes as a consequence of human activity is also shown by the occurrence of *H. katangense* over soil (originally non-mineralised) contaminated by copper-rich emission from the giant smelting works at Lubumbashi (Figure 17).

Figure 17: Carpet of *Haumaniastrum katangense* growing over copper-poisoned soil derived from the stack of the giant copper smelter at Lubumbashi shown in the background. Source: Brooks *et al.* (1992).

AEOLLANTHUS BIFORMIFOLIUS

Aeollanthus biformifolius (Figure 18), another member of the Lamiaceae family, is confined to copper-rich substrates and contains the highest copper concentration ever recorded for any phanerogam (flowering plant). The whole plant contained up to 1.37% copper on a dry weight basis (Malaisse et al., 1979). The plant has a limited distribution (Figure 19). It is found only in the vicinity of the Luiswishi, Ruashi and Etoile mines in the southeastern part of the Shaban Copper Arc. The validity if its classification as a hyperaccumulator is discussed later in this thesis.

Figure 18: The copper hyperaccumulator and indicator plant *Aeollanthus biformifolius* at Etoile mine, Democratic Republic of Congo (Zaïre). Source: Brooks *et al.* (1985).

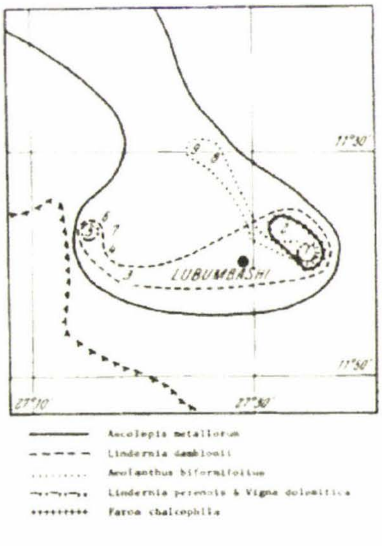


Figure 19: Distribution of Shaban plants endemic to copper/cobalt deposits in the Lubumbashi area. Source: Brooks *et al.* (1985).

ERICA ANDEVALENSIS

Erica andevalensis is a member of the Ericaceae (heath) family and is probably the most recently described woody species from Spain (Cabezudo and Rivera, 1980). This heather is a compact shrub, reaching 1.5 metres tall in favourable localities. *Erica andevalensis* (figures 19 and 20) is an edaphic endemic of the Huelva province in the southwest of Spain. It is found in a geographically defined section of this province (Andévalo) in an area of about 3500km^2 of which this species is found sporadically in about 1500km^2 of this (figure 22).

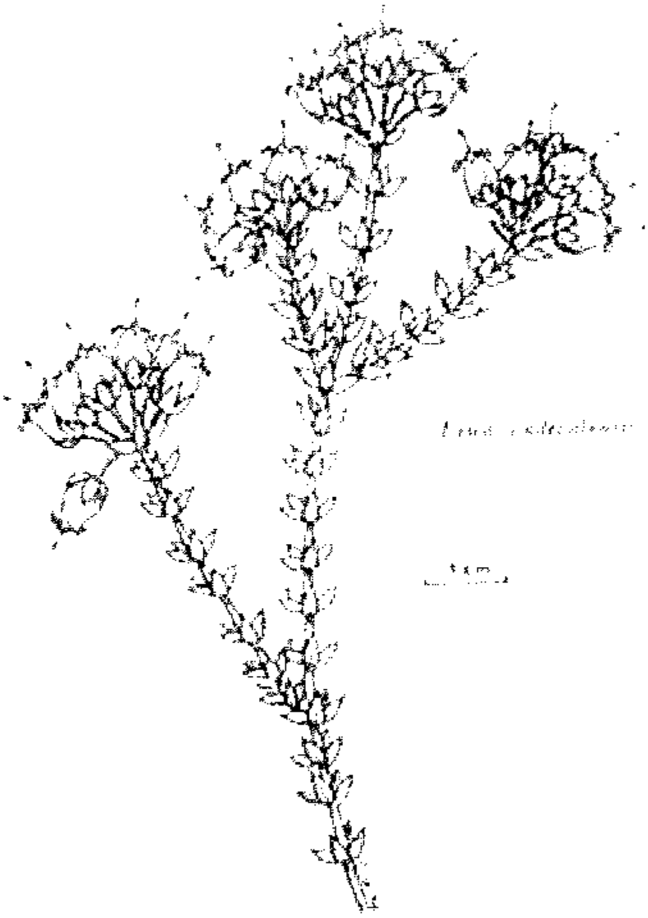


Figure 20: *Erica andevalensis.*

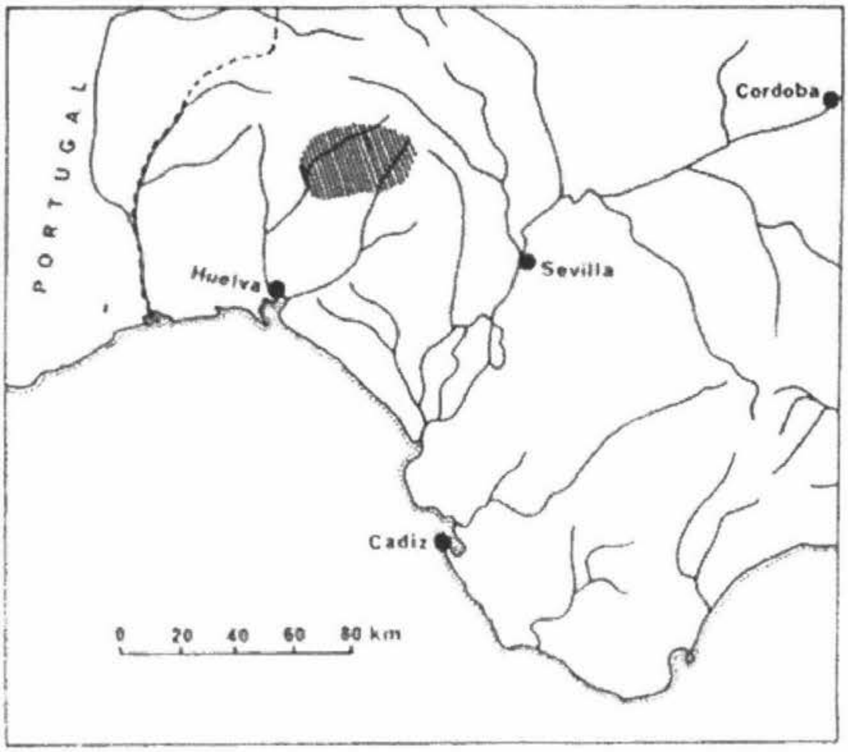
The plant grows on the spoil tips around the great mines of Huelva where pyrite was mined for copper, gold and silver as far back as Roman times. The mine tips are composed predominantly of overburden from open-cast workings and pyroclastic rocks of non-commercial quality, and contain traces of many heavy metals, including copper, lead, manganese and zinc.

Nelson *et al.* (1985) reported populations of *Erica andevalensis* on river banks and gravel which have been contaminated by iron, copper and zinc and believe these to represent the natural habitat of this species. In its riverine habitats, the flow of water is vastly reduced in summer and high concentrations of toxic salts will occur. Nelson *et al.* (1985) believe that it is the ability of *E. andevalensis* to tolerate high concentrations of mineral salts, stagnant water and anaerobic soil conditions that has enabled it to colonise the mine tips.

Figure 21: *Erica andevalensis* in flower during pot trials at Massey University. Photo: R. R. Brooks.

Nelson *et al.* (1980) have analysed the substrate of this plant at three localities and report 4-67 μg/g copper in mine tailings, 11-60 μg/g copper in the riverine soils, and an astonishing 1476 μg/g copper in evaporates of a lake in the Odiel Valley around which the plant was growing.

A related species, *Calluna vulgaris,* found at the Parys Mountain copper mine in Anglesey, Wales, is the only plant capable of colonising significant areas of toxic mine waste. In Huelva *E. andevalensis* fills this niche, for although *Calluna* and other *Erica* species are present in abundance, none grows with *E. andevalensis* in the most toxic habitats.



Map showing distribution (hatched area) of *Erica andevalensis* in S. Spain

Figure 22: Map of the Huelva region of southwest Spain showing the locality of the occurrence of *Erica andevalensis*. Source: Nelson *et al.* (1985).

The aim of the research involving this plant is to establish the degree to which *E. andevalensis* is copper tolerant and at the same time to determine whether it has hyperaccumulator status, here defined as containing > 1000 μg/g copper. If *E. andevalensis* is a hyperaccumulator of copper, this region of southwest Spain would rank as one of only three worldwide that contains plants of hyperaccumulator status for copper. The other two are Democratic Republic of Congo (Zaïre) described by Brooks *et al.* (1992), and Hubei Province, China (X. Yang pers. Comm. To R. R. Brooks 1996).

POT TRIALS

Haumaniastrum katangense, Haumaniastrum robertii, Aeollanthus biformifolius and *Erica andevalensis* were grown in substrates containing varying amounts of copper and/or cobalt (detailed below) to examine the uptake of copper and cobalt. Two chelating agents, ethylenediaminetetraacetic acid (EDTA) and citric acid were then added to determine if this would have any affect on the uptake of these metals. Both of these agents are known to establish stable complexes with metals.

Seeds were grown and germinated for around 2 weeks (or until they were large enough to be transplanted) in seed mix and then the seedlings were transplanted singly into cell trays with 150 mL capacity containing one of the substrates detailed below. Over the next 3-4 weeks seedlings that died were replaced by similar sized plants from the seed trays. At the end of the 4 weeks if no seedlings had survived even after successive replanting it was decided that the particular plant would not grow in that substrate.

The plants were watered three times a week. Individual leaves were sampled and analysed when the plants were considered large enough (depending on each plant species). After initial analyses, EDTA or Citric acid (see table 13) was added to each cell and leaves were then sampled and analysed at weekly periods for three weeks or until the plant died.

ANALYTICAL METHODS

Leaf samples (about 100mg) were air-dried and the dry weight of each leaf recorded. The dried samples were placed in 5cm^3 borosilicate test tubes and ashed overnight at 500°C or until the sample is fully combusted. The resulting ash was then redissolved in 5 mL of 2M hydrochloric acid, shaken and analysed by flame atomic absorption spectrophotometry.

RATES OF EDTA AND CITRIC ACID ADDITION.

NUMBER	TREATMENT	RATE (mL)	RATE (g/kg)
1	Control	0	0
2	4% EDTA	2	0.7
3	4% EDTA	4	1.3
4	4% EDTA	6	2.0
5	10% Citric Acid	4	3.2
6	10% Citric Acid	8	6.7
7	10% Citric Acid	12	10.0

Table 13: Rates of EDTA and Citric Acid addition to the test substrates (based on rates used by Laurie *et al.* (1991), Blaylock *et al.* (1997) and Li *et al.* (1996))

SUBSTRATES:

Plants grown in the initial substrates (1, 2 and 3 below) did not take up a significant amount of metals. This was thought to be due to the metals binding to the organic matter of the seed mix and becoming insoluble. Later trials used pumice, bark and/or peat to dilute the ores and tailings to try to find a substrate that did not make the metals unavailable and yet was still favorable for plant growth.

1. ARTIFICIAL COBALT SUBSTRATE

A 1% Co mixture was made by adding 47.6 g of $CoSO_4$ fertiliser (47.6g $CoSO_4$ contains 10g Co) to 1 kg of silica sand. The large crystals of the fertiliser were dissolved in water, dried at 105°C and crushed with a mortar and pestle to mix with the sand. A 100 μg/g Co substrate was made by adding 10 g of the 1% Co/sand mixture to 1 kg (2L) of sieved 'Liddle Wonder' commercial seed mix (2 mL of dried seed mix weighs 1g). A 500 μg/g Co substrate was made by adding 50g of the 1% Co/sand mixture to 2L of sieved seed mix.

Haumaniastrum katangense and *H. robertii* planted.

2. ARTIFICIAL COPPER SUBSTRATE

A 1% Cu mixture was made by adding 39.3g of $CuSO_4$ fertiliser (39.3g $CuSO_4$ contains 10g Cu) to 1 kg of silica sand. The large crystals of the fertiliser were dissolved in water, dried at 105°C and crushed with a mortar and pestle to mix with the sand. A 100 μg/g Cu substrate was made by adding 10g of the 1% Cu/sand mixture to 2L of sieved seed mix. A 500 μg/g Cu substrate was made by adding 50g of the 1% Cu/sand mixture to 2L of sieved seed mix.

Haumaniastrum katangense and *H. robertii* planted.

3. ARTIFICIAL COPPER/COBALT SUBSTRATE

A 100 μg/g Cu/Co substrate was made by adding 10g of the 1% Cu/sand mixture and 10g of the 1% Co/sand mixture to 4L of sieved seed mix. A 500 μg/g Cu/Co substrate was made by adding 50g of the 1% Cu/sand mixture and 50g of the 1% Co/sand mixture to 4L of sieved seed mix.

Haumaniastrum katangense, H. robertii and *Aeollanthus biformifolius* planted.

4. 1:1 TUI TAILINGS: BARK SUBSTRATE

1:1 tailings: bark + 2% lime + Osmocote. Two and a half litres of tailings were added to 2.5L of sieved bark with 0.05g (2%) lime, 5g of 3-4 month Osmocote and 10g of 8-9 month Osmocote and mixed well.

Haumaniastrum katangense, H. robertii and *Aeollanthus biformifolius* planted.

5. 3:1 TUI TAILINGS: PUMICE SUBSTRATE

3:1 tailings: pumice + 2% lime + Osmocote. Seven and a half litres of tailings were added to 2.5L of sieved pumice with 0.15L of lime (2%) and 10g of 3-4 month Osmocote and 20g of 8-9 month Osmocote and mixed well.

Haumaniastrum katangense planted.

6. 1:1 TUI TAILINGS: PUMICE SUBSTRATE

1:1 tailings: pumice + 2% lime + Osmocote. Three litres of original mixture (3:1 above) contains 2.25L of tailings and 0.75L of pumice. To get 1:1 mixture add 1.5L of sieved pumice to 3L of original mixture.

Haumaniastrum katangense, H. robertii and *Aeollanthus biformifolius* planted.

7. SERPENTINE SUBSTRATE

3:1 bark: serpentine + Osmocote. Six litres of crushed serpentine were added to 18L of sieved bark with 25g of 3-4 month Osmocote and 50g of 8-9 month Osmocote.

Haumaniastrum katangense planted.

8. UNITED ORE SUBSTRATE

Copper ore from the United Mine near Nelson in the South Island was analysed using flame atomic absorption spectrometry and found to contain 6873 μg/g (0.7%) copper, 827 μg/g (0.08%) cobalt, 16134 μg/g (1.6%) magnesium and 300 μg/g nickel. Four litres of ore was sieved to 1mm and added to 4L of sieved bark with 8g of 3-4 month Osmocote and 16g of 8-9 month Osmocote and mixed well.

Haumaniastrum katangense, H. robertii and *Aeollanthus biformifolius* planted.

9. CHAMPION ORE SUBSTRATE

A copper ore (Champion) from the Nelson area of the South Island was analysed using flame atomic absorption spectrometry and found to contain 25000 μg/g (2.5%) copper, 750 μg/g (0.075%) cobalt and 5000 μg/g (0.5%) nickel. Four litres of ore was sieved to 1mm and added to 4L of sieved bark with 8g of 3-4 month Osmocote and 16g of 8-9 month Osmocote and mixed well.

Erica andevalensis planted.

10. CHAMPION ORE - 8 CONCENTRATIONS

Ten litres of sieved pumice was mixed with 10L sieved peat with 20g 3-4 month Osmocote and 40g of 8-9 month Osmocote. One litre of this substrate was used as a control and eight concentrations were made as follows:

		Ni (μg/g)	Co (μg/g)	Cu (μg/g)
1. 1L ore	2L peat/pumice	250	37.5	1250
2. 1L **1**.	1L peat/pumice	125	19	625
3. 1L **2**.	1L peat/pumice	63	9	312.5
4. 1L **3**.	1L peat/pumice	31	5	156
5. 1L **4**.	1L peat/pumice	16	2	78
6. 1L **5**.	1L peat/pumice	8	1	39
7. 1L **6**.	1L peat/pumice	4	0.6	19.5
8.	1L peat/pumice	0	0	0

Haumaniastrum katangense planted.

11. CHAMPION ORE - 8 CONCENTRATIONS

Six litres of sieved seed mix was mixed with 20 g 3-4 month Osmocote and 40 g of 8-9 month Osmocote. One litre of this substrate was used as a control and six concentrations were made as follows:

		Ni (μg/g)	Co (μg/g)	Cu (μg/g)	
1. 1L ore	1L seed mix	2500	375	12500	1:1
2. 1L **1**.	1L seed mix	1250	187.5	6250	4:1
3. 1L **2**.	1L seed mix	625	94	3125	8:1
4. 1L **3**.	1L seed mix	312.5	47	1563	16:1
5. 1L **4**.	1L seed mix	156	23	781	32:1
6.	1L seed mix	0	0	0	control

Erica andevalensis planted.

Soil samples (0.5 g) were digested with 10 mL of a 1:1 mixture of nitric and hydrochloric acids contained in 50 mL polypropylene squat beakers immersed in a water bath. After taking the digestates to dryness, the residues were redissolved in 10 mL of 2M hydrochloric acid. The solutions of soil samples were then analysed for copper and iron by flame atomic absorption spectrometry. The reason for determining iron was that this element (along with chromium and titanium) could serve as an indication of the probable contamination of plant samples by soils and other substrates. Vascular plants usually do not accumulate more than 300 μg/g iron in their leaves via the root systems. Any value higher than this is usually an indication of windborne contamination from the soil.

RESULTS

Haumaniastrum katangense did not survive in straight seed mix, in the serpentine substrate and in the 1:1 Tui tailings: pumice substrate, even after successive replanting. *H. katangense* did grow in all the other substrates it was planted in whereas *H. robertii* proved to be less hardy than its relative and would only grow in the artificial copper substrate (but only half the plants survived) and the United ore substrate.

H. katangense is a much hardier plant (less susceptible to pathogens) and grew faster and larger than *H. robertii.* All the plants grew better (faster, larger and greener) in the United ore than in the artificial substrates although by the end of the test period all the plants had flowered (this could be taken as a sign that the plants were healthy).

Aeollanthus biformifolius also did not survive in the artificial copper/cobalt substrate or in the 1:1 Tui tailings: pumice substrate although it did grow well in the 1:1 Tui tailings: bark substrate. It is probable that the structure of the tailings impeded the growth of plants and although pumice was added to improve this structure the particle size was too fine and anaerobic conditions prevailed. Adding bark did improve the structure of the tailings and drainage was improved enough to allow plant growth.

UPTAKE OF COPPER, COBALT AND NICKEL BY *HAUMANIASTRUM KATANGENSE* AND *HAUMANIASTRUM ROBERTII* *GROWING IN 500 µG/G COPPER AND UNITED ORE.*

COPPER (µg/g)				
Species	Substrate	Before EDTA	1 week EDTA	2 weeks EDTA
H. katangense(1)	500 µg/g copper	13	318	220
H. robertii(2)	500 µg/g copper	16	650	1415
H. katangense(3)	United Ore	128	278	159
H. robertii(4)	United Ore	115	519	Dead
COBALT (µg/g)				
Species	Substrate	Before EDTA	1 week EDTA	2 weeks EDTA
H. katangense(1)	500 µg/g copper	4	0	13
H. robertii(2)	500 µg/g copper	16	8	21
H. katangense(3)	United Ore	44	49	43
H. robertii(4)	United Ore	71	68	Dead
NICKEL (µg/g)				
Species	Substrate	Before EDTA	1 week EDTA	2 weeks EDTA
H. katangense(1)	500 µg/g copper	0	0	0
H. robertii(2)	500 µg/g copper	0	0	0
H. katangense(3)	United Ore	44	15	84
H. robertii(4)	United Ore	192	172	Dead

Table 14: Data for uptake of copper, cobalt and nickel before and after the addition of EDTA (2.0g/kg) for *Haumaniastrum katangense* and *H. robertii* grown in artificial copper substrate (500 µg/g) and United ore substrate.

Table 14 and the three graphs below (figures 23, 24 and 25) show that the uptake of cobalt by each species stays roughly the same before and after the addition of EDTA although *Haumaniastrum robertii* takes up more cobalt than *H. katangense*. The plants growing in the artificial copper substrate did not take up a large amount of cobalt. Identical plants growing in the United ore substrate took up larger amounts which would suggest that this ore contained more cobalt which is borne out by direct analysis of the ore. It could also be that more cobalt was available in a form that could be taken up by the plants.

The plants took up less than 1 μg/g nickel, even after the addition of EDTA, which might indicate that the 500 μg/g copper substrate did not contain very much (if any) nickel. The United ore contains much more nickel and EDTA appears to have the affect of increasing nickel uptake at least in *H. katangense*. The effect of nickel on *H. robertii* is hard to determine due to the fact that the plant died after 1 week and before it could be analysed again but it looked as though a plateau had been reached.

H. katangense took up less copper than *H. robertii* and reached a maximum copper level after one week of EDTA which then decreased at two weeks. *H. robertii* took up much more copper and EDTA had the effect of increasing the copper that was taken up until the plants died at two and three weeks. It looked as though a plateau had not yet been reached and the copper level in the plant would continue to rise.

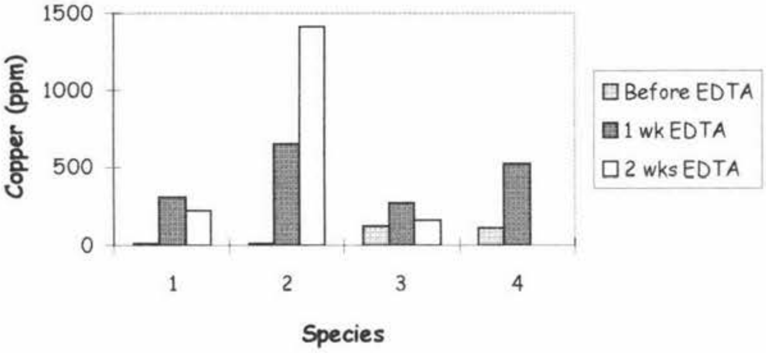


Figure 23: Graph showing effect of EDTA on the uptake of copper by *Haumaniastrum katangense* (1 and 3) and *Haumaniastrum robertii* (2 and 4).

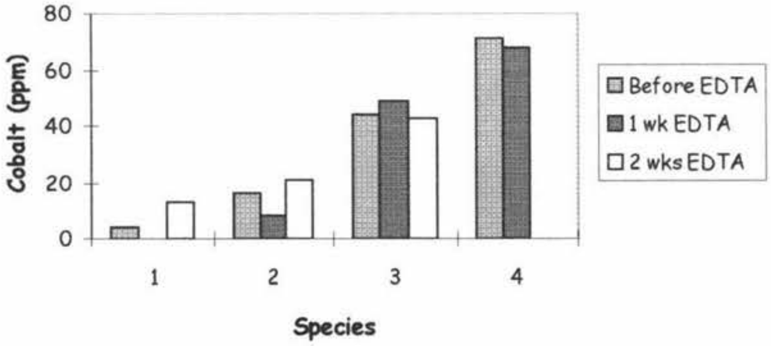


Figure 24: Graph showing effect of EDTA on the uptake of cobalt by *Haumaniastrum katangense* (1 and 3) and *Haumaniastrum robertii* (2 and 4).

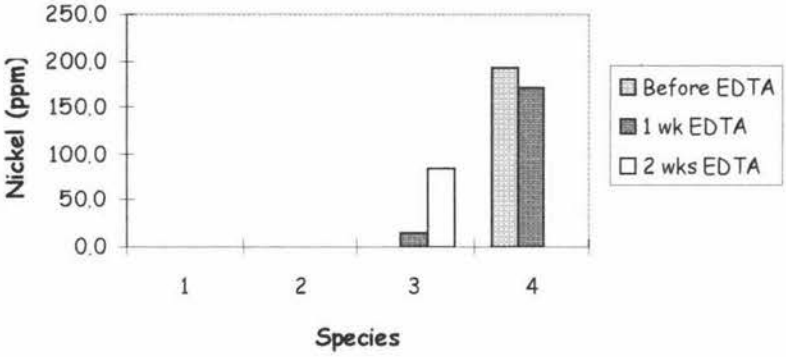


Figure 25: Graph showing effect of EDTA on the uptake of nickel by *Haumaniastrum katangense* (1 and 3) and *Haumaniastrum robertii* (2 and 4).

UPTAKE OF COPPER, COBALT AND NICKEL BY *HAUMANIASTRUM KATANGENSE GROWING IN UNITED ORE SUBSTRATE.*

	Copper			Cobalt			Nickel		
Treatment	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
Control	26	89	76	33	21	18	38	79	29
Control	55	43	57	48	16	23	109	73	33
0.7g/kg EDTA	60	73	145	61	16	22	36	59	34
0.7g/kg EDTA	35	55	70	25	43	30	67	43	61
1.3g/kg EDTA	71	167	130	54	21	33	38	70	48
1.3g/kg EDTA	68	97	824	56	41	17	49	74	39
2.0g/kg EDTA	91	123	577	43	32	24	44	101	38
2.0g/kg EDTA	88	148	311	70	24	15	66	26	25
3.2g/kg Citric	22	107	169	35	17	24	84	41	48
3.2g/kg Citric	25	162	215	24	35	19	55	74	15
6.7g/kg Citric	439	Dead	Dead	25	Dead	Dead	46	Dead	Dead
6.7g/kg Citric	32	71	135	36	18	8	50	64	19
10.0g/kg Citric	1078	Dead	Dead	50	Dead	Dead	102	Dead	Dead
10.0g/kg Citric	728	1044	473	50	55	31	56	114	44

Table 15: Data for uptake of copper, cobalt and nickel after the addition of EDTA and Citric Acid for *Haumaniastrum katangense* grown in United ore substrate.

Table 15 shows that EDTA and Citric Acid did not affect the uptake of cobalt and nickel by *H. katangense* but did increase the uptake of copper. Increasing amounts of EDTA and Citric Acid had greater effects on the uptake of copper (see figure 26) but also had the effect of killing the plant (especially Citric Acid). Addition of 10g/kg Citric Acid increased the uptake of copper to over 1000 μg/g, after which the plant died. Control plants took up an average of around 60 μg/g copper whereas plants treated with EDTA showed a 1.5 to 5-fold increase in copper uptake. Plants treated with Citric Acid showed an increase of 2.5 to 15 times that of the control.

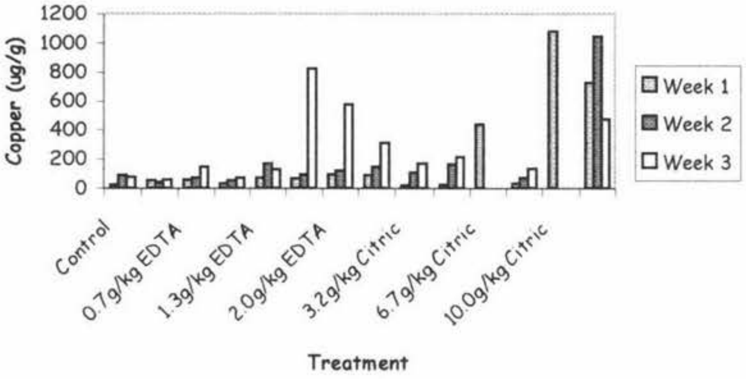


Figure 26: Graph showing effect of EDTA and Citric Acid on the uptake of copper by *Haumaniastrum katangense*.

UPTAKE OF COPPER, COBALT AND NICKEL BY *HAUMANIASTRUM ROBERTII GROWING IN UNITED ORE SUBSTRATE.*

	Copper			Cobalt			Nickel		
Treatment	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
Control	73	49	51	55	44	47	148	119	130
Control	92	40	37	89	11	68	167	150	259
0.7g/kg EDTA	155	75	Dead	82	62	Dead	225	77	Dead
0.7g/kg EDTA	96	127	187	64	10	52	142	162	51
1.3g/kg EDTA	72	181	207	59	31	34	115	93	96
1.3g/kg EDTA	114	174	330	101	23	8	81	85	131
2.0g/kg EDTA	212	113	274	69	55	35	85	104	98
2.0g/kg EDTA	69	35	40	35	43	36	94	132	67
3.2g/kg Citric	426	455	Dead	55	51	Dead	113	134	Dead
3.2g/kg Citric	367	222	485	50	42	64	94	109	142
6.7g/kg Citric	673	Dead	Dead	79	Dead	Dead	107	Dead	Dead
6.7g/kg Citric	693	Dead	Dead	57	Dead	Dead	119	Dead	Dead
10.0g/kg Citric	915	Dead	Dead	80	Dead	Dead	122	Dead	Dead
10.0g/kg Citric	1330	Dead	Dead	64	Dead	Dead	116	Dead	Dead

Table 16: Data for uptake of copper, cobalt and nickel after the addition of EDTA and Citric Acid for *Haumaniastrum robertii* grown in United ore substrate.

Table 16 shows that EDTA and Citric Acid had a similar effect on the uptake of cobalt and nickel (i.e no change) by *H. robertii* although this plant takes up larger amounts of cobalt and nickel than *H. katangense*. Copper level in the plants showed a marked increase with the addition of EDTA and in particular Citric Acid (figure 27). The control plants took up an average of 60 µg/g copper whereas plants treated with EDTA had a 2 to 3-fold increase in copper uptake. Plants treated with Citric Acid showed an increase of 7 to 18 times that of the control although high applications of Citric Acid had the effect of killing the plants.

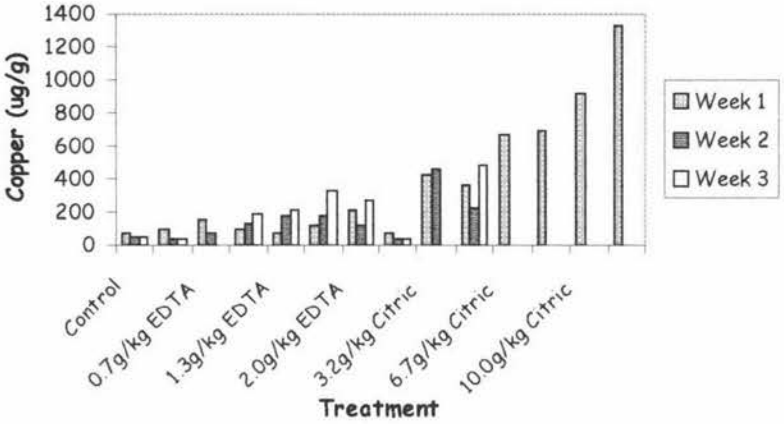


Figure 27: Graph showing effect of EDTA and Citric Acid on the uptake of copper by *Haumaniastrum robertii*.

COPPER, COBALT AND IRON UPTAKE BY HAUMANIASTRUM KATANGENSE GROWN IN CHAMPION ORE

TREATMENT	COPPER (µg/g)	COBALT (µg/g)	IRON (µg/g)
1	n.d	n.d	n.d
2	53	31	88
3	57	31	80
4	41	17	74
5	70	16	64
6	61	6	63
7	53	1	76
Control	17	2	89

Table 17: Mean values for copper, cobalt and iron uptake by *Haumaniastrum katangense* grown in Champion ore: peat/pumice (**1**. 1250 Cu, 37.5 Co; **2**. 625 Cu, 19 Co; **3**. 313 Cu, 9 Co; **4**. 156 Cu, 5 Co; **5**. 78 Cu, 2 Co; **6**. 39 Cu, 1 Co; **7**. 20 Cu, 0.6 Co; **8**. 0 Cu, 0 Co) n.d = plant did not survive.

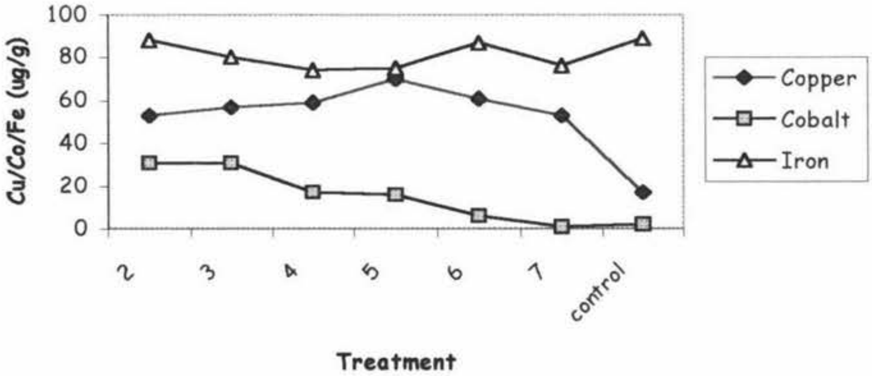


Figure 28: Graph showing mean copper, cobalt and iron values for *Haumaniastrum katangense* grown in 7 Champion:peat/pumice treatments + control (see table 17 for key).

Table 17 and figure 28 show that *Haumaniastrum katangense* did take up more or less copper and cobalt depending on the treatment it was growing in. None of the plants survived in treatment 1 which was a 1:2 mixture of ore: peat/pumice and contained 1250 μg/g copper, 37.5 μg/g cobalt and 250 μg/g iron. The ore is very fine, has poor drainage and tends to be sticky when wet and hard when dry. It is suspected that poor aeration was the reason that the plants did not survive in this treatment.

Cobalt uptake showed a steady decrease with reduction in the amount of cobalt in the substrate. Iron uptake remained fairly constant throughout the entire range of treatments. Copper uptake was fairly constant at between 50-70 μg/g but dropped in the control which had no added cobalt. Copper and cobalt uptake was not as high as expected but the level of copper in the plant was higher than that found in the substrate.

COPPER AND COBALT UPTAKE BY AEOLLANTHUS BIFORMIFOLIUS GROWN IN UNITED ORE

	COPPER (μg/g)			COBALT (μg/g)		
Treatment	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks
Control	10	10	14	23	26	18
0.7g/kg EDTA	7	57	39	32	5	18
1.3g/kg EDTA	89	314	432	24	32	33
2.0g/kg EDTA	55	67	432	13	21	22
3.2g/kg Citric	64	75	45	22	30	28
6.7g/kg Citric	52	197	33	22	18	21
10.0g/kg Citric	191	176	99	27	9	20

Table 18: Mean copper and cobalt uptake by *Aeollanthus biformifolius* grown in United ore after the addition of EDTA and Citric Acid.

Table 18 shows that the cobalt taken up by *Aeollanthus biformifolius* stayed relatively constant (around 20 μg/g) throughout all the treatments and did not increase with time from the addition of EDTA and Citric Acid. The copper uptake did increase with increasing amounts of EDTA and Citric acid (figure 29). The copper uptake increased each week in the EDTA treatments but tended to decrease after 2 weeks of Citric acid. Citric acid also had the effect of killing the plants when it was applied at rates of 6.7 and 10 g/kg.

The chelating agents did not increase the uptake of copper by *A. biformifolius* to the extent that they did with *H. katangense* and *H. robertii.*

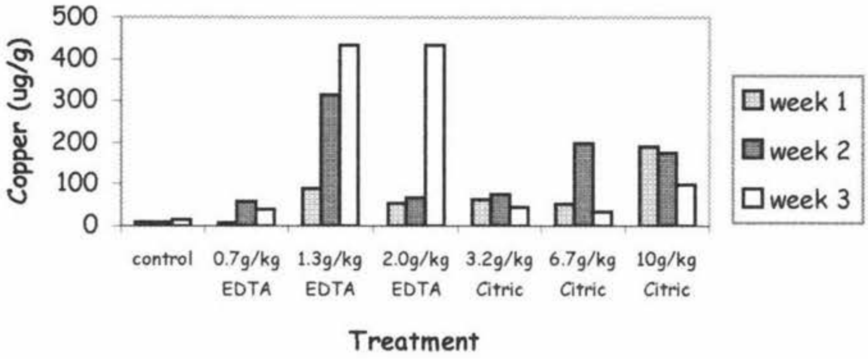


Figure 29: Graph showing mean copper uptake by *Aeollanthus biformifolius* after the addition of EDTA and Citric Acid.

Copper concentrations in ten Andévalo soils in which *Erica andevalensis* was growing are shown in Table 19. They range from 23 to 3676 μg/g, a wide fluctuation. By contrast, iron levels remained relatively constant at around 6000 μg/g (0.6%).

COPPER AND IRON CONCENTRATIONS IN ANDÉVALO SOILS

NUMBER	COPPER	IRON	COPPER/IRON
1	375	5854	0.064
2	547	5883	0.093
3	515	5960	0.086
4	3676	5936	0.620
5	2034	6320	0.320
6	1668	6464	0.250
7	23	6094	0.0038
8	216	5854	0.036
9	177	5638	0.031
10	191	5854	0.032

Table 19: Copper and iron concentrations (μg/g) in mineralised soils from the Andévalo, Huelva Province, Spain.

The copper/iron concentration quotients are also shown to assist in calculation of potential contamination of plant material by wind-blown soil particles. There was no apparent correlation between the copper content of plants and soils. This is not expected because elements that are essential to plant nutrition tend to be internally rather than externally controlled (Timperley *et al.*, 1970). This species is nevertheless a good geobotanical indicator of copper mineralisation because it appears to be confined to this form of mineralisation.

Table 20 shows copper and iron concentrations in *Erica andevalensis* collected at the same sites as the soil samples.

COPPER AND IRON CONCENTRATIONS IN *ERICA ANDEVALENSIS* FROM THE ANDÉVALO

NUMBER	TOTAL COPPER	IRON	CORRECTED COPPER*
1	30	331	2
2	42	588	15
3	43	703	8
4	24	214	24
5	21	239	21
6	28	327	11
7	13	436	12
8	16	94	16
9	10	643	1
10	12	119	12

Table 20: Copper and iron concentrations (μg/g dry weight) in specimens of Andévalo *Erica andevalensis*. *Corrected values calculated by assuming that any iron concentration >300 μg/g in plant material is due to windborne contamination from soil. The copper content of this excess iron is calculated from column 4 in Table 19 and removed from the original total copper in the plant.

The results of pot trials with *Erica andevalensis* grown in Champion ore mixed with seed mix in varying proportions are shown in Table 21. Because of the very high copper content of the ore (2.5%), even a very slight degree of contamination from the soil would greatly affect the apparent copper content of the plant material. The iron content of the plant material was determined and any inordinately high levels were assumed to be due to contamination. Knowing the copper/iron quotient of the ore, it was possible to correct these suspicious copper values.

MEAN COPPER AND IRON CONCENTRATIONS IN *ERICA ANDEVALENSIS* GROWN IN CHAMPION ORE/SEED MIX

Treatment	Copper in substrate	Iron in plants	Apparent Cu in plants	Corrected Cu in plants
Control	10	28	4	4
32:1	781	30	4	4
16:1	1562	254	55	44
8:1	3125	332	79	56
4:1	6250	861	310	215
1:1	12500	877	432	380

Table 21: Copper and iron concentrations (μg/g dry weight) in plants of *Erica andevalensis* grown in varying admixtures of Champion ore and seed mix.

The data are also shown in Figure 30. The shape of the curve is illustrative of the behaviour of non-accumulator plants to elements essential in plant nutrition. As elemental concentrations in the substrate increase, the plant's internal control system is able to restrict uptake of the metal in question until a threshold is reached where the control mechanism starts to break down and unrestricted uptake occurs and is terminated by death of the plant at the limit of tolerance.

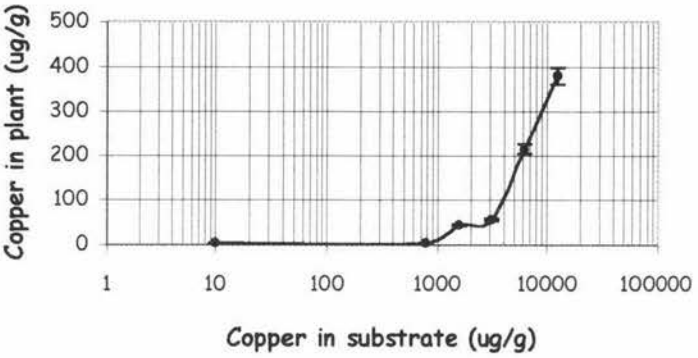


Figure 30: Graph showing copper in *Erica andevalensis* grown in various admixtures of Champion ore and seed mix.

Experiments similar to the above were carried out in which the same plant was grown in various admixtures of Champion ore and a 1:1 (v/v) mixture of peat and pumice. The results are shown in Table 22. It is apparent from this table that copper concentrations in plant material remain low and virtually unchanged despite an increase in the copper content of the substrate. This reflects the effect of the peat with which copper readily complexes and renders this element virtually unavailable to plants. The apparent tolerance of *Erica andevalensis* is therefore much greater than with the previous substrate.

MEAN COPPER AND IRON CONCENTRATIONS IN *ERICA ANDEVALENSIS* GROWN IN CHAMPION ORE: PEAT/PUMICE

TREATMENT	CU IN SUBSTRATE	CU IN PLANT	FE IN PLANT
Control	7	3	53
1:160	156	10	94
1:80	312	14	36
1:20	1250	4	103
1:10	2500	3	41
1:5	5000	1	52

Table 22: Copper and iron concentrations (μg/g dry weight) in *Erica andevalensis* grown in admixtures of champion ore and a 1:1 peat/pumice mixture.

GENERAL DISCUSSION

The plants grown in Tui tailings either did not survive or did not grow as well as plants grown in the two ores from the South Island. The tailings have a fine silt texture with a poorly developed structure and impeded drainage. When wet, the tailings are water-logged and sticky and when dry they harden. This creates anaerobic conditions which limits plant growth and soil organisms (also impeded by the acidity and the heavy metal content). Pumice or bark was mixed with the tailings to try to improve the structure and hence the drainage. The pumice did not in fact improve the structure because it was too fine and the plants died, probably due to the lack of oxygen. The bark improved the structure enough for plant growth although drainage was still impeded as shown by the growth of algae on the soil surface.

The United ore had a much better structure and the plants grew well with no algae present on the surface of the substrate. Plants grown in the artificial substrates were attacked by white fly (and consequently sprayed with insecticide) whereas the plants grown in the ores were not attacked. It is suspected that this could be due to the higher levels of metals in the leaves of the plants grown in the ores and that these higher metal levels also inhibit the growth of algae.

The uptake of cobalt by each species stays roughly the same before and after the addition of EDTA but the amount taken up depends on the amount of cobalt in the substrate and how much is in a form that is available for plant uptake. The plants growing in the artificial cobalt substrate did not take up a large amount of cobalt. Identical plants growing in the United ore substrate took up larger amounts which would suggest that this ore contained more cobalt.

Plants growing in the artificial substrates took up less than 1 μg/g nickel even after the addition of EDTA which might indicate that these substrates do not contain very much (if any) nickel. The United ore contains much more nickel and EDTA appears to have the affect of increasing nickel uptake.

Addition of EDTA has the affect of increasing the uptake of copper by all the plants although *H. katangense* took up less copper than *H. robertii* and *Aeollanthus biformifolius* took up less copper than both these species.

It would appear that growing in their natural habitat, both *H. katangense* and *H. robertii* do not take up a detrimental amount of copper but that the addition of EDTA encouraged *H. robertii* to take up an increasing amount of copper until it killed the plant. *H. katangense*, however, was able to put a limit on its uptake. Malaisse and Grégoire (1979) however observed that *H. katangense* in the wild took up much more cobalt (864μCo) while Brooks *et al.* (1980) observed that *H. katangense* in the wild took up 2140 μg/g Cu and 2240 μg/g Co and Duvigneaud *et al.* (1963) observed that *H. robertii* in the wild took up 1960 μg/g Cu and 10200 μg/g Co. This conflicting evidence with the above test results might indicate that it was their respective tolerance for EDTA that affected the growth and vitality of the plants. This may also relate to the amount and kind of organic matter present in the substrate. Bark and seed mix may interfere differentially with metal complexing and availability.

The *Erica andevalensis* plants would not grow in 100% Champion ore (2.5% Cu) but were able to tolerate copper concentrations half of this level (12500 μg/g). this degree of copper tolerance is almost unequalled in the plant kingdom and is paralleled only in the specialised copper-tolerant flora of The Democratic Republic of Congo (Zaïre) (Brooks *et al.* 1992).

The apparent dichotomy between the two sets of data (*E. andevalensis* grown in ore: seed mix and the same plant grown in ore: peat/pumice) clearly emphasises the importance of assessing tolerance to heavy metals on the basis of the plant-available fraction rather than on the total heavy metal concentration. It is probable that in the Andévalo, the copper is tightly bound into organic matter because the plant will tolerate apparently very high levels of heavy metals in the damp humus-rich environments in which they grow. For example Nelson *et al.* (1985) found it growing in evaporates with as much as 1500 μg/g copper.

Erica andevalensis is clearly not a hyperaccumulator (>1000 μg/g) of copper. Uptake of copper follows the pattern of plant-essential elements where uptake is restricted until the threshold of metal tolerance is exceeded. Thereafter there is a rapid exponential increase over a short range until the plant will no longer grow in the copper-rich substrate. The copper content of the plant does not reflect the copper content of the substrate until the tolerance threshold is exceeded therefore *Erica andevalensis* has no use for biogeochemical prospecting. The plant however is an excellent geobotanical indicator of copper mineralisation as it appears to be restricted to copper-rich substrates.

6 REALITY OF HYPERACCUMULATORS

Professor J.A.C. Smith of Oxford University (Pers. comm. to R.R. Brooks) has alleged that hyperaccumulation of copper and cobalt does not in fact exist, at least in the case of *Haumaniastrum katangense*. This doubt has been cast on the hyperaccumulation of copper and cobalt because the reported high values (Brooks, 1980 and Malaisse *et al.*, 1979) could not be reproduced in his laboratory.

The results from my experiments indicate that these plants do not take up excessive amounts of copper and cobalt. In the substrates used, *Haumaniastrum katangense* took up a maximum of 128 μg/g copper and 44 μg/g cobalt, *Haumaniastrum robertii* took up a maximum of 115 μg/g copper and 71 μg/g cobalt and *Aeollanthus biformifolius* took up a maximum of 14 μg/g copper and 26 μg/g cobalt. These results concur with Smith's doubt as to the reality of hyperaccumulators of copper and cobalt. However, after the addition of chelating agents *H. katangense* took up a maximum of 1078 μg/g copper and 70 μg/g cobalt, *H. robertii* took up a maximum of 1415 μg/g copper and 101 μg/g cobalt and *A. biformifolius* took up a maximum of 432 μg/g copper and 33 μg/g cobalt.

It is evident that the addition of chelating agents increases the uptake of metals significantly, but nevertheless these results are much lower than values reported in the field, by Brooks in 1980 (*H. katangense* 8400 μg/g copper and 2200 μg/g cobalt; *H robertii* 2000 μg/g copper and 10200 μg/g cobalt) and Malaisse in 1979 (*A. biformifolius* 13700 μg/g copper and 4300 μg/g cobalt).

My experiments did indicate that *H. katangense* and *H. robertii* suffered no obvious harm when their metal uptake was facilitated by addition of chelating agents, ready availability of metals and ideal soil structure. This confirms their reality as hyperaccumulators but there are limiting factors to their efficiency.

During my experiments, the iron content of the plants was measured along with the copper and cobalt content as this element can serve as an indication of the probable contamination of plant samples by soils and other substances. Any iron value over 300 μg/g was considered to be due to windborne contamination by soil because vascular plants do not usually accumulate more than 300 μg/g in their leaves.

High levels of iron also accompanied the high values of copper and cobalt reported by Malaisse and Grégoire (1979) and it is suspected that this is due to contamination which would cast doubt on the validity of their results. If the ratio of iron to copper and cobalt is calculated the actual values of copper and cobalt can be determined. Original results with iron levels over 300 µg/g were recalculated to take into account the iron, copper and cobalt present in the soils (see Table 23 below).

Calculation for a soil with:

Copper = 36000 µg/g (3.6%) Then: 1%Fe=0.95%Cu
Cobalt = 15000 µg/g (1.5%) 1%Fe=0.40%Co
Iron = 37000 µg/g (3.7%)

So for *A. biformifolius* with 0.93%Fe = 0.93*0.95 = 0.8857 copper
= 0.93*0.40 = 0.3690 cobalt

Corrected values: Copper = 13700 - 8857 = 4843 µg/g copper
Cobalt = 4300 - 3690 = 610 µg/g cobalt

Table 23 below shows the iron, copper and cobalt values reported by Malaisse and Grégoire in 1979 and the corrected values for copper and cobalt. The corrected values are a lot lower but the values are still high enough that the plants can be considered hyperaccumulators.

Species	Fe in plant	Apparent Cu in plant	Corrected Cu in plant*	Apparent Co in plant	Corrected Co in plant*
Aeolanthus biformifolius	9300	13700	**4840**	4300	**610**
Lindernia perennis	2000	6000	**5290**	2300	**1880**
Vigna dolomitica	3800	3000	**1640**	600	**<1**
Eragrostis boehmii	9000	2800	**<1**	600	**<1**
Justicia elegantula	2160	864	**	246	**
Lindernia damblonii	760	800	**530**	1000	**841**
Lindernia damblonii	-	650	650	1000	1000
Faroa chalcophila	780	665	**<1**	382	**72**
Hibiscus rhodanthus	1480	590	**<1**	170	**<1**
Hibiscus rhodanthus	810	500	**<1**	170	**<1**
Buchnera metallorum	550	460	**264**	500	**385**
Anisopappus hoffmannianus	420	370	**220**	700	**612**
Rendlia cupricola	300	280	**173**	160	**97**
Rendlia cupricola	80	100	100	50	50
Rendlia cupricola	1583	640	**75**	233	**<1**
Triumfetta digitata	532	306	**116**	161	**50**
Thunbergia oblongifolia	537	300	**	82	**
Triumfetta welwitschi	637	239	**12**	75	**<1**
Haumaniastrum katangense	380	233	**97**	864	**784**
Acalypha cupricola	438	221	**213**	48	**46**
Pteris vittata	532	200	**<1**	73	**<1**
Monocymbium ceresiiforme	130	160	160	50	50
Monocymbium ceresiiforme	60	85	85	30	30
Drimiopsis barteri	180	140	140	400	400
Drimiopsis barteri	306	90	**	21	**
Adenodolichos rhomboideus	232	109	109	14	14
Becium peschianum	224	103	103	164	164
Eriospermum abyssinicum	225	96	96	22	22
Droogmansia munamensis	305	95	**	30	**
Tecoma stans	144	70	70	traces	traces
Cryptosepatum maraviense	183	51	51	15	15
Cryptosepatum maraviense	-8	21	21	15	15
Polygata petitana	50	40	40	130	130
Tristachya thollonii	50	25	25	traces	traces

Table 23: Iron, apparent copper and cobalt and corrected copper and cobalt values (μg/g) for selected plant species from Shaba, Democratic Republic of Congo (Zaïre). *Corrected values calculated by assuming that any iron concentration >300 μg/g in plant material is due to windborne contamination from soil. The copper and/or cobalt content of this excess iron is calculated and removed from the original total copper and/or cobalt in the plant. **No soil data available for this plant.

To further test the hypothesis that these plants may not be hyperaccumulators herbarium specimens collected by R.R. Brooks from the Shaba province, Democratic Republic of Congo (Zaïre) were analysed for copper, cobalt and iron. The results are listed below in Tables 22 and 23.

RESULTS OF ANALYSIS OF HERBARIUM SPECIMENS

Locality	Family	Species	Copper	Cobalt	Iron
Kasonata	*Poaceae*	*Bulbostylis mucronata*	2857	5	696
Kasonata	*Asteraceae*	*Anisopappus davyii*	450	6	116
Kasonata	*Scrophulariaceae*	*Lindernia damblonii*	265	3	173
Lupoto	*Scrophulariaceae*	*Sopubia neptunii*	49	3	36
Lupoto	*Asteraceae*	*Vernonia petersii*	222	5	48
Mine de l'Etoile	*Fabaceae*	*Vigna dolomitica*	206	36	101
Mine de l'Etoile	*Scrophulariaceae*	*Lindernia perennis*	406	207	58
Luishia	*Cyperaceae*	*Bulbostylis spp.*	306	200	157
Kamoya	*Lamiaceae*	*Aeollanthus spp.*	45	3	64
Fungurume	*Lamiaceae*	*Haumaniastrum robertii*	62	2316	39
Kasonata	*Lamiaceae*	*Haumaniastrum katangense*	211	23	194
Mindigi	*Caryophyllaceae*	*Silene cobalticola*	36	1	72
Mindigi	*Lamiaceae*	*Aeollanthus spp.*	218	1641	338
Shinkolobwe	*Asteraceae*	*Anisopappus spp.*	1285	37	194

Table 24: Analyses of herbarium specimens collected by R. R. Brooks in Shaba, Democratic Republic of Congo (Zaïre) in 1992.

These analyses (Tables 22 and 23) have reasonably low iron values, which indicates that the samples are not contaminated, but the copper and cobalt values are not especially high. The highest copper value in Table 24 was 2857 μg/g for *Bulbostylis mucronata* but the iron value for this plant was 696 μg/g indicating contamination. This plant is a small, low growing reed, which is quite easily contaminated with soil particles. The next highest copper value is 1285 μg/g for a species of *Anisopappus* which had an iron content of 194 μg/g. This copper value can be taken as a true indication due to the low iron value.

The highest cobalt value was for *Haumaniastrum robertii* (2316 μg/g) and this also was accompanied by a low iron value (39 μg/g). All the other values are quite low compared with the values reported by Malaisse (1979) and Brooks (1980) although the plants having been sourced from different localities could in part explain this. It can be concluded from these analyses that the original data represented some degree of contamination and the maximum copper and cobalt values are in fact appreciably lower for many of these plants.

More analyses would have to be done to determine which plants are hyperaccumulators but these results suggest that *Haumaniastrum robertii* and possibly *Aeollanthus spp.* (probably *biformifolius*) are hyperaccumulators of cobalt and *Anisopappus spp.* (probably *davyii*) is a hyperaccumulator of copper. The corrected data from Malaisse and Grégoire's original analyses suggest that *Aeollanthus biformifolius* and *Vigna dolomitica* are hyperaccumulators of copper and *Lindernia perennis* is a hyperaccumulator of both copper and cobalt.

Species	Iron	Copper	Cobalt
Haumaniastrum homblei	17	3	12
Haumaniastrum robertii	29	8	403
Aeollanthus biformifolius	629	51	100
Aeollanthus saxatilis	34	3	137
Haumaniastrum robertii	49	16	825
Haumaniastrum robertii	20	20	13
Haumaniastrum robertii	8	5	74
Haumaniastrum robertii	11	12	123
Haumaniastrum robertii	16	11	173
ETOILE Liliaceae	16	6	21
Haumaniastrum homblei	59	6	318
KARAVIA Physalis	78	148	15
KARAVIA Celoniatrigyne	335	192	29
Crotalaria cobalticola	19	19	166
SWAMBO Aeollanthus	22	13	146
CHABARA Asteraceae	10	9	320
KWATEBALA Astereceae	7	3	284
Cyanotis longiflora	35	145	412
KWATEBALA Asteraceae	7	6	85
LUMISBUSHI Liliaceae	19	5	311
Anisopappus davyii	4	9	98
FUNGURUME Astereceae	45	2	312
MINDINGI Anisopappus	82	32	141
Alectra welwitschii	95	118	280
Bulbostylis mucronata	311	665	237
LUPOTO Acalyphapcicola	26	74	3
Triumfetta digitala	3	99	9
KARAVIA Vernonia	39	133	50
Panalioka metallorum	16	24	8
Anisopappus hoffmanianus	60	130	66

Table 25: Analyses of herbarium specimens collected by R. R. Brooks in Shaba, Democratic Republic of Congo (Zaire).

DISCUSSION

In my original hypotheses I postulated the following results:

A. The growth of the plants could in fact be genetically determined and largely unaffected by nutrient levels above the basic minimum.

B. The growth of the plants could be limited by the 'metal stress' they suffer through growing in such an extreme environment - the cost-benefit of their adaptation.

C. The growth of the plants could be considerably improved by an amelioration in the nutrient levels available to them.

D. The addition of extra nutrients to the soil could theoretically render less metals available for uptake by the plants and thus leave them susceptible to attack by pathogens.

E. The biomass of the plants could be considerably increased by the addition of fertiliser, but the rate of metal uptake could diminish, remain constant or increase.

F. The further addition of chelating agents to the soil could possibly increase the absorption of a) the metals, b) the nutrients, c) both or d) neither.

I shall now briefly discuss where my research has proven or falsified each of these hypotheses.

A. My experiments showed that the growth of the plants could be increased through the addition of fertiliser so it would appear that low nutrient status of the substrate is the limiting factor on plant growth of these hyperaccumulators rather than any inherent characteristic. There is good potential for increasing the biomass of these plants by adding nutrients and with little concomitant decrease of metal concentration, thus making them more suitable for phytoremediation.

B. The experiments have shown that the plants would not grow in certain substrates. This was due mainly to the physical structure of the substrate (which impedes drainage and causes anaerobic conditions) rather than the high metal levels. In one case, however, it appears to be the high level of metals in the substrate which restricted growth. *Erica andevalensis* would not grow in 100% Champion Ore due to the very high copper level (2.5%). All the plants used in these experiments tolerated very high apparent metal concentrations but these metals are quite tightly bound to the soil so the plant available metal content was much lower.

C. Increasing the nutrient levels available to the plants considerably improved their growth although it is clear that the level of fertilisation was still suboptimal even at the upper level of amendment used.

D. Martens and Boyd (1994) have shown that *Streptanthus polygaloides* is acutely toxic to potential herbivores (Lepidopteran larvae and grasshoppers) and that fungal and bacterial pathogens are deterred by nickel hyperaccumulation. This has also been demonstrated in these experiments where it was observed that plants growing on substrates with low plant availability of metals were attacked by white-fly whereas plants growing in ores took up higher amounts of metal and were unaffected by the insects. The plants growing in substrates with low plant-available metals also had a higher death rate and it can be postulated that this was due to attack by pathogens. It did not appear that the addition of nutrients increased their susceptibility to pathogens.

E. The rate of metal uptake was not affected by the addition of nutrients although, because the plants grew larger the metal content was somewhat diluted. The larger plants took up metals at the same speed as smaller plants so the percentage nickel content was lower in the high biomass plants but the overall nickel content was higher.

F. The chelating agents used in this experiment (EDTA and Citric Acid) had the effect of increasing the uptake of copper and lead but did not affect cobalt, zinc and cadmium. These experiments have exemplified a well known fact that it is not only the available metals in the soils but the soil structure, organic matter content and chelating agents that can affect the uptake of those same metals. The amount of metal available in the soil (rather than total metal content) correlates quite closely to the amount of metal taken up by the plants.

The most useful information achieved by these results are summed up by hypotheses A, D and F as these would help improve the suitability of hyperaccumulators for phytoremediation and phytomining. The uptake of metals by plants is determined not by the total metal content of the soils but by other factors concerning the soil. Organic matter, clays and other soil particles can bind the metals and make them unavailable for plants. Soil amendments have good potential for increasing metal availability and the addition of nutrients has good potential for increasing the growth of the plants.

There is a good possibility that the previously reported values for copper and cobalt are in some cases erroneous. This research has shown that although the copper and cobalt uptake is lower than previously thought hyperaccumulation of copper and cobalt does in fact exist at least in the case of *Anisopappus spp.* (copper), *Haumaniastrum robertii*, and *Aeolanthus spp.* (cobalt). At this stage all other results are suspect and should be redetermined by analysis for iron.

Future research should determine the actual copper and cobalt content of the Shaban soils that is available to plants and retest the Shaban plants under contaminant-free conditions to ascertain the actual copper and cobalt uptake by these plants. This would indicate which of these plants are in fact hyperaccumulators.

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