Aspects of the Accumulation of Cobalt, Copper and Nickel by Plants

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Abstract

Hyperaccumulation of heavy metals was studied with the intention of elucidating the mechanisms of tolerance of hyperaccumulator plant species. Two main areas are covered; cobalt and copper accumulation by plants from Shaba Province, Zaïre, and nickel accumulation by species of the genus Alyssum.

In surveys of vegetation of metalliferous soils of Shaba, nine or ten new hyperaccumulators of cobalt were discovered along with eight or nine very strong accumulators. For copper, seven hyperaccumulators and five or six very strong accumulators were discovered. Some families contained a higher frequency of hyperaccumulators than others. There is also a difference in superorder classification of cobalt and copper hyperaccumulators on one hand and nickel hyperaccumulators on the other. Surveys of the genera Aeolanthus, Ipomoea and Pandiaka were made but only one new copper hyperaccumulator was found: no new cobalt hyperaccumulators were found. Several species had their abilities to accumulate confirmed.

Pot trials on three hyperaccumulators Aeolanthus biformifolius, Haumaniastrum katangense and H. robertii, showed accumulation of cobalt but not the expected accumulation of copper. The uptake curve was of the exclusion-breakdown form. The limit of breakdown, for each metal, was similar from species to species. Cobalt was less readily excluded than copper. The tolerance tests showed that some species have individuals with greatly enhanced abilities to survive higher metal concentrationsthan is normal for that species, while other species have more uniform tolerances. There appears to be no requirement for large metal concentrations at germination and seeds germinate more readily in the absence rather than the presence of the metals.

The distribution of cobalt and copper within leaf tissues, of five species, appears to be parallel within each species. For each metal, the distribution is parallel between different species with the exception of Buchnera metallorum. More detailed studies on cobalt in H. robertii showed the distribution to be even over the leaf area but with small anomalous regions of high concentration. The possibility that some of the cobalt was precipitated as oxalate crystals is considered. The water-soluble cobalt fraction ligand could not be identified but was not proteinaceous. It has a mass of 5,200 g per mole of cobalt.

A survey of the genus Alyssum revealed thirty-four taxa as hyperaccumulators to add to the fourteen previously known. All the taxa are from section Odontarrhena. The geographical distribution of the hyperaccumulators is discussed as is the possible evolution of hyperaccumulators in subsections Compressa and Samarifera from non-accumulators within them.

Studies of nickel accumulation by eleven Alyssum species and the closely related Bornmuellera tymphaea showed similar characteristics for all hyperaccumulators but two non-accumulators differed. A rise-to-saturation uptake form was noted. In the absence of nickel, cobalt could be accumulated with a similar uptake form. Cobalt accumulation in the presence of nickel is unknown. The rate of uptake is relatively rapid. The tolerance of hyperaccumulators to high nickel concentrations was confirmed in two types of tolerance tests; a substrate medium test and a solution test. The results from the two tests are compared.

The distribution of nickel between the plant organs is discussed. The analysis of mineral elements in leaf material showed interesting differences between hyperaccumulators and non-accumulators for calcium, magnesium and manganese content but these could not be related to differing nickel concentrations. A similar find was made for glucosinolates. An organic acid survey was restricted by the non-identification of many acids.

Separation of the nickel complexes was made. Identification of ligands involved in nickel complexation was attempted but few positive results were found. Two ligands were common in significant quantities for all species studied.

The results of these experiments were used to discuss possible evolution of hyperaccumulator species both in terms of their superorder distribution and their method of metal ion uptake. An equilibrium mechanism of uptake is proposed which involves a multiplicity of complexes for the ion absorbed. The mechanism differs from that which is commonly proposed for micronutrient elemental uptake.

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CHAPTER 1

Introduction

The occurrence of characteristic plants on metal-contaminated soils has been observed for centuries. Thalius (1588) noted that Minuartia verna (L.) Hiern. was an indicator of metal contamination while Caesalpino (1583) noted a species of plant (probably Alyssum bertolonii Desv.) restricted to serpentine rocks in Italy. Agricola (1556) had previously described the anomalous appearance of plants growing over veins and metal ore outcrops. Interest in the plants of these anomalous soils has continued for several reasons: (1) to identify the methods by which the plant is able to adapt to the toxic environment and associated with this;(2) how to make fertile or productive those areas covered by these soils; (3) the use of these plants for geobotanical and biogeochemical prospecting; (4) the interest in very high concentrations of metals in plants and (5) the associated possibility of low-energy extraction of metals from their ores by imitation of nature. Those plants which are restricted to growing on these soils are called metallophytes. However, many variations on the basic description of this term have been used by different authors. Antonovics et al. (1971) have discussed this range of variations. For the purposes of this thesis, a metallophyte is taken as a species of plant with a high tolerance to elevated heavy metal contents in the soil. Such a species is often endemic to those soils which contain elevated quantities of the heavy metals. Most metallophytes survive on metal-rich soils by excluding the metal from entry into the plant. However, there exists a range of species, called accumulators, which accumulate the metal within their tissues. These species must have developed a form of physiological tolerance to the metal absorbed. Metals which have been found in high concentrations in plant tissues but which are normally toxic to plants, at these levels, include aluminium (Chenery, 1948, Moomaw et al., 1959, Jones, 1961,

Chenery & Sporne, 1976), chromium (Lyon et al., 1968, Wild, 1974, Jaffré, 1980), lead (Nicolls et al., 1965, Lag et al., 1969, Shimwell & Laurie, 1972, Johnston & Proctor, 1977, Simon, 1978, Barry & Clark, 1978, Crooks, 1979), manganese (Denaeyer-de-Smet, 1966, Jaffré, 1977, 1979, 1980), zinc (Nicolls et al., 1965, Ernst, 1965, Shimwell & Laurie, 1972, Johnston & Proctor, 1977, Barry & Clark, 1978, Simon, 1978, Crooks, 1979), cobalt, copper and nickel (see detailed discussion below).

The discovery of this metal accumulation by plants has led to biogeochemical and phytochemical investigations of this property. In general these studies have investigated plant-soil relationships, the tolerance of the species to the metals, the distribution and nature (ionic, soluble complex, bound complex, etc.) of the metal in the plant, inter-elemental relationships within the plant and the use of these species for biogeochemical prospecting. Biogeochemical prospecting was developed by Tkalich (1938) and Brundin (1939) and involves analysing plant material for the mineral elements to get an indication of the elemental content of the substrate in which it grew. Reviews of this method can be found in Cannon (1960), Malyuga (1964) and Brooks (1972). General reviews of heavy metals in plants and of metallophytes in general can be found in Bollard and Butler (1966), Antonovics et al., (1971), Proctor and Woodell (1975), and Foy et al., (1978).

(a) Nickel

The toxicity of nickel to plants has been known since the work of Haselhoff (1893). The main symptoms of nickel poisoning are chlorosis or yellowing of the leaves followed by necrosis. Other symptoms recorded include stunting of growth, unusual spottings, growth deformities and either stunting or expansion of the root system (Mishra & Kar, 1974). In extreme cases death of the plant may follow.

Despite this known toxicity of nickel, the cause of toxicity of serpentine soils is far from known. As well as nickel, cobalt and chromium are present in high levels and both are also toxic to plants in high concentration. The cause of toxicity of these metals is believed to be interference with, and poisoning of, enzymes (Bowen, 1966). The calcium/magnesium ratio has been cited as a possible cause of toxicity because of the low calcium, high magnesium content (Loew & May, 1901). However low levels of magnesium have been recorded for tropical serpentine soils (Birrell & Wright, 1945) so that the ratio hypothesis has been modified (Vlamis & Jenny, 1948) to one of low soil saturation by calcium. The low soil saturation makes alternative cations more readily available for uptake. Low levels of major nutrients, nitrogen, phosphorus and potassium, have also been observed in serpentine soils. Agricultural practices of fertilizing serpentine soils with these elements have however failed to make the soil fertile. A comparison of these latter two possibilities as causes of the infertility can be found in Raven et al., (1976). Other possible toxicity causes have also been recorded: Gordon and Lipman (1926) suggested soil alkalinity but although their Californian serpentine soils are alkaline, most are not and this appears unlikely to be a general cause; Walker (1948) suggested low molybdenum levels but later information suggested that it was unlikely to be a dominant cause (Walker, 1954). Physical characteristics of these soils do not appear to be a cause of an unfavourable growth environment (Robinson et al., 1935). Given the complexity and variety of serpentine environments, it is highly unlikely that any single cause for the toxicity will be found.

Biogeochemical prospecting for nickel has been studied by Lyon et al., (1968), Timperley et al., (1970a, 1972a, b), Severne (1972), Severne and Brooks (1972), Cole (1973), Lee et al., (1977a), Brooks and Wither (1977) and Wither and Brooks (1977).

In 1977, Brooks, Lee et al. proposed the term hyperaccumulator for those plants which contain nickel concentrations greater than 1,000 µg Ni/g of dry leaf material. Plants having concentrations of 100-1,000 µg/g were termed strong accumulators. It should be noted that these terms are a statement of a concentration level relative to "normal" (non-enriched) levels in plant material and bear no relationship to nickel concentrations in the substrate. "Normality" is however rather difficult to define: on "normal" (low-nickel) soils the concentration of nickel in the plant (on a dry weight basis) seldom exceeds 5 µg/g (mean approx. 0.5 µg/g) but on nickel-enriched (generically termed "serpentine") soils eg. of serpentinitic and peridotitic parent material, "normal" plant concentrations are 25-50 µg/g and may easily reach 100 µg/g. To add further to the confusion many "normal" (non-tolerant) plants will not grow on nickel-enriched substrates. Indeed it is common to be able to recognize these enriched areas by the sharp discontinuity between the vegetation on and off them.

Hyperaccumulation of nickel was first reported in the Crucifer Alyssum bertolonii Desv. in 1948 (Minguzzi & Vergnano) during a survey of the vegetation of serpentine outcrops at Impruneta, near Florence, Italy. The specimen collected had a nickel concentration of 12,000 µg/g (1.2%) in its leaves. The second recording of a hyperaccumulator was made for another member of this genus; A. murale Waldst. & Kit. (Doksopulo, 1961). Although he reported the nickel concentration on an ash weight basis (over 10%) the species is clearly a hyperaccumulator. In 1969 a third taxon within this genus was discovered : A. serpyllifolium Desf. ssp. lusitanicum T.R. Dudley & P. Silva (Menezes de Sequeira, 1969). This taxon, confined to serpentine soils around Braganca, northeastern Portugal, is a subspecies of a widespread southwestern European and North African species. The first non-Alyssum to be discovered as a hyperaccumulator was Dicoma niccolifera Wild (Wild, 1971, earlier as Dicoma macrocephala ssp., Wild, 1970). The highest nickel concentration recorded for this Zimbabwean species was 2,100 µg/g, dry weight.

Since the mid-seventies, the list of hyperaccumulators has grown rapidly through the work of R.R. Brooks and his co-workers. Their first discovery of a nickel hyperaccumulator was Hybanthus floribundus (Lindl.) F. Muell. (Severne & Brooks, 1972). Although this was the first published reference to this taxon, Cole (1973) had made the discovery earlier but had delayed publishing. The nickel content of this species often exceeds 1%. All three subspecies of this species (described by Bennett, 1969) are hyperaccumulators (Severne, 1972).

In 1974 the number of hyperaccumulators more than doubled. In Zimbabwe (then Rhodesia), Wild (1974) reported very high nickel levels in the ash of Pearsonia metallifera Wild. Lee (1977) gives a dry leaf concentration of 1.06% for this species. The vast serpentine soil complexes of New Caledonia supplied the other species : Geissois pruinosa Brongn. & Gris., Homalium guillainii (Vieill.) Briq., Hybanthus austrocaledonicus Schinz. & Guill., H. caledonicus Turcz., Psychotria douarrei (G. Beauv.) Däniker (all Jaffré & Schmid, 1974) and Homalium kanaliense (Vieill.) Briq. (Brooks, Lee & Jaffré, 1974).

Following up this work by surveying the genera which contain known hyperaccumulators was to prove fruitful. Brooks, Lee et al. (1977) in a survey of the genera Homalium and Hybanthus not only re-identified the previously known hyper-accumulators but added a further five to the list. All five are of the Homalium genus (see Appendix II(a) for species). Jaffré, Brooks and Trow (1979) surveyed the Geissois genus. Of the seventeen species sampled, seven were to prove hyperaccumulators. One, G. pruinosa, had already been reported but the remaining six were all new.

Earlier (Jaffré, Brooks et al., 1976) the New Caledonian studies had resulted in the discovery of the hyperaccumulator Sebertia acuminata Pierre ex Baill. This plant has an extremely

unusual latex containing up to 25% nickel on a dry weight basis (11.2% wet weight). The tree is known to the locals as seve-bleue (blue-sap) from the colour of this latex.

Since Homalium and Hybanthus are in the closely related families of Flacourtiaceae and Violaceae respectively, Brooks and Wither (1977) surveyed these families in South East Asia (see also Wither, 1977). This resulted in the identification of Rinorea bengalensis (Wall.) O.K. (Violaceae) as a hyperaccumulator. Following on from this, the genus Rinorea was surveyed (Brooks, Wither & Zepernick, 1977). Seventy of the approx. 250 recognized species were sampled and a further hyperaccumulator of nickel was found : R. javanica (Bl.) O.K. The Rinorea hyperaccumulators are unusual in one respect; both R. bengalensis and R. javanica also accumulate cobalt (545 µg/g and 670 µg/g respectively maximum values). Although serpentine soils are enriched in cobalt no previous nickel hyperaccumulators had simultaneously accumulated cobalt to this degree. Also in 1977, Wither and Brooks sampled vegetation collected at Jikodolong, Obi Island, Indonesia, a known ultrabasic area, to try and identify further hyperaccumulators. Three species Myristica laurifolia Spruce ex DC var. bifurcata, Planchonella oxyedra Dubard and Trichospermum kjellbergii Burret were found. T. kjellbergii also had an elevated cobalt concentration (350 µg/g). Analysis of further samples of Planchonella and Trichospermum revealed no further hyperaccumulators in these genera. However one specimen of P. oxyedra had an elevated cobalt content (240 µg/g), thus joining the other Indonesian hyperaccumulators in having both abnormally-high nickel and elevated cobalt concentrations.

Continuing the Flacourtiaceae line of development, a survey was made of other members of this family in New Caledonia (Jaffré, Kersten et al., 1979). This survey resulted in the re-identification of the seven Homalium hyperaccumulating species previously known and the discovery of twelve more in the genera Casearia (1), Lasiochlamys (1) and Xylosma (10).

All fifty-three species of Flacourtiaceae listed by Sleumer (1974) were analysed.

The New Caledonian surveys continued with the genus Phyllanthus following the results of spot tests (using dimethylglyoxime) done on specimens at the Noumea herbarium. The spot tests showed the possibility of there being several hyperaccumulators in this genus (Kersten, 1979). The results of the survey (Kersten et al., 1979) revealed ten further hyperaccumulators three of which also had elevated cobalt levels similar to those of the Indonesian nickel hyperaccumulators.

A further seven species of hyperaccumulators from New Caledonia have been reported by Jaffré (1980). These brought to fifty-eight the number of nickel hyperaccumulators reported from this island.

When one considers that the first three hyperaccumulators were all of the genus Alyssum it is surprising that no systematic studies in this genus were reported until 1978 when Brooks and Radford (1978a) surveyed all species listed in Flora Europaea by Ball and Dudley (1964). In addition to the two species and one subspecies previously known, eleven new hyperaccumulators were found. It was noted that all the hyperaccumulators in Alyssum belonged to section Odontarrhena (C.A. Meyer) W. Koch. The five other sections were devoid of hyperaccumulators even for individuals growing on ultrabasic rocks.

Other hyperaccumulators within the Cruciferae (Alyssum is a member of this family) have since been discovered. Vergnano Gambi and Gabbrielli (1979) in a survey of vegetation on Italian ophiolitic outcrops discovered two Cruciferous hyperaccumulators in the Valle d'Aosta : Cardamine resedifolia L. and Thlaspi rotundifolium (L.) Gaud. A survey of genera in Tribus Alysseae has revealed that only one further genus contains hyperaccumulators (Reeves, Brooks & Dudley, 1981). This is the genus Bornmuellera with hyperaccumulation in three species and the hybrid B. x petri Greuter, Charpin & Dittrich. In other Cruciferous genera, nickel hyperaccumulation has been reported for Peltaria emarginata (Boiss.) Hausskn.

(Reeves, Brooks & Press, 1980), Streptanthus polygaloides Gray (Reeves, Brooks & MacFarlane, 1981) (the first American nickel hyperaccumulator discovered) and various Thlaspi species. Owing to a certain amount of confusion in the taxonomy of Thlaspi and related genera in Europe, no firm number has been assigned to the list of hyperaccumulators but it appears that approx. thirty taxa have this character. In addition Thlaspi montanum L. (three varieties) in North America and T. japonicum Boiss. in Japan are hyperaccumulators (Reeves & Brooks, unpublished data).

Among these many species which hyperaccumulate nickel, the majority have maximum concentrations of less than 1%. Nineteen taxa have maximum concentrations from 1-2% : Alyssum argenteum All. (1.08%), A. bertolonii (1.22%), A. heldreichii Hausskn. (1.25%), A. markgrafii O.E. Schulz (1.37%), A. robertianum Bernard ex Gren. & Godron (1.25%), Bornmuellera baldaccii (Degen) Heywood ssp. rechingeri Greuter (1.20%), B. glabrescens (Boiss. & Bal.) Cullen & Dudley (1.92%), B. x petri (1.14%), Geissois pruinosa (1.36%), Homalium francii Guill.(1.45%) Hybanthus austrocaledonicus (1.85%), H. floribundus (1.42%), Lasiochlamys peltata Sleumer (1.10%), Planchonella oxyedra (1.96%), Rinorea bengalensis (1.75%), Sebertia acuminata (1.17%), Streptanthus polygaloides (1.48%) and Thlaspi montanum vars. montanum (1.71%) and californicum (Watson) P. Holmgren (1.16%). Only nine taxa have exceeded a concentration of 2%. These are Bornmuellera baldaccii ssp. baldaccii (2.13%), B. baldaccii ssp. markgrafii (2.73%), B. tymphaea (Hausskn.) Hausskn. (3.12%), Geissois intermedia Vieill. ex Pampan (2.29%), Homalium guillainii(2.90%), Peltaria emarginata (3.44%), Phyllanthus serpentinus Moore (3.81%), top of the table Psychotria douarrei (4.70%) and Thlaspi montanum var. siskiyouense P. Holmgren (2.46%).

Many biogeochemical and phytochemical studies of nickel hyperaccumulators have been reported. These include plant-soil relationships, intraplant distribution of nickel

and complexes of nickel within the plant. Brooks (1980) has reviewed these studies.

The most striking plant-soil elemental relationships for nickel hyperaccumulators are their ability to accumulate nickel relative to the soils, the ability to accumulate calcium and potassium to adequate (if low) physiologic levels from nutrient-deficient soils, and the ability to restrict magnesium uptake to an "acceptable" level. Studies by Lee et al. (1977a) showed leaf-nickel levels in Homalium kanaliense to be strongly related to only manganese and extractable-nickel soil levels and in Hybanthus austrocaledonicus to be strongly related to both total and extractable-nickel soil levels. They used these results to suggest that hyperaccumulation may therefore be controlled by plant organic rather than soil inorganic constituents. Calcium uptake appeared unaffected by other soil element concentrations. Potassium uptake varied : H. austrocaledonicus on a richer soil had no significant potassium-soil element relationships whereas H. kanaliense on a poorer soil had several antagonists to potassium uptake including both calcium and magnesium.

The distribution and nature of nickel in hyper-accumulators has been more widely studied. In Alyssum bertolonii nickel has been shown to be in the epidermis and sclerenchymatic tissues of stems (Vergnano Gambi, 1967). This was done by staining the tissues with dimethylglyoxime. Pelosi et al. (1974) studying the nickel complex of this species deduced that it was an organic acid complex. These workers later concluded that malic and malonic acids along with a third unidentified acid were involved (Pelosi et al., 1976). Pancaro et al. (1977) confirmed the involvement of these two bicarboxylic acids in A. bertolonii. They extended their study to include A. serpyllifolium ssp. lusitanicum and concluded that malic acid was also involved here though malonic acid was not. This latter study was essentially confirmed by Lee et al. (1978).

Farago et al. (1975) showed that nickel in Hybanthus floribundus was located in large epidermal cells by using two stains (dithioxamide and dimethylglyoxime). They also stained cells to detect pectin (using ruthenium red). This was also found in high concentration in the large epidermal cells. Kelly et al., (1975) found no evidence of nickel accumulation in specific tissues of some New Caledonian Hybanthus species. This work was done by differential centrifugation and supported by electron microprobe studies which showed a uniform nickel distribution within the leaves. Sequential extraction series (Farago et al., 1975, Lee, 1977) have shown that water and dilute acid will extract over 80% of nickel from leaves of hyperaccumulators. This shows that nickel in hyperaccumulators tends to be as a highly soluble polar complex or is easily exchangeable. Severne (1972) reported a low molecular weight, water-soluble complex from Hybanthus floribundus but could not complete the identification. Further work (Kelly et al., 1975) found this nickel as both aquo-Ni^{2+} and as a low molecular weight complex. No amino acid relationships could be detected. Farago et al., (1975) showed, by paper chromatography, that nickel pectinate was a possibility. They also inferred some nickel association with aspartic acid and cysteine. Kersten (1979) separated and identified a citratonickelate(II) complex from this species.

Working on the New Caledonian hyperaccumulators Kelly et al. (1975), using Hybanthus austrocaledonicus, H. caledonicus and Psychotria douarrei, showed no association between nickel and amino acids. Nickel was also found to be present as both aquo-Ni^{2+} and a complex of a molar mass of approx. 200. Further work (Lee, 1977, Lee et al., 1977b, Lee et al., 1978) has shown the presence of a negatively charged citratonickelate(II) complex in eleven New Caledonian hyperaccumulating and strongly accumulating species (Sebertia acuminata, Psychotria douarrei, Geissois pruinosa, Hybanthus caledonicus, H. austrocaledonicus and six Homalium species).

Seventeen species (the eleven above plus six further Homalium species) showed a linear relationship for nickel-citric acid concentrations. Three hyperaccumulators from outside New Caledonia (Alyssum bertolonii, A. serpyllifolium ssp. lusitanicum and Pearsonia metallifera) did not fit this relationship. The two Alyssum species have complexes with malic and/or malonic acids while Pearsonia metallifera has a complex with an unidentified trihydroxycarboxylic acid of formula $C_5H_{10}O_5$ (Lee, 1977, Lee et al., 1978). While Psychotria douarrei had a similar nickel-citric acid relationship to other New Caledonian species, Kelly et al. (1975) had shown that the bulk of the extracted nickel in this species appeared to be aquo-Ni^{2+} (94% of total nickel). Lee (1977) has shown the presence of a citratonickelate(II) complex but did not investigate the very large non-citrato-nickelate(II) portion. Kersten (1979) further investigated this species and showed that most of the supposedly aquo-Ni^{2+} is in fact a malatonickelate(II) complex. Kersten (1979) also investigated the nickel complexes of seven other hyperaccumulators. Included in these were Homalium kanaliense, which had been shown by Lee et al. (1977b) to have a citratonickelate(II) complex, and Hybanthus floribundus (see prior discussion). He confirmed the presence of citratonickelate(II) in the Homalium species. Four further species (Casearia silvanae (J.R. & G. Forster) Sleumer, Lasiochlamys peltata Sleumer, Xylosma vincentii Guill. from New Caledonia and Rinorea bengalensis from New Guinea) had citratonickelate(II) complexes. Phyllanthus serpentinus however had both citrato- and malato-nickelate(II) complexes. It is worth noting that the two mixed-complex species (P. douarrei and P. serpentinus) had much higher nickel levels than the other species. Had they saturated one system before starting another?

Studies of inter-elemental relationships involving nickel in leaves have shown few significant relationships.

Lee (1977) studied three New Caledonian species; Hybanthus austrocaledonicus had a positive nickel-phosphorus relationship, Homalium guillainii a positive nickel-magnesium relationship and Homalium kanaliense had positive nickel-copper and nickel-zinc relationships. Lee relates this low number of relationships to the probability that a different uptake mechanism is operating for nickel compared to the other elements. Working on Hybanthus floribundus, Farago et al., (1975) associated a high nickel content with a high calcium content. They also suggested that a relationship between nickel and calcium - magnesium existed. A nickel - calcium - magnesium relationship has also been suggested for Alyssum bertolonii (Vergnano Gambi et al., 1977). Kersten (1979) studying Rinorea bengalensis showed positive nickel-cobalt, nickel-copper and nickel-sodium relationships. R. bengalensis is also a very strong accumulator of cobalt. As the nickel-cobalt relationship in serpentine soils is also generally strong and positive it appears that R. bengalensis reflects this soil relationship. This is at variance with most other studied hyperaccumulators. R. bengalensis appears more sensitive to the environment than the others which, conversely, appear to exercise a greater degree of control over which elements are accumulated. This sensitivity does however make R. bengalensis a better biogeochemical prospecting species (cf. Brooks & Wither, 1977, Kersten, 1979).

(b) Cobalt and Copper

"Normal" plant levels for cobalt and copper are < 1 µg/g and 3-15 µg/g (mean 8 µg/g) respectively on a dry weight basis. These concentrations occur for soils with concentrations of 1-8 µg Co/g and 25-75 µg Cu/g. Poisoning of plants by cobalt and copper results in chlorosis and possibly necrosis. Various spottings may also occur, as can stunting and reduced root growth. Hunter and Vergnano (1953) rated these two metals as less toxic than nickel with copper more toxic than cobalt.

On mineralized soils it becomes more difficult to speak of a "normal" concentration but most plants contain 20-100 µg Co/g and 20-70 µg Cu/g. It is of interest to note the much greater increase in cobalt relative to normal (40-fold) than that for copper (5-7 fold). This is to be expected as copper being an essential element tends to be more tightly controlled in uptake than the non-essential cobalt (Timperley et al., 1970b). The content of cobalt and copper in mineralized soils can be very high. Duvigneaud and Denaeyer-de-Smet (1963) give values for some samples from metalliferous soils in Shaba : cobalt, low 600 - 900 µg/g, high > 40,000 µg/g; copper, low 900 - 2,000 µg/g, high > 90,000 µg/g. Few plants will tolerate such metal levels. Thus, as for nickel, cobalt and copper mineralization can be detected by the changes in flora which accompany them. Indeed a transect across mineralized hills shows regular changes in flora with different metal concentrations (see Duvigneaud, 1958 pp.263 & 265, Malaisse & Grégoire, 1978 p.254).

No concentration criteria have yet been determined for hyperaccumulation of cobalt or copper in plants. In declaring 1,000 µg/g as the hyperaccumulation level for nickel Brooks, Lee et al. (1977) had taken a value an order of magnitude higher than the nickel levels found in "normal" plants growing in nickel-rich substrates. No such easily determined level has been found for cobalt or copper. Work cited in this section and the results within this thesis have been analysed to determine a level for hyperaccumulation of these metals. A cumulative frequency plot against concentration in leaf material shows a discontinuity slightly below 1,000 µg/g for both metals (see fig. 1.1). This has been rounded to 1,000 µg/g for simplicity and set as the level above which hyperaccumulation occurs.

In studies of the "copperbelt" in Shaba (ex-Katanga) Province, Zaïre (ex-Belgian Congo), Duvigneaud (1959) had shown very strong accumulation (ie. greater than 500 µg/g) of cobalt in Crotalaria cobalticola Duvign. & Plancke

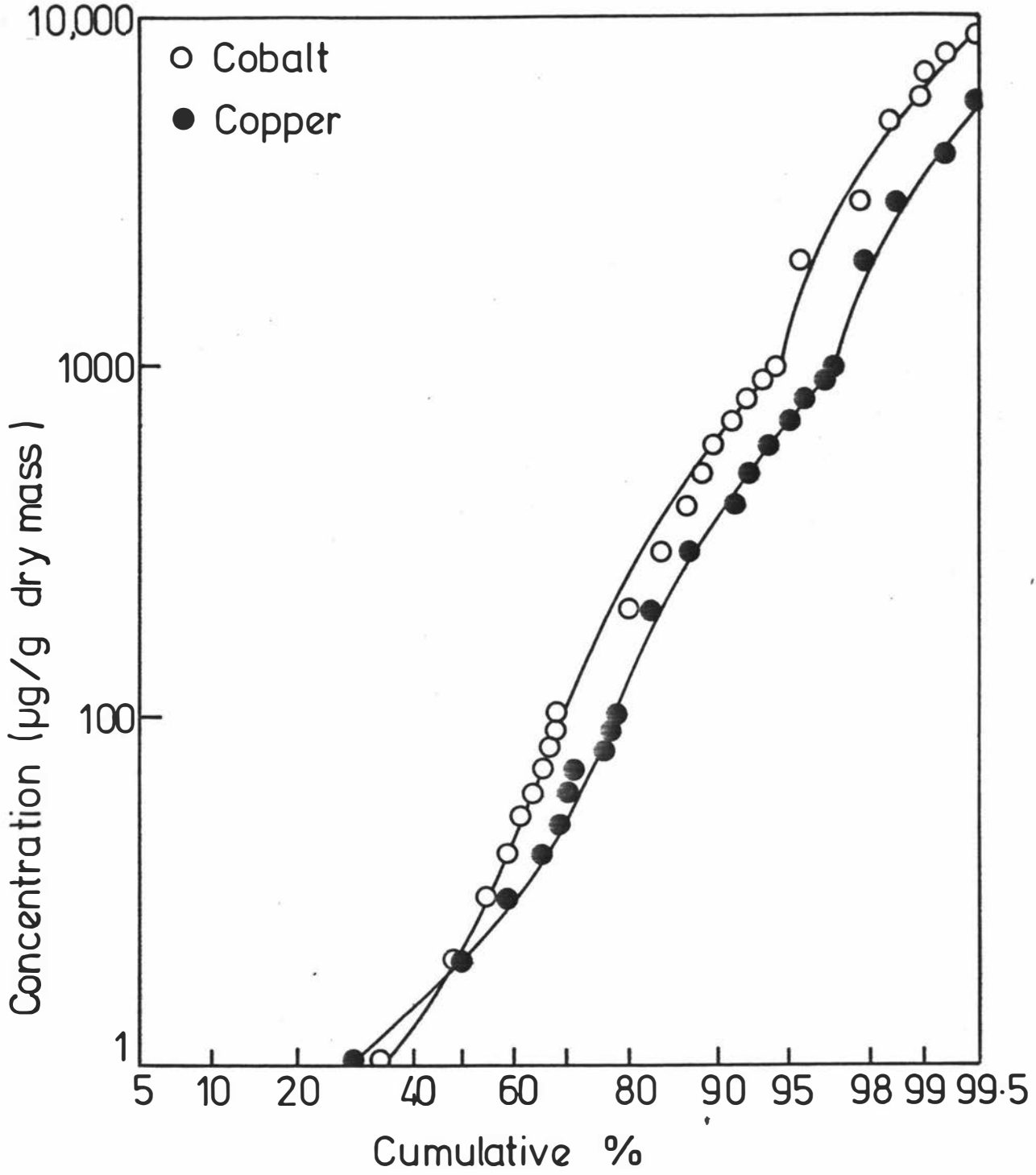


Figure 1·1 Cumulative frequency plot for cobalt and copper in plants from mineralized areas of Shaba Province, Zaïre.

(530 µg/g) while Duvigneaud and Denaeyer-de-Smet (1963) showed both hyperaccumulation and very strong accumulation of copper (Ascolepis metallorum Duvign. & Léonard, 1,200 µg/g, Silene cobalticola Duvign. & Plancke, 1,660 µg/g, Haumaniastrum robertii (Robyns) Duvign. & Plancke, 1,960 µg/g and Pandiaka metallorum Duvign. & van Bock., 740 µg/g).

In 1960, Kubota et al. found very strong accumulation of cobalt in Nyssa sylvatica Marsh. var. biflora (Walt.) Sarg. At 845 µg/g this was the highest cobalt leaf concentration then known. In 1966, Dykeman and De Sousa reported high copper levels in plants growing over the Tantramar copper swamp in Canada. The copper swamp had a concentration of 7% copper. The acidic conditions made copper readily available but chelation by organic material may have lowered the availability and made conditions less toxic. The highest plant levels were 1,120 µg/g in Abies balsamea (L.) Mill., 726 µg/g in Larix laricina (Du Roi) Koch and 500 µg/g in Ledum groenlandicum Oedr. In 1972, Ernst reported a concentration of 890 µg Cu/g in Indigofera dyeri Britt. from the Copper King anomaly in northern Zimbabwe.

In 1977, Malaisse, Brooks and their coworkers started systematic studies of two types : field surveys over specific "copper clearings" in Shaba and genera surveys for those genera in which field surveys show hyperaccumulation. Starting from the genera cited by Duvigneaud (1959) and Duvigneaud and Denaeyer-de-Smet (1963), Brooks (1977) surveyed the genus Haumaniastrum and Brooks, McCleave and Malaisse (1977) the African members of the genus Crotalaria. The results with respect to copper showed no further hyperaccumulator in either genus although C. peschiana Duvign. & Temp. at 705 µg/g is a very strong accumulator. The surveys did, however, reidentify H. robertii as a hyperaccumulator. The cobalt results in Crotalaria failed to show any very strong accumulation. No C. cobalticola specimens were analysed. The survey of Haumaniastrum however led to the first identification of cobalt

hyperaccumulation with a concentration of 10,220 µg/g (1.02%) in H. robertii. This exceeded by over one order of magnitude, the previously recorded highest cobalt levels in dry leaf material.

Also in 1977, Brooks, McCleave and Schofield surveyed the Nyssaceae for cobalt. As well as re-identifying very strong accumulation by Nyssa sylvatica var. biflora they showed very strong accumulation by Nyssa sylvatica var. sylvatica (530 µg/g).

In 1975, Agrostis stolonifera L. was found to hyperaccumulate copper on contaminated soils near a metal refinery at Prescot, U.K. (Wu et al., 1975) while in 1977, Agrostis gigantea Roth. on mine-waste sites near Sudbury, Canada, showed very strong accumulation of this element (Hogan et al., 1977).

The next major report of hyperaccumulation came from Shaba. Malaisse and Grégoire (1978) surveyed the vegetation at the Mine de l'Étoile near Lubumbashi. From this study, four copper and three cobalt hyperaccumulators were discovered (see Appendix II(b) for species). Also discovered were four copper and five cobalt very strong accumulators.

Many serpentine flora surveys have tested for cobalt as well as nickel since serpentine soils tend to be enriched in this element also. Despite this, few "serpentine" plants have elevated cobalt levels and only three have ever reached very strong accumulation levels : Rinorea bengalensis and R. javanica from Indonesia (Brooks, Wither & Zepernick, 1977) and Phyllanthus ngoyensis Schlect. from New Caledonia (Kersten et al., 1979). Work done on copper-lead-zinc enriched areas of Europe have measured copper levels in the vegetation but no copper levels reported have reached those of very strong accumulation.

As for nickel, cobalt and copper accumulating plants can be used for biogeochemical prospecting (Warren et al., 1949, Warren & Delavault, 1950, Riddell, 1952, Brooks, Wither & Westra, 1978). In their discovery of a copper biogeochemical anomaly

on Salajar Island, Indonesia, Brooks, Wither and Westra (1978) found very strong accumulation in three plant species.

Hyperaccumulation of cobalt and copper appears to be much rarer than hyperaccumulation of nickel. Levels reached by those plants which do hyperaccumulate cobalt and/or copper tend to be lower than those reached by "nickel plants". Thus only Haumaniastrum robertii (1.02%) has surpassed the 1% Co level and Aeolanthus biformifolius (1.37%) the 1% Cu level. It should be noted that all the hyperaccumulators of cobalt and all except one of copper come from the Shaban "copperbelt."

Very little information on the biogeochemistry or phytochemistry of accumulators or hyperaccumulators of copper is available.

However some information does exist for the very strong accumulator Indigofera dyeri (Ernst, 1972) and the strong accumulator (max. 324 µg/g, Reilly, 1967) Becium homblei (De Wild.) Duvign. & Plancke. In work on I. dyeri and I. setiflora Bak. (a tolerant but non-accumulating member of this genus) Ernst showed both that populations from mineralized areas had greater tolerance than those from non-mineralized areas and that this tolerance was specific to the minerals present in the original soils rather than a general heavy metal tolerance. Tolerance was tested by the method of comparative protoplasmatology (Repp, 1963, Gries, 1966). A sequential extraction series done on I. dyeri showed that almost half the copper was extracted by water suggesting the location of this copper within the cell vacuole. Very little copper was extracted by an organic solvent (butanol). Most copper not extracted by water could be extracted by exchange processes using sodium chloride, citric acid and dilute hydrochloric acid. Less than 2% remained in the residue. The exchangeable copper was believed to have been bound to the cell wall. No attempt was made to identify the nature of the copper in any fraction.

The "copper flower", Becium homblei has also been studied (Reilly, 1969, Reilly et al., 1970, Howard-Williams, 1969, 1970).

Reilly and co-workers have suggested that copper in this species is complexed to amino acids, particularly cysteine. Evidence (Reilly, 1969) that 50% of the copper is extractable into organic solvents (dioxan, butanol, methanol) is, they suggest, indicative of an organic complex of copper. A further 20% of the copper is soluble in water and dilute acid (ionic and exchangeable copper) and 30% is insoluble (tightly bound copper). Reilly *et al.* (1970) found 17% of the copper associated with the cell wall and suggested that the copper was bound to the polysaccharides, lignins or associated proteins of the wall. By dialysis against water they deduced that 20-25% of the copper was either ionic or in a water-soluble complex. Dialysis against a tartaric acid solution showed a further 10-15% of the copper was only lightly complexed (stability lower than tartaric acid-copper complex). By paper chromatography on water-extracted copper they showed that no inorganic Cu^{2+} was present. Howard-Williams (1969) tested the tolerance of *B. homblei* by the rooting technique and showed that species growing on copper-rich soils had a greater copper resistance than those growing on nickel-rich or normal soils. He also showed in pot trials that *B. homblei* can survive on soils with only a trace of copper. Howard-Williams (1970) gives the field tolerance range as trace to 10,000 µg Cu/g dry soil. Testing of seed germination showed that seeds readily germinated in distilled water; a finding which contradicted that of Horscroft (1961) who found that seeds required solutions of 50-600 µg Cu/cm^3 for germination.

Some studies on the Canadian accumulators have been made. Dykeman and De Sousa (1966) concluded that the copper content of the plants was not related to the total copper content of the soil. They based this conclusion on the fact that vascular plants grew on substrates with a higher total copper than surrounding substrates but whose seedlings died in these surrounding substrates. Hogan *et al.* (1977) compared elemental contents of tolerant and non-tolerant clones of *Agrostis gigantea*. The tolerant clones had higher copper,

iron, nickel and zinc content but manganese, potassium, calcium and magnesium showed no differences. Despite this, rooting tolerance tests showed greater tolerance for only two of five clones growing on the copper-rich substrate compared to the clones growing on surrounding soils. It was shown that these two tolerant clones came from areas with no higher metal content than the other three clones, but that their sites had a significantly lower pH level. At lower pH levels, the metals tend to be more soluble and hence plant-available so that this may have controlled the development of tolerance.

No detailed studies on accumulators or hyperaccumulators of cobalt have yet been made.

The work discussed in the succeeding chapters covers investigations into several aspects of hyperaccumulation. For cobalt and copper hyperaccumulation, field and genera surveys are reported. These lead into biogeochemical and phytochemical studies on some of these plant species. Biogeochemical and phytochemical data are also presented on species of the Alyssum genus which contains a large number of hyperaccumulators of nickel. This work attempts to increase our understanding of the hyperaccumulation of these metals in plants.

PART ONE

Cobalt and Copper
in Vegetation of
Shaba Province, Zaïre

CHAPTER 2

Surveys of Vegetation of Metalliferous Soils

2.1 INTRODUCTION

The "copperbelt" of Shaba Province, Zaïre and northern Zambia forms one of the world's greatest metallogenic provinces. It contains some one hundred mineralized areas with a total area of $20km^2$ dispersed over $22,000km^2$. The mineralized areas often cover several square kilometres. The principal metals of the mineralized areas are copper, cobalt and nickel although others, uranium, zinc, lead, manganese, may also be present (Duvigneaud, 1958). Such mineralized areas are highly toxic to "normal" plants but tolerant populations have evolved on them. Although De Wildeman (1921) mentions this "copper flora", it was Robyns (1932) who began the detailed studies. He noted a herbaceous vegetation zone on rich copper soils surrounded by a transitional zone of stunted woody vegetation extending out to the "normal" open woodland. Duvigneaud (1958, 1959) and Duvigneaud and Denaeyer-de-Smet (1963) give a more detailed study of this flora. They also report concentrations of cobalt (Duvigneaud, 1959) and copper (Duvigneaud and Denaeyer-de-Smet, 1963) in plant material, giving recognition to the extremely high levels some species of this tolerant flora could accumulate.

2.1.1 Physical Environment

(a) Geology, Bowen and Gunatilaka (1976) have recently reviewed the geology of the "copper belt". The geological components of the region have been classified as belonging to either a basement complex or the Katangan sequence.

The basement complex consists of the Lufubu system schists, gneisses and quartzites overlain, unconformably, by the Muva system sediments, and old granites from a post-Lufubu orogenic phase. There is an absence of large scale mineralization in this complex. The Muva sedimentation was ended by an intense orogenic deformation which compressed the sediments into long north-east trending folds.

Subsequent movement now gives the folds a north-west trend. The sediments of the Lower Roan period are believed to have come from this complex.

The Katangan sequence was laid on to this basement complex. At this time the complex had a relief of some 300m. The sequence is of marine sedimentary origin from 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 800-2000m depth, covering the paleoridges. The Lower Roan deposits gradually change to dolomite-rich 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. This group consists of tillite and marine sedimentary horizons laid in Upper Pre-Cambrian times. At least two major tillite horizons exist as testimony to fluctuations in the paleoclimate of Central Africa. Following the Kundelungu sedimentation, the Kundelunguan orogeny rapidly raised the area. This rise was stronger in the north. In Shaba it formed a large arc with a succession of anticlines and synclines.

The ore bodies of the "copperbelt" are confined to the Lower Roan sediments, particularly some 150-200m of sediments near the middle of the series. In relation to the paleoridges, the high-grade ore is found on the slopes with little on either the ridge tops or valley floors. 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. Also unknown are the origins of the metals deposited although some may have come from the basement complex (see Bowen & Gunatilaka, 1976).

Since the final emergence from the sea, Central Africa has been subjected to three peneplanations (Duvigneaud, 1958). The first peneplanation, finishing in the late Cretaceous,

was followed by a general rising of the African continent. The second peneplanation finished in the mid-Tertiary. Today this is the main erosional surface of Shaba. It now has a general altitude of approx. 1,300m. The third peneplanation was more localized, in southern Shaba, and today has an altitude of 1,200-1,300m.

(b) Topography. The landscape of Shaba today is of a series of plateaux, of approx. 1,300m altitude, with few hills or valleys. The highest altitude is approx. 1,700m (Kundelungu Plateau) while the lowest is less than 700m in the larger river valleys (e.g. Lufira Valley). Erosion has uncovered many underlying rocks with the harder rocks, including the metalliferous conglomerate, outcropping as hillocks or crests. The localization of the outcrops is a result of fracturing of the metalliferous belt during the rotation of the belt from a north-east to a south-east trend (fig 2.1). Many of these hillocks are or have been mined over the years and further mining is planned in many areas (fig 2.2). The current largest mines are at Kolwezi (Shewry et al., 1979).

(c) Climate. The Shaban climate is characterized by a hot rainy season and a cooler dry season. The rainy season occurs in summer, lasting from October to April. Rainfall generally exceeds125mm/month from November to March with January having a mean precipitation of 200-300mm. The January mean temperature is 22-24^{o}C with a mean daily maximum of 26-28^{o}C. The dry season in winter is very dry with no measurable precipitation between June and August. The July mean rainfall is less than 0.1mm. The July mean temperature is 15-17^{o}C with a mean daily minimum of 7.5-10^{o}C. 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. Data here are from Schulze and McGee (1978).

2.1.2 Soils and Vegetation

Phytogeographically Shaba belongs to the Zambesian Domain of the Sudano-Zambesian Region (Lebrun, 1947, Duvigneaud, 1958, Werger & Coetzee, 1978). Duvigneaud (1958) recognized three

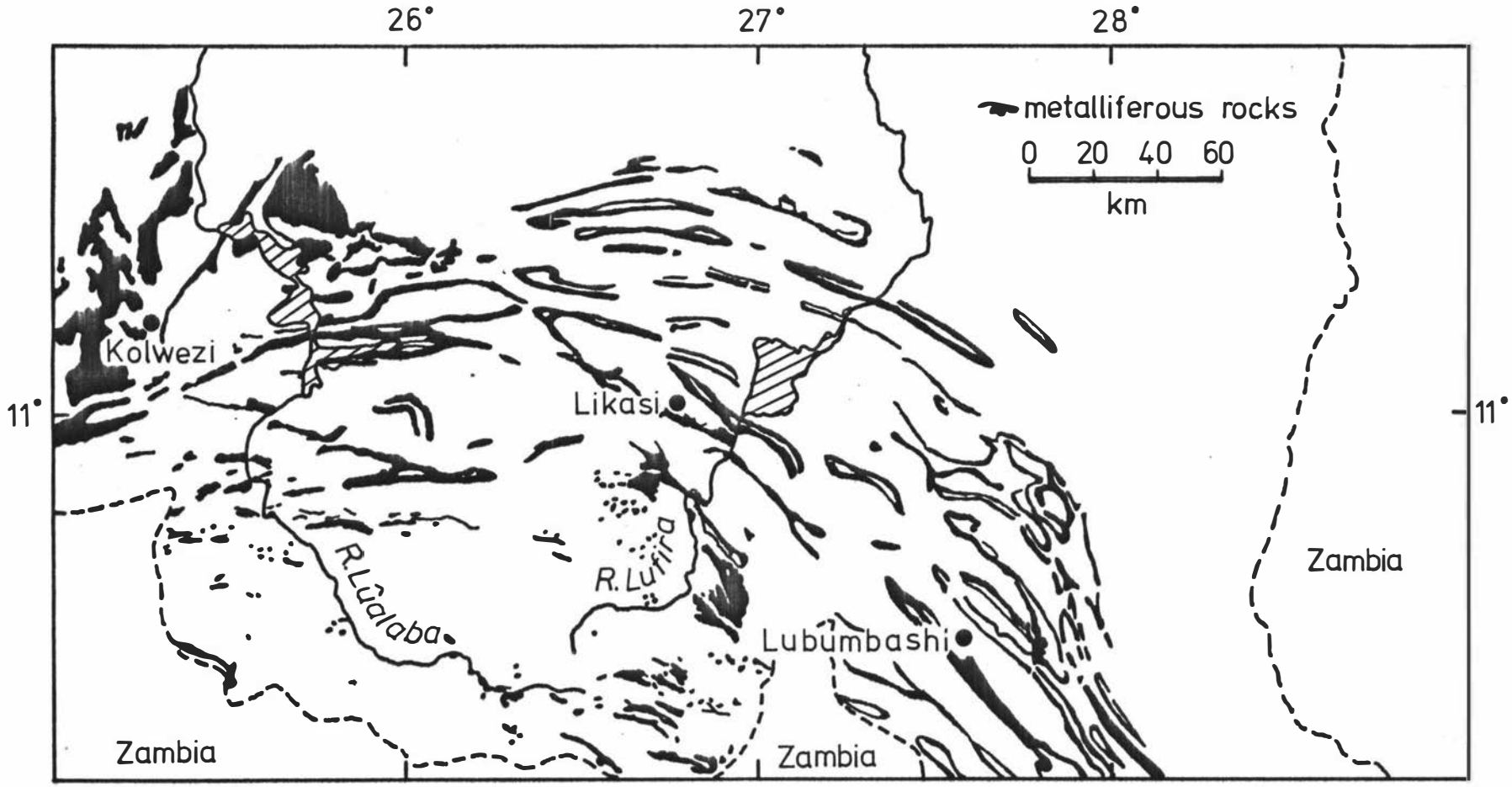


Figure 2·1: Distribution of metalliferous rocks in Shaba Province, Zaïre.

Key to figure 2·2 :

1. Lubumbashi, L'Étoile
2. Ruashi
3. Kasanka north and Niamumenda
4. Luiswishi
5. Lupoto
6. Kipushi
7. Kamwali
8. Luishia
9. Kamatanda
10. Kakonge
11. Likasi
12. Kambove
13. Fungurume
14. Mindingi
15. Swambo
16. Tenke
17. Chabara
18. Menda
19. Kasompi
20. Tilwizembe
21. Kolwezi
22. Dikuluwe
23. Kalongwe

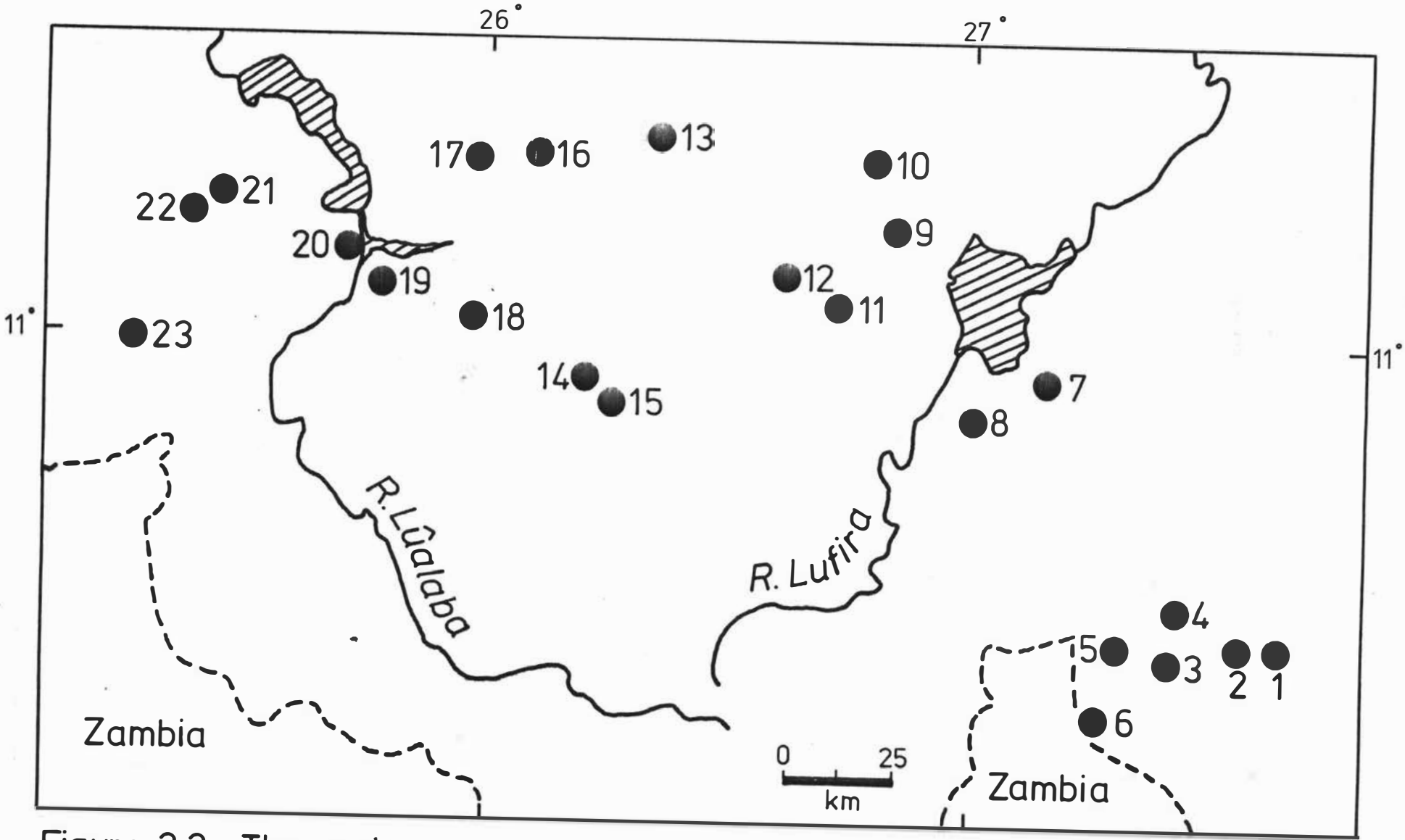


Figure 2·2: The major cobalt and copper mining areas of Shaba Province, Zaïre.

sectors in Shaba; "Lunda" (renamed Central Angolan by Werger & Coetzee, 1978), Lower Katangan and Katango-Zambian. The metalliferous areas of this study are within the Katango-Zambian sector. This sector is characterized by "miombo" woodlands. Miombo has various species of Brachystegia, Julbernardia and Isoberlinia as the major canopy components. On mesic and fertile loamy soils with rainfall above 1,100mm/year, the miombo develops a canopy up to 25m high with a well-developed understorey, at 5-10m height, of shrubs and small trees. A tall and dense herbaceous layer develops between the larger species and this provides good fuel for the dry season fires. Too much heavy firing may however degrade the miombo to grassland (Werger & Coetzee, 1978). On drier, poorer soils the miombo has less height, approx. 17m, and less herbaceous undergrowth. On the poorest soils the undergrowth may be replaced by Cryptosepalum maraviense Oliv.

On deep soils with little compaction Brachystegia longifolia Benth. and Erythrophleum africanum (Welw.) Harms. dominate. On light sandy soils of this nature, they are joined by Guibourtia coleosperma (Benth.) J. Léonard and Burkea africana Hook. while on the rich heavy red earths Brachystegia spiciformis Benth. is common. On shallower, gravelly soils with greater compaction and yellow or grey colouration, these species give way to Brachystegia utilis Burtt, Davy & Hutch. These conditions are often found on slopes. The areas of very shallow or skeletal soils, generally on crests of rocky massifs, are dominated by Brachystegia microphylla Harms. and B. bussei Harms. On lateritic soils, waterlogged in the rainy season, often gravelly and compacted, with grey or white colouration, the dominant species are Brachystegia stipulata De Wild. and Isoberlinia tomentosa (Harms.)Craib. & Stapf.

Within the open forest (miombo) are areas of termite hills ("termiteria"). A special flora has become associated with these termiteria (Malaisse, 1978). The flora tends to be sclerophyllous in nature with a widely variable species range. The "soil" tends to be abnormally dry but nutrient rich.

On alluvial soils along the major river valleys, riverine forests are found. Riverine forest is a rich and varied vegetational grouping with species of other phytogeographical areas (particularly Guineo-Congolian) appearing. The soils are generally moist and rich.

On badly drained or inundated soils in valleys, the miombo vegetation gives way to savannas: thorny, microphyllous savanna, broadleaf savanna or mixed savanna. The most important species are herbaceous Hyparrhenia spp. and woody Acacia spp.

Grasslands may develop on sandy or shallow rocky soils, dilungus and dambos. Dilungus are areas of poor, waterlogged or wet soils with little or no aeration. Such aeration is insufficient for woody species to grow so that these areas have steppes of various grasses and sedges as their principal vegetation elements. Dilungus are most frequently found on old peneplain surfaces. Dambos are flat, wide oval depressions characterized by poor, badly drained or impermeable clay soils. The vegetation is again dominated by grasses and sedges although some dambos may have woody species present. Dambos with woody species are "bushy dambos" while those without are "herbaceous dambos". A specialized grassland community has developed on metalliferous soils (see section 2.1.3). A recent review of the phytogeography of Shaba can be found in Werger and Coetzee (1978) while a more detailed account can be found in Duvigneaud (1958).

2.1.3 Metalliferous Soils and Vegetation

The metalliferous soils of Shaba occur, generally, on hillocks 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-mineralized mother rock the heavy metal content of the colluvion is diluted. Illuvial deposits may, however, enrich such non-mineralized areas, 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. Duvigneaud and Denaeyer-de-Smet (1963) divide the vegetational groups into five forms. Each form exists on different zones of each metalliferous area. The five forms are: (1) the swards, usually open, composed of grasses (Sporobolus, Eragrostis, Monocymbium, etc.spp.), sedges (Bulbostylis, Ascolepis, etc. spp), annual dicotyledones (Haumaniastrum, Crotalaria, etc. spp.), monocotyledones with corms or bulbs (Gladiolus, Lapeyrousia, Eriospermum, Dasystachis, etc. spp.) and perennial dicotyledones with thick underground stocks (Icomum, etc. spp.); (2) the steppes, closed formation, with grasses (Loudetia, Tristachya, Andropogon, etc. spp.) predominating and a characteristic geofrute (Cryptosepalum sp.) associated with various perennials with bulbs or corms (Haumaniastrum, Commelina, etc. spp.) or woody stocks (Becium, Acalypha, etc. spp.); (3) thickets of Uapaca robynsii De Wild; (4) bush savanna of transition to the surrounding open forest; and (5) brush on rocky ground with small trees, bushes and shrubs.

The flora of these metalliferous soils is generally derived from the areas surrounding them. Duvigneaud (1958) considers that it may be derived from: (1) open forest and pioneer plants of rocky hills; (2) open forest on compacted yellow earths; (3) bushy dambos; (4) herbaceous dambos; (5) Uapaca robynsii belt around dilungus; (6) dry steppes with geofrutes of dilungus; and (7) moist, grassy steppes of dilungus. Duvigneaud (1958) further considered that the flora is not derived from: (1) open forest on fertile, deep red earths; (2) sclerophyllous vegetation of termiteria; (3) riverine forests; or (4) alluvial savanna of Acacia spp.

The metalliferous flora may also be divided on an ecological basis (Duvigneaud and Denaeyer-de-Smet, 1963). They recognize four major divisions: metallophytes, metallophiles, metalloresistants and metallifuges. Metallophytes grow on the richest metalliferous soils and, indeed, may be confined to such soils. Such species include Haumaniastrum spp., Bulbostylis spp., Icomum spp., and Gramineae species. Metallophiles are found on soils of lower heavy metal content and are not confined to metalliferous soils. Examples include Becium homblei, Olax obtusifolia De Wild., and Uapaca robynsii. Metalloresistants are ubiquitous species which are indifferent to the presence of heavy metals in the soil. They include Loudetia simplex (Nees) C.E. Hubb., Crotalaria cornetii Taub. & Dewèvre, Andropogon filifolius (Nees) Steud., Xerophyta spp., Aeolanthus spp., and Tithonia spp. Metallifuge species do not occur on metalliferous soils. Their presence indicates a lack of heavy metals in the soil. Hyparrhenia spp. are in this category. For a review of these ecological divisions see Wild (1978).

2.2 ANALYTICAL METHODS

Vegetation samples from metalliferous hillocks in Shaba were collected by Prof. F. Malaisse and Monsieur J. Grégoire of the Université Nationale du Zaïre at Lubumbashi. These samples were washed and air-dried. Soil samples were also collected and dried. A summary of the plant communities at Fungurume was also provided by these workers.

Upon arrival the vegetation samples, mainly leaf material, were analysed for their cobalt and copper content. Samples of 0.02-0.04 g, dry weight, were accurately weighed and placed in 5cm^3 borosilicate test-tubes. These samples were ashed in a muffle furnace at 500^{o}C for 2-3 hours. The ash was dissolved in 1cm^3 of 2M hydrochloric acid prepared from redistilled constant-boiling hydrochloric acid. If necessary slight warming was used to ensure dissolution of the ash.

Samples contaminated by soil had a red, insoluble sediment left after heating. These samples were rejected. This method of analysis has been found to be satisfactory for heavy metal determinations in leaf samples (Wither, 1977). The solutions were then analysed for cobalt and copper using a Varian-Techtron model AA5 atomic absorption spectrophotometer. Automatic background correction was made by coupling a Varian-Techtron model BC6 background corrector unit to the spectrophotometer. Analysis was carried out using cobalt lines at 240.8nm and 304.4nm and copper lines at 324.8nm and 218.2nm. For each element, low metal contents were determined at the most sensitive lines, 240.8nm and 324.8nm respectively, and high metal contents at the less sensitive lines, 304.4nm and 218.2nm respectively. Standards containing both cobalt and copper were prepared from BDH 1000 μg/g analytical grade stock solutions by dilution with 2M hydrochloric acid. The standard concentrations ranged from 5 μg/g to 100 μg/g.

Soil samples were dried at 110^{o}C overnight and sieved to -80 mesh size. Samples of 0.1g were placed in 150cm^{3} polypropylene squat beakers and 20cm^{3}of a 1:1 nitric:hydrofluoric acid mixture added. The samples were then taken to dryness over a water-bath and redissolved in 10cm^{3} of 2M hydrochloric acid, prepared as above. This volume was diluted as appropriate (10-100cm^{3}) for the expected concentrations. The solutions were then analysed for cobalt and copper as outlined above.

Extractable cobalt and copper soil concentrations were determined by shaking 0.5g of dry soil, sieved to -80 mesh size, in 5cm^{3} of an ammonium oxalate-oxalic acid buffer, pH5 (Grigg's reagent, Grigg, 1953) for 1 hour. The samples were left overnight to equilibrate before centrifugation. The supernatant was then decanted off for analysis. Analysis for cobalt and copper was carried out as for the other samples.

2.3 COBALT AND COPPER IN VEGETATION OF FUNGURUME

2.3.1 Study Area

The metalliferous hillocks of Fungurume are situated approx. 180km northwest of Lubumbashi (fig 2.2). The terrain is in marked relief to the plateaux which dominate the Shaban area. The herbaceous vegetation of the metalliferous hillocks is in contrast with the open forest of adjacent non-metalliferous hillocks (plates 2.3 & 2.4). The anomalous appearance of the hillocks is accentuated by irregular ridges, composed of cellular siliceous rocks, upon them (plate 2.5). A location map (fig. 2.6) indicates the position of the six metalliferous hillocks (named I-VI) in the study area. Work was however confined to hillocks III-IV and V-VI.

Malachite ($CuCO_3.Cu(OH)_2$) is the principal mineral in the superficial soil. The total copper content in the soil can reach 3.5%, dry weight basis, with associated cobalt at 0.7% (table 2.1). The extractable metal levels reach 3.2% copper and 0.3% cobalt. Percentage extraction by the oxalate buffer is high, indicating a ready availability of the metals to the plants. Copper extraction generally exceeds 80% while cobalt has a lower extractability, 45-80%. Thus copper is more readily available than cobalt.

2.3.2 Plant Communities

The plant communities at Fungurume have been summarized by species present in table 2.2. Schematic representations of these communities and their distribution over the hillocks are shown in fig. 2.7. Further brief comments on the communities are given below.

A-Open forest (miombo). The open forest surrounding the mineralized hillocks intrude only upon the lightly mineralized areas. The composition varies according to its position. A calcicolous open forest dominated by *Brachystegia bussei* extends along the foot of hillocks V and VI.

Plate 2·3 View of the cupriferous hillocks of Fungurume. In the right foreground is a block of cellular siliceous rocks, in the centre is the steppe savanna of the contamination halo while in the background, across the valley of the Dipeta River, the profile of hillock I is seen.

Plate 2.3

Plate 2.4 The contrast between non-cupriferous and cupriferous hillocks. To the left, a non-cupriferous hillock covered in open forest and in the centre, cupriferous hillock III covered in short herbaceous vegetation.

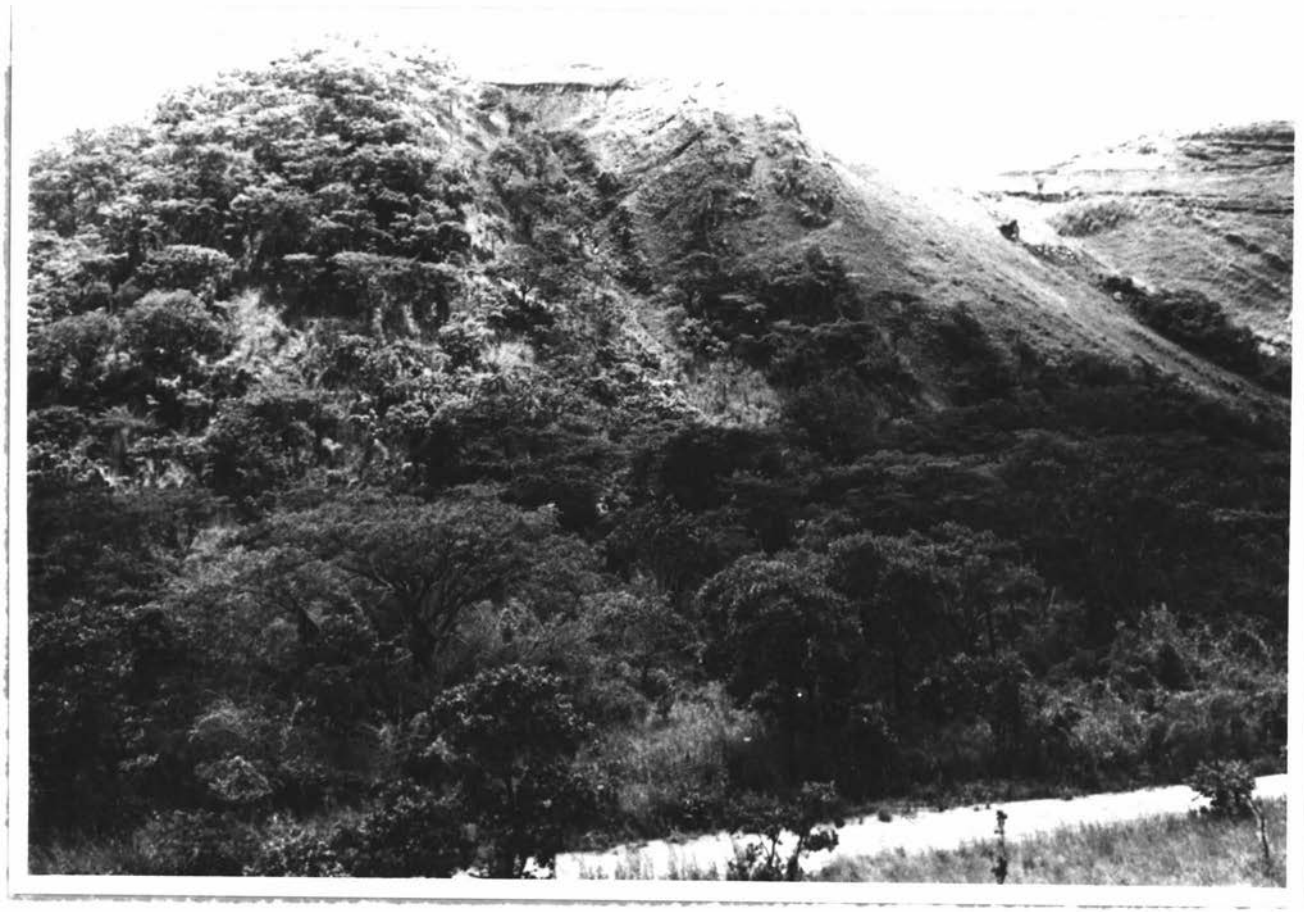


Plate 2.4

Plate 2.5 View of cellular siliceous rocks. Outcrops of cellular siliceous rocks as seen on the lower slopes of hillock V. The rocks carry a special flora; the Velloziaceae steppe is seen between the rocky outcrops.

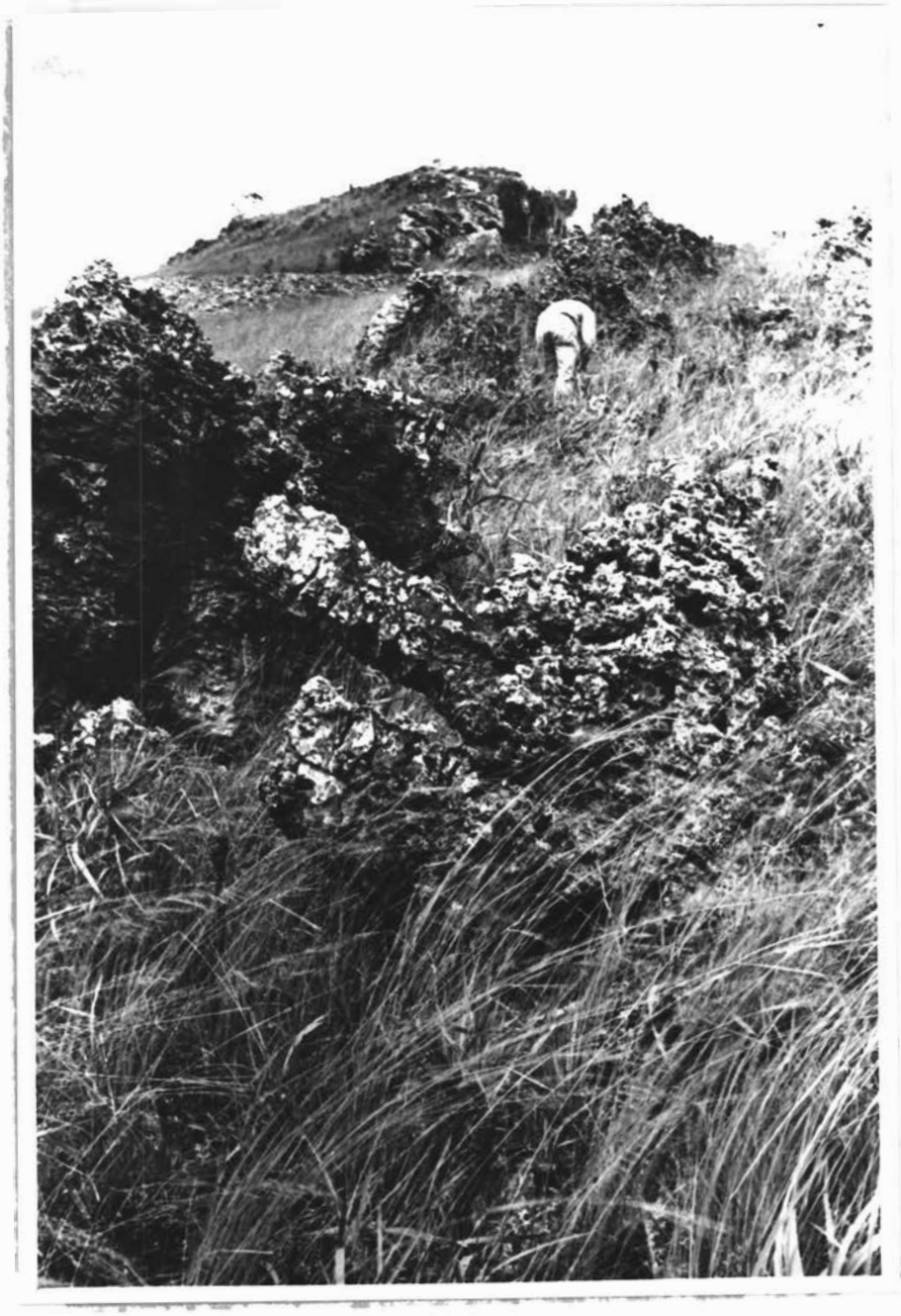


Plate 2.5

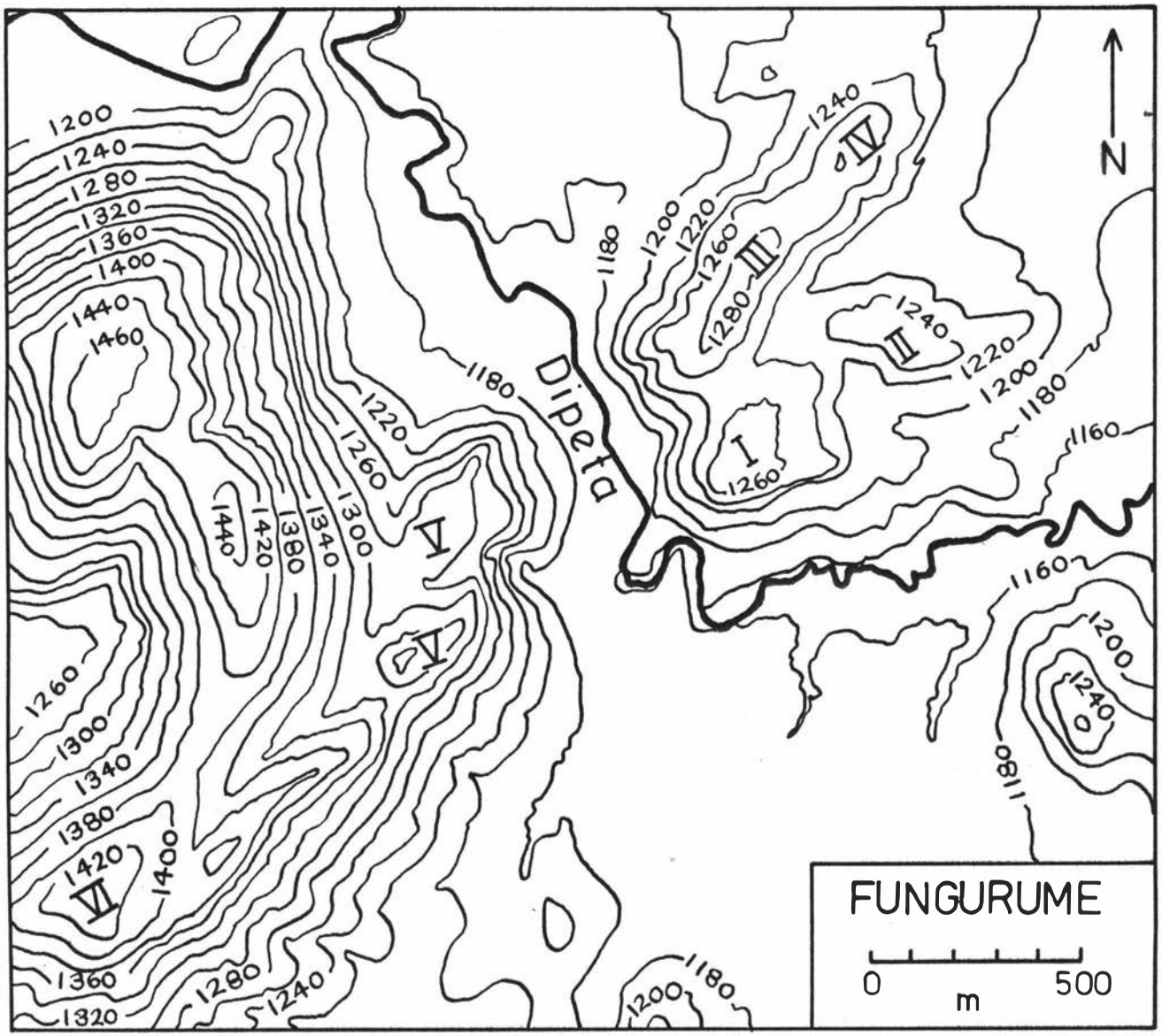


Figure 2.6
Location map of the cupriferous hillocks of Fungurume.

TABLE 2.1

Total and extractable soil cobalt and copper in some Shaban soils.

		COBALT *			COPPER *		
Soil Source		T	E	%E	T	E	%E
Fungurume	A	923	780	85	1892	1830	97
	B	93	61	66	280	235	84
	C	67	51	76	333	314	94
	D	387	205	53	5469	2827	52
	E	1289	862	67	9206	7088	77
	F	6867	2657	39	35200	32480	92
	G	1416	491	35	361	285	79
	H	991	769	78	3119	2564	82
	I	334	163	49	4619	3077	67
	J	1422	680	48	11939	10728	90
Lupoto	A	169	163	96	5215	2885	55
	B	163	108	66	4498	3550	79
	C	124	71	57	4751	1885	40
Ruashi	A	467	340	73	6667	4669	70
	B	164	82	50	3284	1799	55
	C	10309	2588	25	21556	16113	75
Mindingi	A	583	499	86	3107	2642	85
	B	1981	1594	80	39394	30876	78
	C	2549	1846	72	15496	14970	97
	D	4800	3984	83	44748	36853	82

*All concentrations as µg/g dry soil.

T = Total concentration.

E = Extractable concentration.

%E = Percentage of metal extracted.

On hillock IV the open forest thins and changes, progressively, to an arboraceous savanna. Albizzia adianthifolia, Brachystegia spiciformis, Ochna schweinfurthiana and Vitex madiensis ssp. milanjensis occur in the less vegetated stages. The copper content of the soil reaches 280 µg/g which is seven times the normal level for copper in forested areas but similar to that of an open forest, dominated by Brachystegia microphylla, at Likasi (Duvigneaud & Denaeyer-de-Smet, 1963). The open forest near hillock III is the richest floristically with all the usual elements of a miombo.

B-Uapaca robynsii thickets on steep slopes. These thickets occur at the base of the hillocks. The presence of Loudetia superba D.N. signals the transition to an arboraceous savanna.

C-Steppe-savanna within the dispersion halo. As is common for ore deposits, a dispersion halo of heavy metal contamination exists. As copper is more mobile than cobalt, at Fungurume, this halo has a much greater content of copper than cobalt.

D-Velloziaceae steppe. In Shaba, Velloziaceae populations are usually confined to rocky slopes or, more rarely, quartzitic slabs (Malaisse, 1975). At Fungurume they are found on soils rich in cobalt and copper. Xerophyta spp. are the dominant species of Velloziaceae.

E-Commelinaceae and Convolvulaceae steppe. These occur on soils with very high heavy metal concentrations. The prime representatives of the families are Commelina zigzag and Ipomoea alpina respectively. Small shrubs with woody stems are common.

F-Rendlia cupricola swards. The access tracks are bordered by a thick sward of Rendlia cupricola existing in the form of bell-shaped cushions. These swards are monospecific and of only a few metres dimension.

G-*Oxytenanthera abyssinica* (A. Rich) Munro thickets. This formation is found on alluvia of the Dipeta River and represents the transition from the forest (often degraded to *Parkia filicoides*) to the vegetation of the mineralized hillocks.

H-*Haumaniastrum robertii* carpet on mining works. The excavations resulting from mining activities and the residual soils, rich in minerals, are favoured localities for the occurrence of a carpet of *Haumaniastrum robertii*. Grasses and sedges may accompany this species.

I-Loose thickets of *Euphorbia ingens*. A steep face oriented towards the north and hence subjected to sunshine during the cool dry season supports a particular vegetation. *Euphorbia ingens* (a cactiform plant of candelabra-like aspect) and *Sarcostemma viminale* (a liana with crassulescent stem) characterize these thickets. Plants typical of termiteria are also present. Their xerophilic and crassulescent tendencies have been previously noted (Wild, 1952, Aubréville, 1957, Colonval-Elenkov & Malaisse, 1975, Malaisse, 1978).

J-Denuded vertical faces. Several vertical faces denuded of vegetation were observed on the southeast slopes of hillocks V and VI (fig. 2.7).

K-Vegetation of outcrops of cellular siliceous rocks. This vegetation grouping has been termed "chasmophytic" because of the dissected nature of the substrate. Rocks receiving sunshine are covered by two lichens, one fruticulous and the other foliar. In the cavities of the siliceous rocks is found *Euphorbia fanshawei*, a cactus-like plant with napiform roots. The accumulation of a little soil in the cavities results in the appearance of *Aeolanthus rosulifolius*, *Faroa acaulis*, *Monadenium* sp. and several Pteridophytes. The vertical walls of the rocks have cushions containing *Aeolanthus saxatilis* on them. The shaded cavities are covered with a carpet of Hepaticas dominated by *Plagiochasma eximium*. A number of other species also tolerate this environment.

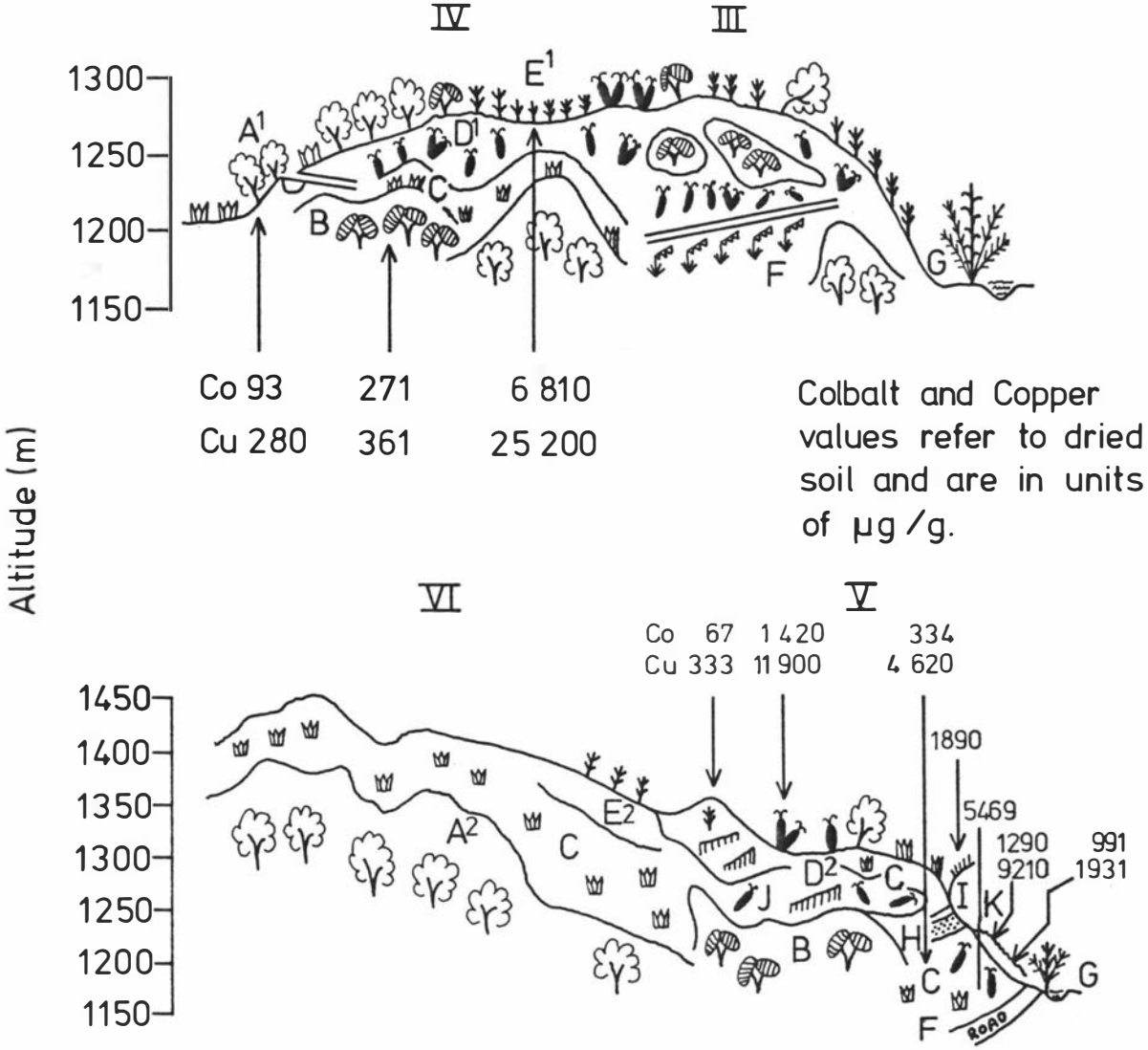
TABLE 2.2

Plant communities on mineralized hillocks at Fungurume, Zaïre. *

Community	Dominant Species	Associated Species
A-Open forest (up to 280 µg/g Cu in soil).	Brachystegia bussei	Albizzia adianthifolia A. antunesiana Brachystegia spiciformis Cussonia arborea Dalbergia boehmii Diplorynchus condylocarpon Kirkia acuminata Ochna schweinfurthiana Pseudolachnostylis maprouneifolia Sterculia quinqueloba Steganotaenia araliacea Psychotria sp. Vitex madiensis ssp. milanjensis
B-Steep thickets (250 µg/g Co and 350 µg/g Cu soil).	Uapaca robynsii	Loudetia superba Phragmanthera rufescens var. cornetii
C-Steppe-savanna (up to 350 µg/g Co and 5000 µg/g Cu in soil).		Haumaniastrum rosulatum Loudetia simplex Polygala petitiana P. usafuensis
D-Velloziaceae steppe (up to 1500 µg/g Co and 12000 µg/g Cu in soil).	Xerophyta retinervis var. equisetoides X. demeesmaekeriana	Crassula alba Lapeyrousia erythranthra var. welwitschii Moraea carsonii Pandiaka metallorum Spuriodaucus marthozianus
E-Commelinaceae and Convolvulaceae steppe (700 µg/g Co and 25000 µg/g Cu in soil).	Commelina zigzag Ipomoea alpina	Alectra sessiliflora Anisopappus davyi Bulbostylis abortiva B. mucronata Crotalaria cornetii Cryptosepalum dasycladum Cyanotis longifolia Hibiscus rhodanthus Sopubia dregeana

F-Rendlia cupricola swards	Rendlia cupricola	
G-Oxytenanthera abyssinica thickets	Oxytenanthera abyssinica	Parkia filicoides
H-Haumaniastrum robertii carpet	Haumaniastrum robertii	Bulbostylis abortiva Eragrostis boehmii Rendlia cupricola
I-Loose thickets of Euphorbia ingens	Euphorbia ingens Sarcostemma viminale	Annona senegalensis Cussonia arborea Dioscorea bulbifera Selaginella abyssinica Steganotaenia araliacea Tacca leontopetaloides
J-Denuded vertical faces		
K-Vegetation of outcrops of cellular siliceous rocks.	Various	Aeolanthus rosulifolius A. saxatilis Aneimia angolensis Anthoceros punctatus A. mandoni Euphorbia fanshawei Faroa acaulis Fossombronia sp. Gongylanthus ericetorum Mohria caffrorum Monadenium sp. Pellaea pectiniformis P. goudotii Plagiochasma eximium Riccia sp. Targionia hypophylla

* See also Figure 2.7



A¹ = Open forest and arboraceous savannah dominated by *Brachystegia spiciformis* and *Albizzia adianthifolia*.

A² = Open forest dominated by *Brachystegia bussei*.

B = *Uapaca robynsii* thickets on steep slopes.

C = *Loudetia simplex* steppe savannah within the dispersion halo of mineralization.

D¹ = *Xerophyta equisetoides* steppe.

D² = Velloziaceae steppe with *Pandiaka metallorum*.

E¹ = *Commelina zigzag* steppe.

E² = *Bulbostylis* spp. *Ipomoea alpina* steppe.

F = *Rendlia cupricola* sward.

G = *Oxytenanthera abyssinica* thickets along the course of the Dipeta River.

H = Sward of *Haumaniastrum robertii* on mining works.

I = Loose thickets of succulent plants (*Euphorbia ingens* and *Sarcostemma viminale*)

J = Vertical denuded rock faces.

K = Vegetation of outcrops of cellular siliceous rocks dominated by *Euphorbia fanshawei* and *Plagiochasma eximium*.

Figure 2·7: Representation of vegetation on metalliferous hillocks at Fungurume.

2.3.3 Cobalt and Copper Biogeochemistry

The cobalt and copper content of 152 plant specimens from Fungurume were determined. The results of these analyses are shown in table 2.3. From this table it can be seen that seven species sampled hyperaccumulate cobalt and two hyperaccumulate copper. In addition nine species are very strong accumulators of cobalt and two are very strong accumulators of copper.

Among the hyperaccumulators of cobalt, Haumaniastrum robertii has previously been recorded (Brooks, 1977) but the other six are new species: Anisopappus davyi, Crassula alba, C. vaginata, Cyanotis longifolia, Haumaniastrum homblei and Sopubia dregeana. Of the nine very strong accumulators of cobalt, eight are new discoveries (Aeolanthus saxatilis, Becium sp.1, Begonia princeae var. princeae, Commelina zigzag, Ipomoea alpina, Monadenium aff. chevalieri, Phragmanthera rufescens var. cornetii and Spuriodaucus marthozianus) while the status of the other, Commelina sp., must wait until more fully identified. Becium sp.1 is a new discovery because no Becium species have shown this character before. Most of these species are found in either the Vellosiaceae steppe or the Commelinaceae and Convolvulaceae steppe. This is not surprising given that the highest cobalt content of the soil is likewise found in these steppes. Some species, however, come from other communities eg. Haumaniastrum robertii from a carpet on old workings and Aeolanthus saxatilis and Monadenium aff. chevalieri from cellular siliceous rocks. Special mention must be made of the cobalt concentration in Phragmanthera rufescens var. cornetii. This epiphyte, cobalt content 765 µg/g, is hosted by Uapaca robynsii in thickets growing over only weakly mineralized substrates (up to 250 µgCo/g). The cobalt content of this host barely exceeds 100 µg/g.

Both of the two hyperaccumulators of copper (Commelina zigzag and Pandiaka metallorum) are previously unknown with this character although P. metallorum has shown very strong accumulation (740 µg/g, Duvigneaud and Denaeyer-de-Smet, 1963). Commelina zigzag is also a very strong accumulator of cobalt.

TABLE 2.3

Cobalt and copper concentrations in plants from mineralized hillocks at Fungurume, Zaïre.

Family	Species	Cobalt*	Copper*
PTERIDOPHYTES			
	Actiniopteris pauciloba Pic.-Ser.	6	6
	idem	15	10
	idem	7	12
	Adiantum lunulatum Burm.	25	70
	Aneimia angolensis Alston	41	8
	Asplenium buettneri Hier.	14	12
	Mohria caffrorum (L.) Desv.	196	455
	idem	143	17
	Pellaea longipilosa Bonap.	18	15
	Pellaea pectiniformis Bak.	40	5
	Selaginella abyssinica Spring.	18	21
	idem	10	3
DICOTYLEDONES			
Acanthaceae	Barleria descampsi Lindau	295	27
	Thunbergia oblongifolia Oliv.	10	82
Amaranthaceae	Pandiaka metallorum Duvig. & Van Bock.	448	2269
Anacardiaceae	Ozoroa reticulata (Bak.f.) R.&A. Fernandes ssp. foveolata R.&A. Fernandes var. cinerea R.&A. Fernandes	1	12
Annonaceae	Annona senegalensis Pers. ssp. senegalensis	3	7
Apocynaceae	Strophanthus welwitschii (Baill.) K. Schum.	8	76
		3	22
Araliaceae	Cussonia arborea Hochst. ex A. Rich.	1	17
Asteraceae	Anisopappus davyi S. Moore	2646	81
	Dicoma anomala Sond.	217	17
	idem	153	32
	Pleiotaxis pulcherrima Steetz	142	14
	Vernonia sp.	6	28
Begoniaceae	Begonia princeae Gilg. var. princeae	813	32
	idem	169	14
Burseraceae	Commiphora madagascariensis Jacq.	2	5

Caesalpinaceae	Brachystegia spiciformis Benth. var. latifoliata (De Wild.) Hoyle	3	10
	Cryptosepalum dasycladum Harms.	19	16
Campanulaceae	Wahlenbergia capitata (Bak.) Thulin	180	3
Convolvulaceae	Ipomoea alpina Rendle	641	356
	idem	65	229
	idem	33	12
	Ipomoea debeerstii De Wild.	26	35
	Ipomoea linosepala Hall. f.	26	31
	Ipomoea sp. 1	284	745
	Ipomoea sp. 2	182	17
	Ipomoea sp. 3	159	42
	Ipomoea sp. 4	67	24
	Ipomoea sp. 5	16	47
Crassulaceae	Crassula alba Forsk.	1625	28
	Crassula vaginata Eckl. & Zeyh.	785	343
	idem	1185	333
Euphorbiaceae	Acalypha cupricola Robyns	282	32
	Euphorbia fanshawei Leach	182	21
	Euphorbia ingens E.Mey. ex Boiss.	30	26
	Monadenium aff. chevalieri N.E.Br.	526	90
	idem	340	49
	idem	584	19
	idem	324	30
	Phyllanthus ninuroides Müll.Arg.	47	8
	Uapaca robynsii De Wild.	115	22
	idem	118	10
Lamiaceae	Aeolanthus adenotrichus Gürke	22	14
	Aeolanthus affinis De Wild.	24	7
	Aeolanthus rosulifolius Duvig.&Denaeyer	371	13
	Aeolanthus saxatilis Duvig. & Denaeyer	996	16
	idem	682	15
	idem	428	16
	idem	110	20
	Becium sp. 1	552	174
	Becium sp. 2	288	13
	Becium sp. 3	190	82
	Haumaniastrum homblei (De Wild.) Duvig. & Plancke	667 481	4 16
	idem	1625	28

	Haumaniastrum robertii (Robyns) Duvig. & Plancke	6159 2296	326 107
	idem	862	29
	idem	62	62
	Haumaniastrum rosulatum (De Wild.) Duvig. & Plancke	276	103
	Haumaniastrum sp.	197	11
	Tinnaea apiculata Robyns & Lebrun	26	21
Lobeliaceae	Cyphia erecta De Wild.	65	8
	idem	19	33
	idem	15	9
Loranthaceae	Phragmanthera rufescens (DC) Balle var. cornetii (Dewèvre) Balle	765	30
Malvaceae	Hibiscus rhodanthus Gürke	172	17
Melastomataceae	Antherotoma naudinii Hook f.	31	36
	idem	1	27
	Dissotis sp.	12	19
Mimosaceae	Albizzia adianthifolia (K.Schum.) W.G. Wight	8	18
	Albizzia antunesiana Harms.	2	22
Ochnaceae	Ochna schweinfurthiana F. Hoffm.	29	16
Olacaceae	Olax obtusifolia De Wild.	175	13
Oxalidaceae	Biophytum sp.	17	11
	Oxalis obliquifolia Steud.ex A. Rich.	100	77
	idem	217	30
	Oxalis semiloba Sond. ssp. uhehensis (Engl.) Exell	110	199
Papilionaceae	Crotalaria cornetii Taub.	8	10
	Crotalaria sp. 1	99	5
	Crotalaria sp. 2	5	10
	Droogmansia sp.	21	3
	Kotschya strigosa (Benth. ex Bak.) Dewitt & Duvig.	14	6
	Eriosema sp. 1	17	15
	Eriosema sp. 2	37	5
Polygalaceae	Polygala albida Schinz. var angustifolia (Chod.) Exell	1	27
	Polygala petitiana A. Rich.	352	12
	idem	132	26
	idem	49	11

	Polygala usafuensis Gürke	1	24
Rubiaceae	Canthium huillense Hiern.	1	10
	Tapiphyllum kaessneri Robyns	10	13
Santalaceae	Thesium sp. 1	41	25
	Thesium sp. 2	218	48
	Thesium sp. 3	328	99
	Thesium sp. 4	115	25
Scrophulariaceae	Alectra sessiliflora (Vahl) O. Ktze. var. senegalensis (Benth.) Hepper	371	13
	Sopubia dregeana Benth.	883	7
	idem	1090	58
	idem	309	60
Umbelliferae	Spuriodaucus marthozianus (Duvig.) Duvig.	392	4
	idem	768	4
	Steganotaenia araliacea Hochst.	1	7
Verbenaceae	Clerodendrum capitatum (Willd.) Schum. & Thonn.	4	13
	Lantana mearnsii Moldenke	37	75
	Vitex madiensis Oliv. ssp. milanjensis (Britten) Pieper	16	22
Vitaceae	Cissus sp.	95	44
MONOCOTYLEDONES			
Commelinaceae	Commelina zigzag Duvig. & Dewit	654	1214
	Commelina sp.	939	102
	Cyanotis longifolia Benth.	4197	608
Cyperaceae	Ascolepis metallorum Duvig. & G. Léon.	84	30
	Bulbostylis abortiva (Steud.) C.B.Cl.	41	27
	idem	37	5
	Bulbostylis mucronata C.B.Cl.	103	19
	Scleria sp.	19	9
Gramineae	Eragrostis racemosa (Th.) Steud.	61	195
	Loudetia simplex (Nees) C.E.Hubb.	19	3
	idem	11	3
	Rendlia cupricola Duvig.	83	14
	idem	22	34
	Trichopteryx elegantula Stapf	5	5

	Tristachya sp. 1	25	37
	Tristachya sp. 2	16	3
	Tristachya sp. 3	9	2
	Tristachya sp. 4	49	40
Iridaceae	Gladiolus gregarius Welw. ex Bak.	87	12
	Gladiolus ledoctei Duvig. & Van Bock.	188	3
	Gladiolus sp. 1	74	11
	Gladiolus sp. 2	150	12
	Gladiolus sp. 3	157	5
	Gladiolus sp. 4	175	49
	Lapeyrousia erythranthra (Klot. ex Klatt) Bak. var. welwitschii (Bak.) Marais	112	4
	ex Gerrinch et al.	170	14
	Moraea sp. 1	219	23
	Moraea sp. 2	204	9
	Moraea carsonii Bak.	4	7
Liliaceae	Acrospira asphodeloides Welw. ex Bak.	24	8
Taccaceae	Tacca leontopetaloides (L.) O.Kuntze	1	6
Velloziaceae	Xerophyta retinervis Bak. var. equisetoides (Bak.) Coetzee	126	5
	Xerophyta aff. demeesmaekeriana Duvig. & Dewit	391	18

*Concentrations expressed in µg/g dry weight.

The two new very strong accumulators of copper are Cyanotis longifolia and Ipomoea sp. 1. C. longifolia is a hyperaccumulator of cobalt. As for the cobalt accumulating species, the copper accumulators come from the Velloziaceae and the Commelinaceae and Convolvulaceae steppes.

Other more general points can be noted. The Pteridophytes, except Mohria caffrorum, Gramineae and Cyperaceae appear to have a strong resistance to cobalt and copper uptake. Mohria caffrorum is a small fern which forms lawns on cellular siliceous rocks (Duvigneaud, 1958). Other families eg. Commelinaceae, Crassulaceae, Lamiaceae and Scrophulariaceae, however, have several species with a tolerance of strong internal concentrations of these metals. It can be seen from the concentrations listed (table 2.3) that uptake of copper is more tightly controlled than uptake of cobalt. Thus, despite greater copper concentrations and availability in the soil, cobalt concentrations have a tendency to exceed copper concentrations in the plant. This is presumably a result of the need to more closely regulate the uptake of an essential element (copper) in comparison to a non-essential element (cobalt)(Timperley et al., 1970).

Plants of the open forest assemblage (community A, table 2.2) do not intrude upon the mineralized areas and, hence, have low cobalt and copper concentrations. The general range for these species at Fungurume is 1-29 µg/g for cobalt and 6-22 µg/g for copper. For species of plants typical of termiteria eg. Cussonia arborea, Euphorbia ingens, Selaginella abyssinica and Steganotaenia araliacea, the cobalt and copper content is also low (1-30 µg/g and 7-26 µg/g respectively).

The behaviour of Phragmanthera rufescens var. cornetii in concentrating cobalt relative to its host, Uapaca robynsii, has already been noted. The difference in copper concentrations between the epiphyte and host is not however so marked. A similar situation exists for the hemiparasite of roots, Alectra sessiliflora var. senegalensis. This species is parasitic on Gramineae species (particularly Loudetia spp.). Again while the cobalt content is much greater (371 µg/g in A. sessiliflora var. senegalensis to less than 100 µg/g in the Gramineae), there is no significant difference in the copper content.

2.4 OTHER FLORAL SURVEYS

2.4.1 Lupoto

Lupoto is approx. 50km west of Lubumbashi in southern Shaba (fig 2.2). Thirty-two plant specimens from this area were analysed. The results are shown in table 2.4. The cobalt contents of these plant samples is comparatively low (nothing greater than 50 µg/g) for Shaban metalliferous areas. This is, however, just a reflection of a comparatively low cobalt content of the soil (table 2.1). The copper content has more interest. Two specimens hyperaccumulated copper: Haumaniastrum katangense and an unidentified Asteraceae species. Haumaniastrum katangense is the second hyperaccumulator of copper in this genus, the other being H. robertii (Duvigneaud & Denaeyer-de-Smet, 1963, Brooks, 1977). A further unidentified Haumaniastrum specimen is a very strong accumulator of copper at Lupoto. Four other specimens showed very strong accumulation: Acalypha cupricola, Triumfetta digitata and the two unidentified Becium spp. The general copper levels in plants at Lupoto is very high even for a metalliferous area although the soil levels are not excessively large. Even with omission of those plants showing very strong accumulation or hyperaccumulation the average leaf concentration of copper is 240 µg/g. Specimens of Cryptosepalum dasycladum and Olax obtusifolia have copper levels at Lupoto ten times those at Fungurume.

2.4.2 Ruashi

The abandoned Ruashi mine is on the outskirts of Lubumbashi. The results of analyses of fifteen specimens from this mine area are shown in table 2.5. The soil has a copper content range of 0.3 - 2.2% and a cobalt range up to 1%. The 1% cobalt soil is the richest cobalt soil tested (table 2.1). One new hyperaccumulator of copper was found: Triumfetta digitata. This species has shown very strong accumulation at Lupoto (Section 2.4.1). Acalypha cupricola and Faroa chalcophila were very strong accumulators of copper. Both have shown this property before:

TABLE 2.4

Cobalt and copper concentrations in plants from Lupoto.

Family	Species	Cobalt*	Copper*
PTERIDOPHYTE			
	Unidentified sp.	5	197
DICOTYLEDONES			
Acanthaceae	Thunbergia acutibracteata De Wild.	17	394
	idem	17	227
Asteraceae	Unidentified sp.	38	1487
Caesalpinaceae	Cryptosepalum dasycladum Harms.	8	207
Euphorbiaceae	Acalypha cupricola Robyns	26	905
Lamiaceae	Becium sp.1	18	766
	Becium sp.2	23	884
	Haumaniastrum katangense (S. Moore) Duvign.& Plancke	49	2135
	Haumaniastrum sp.	44	542
Moraceae	Ficus sp.	4	152
Olacaceae	Olax obtusifolia De Wild.	13	129
Papilionaceae	Adenodolichos rhomboideus (O. Hoffm.) Harms.	12	432
	Kotschya sp.	14	405
Santalaceae	Thesium sp.	16	151
Scrophulariaceae	Sopubia densiflora Skan.	11	22
	Sopubia neptuneii Duvign. & Van Bock.	17	125
	Sopubia sp.	24	341
Tiliaceae	Triumfetta digitata (Oliv.) Hutch,& Sprague	33	694
	Triumfetta welwitschii Mast.	14	369
MONOCOTYLEDONES			
Gramineae	Eragrostis boehmii Hack.	5	255
	Monocymbium ceresiiforme (Nees) Stapf	8	217
Liliaceae	Eriospermum welwitschii	13	232

* Concentrations expressed in µg/g dry weight.

TABLE 2.5

Cobalt and copper in vegetation from Ruashi.

Family	Species	Cobalt*	Copper*
DICOTYLEDONES			
Caesalpinaceae	Cryptosepalum dasycladum Harms.	21	45
Combretaceae	Combretum sp.	26	210
Euphorbiaceae	Acalypha cupricola Robyns	77	617
Gentianaceae	Faroa chalcophila P. Taylor	126	239
	idem	104	642
Lamiaceae	Becium cf. obovatum (E.Mey.) N.E.Br.	25	164
	Haumaniastrum katangense (S. Moore) Duvign.& Plancke	643	257
Malvaceae	Hibiscus rhodanthus Gürke	105	224
Scrophulariaceae	Buchnera metallorum Duvign.& Van Bock.	368	186
	Lindernia damblonii Duvign.	321	207
Tiliaceae	Triumfetta digitata (Oliv.) Hutch.& Sprague	256	1057
MONOCOTYLEDONES			
Gramineae	Eragrostis boehmii Hack.	19	86
	Loudetia simplex (Nees) C.E. Hubb.	13	76
	Monocymbium ceresiiforme (Nees) Stapf	20	75
	Rendlia cupricola Duvign.	16	75

*Concentrations expressed in µg/g dry weight

A. cupricola at Lupoto (Section 2.4.1) and F. chalcophila at the Mine de L'Étoile (Malaisse & Grégoire, 1978). Haumaniastrum katangense was the only very strong accumulator of cobalt found: no hyperaccumulators were found. H. katangense has been recorded, previously, as a very strong accumulator of cobalt at the Mine de L'Étoile (Malaisse & Grégoire, 1978).

2.4.3 Lubumbashi

Fourteen specimens of plants growing in the general area of Lubumbashi were analysed. Most of these specimens had low cobalt and copper contents but a few specimens have noticeably higher contents (table 2.6). These plants with high metal contents are growing over either mineralized areas or areas polluted by these metals (from smelters, water run-off, streams from mineralized areas, etc.). No soils were analysed. Three species hyperaccumulated cobalt: Haumaniastrum katangense (previously a very strong accumulator at the Mine de L'Étoile, Malaisse & Grégoire, 1978, and Ruashi, Section 2.4.2), Alectra welwitschii and Bulbostylis mucronata. B. mucronata was the only copper hyperaccumulator. No very strong accumulator of either metal was found. The metal content of B. mucronata is surprisingly high as previously this ephemeral sedge had appeared to resist heavy metal uptake (cf. tables 2.3 and 2.6). Haumaniastrum katangense is the third cobalt hyperaccumulator of this genus. The other two hyperaccumulating species are H. homblei (Section 2.3.3) and H. robertii (Brooks, 1977, Section 2.3.3).

2.4.4 Mindingi

Mindingi is a metalliferous area 170km northwest of Lubumbashi and 55km south of Fungurume (fig 2.2). It is the richest metalliferous area sampled in this survey (table 2.1), although only eleven specimens were collected for analysis. The results of the analyses are shown in table 2.7. An Anisopappus sp. showed hyperaccumulation of cobalt and Bulbostylis abortiva showed hyperaccumulation of copper. No very strong accumulators of either metal were discovered. Anisopappus davyi is a

TABLE 2.6

Cobalt and copper in vegetation from Lubumbashi.

Family	Species	Cobalt*	Copper*
DICOTYLEDONES			
Asteraceae	Laggera sp.	440	31
Bignoniaceae	Tecomaria cf. capensis (Thunb.) Fenzl.	4	50
Lamiaceae	Becium sp.	120	20
	Haumaniastrum katangense (S. Moore) Duvign.& Plancke	2241	166
Malvaceae	Hibiscus rhodanthus Gürke	59	16
Papilionaceae	Adenodolichos rhomboideus (O. Hoffm.) Harms.	18	37
Scrophulariaceae	Alectra welwitschii Hemsl.	1589	78
Verbenaceae	Lippia sp.	12	20
MONOCOTYLEDONES			
Cyperaceae	Bulbostylis abortiva (Steud.) C.B.Cl.	139	26
	Bulbostylis mucronata C.B.Cl.	2127	5701
Gramineae	Arthraxon quartinianus (A. Rich.) Nash	51	36
	Eragrostis boehmii Hack.	44	43
	Monocymbium ceresiiforme (Nees) Stapf	31	31
	Rendlia cupricola Duvign.	37	31

*Concentrations expressed in µg/g dry weight.

TABLE 2.7

Cobalt and copper in vegetation from Mindingi.

Family	Species	Cobalt*	Copper*
DICOTYLEDONES			
Acanthaceae	Unidentified sp.	41	43
Asteraceae	Unidentified sp.	26	21
	Anisopappus sp.	1211	448
Caesalpinaceae	Cryptosepalum maraviense Oliv.	9	35
Lamiaceae	Haumaniastrum polyneurum (S. Moore) Duvign.& Plancke	42	53
	Haumaniastrum robertii (Robyns) Duvign.& Plancke	251	95
Malvaceae	Hibiscus rhodanthus Gürke	27	34
Papilionaceae	Adenodolichos rhomboideus (O. Hoffm.) Harms.	12	22
Proteaceae	Protea hirta Klotz. ex Krauss	1	10
Umbelliferae	Spuriodaucus sp.	14	45
MONOCOTYLEDONES			
Cyperaceae	Bulbostylis abortiva (Steud.) C.B.Cl.	329	1523

* Concentrations expressed in µg/g dry weight.

hyperaccumulator of cobalt at Fungurume (Section 2.3.3) so that if this specimen is not A. davyi, a second hyperaccumulator has been found in this genus. A. hoffmannianus has shown very strong accumulation of cobalt at the Mine de L'Étoile (Malaisse & Grégoire, 1978). Bulbostylis abortiva joins B. mucronata as a hyperaccumulator of copper within the Bulbostylis genus (Section 2.4.3).

2.5 GENERAL DISCUSSION

The surveys of metalliferous soil flora have revealed nine, possibly ten (the identification of one specimen is incomplete) hyperaccumulators of cobalt and seven hyperaccumulators of copper. The counts for very strong accumulators discovered are eight, possibly nine, for cobalt and five, possibly six, for copper (see Appendix III(b)&(c)). The lack of specific identification has a restricting effect on the discussion.

One of the reasons for this identification difficulty is the ecotype problem. The nature of the environment, ie. the heavy metal content of the soil, subjects the plants to physiological stresses which may show as morphological variations to the "normal" plant species form. This can obviously lead to problems at the specific and infra-specific levels of identification. The problem is compounded by the non-continuous nature of the metalliferous soils. Thus many variations of a species present in the surrounding vegetation communities can be seen on individual hillocks or on restricted groups of hillocks (Duvigneaud & Denaeyer-de-Smet, 1963 - Gladiolus spp. p.123, Becium spp. p.130, Buchnera spp. p.132 and Ipomoea spp. p.136). This problem is reflected in the surveys with noticeable numbers of unidentified specifics for the genera Becium, Gladiolus, Ipomoea, Thesium and Tristachya.

The distribution of hyperaccumulation among the plant families should also be noted (tables 2.3, 2.4, 2.5, 2.6, 2.7, Appendix II(b)&(c)). Of the thirteen confirmed species which hyperaccumulate cobalt, eight are members of either Lamiaceae (3 Haumaniastrum spp., 1 Aeolanthus sp.) or Scrophulariaceae

(2 Lindernia spp., 1 Sopubia sp., 1 Alectra sp.). The distribution of copper hyperaccumulation is a little more even although six of the fifteen species belong to either Cyperaceae (2 Bulbostylis spp., 1 Ascolepis sp.) or Lamiaceae (2 Haumaniastrum spp., 1 Aeolanthus sp.). The three Lamiaceae hyperaccumulators of copper are also hyperaccumulators of cobalt. Two other species are also double hyperaccumulators: Lindernia perennis (Scrophulariaceae) and Bulbostylis mucronata (Cyperaceae). Of these five double hyperaccumulators, four are simultaneous hyperaccumulators (ie. take up cobalt and copper to hyperaccumulation level simultaneously) while the other, Haumaniastrum katangense is a differential hyperaccumulator (hyperaccumulation of cobalt and copper occurs but not simultaneously).

It is noteworthy that the majority of the dicotyledonous hyperaccumulators of cobalt and copper belong to the superorder Asteridae (table 2.8). Furthermore it is notable that all monocotyledonous hyperaccumulators are of the superorder Commelinidae. Cobalt and copper hyperaccumulation in the dicotyledones are thus in variation to the nickel hyperaccumulators which are found, predominantly, in the superorder Dilleniidae. Dilleniidae includes the families Cruciferae, Flacourtiaceae, Sapotaceae, Tiliaceae and Violaceae, all of which contain nickel hyperaccumulators (Jaffré, Kersten, et al., 1979, Kersten, 1979). No monocotyledonous hyperaccumulators of nickel have been found for any comparison to be made.

The other point to note from table 2.8 is the relatively advanced characters of the families with hyperaccumulators. It is further noticed that those families with the greater numbers of hyperaccumulating species are generally those with the higher advancement indices. This observation holds for both monocotyledones and dicotyledones. The advancement indices used were devised by Lowe (1961) for the monocotyledones and Sporne (1969) for the dicotyledones and are based on series of morphological characteristics (12 for monocotyledones, 22 for dicotyledones). The index is expressed as a percentage. Among the monocotyledones, the range is from 5 for Velloziaceae (Liliales, Liliidae) to 95 for Zosteraceae (Najadales, Alismatidae) (Lowe, 1961). Within the

TABLE 2.8

Distribution and advancement of cobalt and copper hyperaccumulators.

Family *	Index**	Order	Superorder	Class ***
COBALT HYPERACCUMULATORS				
Asteraceae (1 or 2)	78	Asterales	Asteridae	Dicotyledonae
Crassulaceae (2)	56	Rosales	Rosidae	Dicotyledonae
Lamiaceae (4)	90	Lamiales	Asteridae	Dicotyledonae
Scrophulariaceae (4)	83	Scrophulariales	Asteridae	Dicotyledonae
Commelinaceae (1)	50	Commelinales	Commelinidae	Monocotyledonae
Cyperaceae (1)	79	Cyperales	Commelinidae	Monocotyledonae
COPPER HYPERACCUMULATORS				
Amaranthaceae (1)	72	Caryophyllales	Caryophyllidae	Dicotyledonae
Asteraceae (1)	78	Asterales	Asteridae	Dicotyledonae
Caryophyllaceae (1)	58	Caryophyllales	Caryophyllidae	Dicotyledonae
Lamiaceae (3)	90	Lamiales	Asteridae	Dicotyledonae
Leguminosae (1)	42	Fabales	Rosidae	Dicotyledonae
Scrophulariaceae (1)	83	Scrophulariales	Asteridae	Dicotyledonae
Tiliaceae (1)	40	Malvales	Dilleniidae	Dicotyledonae
Commelinaceae (1)	50	Commelinales	Commelinidae	Monocotyledonae
Cyperaceae (3)	79	Cyperales	Commelinidae	Monocotyledonae
Gramineae (1)	87	Poales	Commelinidae	Monocotyledonae

* Number of taxa given in parentheses.

** Advancement indices from Sporne (1969) for Dicotyledonae and Lowe (1961) for Monocotyledonae.

*** Classification according to Heywood (1978).

dicotyledones, the range is from 21 in Rhizophoraceae (Myrtales, Rosidae) to 100 in Callitrichaceae (Lamiales, Asteridae), Hippuridaceae (Haloragales, Rosidae), Hydrostachydaceae (Scrophulariales, Asteridae), and Phrymaceae (Lamiales, Asteridae) (Sporne, 1969). Cronquist (1968) considers the Asteridae to be the most advanced of the superorders of the dicotyledones. The Commelinidae are also among the most advanced superorders of the monocotyledones. Despite this, families in any given superorder can vary widely in their advancement indices. Reasons for the spread of indices must include an acknowledgement that Cronquist (1968) followed the evolutionary development of the superorders rather than a direct comparison among superorders which have continued to evolve since their various paths diverged. Secondary returns to "primitive" characters must also act as a complication to the development of an advancement index. Nevertheless the index system does appear to give a reasonable scale of evolutionary distance from the "earlier" primitive plants to the "later" advanced plants. Consequent to this it would appear that hyperaccumulation of cobalt and copper is more characteristic of advanced rather than primitive plants. It remains open as to whether this is because the ability to tolerate these metals has been confined to advanced plants or whether the general flora surrounding those metalliferous areas with cobalt and copper hyperaccumulators is itself composed of the more advanced plants with subsequent development of the hyperaccumulators from this source.

CHAPTER 3

Surveys of the Genera *Aeolanthus*, *Ipomoea* and *Pandiaka*

3.1 INTRODUCTION

The discovery of a hyperaccumulator within any genus leads to speculation as to whether or not this genus has a particular tolerance to heavy metals. To investigate this possibility heavy metal determinations have been made on other species within the genus. One problem which arises is the obtaining of samples for analysis. Recently the use of herbarium specimens for this purpose has been developed by R.R. Brooks.

The world's herbaria are a vast repository of plant samples with more than 200million specimens deposited over the last 150 years. The collecting of these specimens has generally been for taxonomic purposes so that their curators have been wary of damaging them for use for other purposes. Certainly the sampling of such specimens for their trace element content by classical analytical methods, which require 5-10g of leaf sample, was impossible. Even the amount required for emission spectrographic analysis was too large for the curator of Florence herbarium to allow sampling by Minguzzi and Vergnano (Pers. com. O. Vergnano Gambi to R.R. Brooks). The more recent development of atomic absorption spectroscopy with its much greater sensitivity appears to have solved many of these worries. Analysis of herbarium material for trace metals at µg/g concentrations requires a sample no greater than 0.03g and may be carried out routinely on samples of less than 0.01g although at this level the limit of detection is rising. Use of a carbon rod furnace can lower the sample requirement even further since all the sample is analysed whereas in flame analysis only five percent of the aspirated sample is actually analysed.

Even without analysis, herbarium specimens had been used for prospecting purposes. Persson (1956) examined "copper mosses" in Swedish herbaria noting their original locality which he then re-examined by other prospecting methods. Cole (1971) in Namibia and Botswana identified cuprophytes over a known copper deposit then checked herbaria for the localities of other specimens of these species.

The first reported analysis of herbarium material appears to be by Chenery (1948). He semiquantitatively determined the aluminium content of approx. 4,000 dicotyledone specimens. Even this required 6cm^2 of leaf sample. Later Rühling and Tyler (1969) and Tyler (1970) used analyses of herbarium specimens of mosses to show historical trends in concentrations of heavy metals within the area of collection. In 1971, Goodman and Roberts monitored atmospheric pollution by analysis of herbarium specimens of bryophytes.

In the late 1970's, use of herbarium samples has become more common. Chenery and Sporne (1976) completed sampling of 259 dicotyledone families for aluminium, finding that 37 families contained at least one accumulator of this element. Brooks, Lee, et al. (1977) carried out the first survey of nickel hyperaccumulating genera from herbarium samples. This has now become a recognized method for surveying hyperaccumulating genera (Brooks, 1977, Brooks, McCleave & Schofield, 1977, Brooks, McCleave & Malaisse, 1977, Brooks, Wither & Zepernick, 1977, Brooks & Radford, 1978a, Jaffré, Brooks & Trow, 1979, Kersten, et al, 1979). Herbarium specimens have also been used to survey families or species in various geographical regions (Brooks & Wither, 1977, Jaffré, Kersten, et al., 1979, Brooks, Trow & Sølviken, 1979), samples from restricted areas (frequently metalliferous) (Wither & Brooks, 1977, Brooks, Wither & Westra, 1978), and genera of tolerant but essentially non-accumulating species (Brooks & Radford, 1978b).

Two of the herbarium surveys are of particular interest: Brooks (1977) surveyed Haumaniastrum species and Brooks, McCleave and Malaisse (1977) the African species of Crotalaria. In Brooks, McCleave and Malaisse (1977) highly-anomalous values, for dried leaf material, are designated as equal to or exceeding 5 μgCo/g and 50 μgCu/g. These values correspond to a level ten times normal values on non-metalliferous soils. On this basis, Brooks (1977) showed highly anomalous concentrations of both cobalt and copper in three out of nineteen Haumaniastrum species although only one, H. robertii, hyperaccumulated these elements. The other two species, H. homblei and H. katangense, have both since shown

hyperaccumulation of cobalt and H. katangense has also shown hyperaccumulation of copper (Chapter 2). The Crotalaria survey showed highly anomalous values in thirteen of 284 species analysed, excluding C. cobalticola from Duvigneaud's (1959) analysis (Brooks, McCleave & Malaisse, 1977). Of these fourteen species, three had anomalous cobalt only, six anomalous copper only and five had both anomalous cobalt and copper. No species were hyperaccumulators although C. peschiana showed very strong accumulation of copper. C. cobalticola had previously shown very strong accumulation of cobalt (Duvigneaud, 1959) and has since shown hyperaccumulation of this element (Brooks, unpublished data).

In a continuation of these surveys, the results are reported here for the genera Aeolanthus, Ipomoea and Pandiaka. The Ipomoea survey has been restricted to species from Central Africa.

3.2 ANALYTICAL METHODS

Specimens from the genera Aeolanthus, Ipomoea and Pandiaka were supplied by the herbarium of the Jardin Botanique National de Belgique at Brussels (BR). On arrival, the collection details (collector and locality) were noted. Samples, of weight 0.01-0.03g were placed in $5cm^3$ borosilicate test-tubes and ashed for two hours at 500^oC in a muffle furnace. The ash was then dissolved in $1cm^3$ of 2M hydrochloric acid prepared from redistilled constant-boiling hydrochloric acid. Gentle heat was applied, if necessary, to ensure dissolution. Any sample which was contaminated by soil, revealed by the presence of a red sediment, was rejected.

The solutions were analysed for cobalt and copper by atomic absorption spectrophotometry as in Chapter 2. The lines used for analysis were 240.8nm and 304.4nm for low and high cobalt concentrations respectively and 324.8nm and 218.2nm for low and high copper concentrations respectively. The results of all analyses are expressed as µg/g dry weight.

3.3 AEOLANTHUS MART. (LAMIACEAE)

The genus Aeolanthus Mart. (Lamiaceae) contains approx. fifty tropical or subtropical species (Willis, 1973). The species are generally perennial herbs. Sixty-four specimens covering forty-eight species of Aeolanthus were analysed and the results are shown in table 3.1.

The most striking features of the results are the hyperaccumulating abilities of A. biformifolius and A. rosulifolius. A. biformifolius has previously shown hyperaccumulation at the Mine de l'Étoile (Malaisse & Grégoire, 1978). This species is a simultaneous hyperaccumulator of cobalt and copper. A. rosulifolius showed only hyperaccumulation of copper with cobalt restricted to very strong accumulation. A. saxatilis also had a high cobalt content although at 428 µg/g the sample did not reach very strong accumulation. The field survey at Fungurume (Chapter 2) had a sample with a higher cobalt content (996 µg/g). Accumulation of copper by this species is virtually unknown. Given that cobalt is generally at a level of less than 1 µg/g in plant material, the concentration in Aeolanthus species is elevated even for those not from the "copperbelt". The mean cobalt content for these non-"copperbelt" specimens is 9.2 µg/g. This degree of cobalt accumulation over a single genus has previously been reported only for Nyssa (Brooks, McCleave & Schofield, 1977). The mean copper content of the non-"copperbelt" specimens is, however, normal at 5.4 µg/g. The two hyperaccumulating species, A. biformifolius and A. rosulifolius, are superficially similar in morphology (see leaf sections, figs. 3.1 and 3.2 for comparisons of A. biformifolius, A. rosulifolius and A. saxatilis). Further study (by a botanist) would be required before any further comments could be made but it is worth noting that A. biformifolius is found only in the Lubumbashi area (Luiswishi, Mine de l'Étoile, Ruashi) while A. rosulifolius is found at Fungurume (see fig. 2.2 for locations). Could this be an example of ecotypic differentiation?

A further difficulty involved in the study of the genus Aeolanthus is the need for a revision of this genus and the genus Icomum Hua. Icomum, as listed by Willis (1973), consists of ten

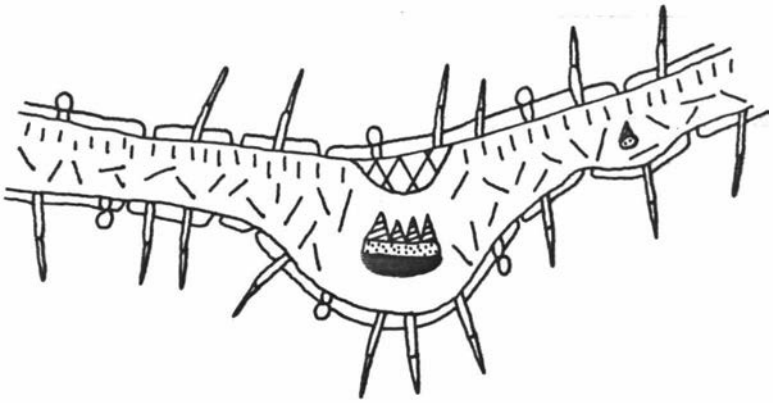
TABLE 3.1

Cobalt and copper concentrations in species of *Aeolanthus*.

Species	Location	Cobalt*	Copper*
A. abyssinicus Hochst. ex Benth.	Addis Ababa, Ethiopia	12	12
A. adenotrichus Gürke	Shaba, Zaïre	22	17
A. affinis De Wild.	Lake Albert, Zaïre	8	7
A. bequaertii De Wild.	Bogoro, Zaïre	35	49
A. biformifolius De Wild.	Étoile, Zaïre	2383	3923
	Luiswishi, Zaïre	2520	432
A. breviflorus De Wild.	Katuba, Zaïre	12	8
A. butaguensis De Wild.	Kasindi, Zaïre	13	8
	Mobenga, Zaïre	13	2
A. caliris Briq.	Upper Oubangui, Zaïre	13	10
A. cameronii Burk.	Gwanda, Zambia	50	10
	Mtoko, Zambia	26	3
A. canescens Gürke	Rusapi, Zambia	4	4
A. claessensi De Wild.	Lake Albert, Zaïre	7	5
A. conglomeratus Bak.	Mwinilunba, Zambia	4	3
A. elongatus De Wild.	Kambove, Zaïre	28	11
A. engleri Briq.	Tanzania	6	2
	Isoka, Zambia	5	3
A. elsholtzioides Briq.	Solwezi, Zambia	20	5
A. ganwellae Taylor	Ufipa, Tanzania	5	10
A. glabrifolius De Wild.	Irumu, Zaïre	18	4
A. glandulosus Gürke	Ussangu, Zaïre	12	1
A. goetzei Gürke	Tanzania	5	3
A. heliotropioides Oliv.	Dawo, Nigeria	10	3
	Uganda	13	3
	Chingola, Zambia	7	3
A. holstii Gürke	Tanzania	8	4
A. homblei De Wild.	Katentania, Zaïre	6	7
A. lamborayi De Wild.	Basankusu, Zaïre	9	6
A. linearis Hua & Briq. ex Briq.	Kasungu, Zaïre	7	3
A. lobatus N.E. Br.	Vila-Aniaga, Angola	10	1
A. lugai De Wild.	Sankuru, Zaïre	6	3
	Shaba, Zaïre	22	17

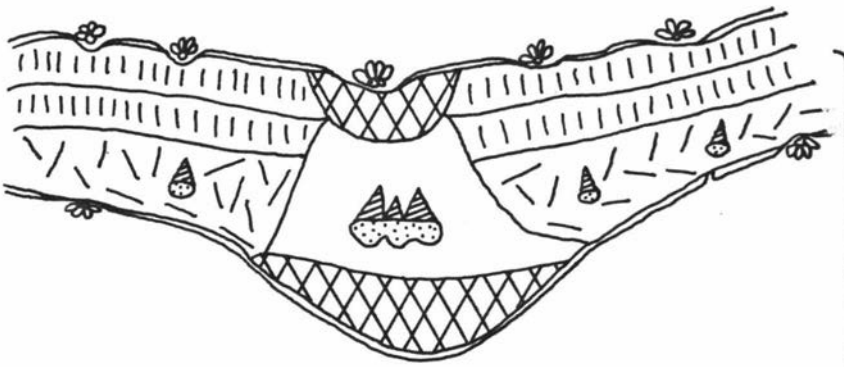
A. myrianthus Bak.	Zambia	1	1
A. nyassae Gürke	Tanzania	4	4
A. nykensis Bak.	Tanzania	6	2
A. parvifolius Benth.	Rhodesia	7	1
A. petasatus Briq.	Eala, Zaïre	4	9
A. pinnatifidus Hochst.	Ethiopia	8	8
A. pubescens Benth.	Cameroun	10	2
A. rehmannii Gürke	Zambia	8	4
A. repens Oliv.	Niragongo, Zaïre	4	2
	Ineac-Kisoro, Zaïre	5	2
	Yakahondo, Zaïre	9	3
	Gabiro, Ruanda-Urundi	6	3
	Kisosi, Ruanda-Urundi	4	2
A. robyns De Wild.	Kasenga, Zaïre	8	76
A. rosulifolius Duvig. et Denaeyer.	Fungurume, Zaïre	20	15
	Fungurume, Zaïre	753	1113
A. saxatilis Duvig. et Denaeyer.	Fungurume, Zaïre	428	16
A. schliebenii Mansf. et Mildbr.	Tanzania	5	3
A. semicylindriens De Wild.	Busiga, Ruanda-Urundi	3	7
A. stormsioides sp. nov.	Sandoa, Zaïre	4	2
A. tuberculatus De Wild.	Luiswishi, Zaïre	79	2
A. tuberosis Hiern.	Gitega, Burundi	6	4
A. ukambensis Gürke	Tanzania	3	3
	Zambia	3	50
A. violacens De Wild.	Shaba, Zaïre	7	4
	Pweto, Zaïre	5	1
A. virgatus Gürke	Ango, Zaïre	5	9
	Tukwo, Zaïre	5	2
A. xerophytiens Lebrun	Faradje, Zaïre	3	1
A. zanzibarensis S. Moore	Tanzania	13	3
	Kenya	7	4

* Concentrations expressed as µg/g dry weight.

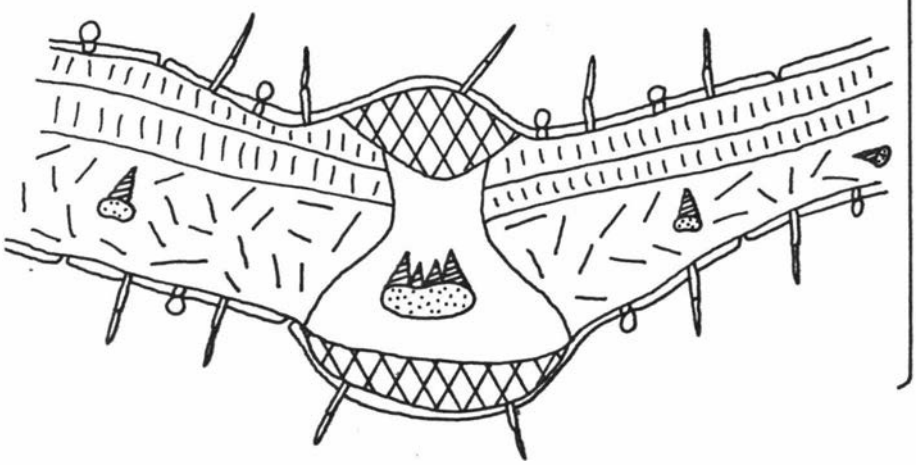


A. *A. saxatilis*

- palisade parenchyma is a poorly-differentiated layer.
- sclerenchymatous tissue supports the main vein.



B. *A. rosulifolius*



C. *A. biformifolius*

- palisade parenchyma consists of two distinct layers.
- collenchyma strands support the main vein.

All three species:

- show a tendency to succulence.
- have stomata present on both surfaces.
- have striated epiderma.

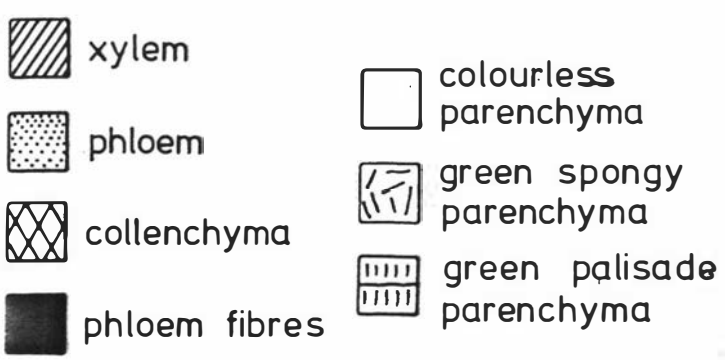


Figure 3.1 Transverse sections through the laminae of three *Aeolanthus* species.

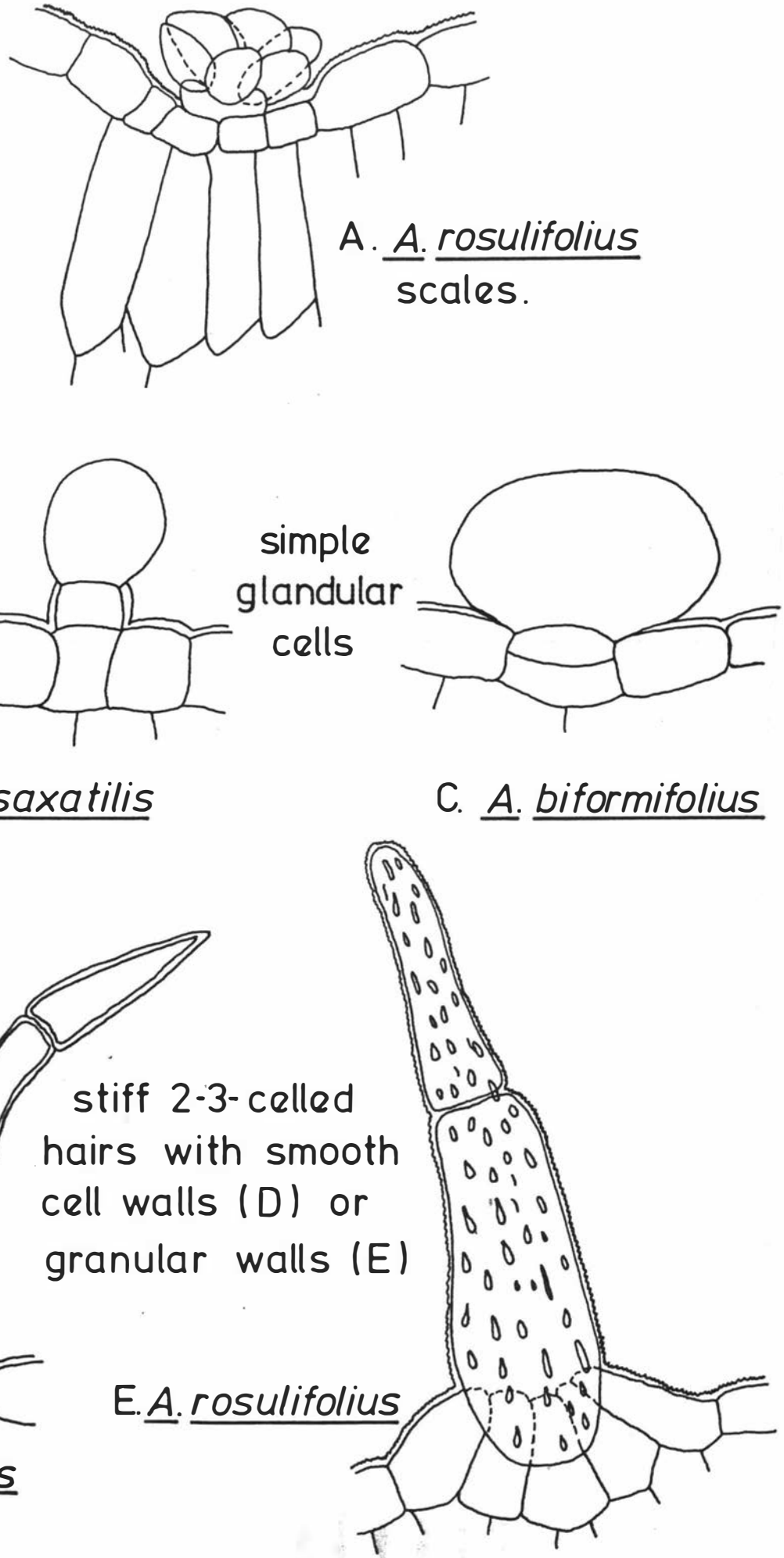


Figure 3.2 Foliar idumenta for three metal-tolerant species of *Aeolanthus*.

tropical African species. Several species appear to be double-listed by Index Kewensis. A. biformifolius is one such species, being listed as I. biformifolium De Wild. as well. The original reference cited for both names is De Wildeman (1927). De Wildeman (1927) formed A. biformifolius by splitting A. linearis (also known as I. lineare Burk.) into three new species. The other two species formed being A. elongatus (I. elongatum De Wild.) and A. tuberculatus (I. tuberculatum De Wild.). None of A. linearis, A. elongatus or A. tuberculatus show levels of cobalt or copper in leaf tissues above those "normal" to mineralized areas.

The A. group linearis species are found on colluvion which is rich in copper or in crevices on cellular siliceous rocks. The other copper hyperaccumulator, A. rosulifolius, and the very strong cobalt accumulator, A. saxatilis, are both found on cellular siliceous rocks. The metalliferous species of Aeolanthus are generally geophytes with corms or hemicryptophytes with woody stems. The growth cycle of these species is often short: at Mine de l'Étoile, A. biformifolius has a three month "rainy season" growing cycle (Malaisse & Grégoire, 1978).

3.4 IPOMOEA L. (CONVOLVULACEAE)

Ipomoea L. (Convolvulaceae) is a large genus (approx. 500 species) of tropical and warm temperate regions (Willis, 1973). The plants themselves are often vines or creepers. The genus is probably best known as the genus of the sweet potato, I. batatas Poir. The genus is well represented in Shaba although difficulties in specific identification do occur (Duvigneaud & Denaeyer-de-Smet, 1963). Thirty-eight specimens covering twenty-seven taxa were analysed for their cobalt and copper content in this survey. All taxa are from the Central African region. The results of the analyses are shown in table 3.2.

No hyperaccumulation of either cobalt or copper has been recorded for this genus either before, or during, this survey. However the fact that very strong accumulation of cobalt has been shown (Chapter 2) and thatthe distribution of taxa over the various

TABLE 3.2

Cobalt and copper concentrations in some Ipomoea species.

Species	Location	Cobalt*	Copper*
I. alpina Rendle	Gitega, Burundi	xx	40
	Étoile, Zaïre	178	63
I. alpina Rendle ssp. hockii (De Wild.)Duvig. et Dewit.	Étoile, Zaïre	116	489
I. aquatica Forsk.	Kambove, Zaïre	xx	22
I. arachnosperma Welw.	Kasenga, Zaïre	xx	10
I. barteri Bak.	Kasapa, Zaïre	xx	17
	Lubumbashi, Zaïre	xx	29
I. blepharophylla Hall.	Kantala, Zaïre	xx	20
I. cairica (L.) Sweet	Étoile, Zaïre	4	49
I. crassipes Hook. var. crassipes	Mwinilunga, Zambia	xx	7
I. crepidiformis Hall. f. var microcephala (Hall.f.) Verdc.	Abercorn, Zambia	xx	10
I. dammeriana De Wild.	Manono, Zaïre	2	36
I. debeerstii De Wild.	Katuba, Zaïre	xx	68
I. digitata L.	Kakonkania, Zaïre	xx	28
I. eriocarpa R. Br.	Bunkeya, Zaïre	xx	12
I. fulvicaulis (Choisy) Hall.f. var. asperifolia (Hall.f.) Verdc.	Muhila, Zaïre	xx	15
	Lubumbashi, Zaïre	4	12
I. involucrata Beauv. var. involucrata	Lakulu, Zaïre	xx	17
	Keyberg, Zaïre	xx	55
I. lapathifolia Hall. f. var lapathifolia	Rusizi, Burundi	xx	14
I. linosepala Hall. f.	Kafubu, Zaïre	5	37
	Lubumbashi, Zaïre	2	44
	Luiswishi (Mine), Zaïre	44	57
I. lukafuensis De Wild.	Lutshipuka, Zaïre	xx	15
	Kibondja, Zaïre	xx	110
	Lubumbashi, Zaïre	6	119
I. ochracea (Lindl.) G. Don. var. ochracea	Lofoi, Zaïre	xx	22
I. pes-caprae (L.) R. Br. ssp. brasiliensis (L.)van Ooststr.	Uvira, Zaïre	1	18
I. pharbitiformis Bak.	Katshupa, Zaïre	xx	6
	Mutombo-Mukulu, Zaïre	xx	19

	Kisanga, Zaïre	xx	12
I. pes-tigridis L. var. pes-tigridis	Upemba, Zaïre	xx	4
I. pileata Roxb.	Keyberg, Zaïre	xx	21
I. prismatosyphon Welw.	Maseba, Zaïre	xx	5
	Lungulungu, Zaïre	2	8
I. rubens Choisy	Dikuluwe, Zaïre	xx	11
I. shupangensis Baker	Lubumbashi, Zaïre	3	66
I. vernalis R.E. Fr.	Kapiri, Zaïre	1	17

* Concentrations expressed as µg/g dry weight.

xx Cobalt concentration less than 1 µg/g.

hillocks has been studied by Duvigneaud and Denaeyer-de-Smet (1963, pp.136-144) makes a study of the cobalt and copper content of interest. Many of the taxa on metalliferous soils have discontinuous ranges over the hillocks. The highest cobalt content in this survey was found in I. alpina (178 µg/g) with I. alpina ssp. hockii having a slightly lower content. I. linosepala and I. lukafuensis also show anomalous values (> 5 µg/g). I. alpina ssp. hockii had the highest copper content(489 µg/g), just below very strong accumulation content. I. lukafuensis had the second highest copper content but barely passed the 100 µg/g level. Other species to record anomalous copper concentrations (> 50 µg/g) were I. debeerstii, I. shupangensis, I. alpina, I. linosepala and I. involucrata var. involucrata. In the field surveys (Chapter 2), I. alpina had shown very strong accumulation of cobalt (table 2.3) but the subspecies for this specimen is not known. Duvigneaud and Denaeyer-de-Smet (1963) have recorded I. alpina ssp. argyrophylla Duvign.& Dewit. at Fungurume. They found this taxa growing on soils low in copper with I. debeerstii ssp. debeerstii replacing it on soils rich in copper. I. debeerstii does not appear to be a strong accumulator of either metal although it can reach anomalous levels for both. Anomalous levels are however "normal" for plants on metalliferous substrates. Further research combining botanical studies of metalliferous-soil taxa and biogeochemical studies of their cobalt and copper uptake may help in understanding the causes of the discontinuous ranges of the various taxa and the nature of their evolution.

3.5 PANDIAKA (MOQ.) HOOK. f. (AMARANTHACEAE)

Pandiaka (Moq.) Hook. f. (Amaranthaceae) is a genus of some twenty species from tropical and southern Africa. Members of the genus tend to be herbaceous in growth. Fifteen samples covering eight taxa of Pandiaka were analysed for cobalt and copper. The results of these analyses are shown in table 3.3.

The most striking feature of table 3.3 is the hyperaccumulation of copper by P. metallorum. This species has also shown copper hyperaccumulation in the Fungurume field survey (Chapter 2). The cobalt content is also markedly elevated, reaching the level of very

TABLE 3.3

Cobalt and copper concentrations in *Pandiaka* species.

Species	Location	Cobalt*	Copper*
P. andongensis Hiern.	Kasenga, Zaïre	xx	18
	Marungu, Zaïre	xx	15
P. carsoni (Bak.) Clarke	Manika, Zaïre	xx	5
	Lubumbashi, Zaïre	xx	79
P. carsoni (Bak.) Clarke var. *linearifolia* Hauman	Kalashie, Zaïre	xx	7
	Luiswishi, Zaïre	134	123
P. glabra (Schinz.) Hauman	Mukuen, Zaïre	xx	39
	Keyberg, Zaïre	4	120
P. kassneri Suessenguth	Kala, Zaïre	xx	8
P. metallorum Duvig. et Van Bock.	Fungurume, Zaïre	570	6270
	Fungurume, Zaïre	161	1130
	Fungurume, Zaïre	101	629
P. obovata Suess.	Katuba, Zaïre	25	27
P. polystachya Suess.	Katema, Zaïre	xx	13
	Kansenia, Zaïre	8	8

* Concentrations expressed as µg/g dry weight.

xx Concentration less than 3 µg/g.

strong accumulation. The field survey sample at 448 µg/g was only just below this level. Other anomalous values were recorded for both cobalt and copper in P. carsoni var. linearifolia, for cobalt only in P. obovata and P. polystachya, and for copper only in P. carsoni and P. glabra. All samples, anomalous or not, were from Zaïre. P. carsoni, P. carsoni var. linearifolia and P. glabra may be locally useful for indicating mineralization but none are universal indicators. Thus for P. carsoni var. linearifolia, a specimen from Luiswishi Mine showed significant heavy metal levels while a specimen from the Kalashie salt pan showed only trace amounts. It is necessary to note that P. metallorum had previously been considered to be a variant of P. carsoni (Ernst, 1974). Duvigneaud and Denaeyer-de-Smet (1963) considered that P. metallorum probably has many varieties, basing their supposition on the wide variability in leaf forms and sizes. They also considered the species to be little different from other species found in dilungus and dambos and was probably derived from them by ecological isolation. Unlike most other species which only flower in either the rainy or the dry season, P. metallorum flowers throughout the year. The species is widespread in Shaba, being present on virtually all the cupriferous outcrops.

3.6 GENERAL DISCUSSION

The surveys of Aeolanthus, Ipomoea and Pandiaka have revealed only one new hyperaccumulator: A. rosulifolius showed hyper-accumulation of copper. The hyperaccumulation of cobalt and/or copper in A. biformifolius and P. metallorum was confirmed. Also revealed was the ability of Aeolanthus species, in general, to accumulate significant quantities of cobalt even when not growing over notably rich substrates.

The use of herbarium specimens has greatly aided the rapidity with which the systematic surveys of genera for metal accumulation can be carried out. This information may be useful in helping with the delineation of species, or lower taxonomic ranks, for plants found on metalliferous soils. Undoubtedly the final delineations will be made on floristic characters but how should the effect of physiological stress from an adverse (metal-rich) environment as a

cause of morphological differences be noted in the ranking? Such problems are not unknown among nickel hyperaccumulators: Dicoma niccolifera was originally described as a Dicoma macrocephala subspecies (Wild, 1970, 1971) and the Alyssum taxon, A. serpyllifolium ssp. lusitanicum shows hyperaccumulation while the taxon A. serpyllifolium ssp. serpyllifolium does not (Brooks & Radford, 1978a). This is again a form of ecotypic differentiation but between taxa of metalliferous and non-metalliferous soils. The existence of ecotypic differentiation between taxa of various metalliferous soils only serves to compound the issue. It has been noted in this chapter that P. metallorum appears to have ecotypic differentiation between taxa on metalliferous or non-metalliferous soils and between the different metalliferous outcrops. Ecotypic differentiations within Aeolanthus and Ipomoea however are generally between taxa on the different metalliferous outcrops.

Finding causes for these ecotypic differentiations will have to involve widespread samplings of specimens from both metalliferous and non-metalliferous soils and experimental work on their physiology and morphology. Shewry et al. (1979) have shown morphological differences between Cryptosepalum maraviense specimens on a rocky, cupriferous soil at Kazinyanga and a non-cupriferous soil near Dikuluwe. They have also investigated Xerophyta specimens, at Dikuluwe, on a rocky, cupriferous soil, a rocky, non-cupriferous soil and in a cupriferous dambo and found that two basic morphological forms existed: form one on rocky sites with or without copper enrichment; form two on deep, fine, dry soil in the cupriferous dambo. There were smaller differences between the specimens on the rocky, cupriferous soil and the corresponding non-cupriferous soil. Thus it can be seen that it is not only the metal content which has an effect on the plant morphology but that other soil factors are also involved. Howard-Williams (1970) found significant differences in leaf shapes of Becium homblei specimens collected on soils containing over 3,000 µg Cu/g compared with those growing on soils with less than 1,000 µg Cu/g. These results, from field sample analysis, could not be reproduced in shade-house experimental trials. From this, Howard-Williams suspected that the differences were due to microclimatic factors rather than soil-metal levels. Thus variations between plant specimens of the same or closely-related species can make specific identification difficult because of the

wide range of variations possible as responses to many and varied environmental factors. Wild and Bradshaw (1977) list ten species which show some degree of morphological differentiation between tolerant and non-tolerant populations on toxic (copper-rich, nickel-rich or serpentine) soils in Zimbabwe. In the final analysis, ecotypic differentiation becomes a question as to whether the differences are "real" (genetically inherited) or merely a result of stress and this can only be determined by further experimental work.

CHAPTER 4

Biogeochemical Studies on Some Metallophytes from Shaba

4.1 INTRODUCTION

The existence of metallophytes leads inevitably to questions concerning the abilities of these plants to tolerate the metals. For those metallophytes which are also hyperaccumulators, a further question arises as to the maximum amount which can be accumulated without killing the plant. Few studies have been made of cobalt and copper metallophytes however, and none have been made of cobalt and copper hyperaccumulators. Among the copper-tolerant species studied are several from the Dugald River area of Australia (Nicolls *et al.*, 1965), *Becium homblei* (Reilly, 1969, Howard-Williams, 1970), *Mimulus guttatus* DC. (Allen & Sheppard, 1971), *Indigofera* spp. (Ernst, 1972), *Agrostis stolonifera* L. (Wu *et al.*, 1975), *Agrostis gigantea* (Hogan *et al.*, 1977) and two Fennoscandian species (Crooks, 1979). Most of these studies have investigated tolerance to metals with a more limited number involving uptake characteristics.

In studies of uptake characteristics, three general forms of uptake can be distinguished: the exclusion-breakdown form (Nicolls *et al.*, 1965, Wu *et al.*, 1975, Crooks, 1979), the rise-to-saturation form (Reilly, 1969) and the linear form (Nicolls *et al.*, 1965). The exclusion-breakdown form, characterized by entry restrictions at low soil metal levels with an apparent breakdown of the exclusion mechanism with consequent accumulation of the metal on soils with a higher metal content, can be found for almost any metal studied (eg. nickel, copper, zinc, lead) irrespective of essentiality or non-essentiality. This contrasts with the work of Timperley *et al.*(1970b) who suggest that different uptake patterns could be expected for essential and non-essential elements. In their studies, essential elements appeared to have the exclusion-breakdown form and non-essential elements the linear form. Further contrast to Timperley *et al.* (1970b) comes from a study of *Becium homblei* (Reilly, 1969) in which copper uptake shows not the exclusion-breakdown form but the rise-to-saturation form. This form is characterized by a linear rise in the metal content of the plant at low soil metal levels with a flattening of this curve at higher soil metal levels. Whether a real saturation level

of some kind is reached or some other effect gives an apparent saturation level is unknown. It should be noted that in their studies on Agrostis stolonifera, Wu et al. (1975) showed both these forms of uptake: the exclusion-breakdown form for leaves and the rise-to-saturation form for roots. As could be expected the point of exclusion-breakdown (ie. the point where the copper concentration begins to rise) in the leaves corresponded well with the saturation point in the roots. No other study has involved both root and leaf uptake patterns. The linear form of uptake, characterized by a purely linear curve, has been observed for zinc in three Australian metal-tolerant species (Nicolls et al., 1965) and for non-essential elements in non-tolerant plants (Timperley et al., 1970b, Beckett & Davis, 1977).

The tolerance of a species to a particular metal can be tested in various ways. In 1957, Wilkins conceived the solution tolerance test in which the plant to be tested was rated on root growth in control and experimental solutions. From the root growth, a tolerance index, I, was calculated:

$$I = \frac{\text{length of root in experimental solution}}{\text{length of root in control solution}} \times 100$$

For his control solution Wilkins (1957) tested both distilled water and a full nutrient solution. He found that the concentration levels required for lead studies were too low in aqueous solution to be easily handled but were higher and, hence, more easily handled in the full nutrient solution. By testing the components of the full nutrient solution individually, he found that calcium ameliorated the toxicity of the lead. For his subsequent work, and for most other workers (Jowett, 1958, 1964, McNeilly, 1968, Allen & Sheppard, 1971, Wu & Antonovics, 1976, Craig, 1977), calcium nitrate at 0.5-1g/l has been added to distilled water to form the control solution. This test can not only show tolerance to one metal but can also be used to arrange orders of tolerance (or conversely orders of toxicity) for various metals within a given species (Jowett, 1958, Craig, 1977). A second technique, comparative protoplasmatology, has been used by Repp (1963), Gries (1966) and Ernst (1972). This method uses cells, generally from shoots, and places them in graduated metal solutions for a given time before

being tested for vitality (the ability to plasmolise in sugar solution). This method gives only a limit of tolerance. Allen and Sheppard (1971) developed a soil rooting method as well as using the solution rooting method above. This new method involved potting cuttings into a copper-rich soil and an uncontaminated soil, leaving them to develop roots and then calculating a tolerance index as for the solution rooting technique. The soil rooting technique appeared to work as well as the solution rooting technique.

Horscroft (1961) reported germination tests on Becium homblei in a solution culture experiment and found that the species required 50-600 µg Cu/g for germination which suggests a physiological requirement for high copper concentrations. Howard-Williams (1970), however, found that the same species germinated readily in distilled water. Allen and Sheppard (1971) tested the germination and establishment of Mimulus guttatus seedlings in contaminated and uncontaminated soils. They found that this test easily distinguished between the tolerant and non-tolerant populations. All tolerant populations germinated readily in uncontaminated soils.

The studies reported here look at the uptake and accumulation of cobalt and copper in three Shaban hyperaccumulators: Haumaniastrum katangense, H. robertii and Aeolanthus biformifolius. The tolerance of two of these species, H. katangense and A. biformifolius, was determined by a soil culture method. Germination experiments were also carried out on two species; H. robertii and A. biformifolius. Finally, the uptake of copper by A. biformifolius was followed through a growing season.

4.2 EXPERIMENTAL METHODS

Seeds of three metallophytes (Haumaniastrum katangense, H. robertii and Aeolanthus biformifolius) and corms of A. biformifolius were collected in Shaba Province, Zaïre (Fungurume or Mine de L'Étoile) by Prof. F. Malaisse of the Université Nationale du Zaïre at Lubumbashi. The seeds were stored under refrigeration until used.

Before any germination could proceed, the seeds had to be washed in running water for 4-7 days. This is presumed to be necessary because of an adaptation to the climatic conditions of their homeland. The seeds germinate at the beginning of the rainy season since most plants there grow during the rainy season and die-back during the dry season. It is necessary therefore that seeds do not germinate too early. It appears that some inhibitor of germination has been developed and only the continued presence of excess water overcomes this inhibition, probably by leaching the inhibitor from the seed.

4.2.1 Uptake and Accumulation Trial

A potting mixture of 50% peat - 50% perlite with added nutrients was used in this trial. To the basic mixture was added cobalt and/or copper (as nitrates) to give the following conditions; 100, 1,000 and 10,000 µg/g of cobalt, of copper and of cobalt + copper. Background mixture (ie. no additives) was used for control purposes. The potting mixture was placed in pots each of which contained approx. 200g. Each concentration of heavy metals was replicated five times. One seedling was planted per pot. The seedlings had been germinated on a Copenhagen table after washing and then planted into background potting mixture until the second pair of leaves developed. At this time the seedlings were transplanted into the experimental pots. All pots were watered from beneath. All three metallophytes were grown. Leaf samples were collected for analysis after five weeks and every second week thereafter. All survivors were sampled in each condition and a composite sample was analysed. The soils were sampled to determine their cobalt and copper content after two months.

A supplementary uptake trial was made using corms, rather than seeds, of A. biformifolius. The same conditions with respect to the potting mix and metal content applied. Five replicates were also used at each concentration. The corms were planted directly into the potting mix with one corm per pot. After three months, the leaves and the soils were sampled for analysis.

All leaf analyses were performed by the method used for the field survey samples in Chapter 2. The soil analyses were performed by digesting 0.1g of oven-dried soil with $20cm^3$ of aqua regia. This digest was taken to dryness over a water-bath and then redissolved in $10cm^3$ of 2M hydrochloric acid (prepared from redistilled constant-boiling hydrochloric acid). This solution was centrifuged to remove the unreacted silicates and the supernatant decanted. The supernatant was then diluted as necessary (up to $100cm^3$). Analysis was performed by atomic absorption spectrophotometry as for Chapter 2.

4.2.2 Tolerance Tests

A tolerance test was devised which used the peat-perlite potting mix of the uptake trial. Seeds of *H. katangense* and *A. biformifolius* were germinated on a Copenhagen table and then planted into bedding trays. After the development of a second pair of leaves, the seedlings were transplanted into vials containing approx. 2g of background potting mix. After a further ten days, to allow for recovery after the transplant, solutions of cobalt or copper were added to give the desired concentrations: for *H. katangense*, background and 100-64,000 µg/g of either cobalt or copper with four replicates per concentration; for *A. biformifolius*, background, 100-16,000 µg/g of cobalt and 100-64,000 µg/g of copper also with four replicates per concentration. The tests were continued until stable results were achieved (ie. no change over three weekly readings). The tolerance level was taken as the highest concentration with at least half the seedlings surviving. Other concentrations for which any individual specimen survived were also noted. All soils were analysed for cobalt and copper content after the test. The analyses were performed as for the soils in the uptake trials. Leaves of *H. katangense* were also analysed for their cobalt and copper content. Analysis of these samples was performed as for field survey samples in Chapter 2. Leaves of *A. biformifolius* were not analysed because of algal problems encountered in this test.

4.2.3 Germination Tests

After the washing period, seeds of two species, H. robertii and A. biformifolius, were placed on filter papers within petri dishes. The filter papers had previously been soaked in solutions of cobalt, of copper or of cobalt + copper over a range from 0.1 - 3%. A control was kept by germinating seeds on a filter paper soaked in deionized water. Twenty seeds were used per concentration. All filter papers were kept moist; if necessary deionized water was added to maintain the moisture level. The test was continued for three weeks. Both the rate and the percentage of germinations were recorded.

4.2.4 Seasonal Copper Uptake in Aeolanthus biformifolius

Samples from a specimen of A. biformifolius growing at Mine de L'Étoile were collected by Prof. F. Malaisse throughout the plant's three month growing season. These samples were analysed for copper by the method employed for field survey samples in Chapter 2.

4.3 COBALT AND COPPER UPTAKE

The results of the seedling uptake trials are shown in figs. 4.1 for H. katangense, 4.2 for H. robertii and 4.3 for A. biformifolius. It should be noted that each point on the diagrams is a mean of values over four analyses. No differences in the leaf concentrations, at each soil concentration, were found over the weeks that they were tested, indicating that the uptake of cobalt and copper had reached a limiting value before sampling began at five weeks in the trial substrates. Only H. robertii survived in all concentrations. H. katangense failed to survive in any 10,000 µg/g pot irrespective of the metal present;

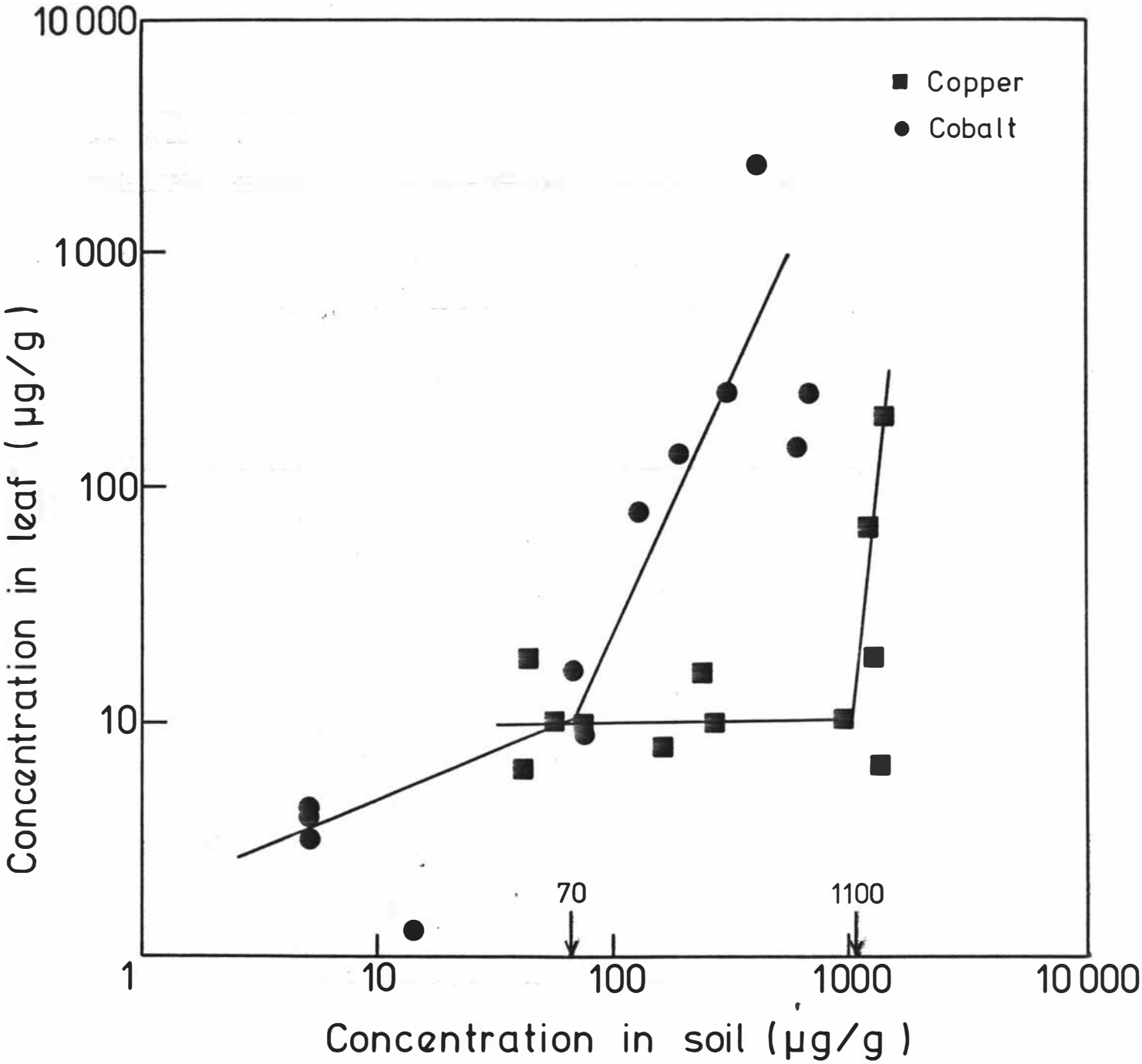


Figure 4.1 Accumulation curve for *Haumaniastrum katangense* seedlings.

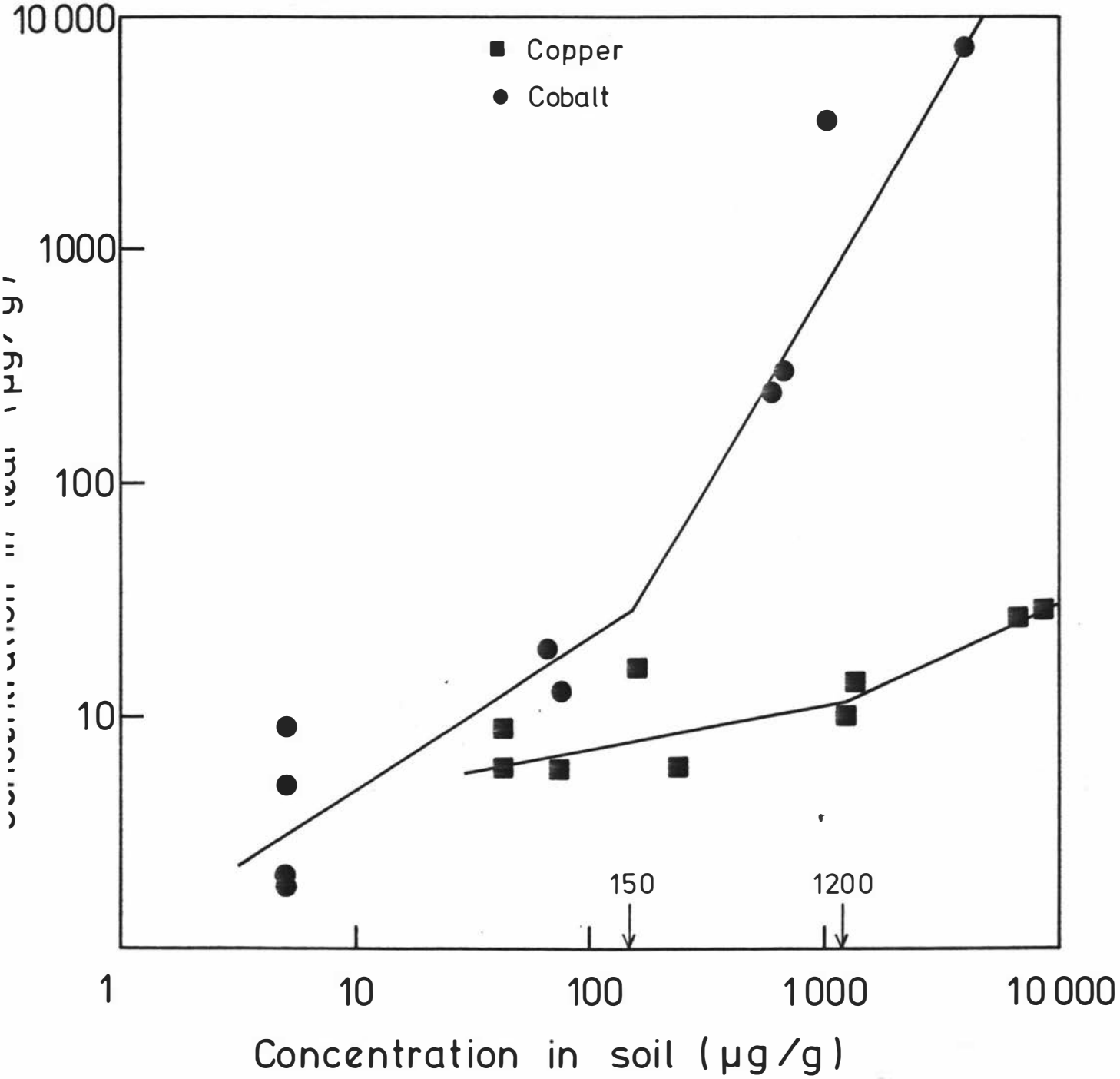


Figure 4.2 Accumulation curve for *Haumaniastrum robertii* seedlings.

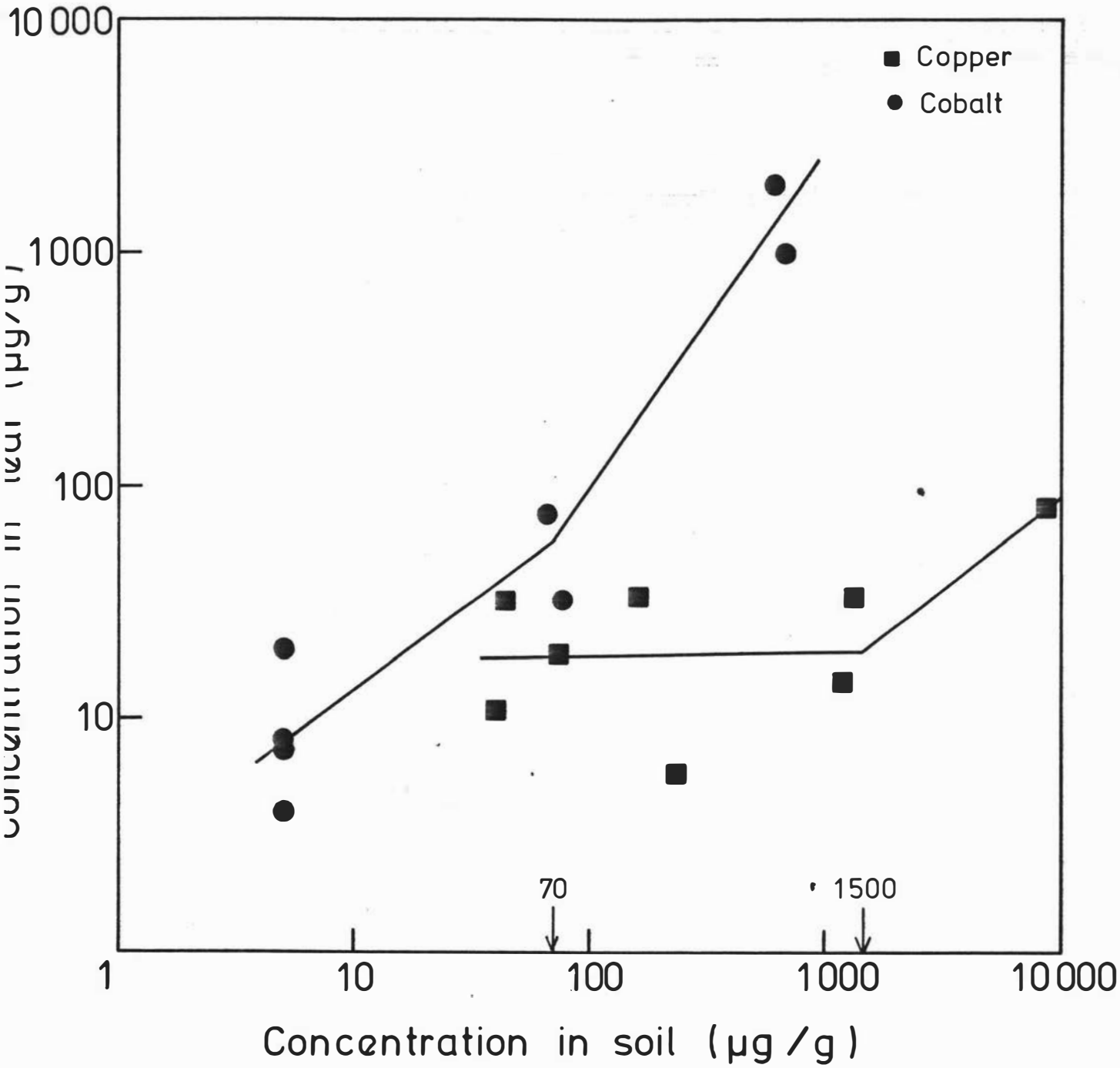


Figure 4.3 Accumulation curve for *Aeolanthus biformifolius* seedlings.

A. biformifolius failed to survive in the 10,000 µg/g cobalt and cobalt + copper pots. Significant quantities of the cobalt salts had been leached from the 10,000 µg/g pots. The leaching of copper from the 10,000 µg/g pots was much less. Some cobalt was also lost from the 1,000 µg/g pots but lower concentrations were not affected. Copper concentrations below 10,000 µg/g were not affected by leaching.

H. robertii survived in 4,000 µg Co/g and 8,500 µg Cu/g soil concentrations. The corresponding leaf concentrations were 7,380 µg Co/g and 29 µg Cu/g respectively. The best growth of the seedlings occurred in the pots with the highest metal concentrations. There was a heavy loss of seedlings at low metal concentrations, particularly in the control group.

H. katangense survived in much lower concentrations with a maximum of 660 µg Co/g and 1,320 µg Cu/g. The corresponding leaf concentrations were 260 µg Co/g and 6 µg Cu/g (the higher concentrations on fig 4.1 come from the tolerance tests). The best growth occurred at the 100 µg/g metal concentrations. The control group specimens were only slightly less in growth. Growth in 1,320 µg Cu/g was better than in 660 µg Co/g with specimens in the latter showing signs of chlorosis. For the 620 µg Co/g + 1,240 µg Cu/g (added 1,000 ug/g cobalt + copper) only one stunted specimen survived.

A. biformifolius survived in 8,500 µg Cu/g but only 660 µg Co/g. The copper concentration in the leaves never exceeded 85 µg/g compared to the very high values found in wild specimens at Mine de L'Étoile (Malaisse & Grégoire, 1978). The highest cobalt concentration in leaf material was 2,000 µg/g. The best growth was at 8,500 µg Cu/g. At this concentration the plants were darker in colour and more steady in growth. Flowering was first observed in a specimen at this level. Flowering was slower at lower copper concentrations and no flowering was observed in specimens on cobalt-enriched substrates.

The accumulation curves (figs. 4.1, 4.2 & 4.3) do have some characteristics in common. All show discontinuities. This gives the accumulation curves the exclusion-breakdown form discussed in the introduction to this chapter. For cobalt, the discontinuities are at 150 µg/g for H. robertii and 70 µg/g for H. katangense and A. biformifolius. The corresponding copper values are 1,200 µg/g for H. robertii, 1,100 µg/g for H. katangense and 1,500 µg/g for A. biformifolius. The most immediate observation is the much lower soil concentrations at which cobalt exclusion breaks down in comparison to copper exclusion. This is not surprising since plants keep a tighter control over essential elements (eg. copper) compared to non-essential elements (eg. cobalt). A further piece of evidence for tighter control of the essential element is the greater gradient of the pre-breakdown curves for cobalt indicating a less pronounced exclusion mechanism for this element.

In the wild, all three species are confined to mineralized soils although the pot trials reported here show that all three can exist on substrates with only traces of cobalt or copper. Losses at lower concentrations were high however, with fungal attacks the predominant apparent cause. When one considers the toxic nature of the natural soils of these species, particularly the high copper content which inhibits fungal growth, it is not surprising that the plants have a low natural resistance to fungal attacks. H. katangense, the least tolerant of the three species, did however have a greater resistance to the fungi.

The results of the uptake of copper in A. biformifolius were surprising. The copper content of leaves in the wild contain approx. 2,000 µg/g plus, yet the highest content recorded here was only 85 µg/g. It was considered that this might be because the specimens here were grown from seed rather than from corms which have an inbuilt store of copper which might be mobilized to give higher leaf concentrations. To test this theory corms were grown over a similar concentration range. The results of this trial are shown in fig. 4.4. The results showed no significant difference in copper levels to the seedling trial, with a maximum leaf concentration of 151 µg/g at a soil concentration of 9,000 µg/g.

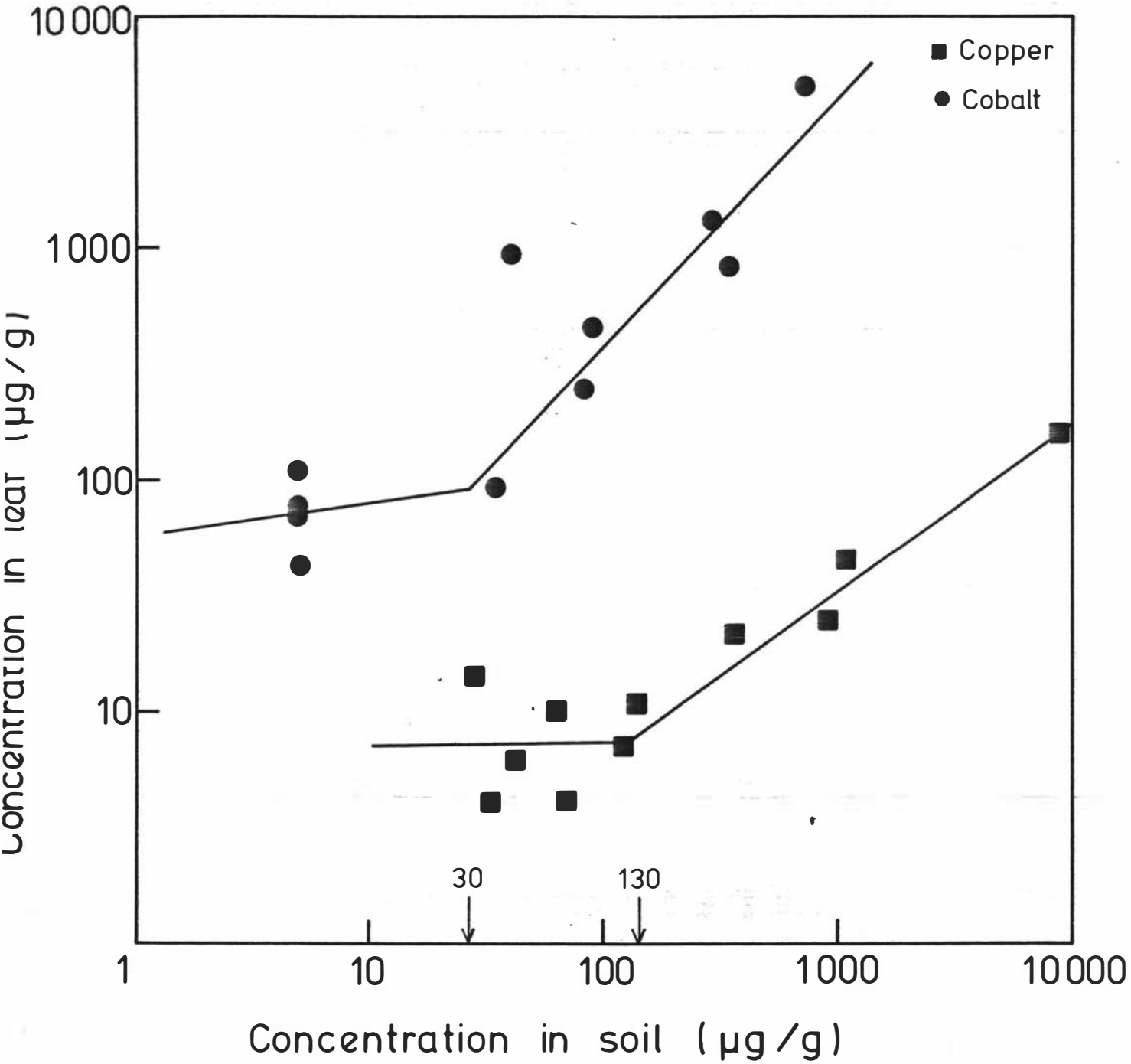


Figure 4.4 Accumulation curve for *Aeolanthus biformifolius* grown from corms.

This difference in behaviour between the wild and cultivated samples must therefore be due to other reasons. With the considerable differences in soils, this is perhaps understandable but at this time the exact reason(s) are unknown. It is noteworthy that the point of the discontinuities for both cobalt and, more particularly, copper are at much reduced soil concentrations for the corms when compared to those of the seedlings. It appears that corms have a reduced ability to exclude these metals. The reason for the difference in behaviour is unknown but obviously worthy of further study. The growth of all the corm specimens was similar although the specimen at 9,000 µg/g was smaller than the others.

4.4 TOLERANCE TESTS

Only two species were tested for tolerance: H. robertii was omitted when the mortality rate among the seedlings was found to be too high to allow any meaningful results to be obtained.

The tolerance levels (greater than 50% survival) for H. katangense were 300 µg/g for cobalt and 1,380 µg/g for copper. As the cobalt content of the soils used in the copper tests averaged 12 µg/g and the copper content of the soils in the cobalt test averaged 60 µg/g, no interference effects were likely. At the tolerance levels, the leaves had concentrations of 259 µg Co/g and 201 µg Cu/g respectively. The data for soil and leaf concentrations have been included in fig. 4.1. One individual specimen survived in a cobalt concentration above the tolerance level, at 370 µg/g with a leaf concentration of 2,400 µg/g. The copper tolerance level was sharply defined with death occurring rapidly above this level while the plants at 1,380 µg/g were growing well. The cobalt level was much less sharply defined. The specimens at the two concentrations immediately above the tolerance level (370 and 800 µg/g) were very slow in dying (the survivor excepted).

The tolerance levels for A. biformifolius were 610 µg/g for cobalt and 870 µg/g for copper.

Background levels of cobalt in the copper-test soils and copper in the cobalt-test soils were the same as for H. katangense (12 µg Co/g and 60 µg Cu/g respectively). Unlike H. katangense, A. biformifolius had several individuals surviving higher levels of both metals: 1,670 and 9,740 µg/g for cobalt and 2,460, 7,780 and 16,700 µg/g for copper. A further difference between the two species is that for A. biformifolius, cobalt appeared to be more toxic than copper. While the tolerance levels of both species is of the same order of magnitude, the tolerance of individual specimens of A. biformifolius is such that this species appears capable of surviving in more toxic substrates than H. katangense.

4.5 GERMINATION TESTS

Both H. robertii and A. biformifolius were tested for their ability to germinate in the presence of cobalt and copper. The results are recorded in tables 4.1 (H. robertii) and 4.2 (A. biformifolius).

In the H. robertii test, it was observed that germination in control (distilled water) did not differ from germination in the presence of 0.1% cobalt or 0.1% copper. Germination in 0.1% cobalt + copper was however lower. Germination can occur in up to 1% cobalt, although the percentage here is low, but no germination occurred for copper concentrations greater than 0.1%. This difference could be used to reinforce the designation of the species as a "cobalt-flower" rather than a "copper-flower" (Brooks, 1977). In the presence of cobalt, germination at a low percentage can occur with copper concentrations above 0.1%. The overall germination rate of H. robertii is low, being not more than 55% under any of the conditions tested.

A. biformifolius is a very ready germinator, with up to 95% germination being observed. In this test, the control germination level was most closely matched by the 0.1% cobalt + copper germination level, although the 0.1% copper and 0.1% cobalt were only marginally lower. Lower germination levels were recorded in higher concentrations of cobalt (0.5%, 1%), copper (0.5%) and cobalt + copper (0.5%, 1%). As for H. robertii,

TABLE 4.1

Germination of Haumaniastrum robertii seeds. *

Time	Control	Cobalt					Copper					Cobalt + Copper				
(Weeks)	0	0.1	0.5	1	2	3	0.1	0.5	1	2	3	0.1	0.5	1	2	3
0.5	40	25	-	-	-	-	10	-	-	-	-	-	-	-	-	-
1	50	55	20	5	-	-	50	-	-	-	-	25	5	-	-	-
1.5	50	55	20	5	-	-	55	-	-	-	-	35	5	-	-	-
2	50	55	20	10	-	-	55	-	-	-	-	35	5	-	-	-
2.5	55	55	25	10	-	-	55	-	-	-	-	35	5	-	-	-
3	55	55	25	10	-	-	55	-	-	-	-	35	10	-	-	-

*Metal concentrations in test conditions expressed as percent.
Results are expressed in percentage germination.

TABLE 4.2

Germination of Aeolanthus biformifolius seeds. *

Time	Control	Cobalt					Copper					Cobalt + Copper				
(Weeks)	0	0.1	0.5	1	2	3	0.1	0.5	1	2	3	0.1	0.5	1	2	3
0.5	45	35	-	-	-	-	15	-	-	-	-	45	5	-	-	-
1	75	65	5	-	-	-	75	5	-	-	-	80	5	-	-	-
1.5	90	75	10	5	-	-	80	5	-	-	-	95	10	5	-	-
2	95	80	10	5	-	-	80	5	-	-	-	95	10	5	-	-
2.5	95	85	10	5	-	-	90	10	-	-	-	95	10	5	-	-
3	95	85	15	10	-	-	90	15	-	-	-	95	15	5	-	-

*Metal concentrations in test conditions expressed as percent.
Results are expressed in percentage germination.

germination will occur at higher cobalt + copper than copper concentrations. The overall tolerance to the presence of copper during germination is however higher for A. biformifolius than for H. robertii while the tolerance to cobalt at high concentrations may be marginally lower.

The rate of germination is relatively rapid. There were few new germinations after two weeks of the three week test. There are indications in the results that the higher the metal concentration, the longer the germination time required. The healthiest seedlings germinated were under the control conditions. The seedlings were both larger and had a greater root development. At the higher metal concentrations seedlings were very small and root development was inhibited. It must be doubtful whether many of the seedlings that germinated at higher metal concentrations would have survived to establish themselves if in metal-rich soils. It should be noted here that plants in Shaba, and more particularly the metallophytes studied here, begin growth with the onset of the rainy season when the volume of rain is such that the soil-water concentrations of metals will be lowest.

4.6 COPPER ACCUMULATION BY AEOLANTHUS BIFORMIFOLIUS

A. biformifolius was analysed for its copper content through its three month growing season at Mine de l'Étoile. The results of these analyses are shown in table 4.3. It is obvious that this species is a hyperaccumulator of copper (Malaisse & Grégoire, 1978). It was observed that the concentration of copper in the aerial parts (basal leaves, flowers and stems) decreased during the growing season whereas the corm, which developed during the growing season, increased its copper content. The level reached by the corm at the end of the growing season is the highest plant copper concentration currently recorded.

TABLE 4.3

Copper accumulation in *Aeolanthus biformifolius* during the growing season. *

Date	Basal Leaves	Flowers and Stems	Corms
7/1	2,600	3,500	2,600
2/2	2,150	2,150	11,800
24/3			13,700

* All results expressed as µg/g dry weight.

4.7 GENERAL DISCUSSION

The similarities of the accumulation curves of the three metallophytes studied, for both cobalt and copper, are notable. Furthermore the level of the exclusion limit for copper in these species (*A. biformifolius* corms excepted) corresponds well with the levels in other tolerant species; *Triodia pungens* R. Br., *Tephrosia* sp. nov., *Eriachne mucronata* R. Br. (Nicolls *et al.*, 1965), *Lychnis alpina* L. and *Silene dioica* (L.) Clairv. (Crooks, 1979). It remains to be seen just how widespread this limit (1,000 - 2,000 µg Cu/g soil) actually is. The study of *Becium homblei* with its rise-to-saturation form does appear to be different (Reilly, 1969). However the uptake was studied at only low copper concentrations (less than 140 µg/g). The possibility that the "rise" is the filling of a requirement not met until the copper content of the soil is approx. 70 µg/g cannot be dismissed. Had this species been studied at higher soil copper contents, might it not have moved upwards from the plateau as some exclusion mechanism broke down?

It is impossible to compare our tolerance test results with other published data. Most tolerance tests have been done in solutions. As soils fix a certain quantity of the added metal salts, it is impossible to make comparisons between tolerance limits obtained by the two methods. The only other soil tolerance test on a copper-tolerant species (Mimulus guttatus, Allen & Sheppard, 1971) was to look at indices of tolerance of several populations at a given soil copper content rather than an attempt to find a limit of tolerance for the species.

The germination tests on H. robertii and A. biformifolius confirm the conclusions of most studies on other tolerant species. With the exception of Horscroft (1961) for Becium homblei, no studies of species tolerant to heavy metals have shown any essentiality for that heavy metal before germination (or growth in general) will occur (Howard-Williams, 1970, Allen & Sheppard, 1971, Shaw, 1980). The data of Horscroft (1961) are in conflict with data on the same species given by Howard-Williams (1970).

From this discussion it can be seen that the character of tolerance to copper has many similarities despite the wide variety of plant species which have been studied. It appears that there may be a case for parallel adaptation to the toxicity of copper contaminated soils among these different plant species. The lack of studies on other cobalt tolerant species make comparative discussions for this element impossible.

CHAPTER 5

Phytochemical Studies on Some Metallophytes from Shaba

5.1 INTRODUCTION

The presence in cells of large amounts of what are normally trace elements generally results in the ill-health or death of the plant (Hunter & Vergnano, 1953). The existence of metallophytes which can accumulate these elements without such effects raises questions as to the nature of the excess amount within the plant. The existence of free metal ions could be expected to interfere severely with the physiological processes of the plant because of the fact that they can readily complex with many organic functional groups (alcoholic, amino, carboxyl, disulphide, imidazal, phenolic and sulphydryl groups - Gilbert, 1951, Mills, 1954, Timberlake, 1959, Ennis, 1962, Rasheed & Seely, 1966, Vallee & Ulmer, 1972). These functional groups are all important components of compounds within the plant's metabolic cycles.

Copper is an essential element in plants where it is an activator of several enzymes (Nason, 1958). The requirement for copper to fulfill these functions does not however appear to exceed 20 µg/g (Piper, 1942, Beeson *et al.*, 1956, Rasheed & Seely, 1966). The ability of certain cuprophytes to accumulate this element greatly exceeds this basic requirement of most plants. Since excess copper in "normal" plants leads to necrosis, the healthiness of these accumulators requires an explanation. As the necrosis in "normal" plants grown on copper-rich soils is due to the interference with, and breakdown of, physiological functions within the plant, it is obvious that cuprophytes have developed methods of avoiding such interference. The method most commonly invoked to "remove" the copper from circulation is complexation (Mills, 1954, Gilbert, 1951, Reilly, 1969). The evidence for complexation is certainly strong in the studies of the cuprophyte *Becium homblei* (Reilly, 1969, 1972, Reilly *et al.*, 1970). Reilly (1969) showed a strong correlation between copper and total nitrogen in leaf samples which suggested that the copper might be bound in a proteinaceous complex. Furthermore the fact that almost

50% of the copper was soluble in a series of organic solvents (dioxan, butanol, methanol) is certainly consistent with an organic-copper complex. Reilly et al. (1970) found that approx. 17% of the copper was bound with structural components of the cell wall (lignins, polysaccharides or proteins). By a dialysis experiment, these workers found that 20-25% of the copper existed as either free ions or was loosely complexed while a further 10-15% was only lightly complexed. Using paper chromatography they showed that no free ions wera detectable and that at least two amino acid or peptide complexes were present. The number of copper-containing spots found was however dependent on the solvents used. Reilly (1972) similarly found that the number of spots in chromatographic experiments varied with the solvents used when he compared copper-tolerant and non-tolerant specimens of Becium homblei. Studying the two Indigofera species, I. dyeri and I. setiflora, Ernst (1972) showed by a sequential solvent extraction process that the bulk of the copper was soluble in either water or dilute hydrochloric acid. Little copper was soluble in butanol, in two reagents capable of exchange processes (sodium chloride and citric acid) or in the residue remaining after the extraction series. He considered that the water-soluble copper was probably located in the cell vacuole but made no attempt to identify the nature of the copper. The hydrochloric acid-soluble fraction was considered as being exchangeable copper and most probably located on the cell wall. The small amount of copper in the residue was considered to be strongly bound to the cell wall.

Studies of cobalt in plants have yet to prove that it is an essential element for them all. Nason (1958) considers cobalt to be an activator of some enzymes. A need for cobalt in nodulated leguminous plants is well known but several groups have considered that it may also be an essential element for other plants (Ahmed & Evans, 1960, Wilson & Nicholas, 1967). Certainly cobalt complexes have now been recorded in many plant species used by man (Ballentine & Stevens, 1951, Bowen et al., 1962, D'Souza & Mistry, 1979). The "normal" content of cobalt in plants is less than 1 µg/g so that the values recorded for many cobaltophytes in Shaba easily exceed

normal toxicity levels. As for copper, the existence of complexes has generally been considered as the method by which these species avoid the disruption of their physiological functions that free cobaltous ions would create. No specific studies on cobaltophytes have however been carried out. Evidence from non-tolerant species has suggested that cobalt is complexed with proteins and peptides (Ballentine & Stevens, 1951, D'Souza & Mistry, 1979). The possibility that the cobalt was in the form of vitamin B_{12} was dismissed on the evidence available to these researchers and also to that available to Bowen et al. (1962).

The work presented in this chapter investigates the distribution of cobalt and copper between the various cell components by a sequential solvent extraction method. This allows some speculation on the nature of these elements within the leaves of five metallophytes. Studies in greater detail on the nature of cobalt in Haumaniastrum robertii are also reported. These studies involved proton probe analyses for elemental distribution within the leaf and isolation of the water-soluble cobalt for tests to determine the complexing agent. The nature of the acid-soluble cobalt is also subjected to speculation.

5.2 EXPERIMENTAL METHODS

Leaf material of five metallophytes (Aeolanthus biformifolius, Buchnera metallorum Duvign.& Van Bock., Faroa chalcophila, Haumaniastrum robertii and Silene cobalticola) were collected at various Shaban localities by Prof. F. Malaisse of the Université Nationale du Zaïre at Lubumbashi. The material was freeze-dried and freighted to New Zealand. Upon arrival, all the material was placed in a refrigerator until used.

5.2.1 Sequential Solvent Extraction

A sequential solvent extraction method was used to investigate the nature of cobalt and copper in the five metallophytes. The sequence used was developed by Lee (1977) from the method of Bowen et al. (1962). The sequence began by macerating 2g of freeze-dried leaf material with $10cm^3$ of 95% ethanol in an

homogenizer for five minutes. The resultant slurry was centrifuged and the supernatant decanted. The residue was washed with two further portions of ethanol which were similarly centrifuged and decanted. The three ethanol supernatants were filtered, combined and labelled fraction A. The residue was further extracted with two $10cm^3$ portions of deionized water in the homogenizer. The supernatants, after centrifuging, were filtered and combined. The resultant solution was labelled fraction B. The residue was then similarly extracted with three $5cm^3$ portions of 0.2M hydrochloric acid (prepared from redistilled constant-boiling hydrochloric acid). The combined supernatants were filtered and then treated with an equal volume of acetone to precipitate the proteins and pectates. These precipitates were labelled fraction D. The supernatant after acetone-precipitation was labelled fraction C. The residue from the hydrochloric acid treatment was then treated with three $5cm^3$ portions of 0.5M perchloric acid at $80^{o}C$. These digests were also centrifuged and the supernatants combined. The supernatant was again treated with an equal volume of acetone to precipitate the nucleic acids. This precipitate was labelled fraction F and the remaining supernatant fraction E. The residue from the perchloric acid treatment was finally digested for ten minutes with boiling 2M sodium hydroxide. The supernatant collected from this was labelled fraction G while the residue was labelled fraction H.

All cobalt, copper and manganese determinations were made by atomic absorption spectrophotometry. The instrument used was the Varian-Techtron model AA5 with automatic background correction as in Chapter 2. The lines used were 240.8nm and 304.4nm for cobalt, 324.8nm and 218.2nm for copper and 279.5nm for manganese. Where the fractions consisted of aqueous solutions they were analysed directly. The residue, precipitates and organic solutions were treated as follows: they were taken to dryness, ashed at $500^{o}C$ in a muffle furnace and then redissolved in 2M hydrochloric acid before analysis.

5.2.2 Proton Microprobe Analysis

The proton microprobe analyses were performed at Harvard University and the MIT Lincoln Laboratory at Cambridge, Massachusetts, USA. The proton microprobe operates on a principle similar to the electron microprobe (Horowitz & Grodzins, 1975, Horowitz et al., 1976). Thus a sample is bombarded by a microbeam of protons which causes excitation of the nuclei within the sample. These nuclei emit characteristic X-rays as they return to the unexcited state. The X-rays are then analysed to determine the composition of the sample. The proton microprobe has two important advantages over the electron microprobe : sensitivity is much improved being of the order of 1-10 μg/g compared to approx. 1,000 μg/g; and measurements do not need to be made in a vacuum. The instrument used here utilized a 2MeV emergent proton beam from a Van de Graaf accelerator. The samples were mounted and then scanned past the fixed microbeam by a stepping-motor driven XY stage. In all cases the characteristic X-rays produced by the elements within the top approx. 20 μm of the sample were detected by a Si(Li) detector and sorted according to pulse height (energy) to determine the composition. Both the sample motion and the data collection were controlled by minicomputer (Aronson & Horowitz, 1980).

Only H. robertii was subjected to proton probe analysis. Two sets of information were gathered: two-dimensional photographic scans and one-dimensional line scans. The two-dimensional scans were done on five-ten elements simultaneously at lower resolution (approx. 150 μm) forming images of 10,000 pixels to show the elemental distributions. From these micrographs, areas of interest (high cobalt content) were investigated more closely by use of higher resolution (approx. 100 μm) line scans. These scans were sensitive to all elements under investigation simultaneously.

5.2.3 Cobalt Complexes in Haumaniastrum robertii

(a) Acid-soluble cobalt. An attempt was made to identify, speculatively, the nature of the acid-soluble cobalt found during

the solvent extraction sequence. A further 2g of leaf material was treated as for the solvent extraction sequence until fraction C was obtained. This was analysed for oxalate by a method similar to that used by Bornkamm (1965) and Mathys (1977). The solution, fraction C, was neutralized by the addition of a few drops of 2M sodium hydroxide. This precipitated the oxalate which was collected by filtering through a Whatman 542 filter paper. The precipitate was washed with warm deionized water before being redissolved in a minimum quantity of concentrated sulphuric acid. The redissolved oxalate solution was diluted until the acid strength was approx. 1M. This solution was then titrated at $60^{o}C$ with 0.02M potassium permanganate solution.

(b) Water-soluble cobalt. The water-soluble cobalt complex was separated with the intention of identifying the ligands. The method of separation is similar to that used by Lee et al. (1977b) in the separation of water-soluble nickel complexes from nickel hyperaccumulators.

Extraction of the complex required homogenization of 25g of freeze-dried leaf material, containing 0.45% cobalt, with two $100cm^3$ portions of deionized water. These extracts were filtered and combined. To remove lipids, proteins and other large organic molecules, the filtrate was washed with a 10:1 chloroform : n-butanol mixture until no further precipitation occurred at the solvent interface. The precipitated material was analysed but contained a negligible amount of cobalt. After refiltering the aqueous solution, it was reduced in volume to approx. 20 cm^3. This was run in 2 cm^3 portions, through a 30 cm x 2 cm Sephadex G-75 gel filtration column. The elutant was collected in 5 cm^3 fractions and the cobalt fractions combined. The presence of cobalt was determined by atomic absorption spectrophotometry. The recombined cobalt fractions were then recycled through the column. After recombining the cobalt fractions from the second filtration, the sample was run through a 60cm x 1cm Sephadex G-10 column but as no retardation was found, this was unsatisfactory as a means of separating the cobalt complex from other substances and was discontinued. At this point the cobalt had not been greatly concentrated.

Further cleaning was initially tried with ion-exchange resins but those tested, Dowex 1-X8 in hydroxide, carbonate or acetate form, Sephadex CM-25 in ammonium form, and Amberlite IR-45 in hydroxide form, all failed to exchange with the complex. A precipitation cleansing process was then tried. Beginning with a 50:50 methanol : water solution (made by adding an equal volume of methanol to the solution containing the cobalt complex) and increasing the methanol content in 10% steps (ie. 60:40 and 70:30) much precipitation occurred. For each pair of precipitate and solution, the cobalt was traced by atomic absorption spectrophotometry. Prior to this tracing analysis, the filtrate was reduced in volume to remove the methanol and the precipitate was redissolved in deionized water. The cobalt complex began precipitating in the 70:30 methanol:water solution. Both the precipitate and the supernatant were taken to dryness by slow evaporation. This precipitate was analysed for its cobalt content and used for microanalysis and electrophoresis.

Microanalytical analyses for carbon, hydrogen and nitrogen were performed by the microanalytical chemistry laboratory at Otago University. Oxygen could not be determined because of the presence of cobalt in the complex.

Electrophoresis was performed on a water-cooled Savant-type apparatus in which two buffer reservoirs are separated by a low flash point petroleum spirit. The buffer used was 500:20 : 4,500 pyridine:acetic acid:water at pH6.5. The operation was done at 3kV which gave a current of approx. 40mA. The average time length of a run was one hour. The paper used was Whatman No.1 chromatographic paper. The electrophoretogram was analysed for cobalt by dividing the column of paper which contained the original "spot" of the complex into 1cm x 1cm squares, ashing the squares at $500^{o}C$ in a muffle furnace, redissolving the ash in 2M hydrochloric acid and determining the presence of cobalt by atomic absorption spectrophotometry.

For preparative electrophoretic runs, the origin was a line across the centre of the paper rather than a "spot" on this line. After the cobalt had been located by using the strip analysis,

bands of paper containing the cobalt complexes were soaked in water overnight to leach out the complexes. The resultant solutions were filtered to remove the paper and then dried to obtain the precipitates which were used in both HPLC and GLC.

High performance liquid chromatography (HPLC) was performed on a Waters Associates model 660 solvent programmer coupled to a μ-bondpak C18 column. The buffer used was 2mM tetra-n-butylammonium phosphate at pH 3.2. The flow rate used was 1.5 cm^3/min and the detector, a Cecil CE 212 A spectrometer, was set at 220nm since most substances absorb around this wavelength. The analysis was done by redissolving 10mg of the cobalt precipitates, obtained by preparative electrophoresis, in 0.5cm^3 of water and directly injecting 25 μl of this solution into the system.

Gas-liquid chromatography (GLC) was performed by a Pye model 104 chromatograph equipped with a 2.8m x 4mm column packed with 3% SP2340 liquid phase on a supelcoport, 100-220 mesh, PB 34 support. The column was operated at 180^{o}C with dry nitrogen, at a flow rate of 30 cm^3/min, as the carrier gas. The detector was a flame-ionization detector with an air-hydrogen flame. The flow rates for these gases were 350 cm^3/min and 30 cm^3/min respectively. The cobalt fractions from the preparative electrophoresis were derivatized by diazomethane to produce the methyl esters of the complexing agents. The diazomethane was prepared by the method of Werner (1919). The cobalt complexes (10mg) were destroyed by the addition of dilute hydrochloric acid and dried on a water-bath under a stream of nitrogen. The precipitate was then dissolved in 0.5cm^3 of ether and the diazomethane added. Further additions of diazomethane were made until effervescence stopped. The ether was then evaporated by flushing the vial containing the sample with nitrogen. The precipitate which remained was redissolved in 0.5cm^3 of redistilled chloroform and analysed in 2 μl samples.

5.3 DISTRIBUTION OF HEAVY METALS IN PLANT TISSUE EXTRACTS

The distribution of cobalt and copper in the various fractions of plant tissue extracts is shown in table 5.1. It can be seen that most of the heavy metals are found in fractions B, C and E. These fractions contain predominantly polar compounds.

Fraction B contains the water-soluble, low molecular weight polar compounds eg. organic acids and inorganic salts. For all the species analysed, the cobalt percentage extraction exceeded that of the copper. This is most particularly evident in H. robertii and A. biformifolius. The percentage extracted of these metals is much lower than that found for nickel in nickel hyperaccumulators. Kelly et al. (1975) found 65-94% of the nickel to be extractable while in these studies the maximum cobalt extraction was 42.9% (H. robertii) and the corresponding copper extraction was 22.2% (S. cobalticola).

Fraction C contains acid-soluble polar compounds (eg. salts of organic acids, phosphates, carbonates) and ions released by exchange processes with the cell walls and some proteins. With the exception of cobalt in A. biformifolius and H. robertii, the percentage of extracted metals exceeded that of fraction B. This increased percentage extraction is considerably more noticeable for copper than for cobalt. The maximum cobalt extraction was 53.4% (S. cobalticola) and the copper extraction was 59.3% (B. metallorum).

Fraction E contains most remaining polar compounds and some structural groups eg. some celluloses and lignins. The copper percentage extraction in this fraction always exceeded the percentage extracted in fraction B but never that of fraction C. The cobalt percentage extraction varies much more considerably but only in B. metallorum is the percentage extracted here the highest in any fraction for a given species. Maximum extractions were 39.6% for cobalt (B. metallorum) and 26.0% for copper (A. biformifolius).

TABLE 5.1 The fractionation of cobalt and copper in plant tissue extracts.

	Aeolanthus biformifolius		Buchera metallorum		Faroa chalcophila		Haumaniastrum robertii			Silene cobalticola	
	Co	Cu	Co	Cu	Co	Cu	Co	Cu	Mn	Co	Cu
Total Concentrations (µg/g)	2380	3920	1510	3520	134	700	4690	489	198	233	33
% in fractions											
A	0.5	0.8	0.1	0.1	1.2	1.4	0.2	1.7	0.8	0.5	2.5
B	33.8	11.3	10.3	9.1	20.4	16.6	42.9	12.6	21.7	34.9	22.2
C	31.7	47.8	36.3	59.3	48.9	54.6	39.7	39.9	58.7	53.4	38.8
D	1.8	4.2	1.5	4.0	0.9	0.8	1.6	3.0	1.8	1.2	1.9
E	22.0	26.0	39.6	20.7	21.3	17.4	9.9	18.3	11.6	9.0	23.2
F	0.9	1.2	0.7	0.6	0.2	0.2	0.7	2.5	0.8	0.1	0.5
G	8.5	1.8	7.0	2.0	4.4	3.6	4.5	7.1	4.0	0.7	5.4
H	0.9	6.9	4.7	4.3	2.7	5.3	0.5	14.9	0.8	0.4	5.6

A - Neutral small molecules including amino acids and pigments

B - Water-soluble low molecular weight polar compounds.

C - Acid-soluble polar compounds and exchangeable ions

D - Proteins and pectates

E - Polar compounds and some structural groups

F - Nucleic acids

G - Remaining proteins and polysaccharides

H - Cellulose, lignin and immobile fractions of cell walls.

TABLE 5.2

Statistical data for cobalt and copper associations in plant fractions.

Intraspecies Association

Species Code	Species	Variables	r	Significance
I	A. biformifolius	Co vs Cu	0.75	S
II	B. metallorum	Co vs Cu	0.82	S
III	F. chalcophila	Co vs Cu	0.99	S^{xx}
IV	H. robertii	Co vs Cu	0.69	S
		Co vs Mn	0.85	S^{x}
V	S. cobalticola	Co vs Cu	0.91	S^{x}

Interspecies Associations

(values of r with significances in parentheses)

Co				
	II	III	IV	V
I	0.73 (S)	0.87 (S^{x})	0.94 (S^{xx})	0.89 (S^{x})
II	-	0.84 (S^{x})	0.52 (NS)	0.62 (NS)
III	-	-	0.83 (S^{x})	0.93 (S^{xx})
IV	-	-	-	0.96 (S^{xx})

Cu				
	II	III	IV	V
I	0.98 (S^{xx})	0.97 (S^{xx})	0.96 (S^{xx})	0.94 (S^{xx})
II	-	0.98 (S^{xx})	0.95 (S^{xx})	0.91 (S^{x})
III	-	-	0.97 (S^{xx})	0.95 (S^{xx})
IV	-	-	-	0.92 (S^{x})

S^{xx} - very highly significant ($P < 0.001$)
S^{x} - highly significant ($0.001 \leq P < 0.01$)
S - significant ($0.01 \leq P < 0.05$)
NS - not significant ($P \geq 0.05$)

In contrast to the Becium homblei studies of Reilly (1969), very little of the heavy metals in the five metallophytes were found in the fractions containing amino acids (fraction A) or proteins (fractions D and G). Generally (the exception being A. biformifolius) even less metal was associated with the nucleic acids (fraction F). The percentage remaining in the residue (fraction H - celluloses, lignins and cell walls) was generally the fourth highest for copper, after the three polar compound fractions. Thus it may be that a significant portion of the copper is bound to the cell walls. The percentage in the residue ranged from 4.3% (B. metallorum) to 14.9% (H. robertii). Reilly et al. (1970) found 17% of the copper in B. homblei was associated with the cell wall. The percentage of cobalt in the residue was not generally significant and a greater amount was to be found with the proteins and polysaccharides of fraction G.

It is obvious that the cobalt and copper in these plants is generally complexed to organic ligands to form polar compounds. The distribution of the metals among the various fractions would indicate that more than one ligand is involved. This contrasts with most nickel hyperaccumulators which have the nickel bound to only a few simple organic acids (Lee et al., 1977b, Lee et al., 1978, Kersten, 1979). Because vitamin B_{12} is a cobalt complex, the presence of cobalt in a plant has often raised the question as to whether or not the plant is producing this vitamin. No evidence has ever been found to support vitamin production and most available evidence discounts it (Bowen et al., 1962). As vitamin B_{12} is readily soluble in 95% ethanol (fraction A solvent) and very little cobalt is soluble in this solvent, it is highly improbable that the cobalt forms any such vitamin complex in these species. Non-accumulating plant species eg. tomato (Bowen et al., 1962) and red kidney beans (D'Souza & Mistry, 1979) have very different percentage distributions between the various solvent fractions. Fraction A had generally of the order of 30-60% of the cobalt while only slightly less was extracted by 0.2M hydrochloric acid (Note: as neither Bowen et al., 1962, nor D'Souza & Mistry, 1979, did aqueous extractions, their hydrochloric acid fraction

is the equivalent of fraction B + fraction C). The difference between the copper distributions is not so marked although at 9% the fraction A percentage for tomato (Bowen et al., 1952) is significantly higher than our metallophyte percentages.

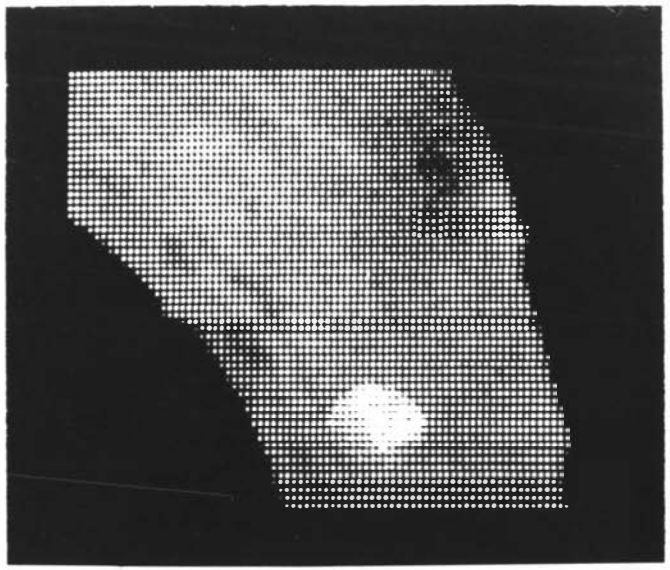
Statistical analysis of the data in table 5.1 is shown in table 5.2. For cobalt and copper within a single species (intraspecies correlations), there are significant to very highly significant correlations for all species. This would appear to indicate a parallel uptake for the two elements within a species. For H. robertii where manganese distribution was also studied, a highly significant correlation was also observed with cobalt. There would appear to be parallel uptake of all three elements in this species.

The interspecies correlations (correlations between the same element in different species) are also generally significant. Certainly the copper distribution between the five species shows an extraordinary similarity ($r > 0.9$ for all correlations). The distribution of cobalt is not so uniform however. Despite this, most of the correlations are highly or very highly significant. B. metallorum does however appear to differ from the other four species in cobalt distribution. Both non-significant and the only significant correlation involve this species and even its highly significant correlation with F. chalcophila is comparatively low for this series.

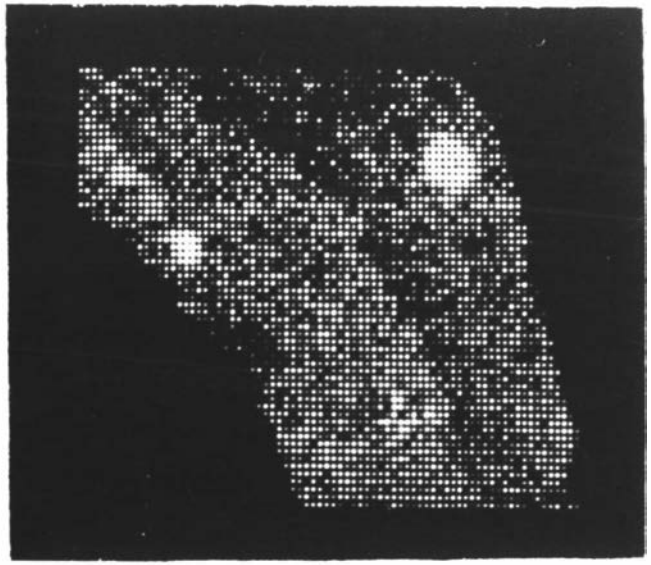
As a footnote, it is recorded that the specimen of B. metallorum used in these experiments was the first of this species to show hyperaccumulation of cobalt and copper.

5.4 PROTON MICROPROBE STUDIES ON HAUMANIASTRUM ROBERTII

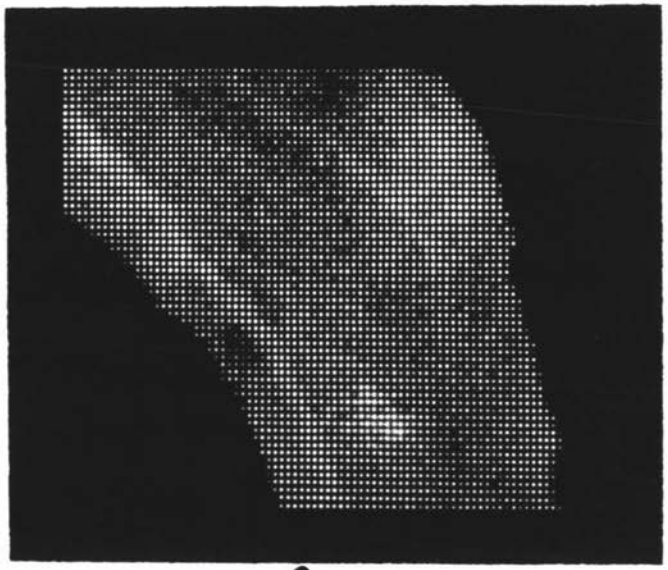
The two-dimensional scans of a leaf of H. robertii revealed several regions in which the cobalt content was higher than the general concentration. Calcium was also elevated in these regions but potassium was deficient. The micrographs of these elemental scans are shown in plate 5.1. From these regions, one was chosen on which to carry out a more detailed line scan.



K



Co



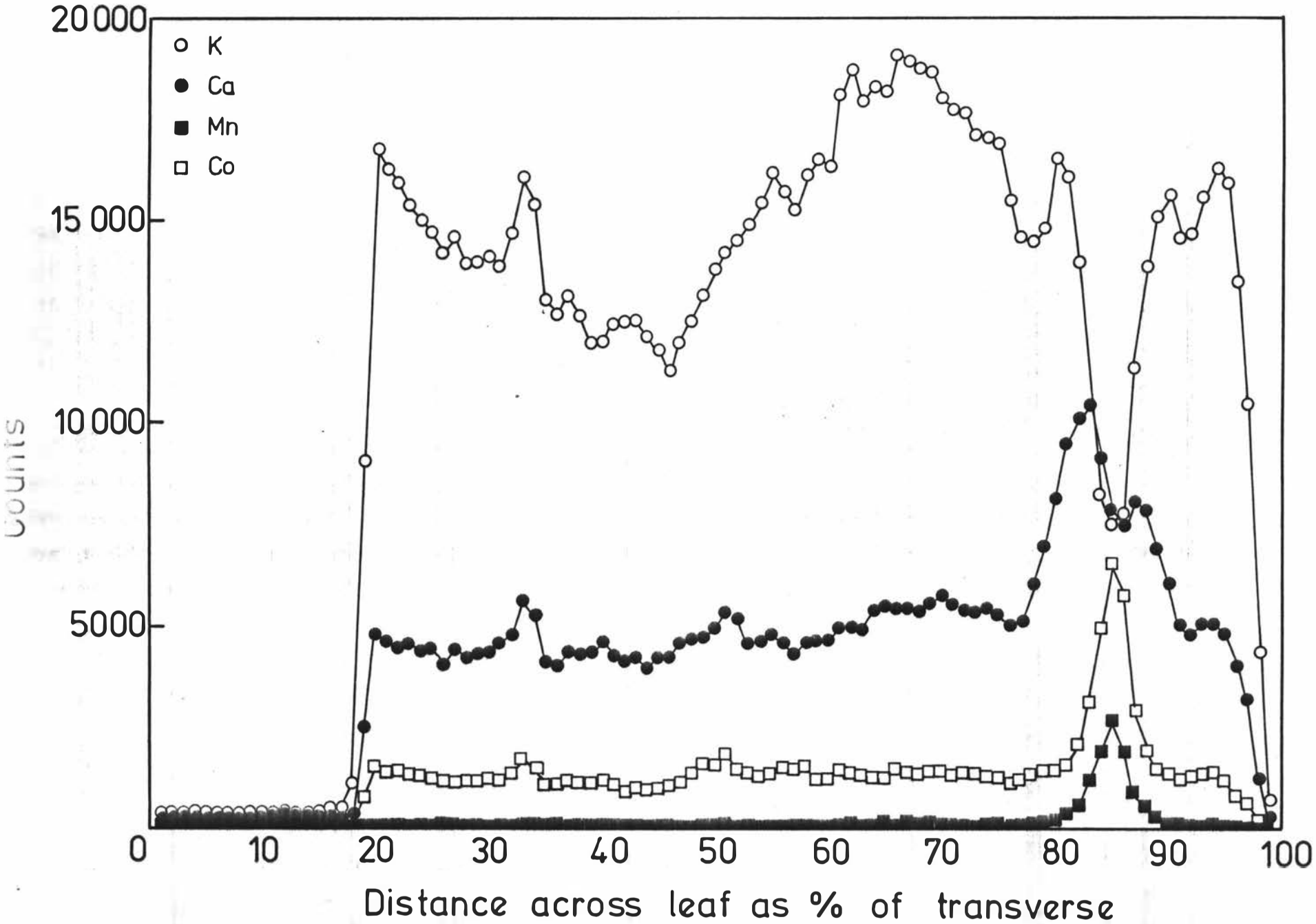
Ca

Haumaniastrum robertii

Plate 5.1

Proton microprobe micrographs showing the distribution of cobalt, calcium and potassium in a leaf. The lighter regions are the regions of greatest concentration.

Figure 5.2 Line scan across leaf of *Haumaniastrum robertii* showing elemental concentrations.


20 000
15 000
10 000
5000
0
Counts
K
Ca
Mn
Co
10
20
30
40
50
60
70
80
90
100
Distance across leaf as % of transverse

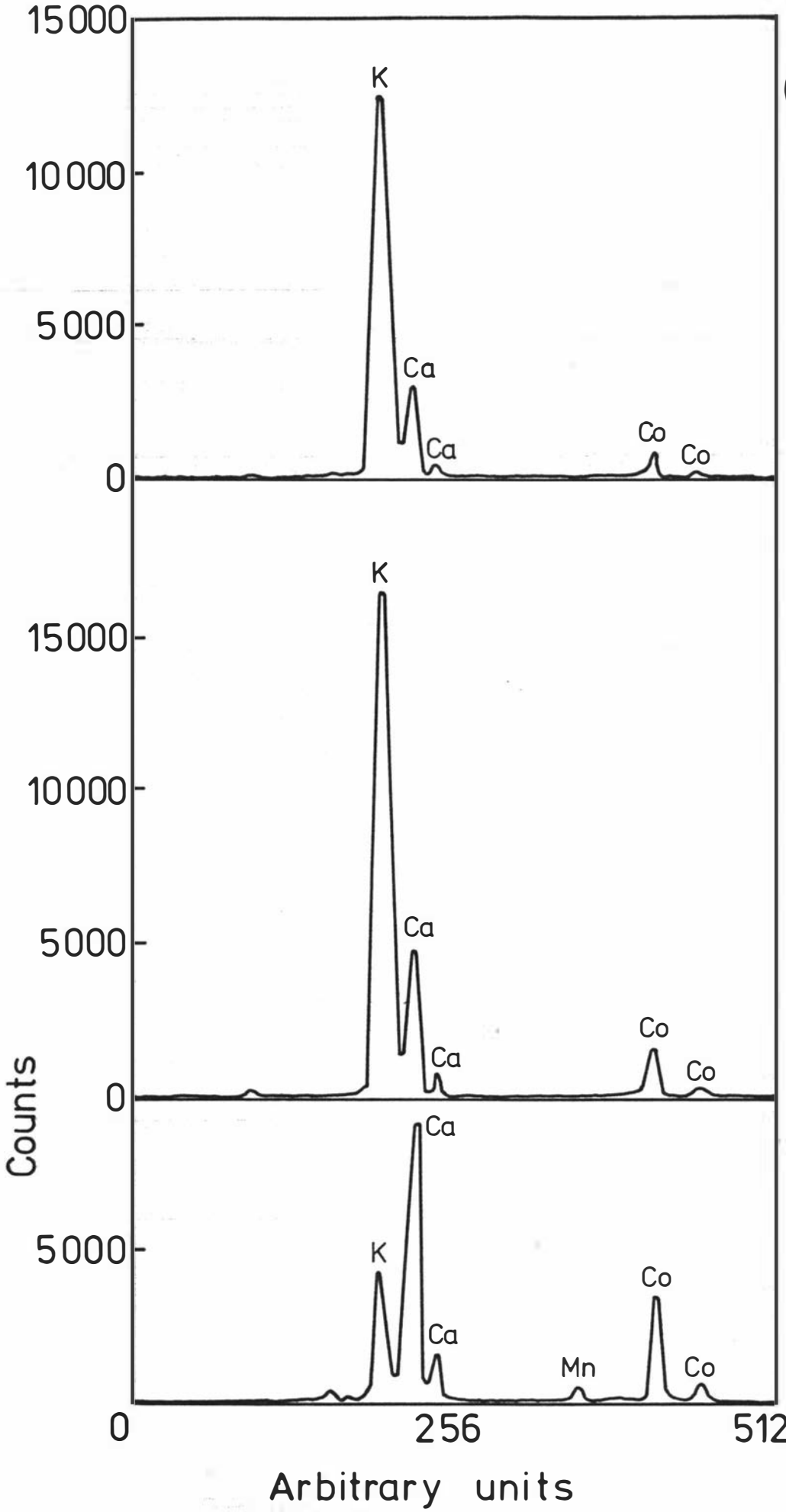


Figure 5.3 X-ray spectra from proton microprobe.

This line scan is shown in fig. 5.2 for cobalt, calcium, manganese and potassium. The distribution of cobalt, calcium and manganese is remarkably uniform across most of the leaf with a variation of no more than ± 10% from the mean concentration. Potassium shows a greater variation being as much as ± 20% from the mean concentration. At the anomaly, the cobalt and manganese concentrations rise sharply, in parallel, while the potassium concentration drops sharply. Calcium rises in concentration immediately adjacent to the cobalt rise but dips below the maximum concentration at the centre of the anomaly. The diameter of this anomaly is approx. 1mm.

A more direct comparison of the concentrations of cobalt, manganese, calcium and potassium between the cobalt anomalies and other regions of the leaf can be seen in the spectra of fig.5.3. It can be observed that manganese shows only in the cobalt anomaly spectrum. Cobalt also shows most strongly in this spectrum but does appear in the other spectra. Potassium and calcium naturally dominate the spectra but it is easily observed that at the anomaly (which is not the same anomaly as in the line scan) the calcium concentration exceeds the potassium concentration. This is the reverse of the normal situation which is observed for the background spectra.

It appears from these results that much of the cobalt is localized within the plant. One possibility for such localization would be the precipitation of cobalt in some crystal form. The rise in calcium concentration in the same general area and the known occurrence of calcium oxalate crystals in plants (Al-Rais *et al.*, 1971) raised the question as to whether the cobalt could be co-precipitating as an oxalate crystal. Certainly cobalt oxalate crystals are insoluble in aqueous media.

5.5 COBALT COMPLEXES IN HAUMANIASTRUM ROBERTII

Following the results of the proton probe studies, it was decided to investigate the relative amounts of acid-extractable cobalt and oxalate. This was done by determining their concentration in fraction C of the solvent extraction sequence.

The results showed that an aliquot of fraction C contained 16.6 μmoles of cobalt and 18.3 μmoles of oxalate. It is thus possible that the acid-extractable cobalt could be present in the leaf as cobalt oxalate crystals. The removal from the cytoplasm of 30-50% of the cobalt as oxalate crystals would obviously be beneficial to the plant. It would also appear that some manganese occurs in these crystals along with the cobalt and calcium. The substitution of other divalent ions for calcium in oxalate crystals extracted from plants has been shown by Al-Rais et al. (1971).

The water-soluble cobalt complex extracted and separated as in subsection 5.2.3 was found to contain only 1.14% cobalt. This would give the complex a molecular weight of 5,200g per mole of cobalt if pure. The microanalysis of the complex gave the results 28.05% carbon, 3.95% hydrogen and 1.04% nitrogen. This gives the complex 120 moles of carbon and 4 moles of nitrogen per mole of cobalt. On the basis of this nitrogen content, it is obvious that the complexing agent is not proteinaceous in nature.

The electrophoretogram run on the complex revealed a rather complex pattern of cobalt distribution (fig. 5.4). The dominant peak was an anionic peak. The cationic fraction appeared to contain three overlapping peaks. To attempt further identification, several preparative-scale electrophoretograms were run and the cobalt soaked off as two fractions, anionic and cationic. These fractions were analysed by HPLC. The anionic fraction gave rise to two peaks which did not coincide with any of the seven common organic acids run as standards (aconitic, citric, isocitric, malic, malonic, quinic and tartaric acids). The conditions used in HPLC split up the complex, releasing free aquo-cobalt-(II) ions and the complexing agent(s). This allowed direct comparisons between the standards and the samples. The cationic fraction showed a very strong cobalt peak but no other significant peaks which suggested that much of the cobalt in this fraction might have been aquo-cobalt-(II) ions.

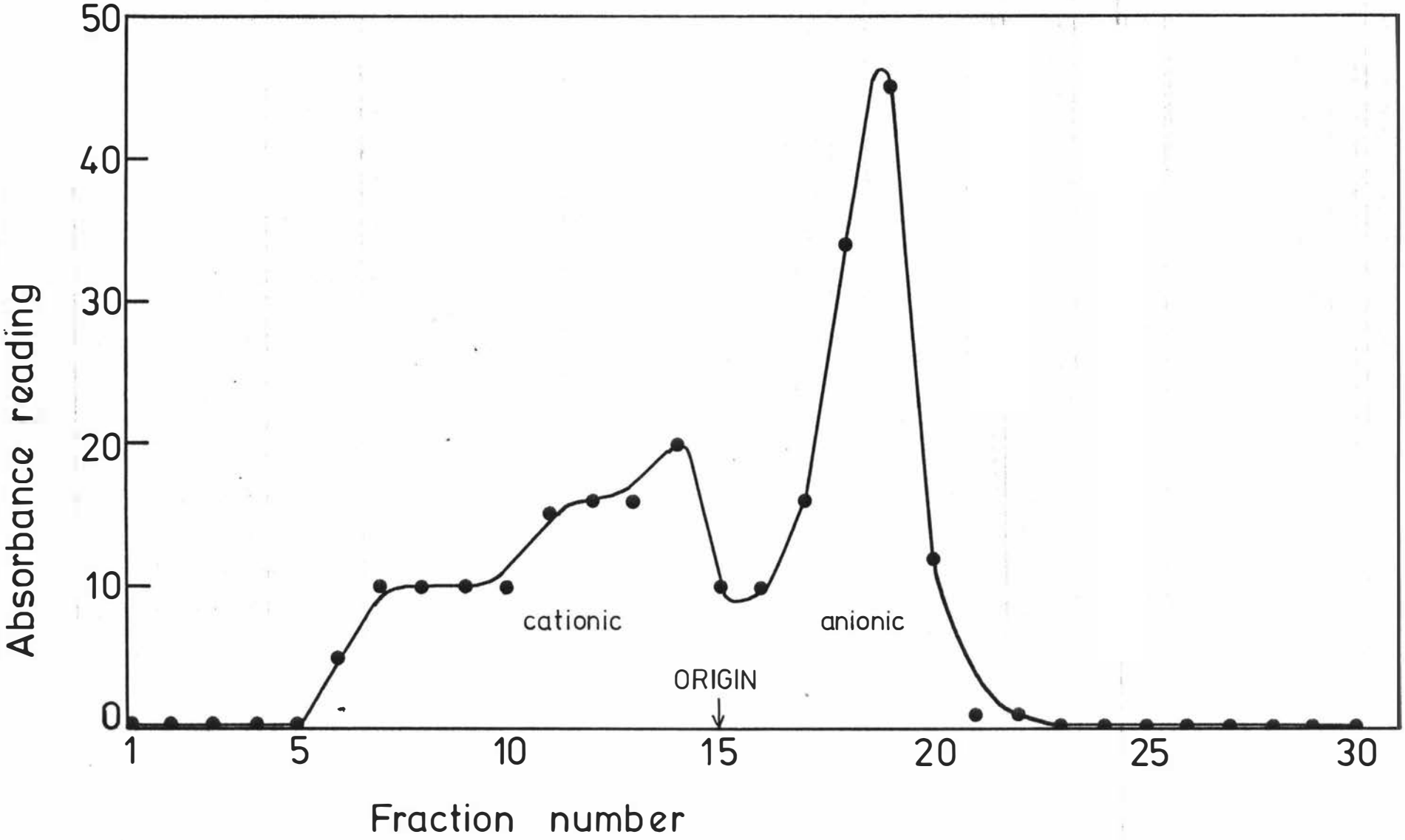


Figure 5.4 Electrophoretogram of water-soluble cobalt complex extracted from *Haumaniastrum robertii*.

Further samples of these fractions were methylated for analysis by GLC. The results for the anionic fraction were similar to those for HPLC ie. there were two peaks which did not correspond with those of the standards used (aconitic, citric, α-ketoglutaric, malic, malonic, oxalic, oxaloacetic, succinic and tartaric acids). The cationic fraction showed a single peak which also did not correspond with any of the standards. It must be noted that as the "molecular weight" of the cobalt complex is of the order of 5,200g per mole of cobalt, the complexing agent(s) may have been too large to be satisfactorily identified by the methods or conditions used. Furthermore if the complexing agent is small and much of the mass is in fact some contamination, it will be difficult to decide whether any substance detected is the complexing agent or the contaminant. Much further work will be required before any identification of the complex can be made and this will require the separation of a larger quantity of the complex from the leaf.

PART TWO

Alyssum robertianum

Nickel Accumulation

by the

Genus *Alyssum*

CHAPTER 6

Survey of the Genus *Alyssum*

6.1 INTRODUCTION

Alyssum L. is one of the larger genera in the family Cruciferae (= Brassicaceae). Schulz (1936) divided this family into nineteen tribes one of which is named Alysseae after its principal component genus, Alyssum. The family, tribe and genus have been described as Irano-Turanian in phytogeographical origin (Hedge, 1976). Alyssum is however also widely represented in the Mediterranean and Saharo-Sindian regions. Outside these regions, Alyssum species can be found in a belt through Siberia to the Yukon district in North America. The genus has its greatest concentration, diversification and proliferation in the eastern Mediterranean and Turkish areas (Dudley, 1964 a, b, 1965 a, b, Persson, 1971). The Yukon representation consists of one species, A. americanum Greene, in a disjunct population. This species closely resembles two Siberian species, A. obovatum (Meyer) Turcz. and A. biovulatum Busch, and may be conspecific with them (in which case A. obovatum would be the specific epithet)(Dudley, 1964a). The distribution of a species through Siberia to North America has been recorded in other genera of the Cruciferae (Hedge, 1976).

The genus Alyssum consists of approx. 170 species. Dudley (1964a) recognizes 168 species in his revision of the genus but maintains the possibility of several more because he was unable to examine the type material of some other previously-named species. The genus has been divided into various sections, subsections and series. There are six sections: (1) section Meniocus (Desv.) Hook.; (2) section Psilonema (Meyer) Hook.; (3) section Alyssum; (4) section Gamosepalum (Hausskn.)Dudley with the series Connata Dudley and Libera Dudley; (5) section Tetradenia (Spach.) Dudley; (6) section Odontarrhena (Meyer) Koch with the subsections Inflata Dudley, Compressa Dudley comprising the series Integra Dudley and Crenulata Dudley, and Samarifera Dudley. The plants themselves are herbaceous in character and may be annuals, biennials or perennials. Within any given section, however,

the life period is often more clearly defined: Meniocus and Psilonema are composed wholly of annuals, Alyssum has all three, Gamosepalum and Tetradenia perennials only and Odontarrhena mostly perennials with, rarely, biennials.

The genus Alyssum holds a special place in the study of nickel hyperaccumulation. The first three species identified as hyperaccumulators of this element were all from this genus : A. bertolonii (Minguzzi & Vergnano, 1948), A. murale (Doksopulo, 1961) and A. serpyllifolium ssp. lusitanicum (Menezes de Sequeira, 1969). The 1.22% nickel reported by Minguzzi and Vergnano (1948) in dried leaves of A. bertolonii was over an order of magnitude higher than that reported for other vegetation from the ophiolitic outcrop at Impruneta, near Firenze (Florence), Italy. This value greatly exceeded any other nickel concentration in plant material recorded at that time. In 1978, Brooks and Radford (1978a) surveyed the nickel content of European species of this genus and discovered eleven new hyperaccumulators (see appendix II (a)). Furthermore all eleven species were from section Odontarrhena. The three previously discovered taxa are all also from this section of the genus.

Alyssum section Odontarrhena was originally described as a separate genus, Odontarrhena Meyer (Meyer, 1831). This was because its member species had several important and contrasting differences compared with species of the genus Alyssum as it was then delimited (Dudley, 1964a). This section has a large proportion of species which are ecologically endemic, and restricted solely to serpentinitic and other ultrabasic substrates. Of the fourteen known hyperaccumulators of nickel within this section, eleven are endemic to such substrates, while three species, A. alpestre, A. argenteum and A. obovatum are not (Brooks & Radford, 1978a). Not only is section Odontarrhena the sole section with nickel hyperaccumulators but the fourteen taxa with this property come from a survey of only twenty-three taxa recorded by Ball and Dudley (1964) in Flora Europaea. This gives a remarkably high proportion of hyper-accumulators in this section. A similar proportion exists for

species of the genus Homalium in New Caledonia (Brooks, Lee, et al., 1977). The situation in which hyperaccumulation of nickel is restricted to a particular section has not been recorded for any other large genus but in Phyllanthus there are some sections which have hyperaccumulators and others which do not (Kersten et al., 1979). Species of other sections of Alyssum showed no hyperaccumulation even for species growing on serpentinitic substrates (Brooks & Radford, 1978a). Thus A. densistellatum (section Alyssum) growing over serpentine did not accumulate more than 40 µg Ni/g, a concentration which is "normal" for any plant growing over such substrates.

Despite the fact that serpentinitic substrates are also relatively rich in cobalt and chromium, uptake of these two elements is not notable. Indeed Brooks and Radford (1978a) used the chromium content of specimens as an index of possible contamination by soil. Specimens with greater than 10 µg Cr/g were assumed to be contaminated and hence rejected. If the species were chromium accumulators this would have led to an unacceptably high rejection rate.

In a continuation of the survey of this genus, the non-European species, plus some further European species, were analysed for nickel, cobalt and chromium. As a basis for determining the species to be covered by this survey, it was decided to cover only those species listed in Dudley's synopsis of the genus (Dudley, 1964a). This meant that the survey attempted to cover 168 species of Alyssum.

6.2 ANALYTICAL METHODS

The survey for nickel hyperaccumulation in Alyssum species was carried out on herbarium specimens. Approaches were made to a number of herbaria and those listed in appendix I (a) provided specimens.

From the specimens supplied, samples of 0.01 - 0.03g dry weight were weighed and placed in 5 cm^3 borosilicate test-tubes. The samples were then ashed at 500^{o}C in a muffle furnace. The ash was then dissolved in 1cm^3 of 2M hydrochloric

acid (prepared from redistilled constant-boiling hydrochloric acid). The resultant solution was analysed by atomic absorption spectrophotometry for the elements nickel, cobalt and chromium.

The atomic absorption spectrophotometer used was the Varian-Techtron model AA5 with automatic background corrector as for Chapter 2. The lines used for analysis were 232.0nm and 351.5nm for nickel (low and high concentrations respectively), 240.8nm for cobalt and 357.9nm for chromium.

The chromium content was determined as an indicator of possible contamination although had any species been rejected too frequently further investigations of the chromium contents of that species would have ensued. No species was however rejected with any great frequency. No species in which individual specimens were rejected had their status changed because of that rejection ie. all species which showed high nickel contents in contaminated specimens also had at least one specimen with high nickel content without contamination. As for Brooks and Radford (1978a), a chromium content of 10 µg/g or greater was assumed to be a result of contamination and the sample was rejected. The results of all the analyses are reported on a dry weight basis.

6.3 RESULTS AND DISCUSSION

The results of nickel analyses of several hundred specimens of Alyssum species are recorded in table 6.1. This table also includes data for the fourteen hyperaccumulators discovered by Brooks and Radford (1978a). With these results included, 167 of the 168 species listed by Dudley (1964a) have now been surveyed for their nickel content. Although cobalt analyses were also performed, the data were never anomalous (the majority had concentrations below 1 µg/g) and have been omitted from the table. The relative lack of cobalt in the serpentine-growing specimens indicates that Alyssum species preferentially accumulate nickel relative to cobalt.

Table 6.1

Nickel concentrations in *Alyssum* L. species

Species	Nickel Content (µg/g dry weight)
Section Meniocus (Desvaux) Hooker	
A. aureum (Fenzl.) Boissier	<1, 3
A. blepharocarpum T.R. Dudley & Huber-Morath	8, 9
A. heterotrichum Boissier	6, 12
A. huetii Boissier	<1, <1
A. linifolium Stephan ex Willdenow	9, 9, 16
A. meniocoides Boissier	<1, 2
A. stylare (Boissier & Balansa) Boissier	3, 14
mean nickel content for section	6
Section Psilonema (C.A. Meyer) Hooker	
A. alyssoides (Linnaeus) Linnaeus	2, 4, 4, 5, 6,
A. damascenum Boissier & Gaillardot	<1, 4
A. dasycarpum Stephan ex Willdenow	2, 3, 6
A. granatense Boissier & Reuter	<1, 2, 2, 4, 6
A. homalocarpum (F. Fischer & C.A. Meyer) Boissier	2, 7
mean nickel content for section	3
Section Alyssum	
A. aizoides Boissier	2, 10
A. arenarium Loiseleur-Deslongschamps	<1, <1, 5, 5, 9
A. argyrophyllum Schott & Kotschy	2, 4
A. armenum Boissier	<1, 3
A. artwinense Busch	<1, 9
A. atlanticum Desfontaines	<1, <1
A. aurantiacum Boissier	3, 15
A. bornmuelleri Haussknecht ex Degen	<1, 3
A. bulbotrichum Haussknecht & Bornmüller	<1, 10

A. caespitosum J. Baumgartner	7, 10
A. calycocarpum Ruprecht	<1, 1
A. canescens De Candolle	5, 6
A. cephalotes Boissier	<1, 3
A. contemptum Schott & Kotschy	<1, 3
A. cuneifolium Tenore	<1, <1, <1, 2, 2
A. densistellatum T.R. Dudley	<1, <1, 40, 40
A. desertorum Stapf	<1, 2, 5, 14
A. diffusum Tenore	<1, 3
A. doerfleri Degen	2
A. erosulum Gennari & Pestalozza	<1, 2
A. fastigiatum Heywood	3
A. fischerianum De Candolle	<1, <1, <1, 7
A. foliosum Bory & Chaubard	<1, <1
A. fulvescens Sibthorp & Smith	<1, <1
A. handelii Hayek	<1, 5
A. hirsutum Bieberstein	<1, 2, 3, 9
A. idaeum Boissier & Heldreich	<1, 2, 3
A. iranicum Haussknecht ex J. Baumgartner	2, 4
A. lanceolatum J. Baumgartner	2, 3
A. lassiticum Halácsy	3, 10
A. lenense Adams	1, 2, 13
A. lepidotum Boissier	<1, 7
A. macrocalyx Cosson & Durand	3, 5
A. macropodon Boissier & Balansa	2, 5
A. marginatum Steudel ex Boissier	<1, 22
A. microphyllum (C.A. Meyer) Steudel	70, 152
A. minus (Linnaeus) Rothmaler	<1, <1, 1, 2, 4
A. minutum Schlectendal ex De Candolle	<1, <1, 5
A. moellendorfianum Ascherson ex Beck	<1, <1, 3
A. montanum Linnaeus	<1, <1, 2, 2, 5
A. mouradicum Boissier & Balansa	<1, <1, 12
A. muelleri Boissier & Buhse	2, 11
A. nevadense Wilmott ex P. Ball & T.R. Dudley	<1
A. ochroleucum Boissier & Hueter	<1, 2
A. ovirense Kerner	<1, <1, 2, 2, 5

A. persicum Boissier	2, 3
A. praecox Boissier & Balansa	<1, <1
A. propinquum J. Baumgartner	67
A. pseudo-mouradicum Haussknecht & Bornmüller ex J. Baumgartner	<1, 9
A. pulvinare Velenovsky	1, 2, 5, 6, 9
A. purpureum Lagasca & Rodriguez	<1, 5
A. repens J.C.G. Baumgarten	<1, <1, 2, 2, 6
A. rostratum Steven	<1, <1, 4, 10
A. scardicum Wettstein	7, 26
A. scutigerum Durand	<1, 9
A. smyrnaeum C.A. Meyer	<1, <1, <1, 1
A. sphacioticum Boissier & Heldreich	<1, <1, 2
A. stapfii Vierhapper	<1, 10
A. stribrnyi Velenovsky	1,1, 15
A. strictum Willdenow	2, 3
A. strigosum Banks & Solander	<1, 5
A. szowitsianum F. Fischer & C.A. Meyer	2, 6
A. taygeteum Heldreich	2
A. tenuifolium Stephan ex Willdenow	3, 4
A. trichocarpum T.R. Dudley & Huber-Morath	<1, 14
A. turkestanicum Regel & Schmalhausen	<1, <1, 5
A. umbellatum Desvaux	<1, 1
A. vourinonense Dudley & Rechinger	14
A. wierzbickii Heuffel	<1, <1, <1, 1
A. wulfenianum Schlechtendal	1
A. xanthocarpum Boissier	9, 10, 20
mean nickel content for section	5
Section Gamosepalum (Haussknecht) T.R. Dudley	
(a) Series Connata T.R. Dudley	
A. lepidoto-stellatum (Haussknecht & Bornmüller ex Haussknecht) T.R. Dudley	3, 3
A. paphlagonicum (Haussknecht) T.R. Dudley	2, 60
A. tetrastemon Boissier	3, 25, 75

A. thymops (Huber-Morath & Reese) T.R. Dudley	9, 11, 16
(b) Series Libera T.R. Dudley	
A. baumgartnerianum Bornmüller	<1, 1, 6
A. corningii T.R. Dudley	<1, 3
A. harputicum T.R. Dudley	<1, 6
A. lycaonicum (E.E. Schulz) T.R. Dudley	4, 33
A. niveum T.R. Dudley	1, 5
A. sulphureum T.R. Dudley & Huber-Morath	8, 34
mean nickel content for section	5
Section Tetradenia (Spach) T.R. Dudley	
A. cochleatum Cosson & Durand	2, 3
A. lapeyrousianum Jordan	<1, 7
A. spinosum Linnaeus	<1, 9
mean nickel content for section	4
Section Odontarrhena (C.A. Meyer) W. Koch	
I. Subsection Inflata T.R. Dudley	
A. alpestre Linnaeus	3640 *
A. americanum Greene	7, 11, 21, 36, 147, 184, 381
A. anatolicum Haussknecht ex E. Nyárády	<1, 10, 8170
A. baicalicum E. Nyárády	<1, 4, 15, 27
A. bertolonii Desvaux	13400 *
A. biovulatum Busch	5
A. borzaeanum E. Nyárády	<1, <1, 2
A. bracteatum Boissier & Buhse	<1, <1, <1, 22
A. caliacrae E. Nyárády	2, 2, 2, 15, 32
A. callichroum Boissier & Buhse	33, 146, 1930, 10900
A. chondrogynum Burtt	3980, 7260, 16300
A. condensatum Boissier ex Haussknecht ssp. condensatum	13,15, 19, 81, 700
A. condensatum ssp. flexibile (E. Nyárády) T.R. Dudley	<1, 9, 375, 2330, 4990
A. condensatum ssp. lycium	<1, 119, 159

A. constellatum Boissier	13, 3820, 5380, 18100
A. corsicum Duby	4600, 6170, 11400, 11700, 21500
A. corymbosoides Formánek	2
A. cypricum E. Nyárády	3900, 4140, 7670 14400, 20200, 23600
A. davisianum T.R. Dudley	6500, 11600, 19600
A. discolor T.R. Dudley & Huber-Morath	4450, 6030, 8730, 11700
A. eriophyllum Boissier & Haussknecht	8530, 11500, 11500
A. euboeum Halácsy	4550 *
A. fallacinum Haussknecht	3960 *
A. fedtschenkoanum Busch	6, 7
A. filiforme E. Nyárády	132, 151, 814
A. fragillimum (Baldacci) Rechinger f.	<1, <1
A. gehamense Federov	<1, <1, <1
A. haussknechtii Boissier	<1, <1, 115
A. huber-morathii T.R. Dudley	1220, 3540, 4990, 11800, 13500
A. inflatum E. Nyárády	<1, 20, 54, 63
A. lanigerum De Candolle	<1, 5, 63, 100
A. libanoticum E. Nyárády	<1, <1, <1
A. longistylum (Sommier & Levier) Grossheim	<1, <1, 63, 93
A. markgrafii O.E. Schulz	13700 *
A. masmenaeum Boissier	5480, 15500, 15600, 24300
A. nebrodense Tineo	<1, <1, <1, 11
A. obovatum (C.A. Meyer) Turczaninow	2, 6, 26, 64, 1030, 4590
A. obtusifolium Steven ex De Candolle	60
A. oxycarpum Boissier & Balansa	<1, 10, 4460, 7290
A. pateri E. Nyárády	<1, 30, 44, 53, 61 184, 484
A. penjwinensis T.R. Dudley	<1, 13, 1720, 7860
A. polycladum Rechinger f.	<1, <1, 6, 127
A. robertianum Bernard ex Grenier & Godron	12 500*
A. serpyllifolium Desfontaines ssp. serpyllifolium	<1, <1, <1, 2, 4
A. serpyllifolium ssp. lusitanicum T.R. Dudley & P. Silva	9000*
A. serpyllifolium ssp. malacitanum Rivas Goday	<1, 10, 483, 1760, 8470

A. sibiricum Willdenow	1, 1, 18, 20, 22, 487
A. singarense Boissier & Haussknecht	1, 8, 29, 1280
A. smolikanum E. Nyárády	6600*
A. syriacum E. Nyárády	3380, 5250, 10200
A. szarabiacum E. Nyárády	Not analysed
A. tavolarae Briquet	3
A. tortuosum Waldstein & Kitaibel ex Willdenow	1, 1, 6, 11, 17, 27, 314
A. troodii Boissier	5120, 6560, 9510, 9790
A. turgidum T.R. Dudley	36
II. Subsection Compressa T.R. Dudley (a) Series Integra T.R. Dudley	
A. akamasicum Burtt	3660, 4290, 9090
A. argenteum Allioni	10800*
A. cassium Boissier	5590, 6320, 7250, 9150, 20000
A. janchenii E. Nyárády	6330, 9610
A. murale Waldstein & Kitaibel	7080*
A. subspinosum T.R. Dudley	7
A. tenium Halácsy	3420*
(b) Series Crenulata T.R. Dudley	
A. cilicicum Boissier & Balansa	4260, 13700
A. crenulatum Boissier	7080, 8360, 10400
A. giosnanum E. Nyárády	4170, 4800, 6910, 7390
A. heldreichii Haussknecht	12500*
A. pterocarpum T.R. Dudley	1190, 4860, 6740
III. Subsection Samarifera T.R. Dudley	
A. caricum T.R. Dudley & Huber-Morath	3630, 4780, 6130
A. dubertretii Gombault	16500
A. floribundum Boissier & Balansa	641, 1370, 5620, 7700
A. lesbiacum (Candárgy) Rechinger f.	7920, 8200, 14300, 22400
A. peltarioides Boissier ssp. peltarioides	2, 4, 17
A. peltarioides ssp. virgatiforme (E. Nyárády) Dudley	5300
A. peltarioides ssp. undetermined	7600
A. pinifolium (E. Nyárády) T.R. Dudley	6670, 9950, 12600

A. samariferum Boissier & Haussknecht	4220, 5460, 6650
A. trapeziforme Bornmüller ex E. Nyárády	2820, 5600, 6900, 11900
A. virgatum E. Nyárády	1830, 3080, 5480, 6230
mean nickel content for section (excluding hyperaccumulators)	56

*Highest value reported by Brooks & Radford (1978)

It is observed in table 6.1 that hyperaccumulation of nickel is confined to section Odontarrhena. A total of forty-eight taxa of this section have shown this property. All non-Odontarrhena species listed have now had their nickel content determined and of these the highest content recorded was 152 µg/g in A. microphyllum (section Alyssum). This sample was the only non-Odontarrhena specimen to surpass the 100 µg/g concentration level. A. microphyllum is a species found throughout Siberia. An easy comparison between the nickel concentrations of the Odontarrhena and non-Odontarrhena specimens can be made by viewing the histograms of fig. 6.1. From this it is readily apparent that the bulk of non-Odontarrhena specimens contained less than 10 µg/g. The Odontarrhena specimens show a completely different distribution. Indeed the distribution is indicative of two populations of species with a separation boundary between 320 µg/g and 1,000 µg/g. This boundary coincides well with the value designated by Brooks, Lee et al. (1977) as the concentration criterion for nickel hyperaccumulation. For Alyssum species in section Odontarrhena, 55% of the specimens have nickel levels below 1,000 µg/g and 45% have concentrations above this. The highest concentration was recorded for the Turkish serpentine-endemic, A. masmenaeum (2.43% nickel). Further comparisons between Odontarrhena and non-Odontarrhena species can be made by comparing the mean nickel content for each section (table 6.1).
Even after the values of the hyperaccumulators have been excluded, the arithmetic mean for Odontarrhena at 56 µg/g is an order of magnitude greater than that for any of the other five sections: Meniocus, 5 µg/g ; Psilonema, 3 µg/g ; Alyssum, 5 µg/g ; Gamosepalum, 5 µg/g; and Tetradenia, 4 µg/g. Only eighteen of the seventy-four taxa listed under Odontarrhena failed to reach 100 µg/g in any specimen, whereas, as mentioned above, only one non-Odontarrhena taxon reached this level. Tolerance to high nickel appears to be a common although not invariable property of Odontarrhena species.

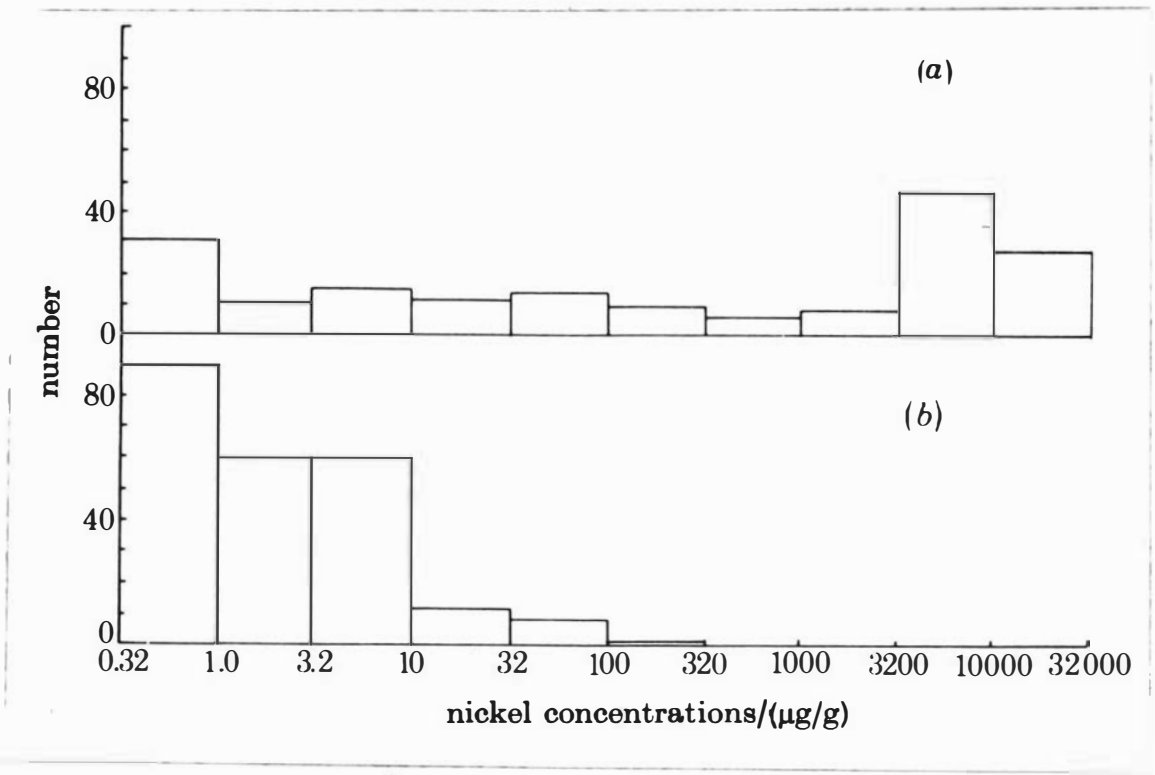


Figure 6.1 Histograms of nickel concentration in *Alyssum* specimens.

(a) Species of section Odontarrhena.

(b) Species of sections Meniocus, Psilonema, Alyssum, Gamosepalum and Tetradenia.

The geographical distribution of the Odontarrhena specimens is shown in figs. 6.2, 6.3 and 6.4. The Turkish specimens (fig. 6.2) are shown distributed by vilayets (provinces) rather than actual localities. A very strong correlation between the existence of ultrabasic rocks and the distribution of nickel hyperaccumulating specimens is clearly seen. Specimens in the western Irano-Turanian phytogeographical region (Iran, Iraq, Transcaucasia and eastern Turkey - fig. 6.3) and the outlying specimens (Ukraine, eastern Irano-Turanian region, Siberia and Yukon district - fig. 6.4) show a much lower hyperaccumulation proportion (for the distribution of the European hyperaccumulators see Brooks & Radford, 1978a). From these maps, it can be seen that almost all hyperaccumulating species are found in southern Europe, the eastern Mediterranean area and Turkey. Only three species, A. obovatum (found in southeast Russia, southern Ukraine and Siberia), A. penjwinensis and A. singarense (both endemic to Iraq), showed hyperaccumulation outside this region. Species which contain over 10,000 µg/g (= 1% nickel) are almost all restricted to eastern Mediterranean lands (Greece, Aegean Islands, Cyprus and the Mediterranean coastal regions of Turkey and northern Syria). Some however occur in central Turkey, northern Italy and Corsica. Species which hyperaccumulate more than 1% nickel (there are eighteen such species) are generally very restricted in their distribution. A. constellatum is the exception to this rule but even this species is found only in Turkey and northern Iraq. Species which do not hyperaccumulate nickel to this extent frequently have much greater distribution ranges. Some of these species are not serpentine endemics, eg. A. obovatum, and may have specimens with low nickel content: 2-4,600 µg/g is the range for A. obovatum. There is little question that many Odontarrhena species are very successful in their adaptation to serpentine environments. Several taxa are found in almost pure populations on serpentine outcrops eg. A. murale in the Balkans and A. corsicum and A. cypricum in southwestern Turkey.

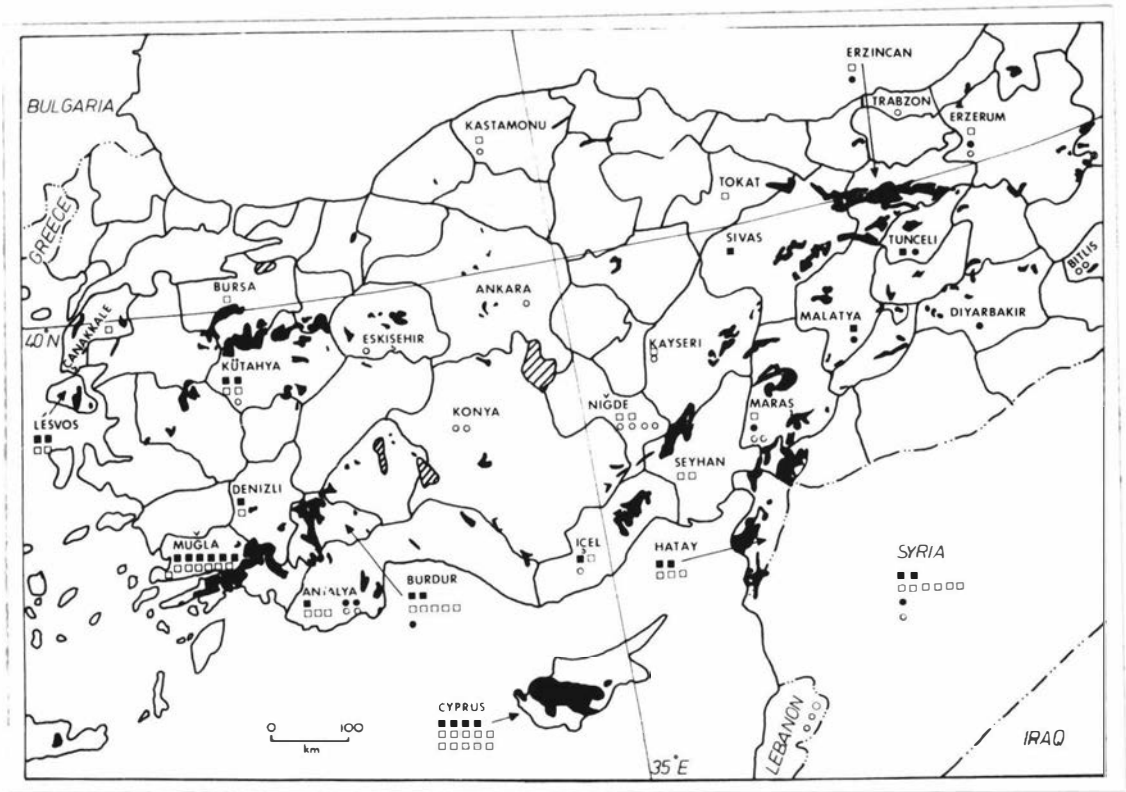


Figure 6.2 Distribution, by vilayets, of Turkish species of section Odontarrhena.

Key to Figures 6.2, 6.3 + 6.4

- ■ Specimens containing nickel concentrations >10,000 µg/g
- □ Specimens containing 1,000 - 10,000 µg/g
- ● Specimens containing 100 - 999 µg/g
- ○ Specimens containing <100 µg/g

The black areas in Figure 6.2 are ultrabasic rocks.

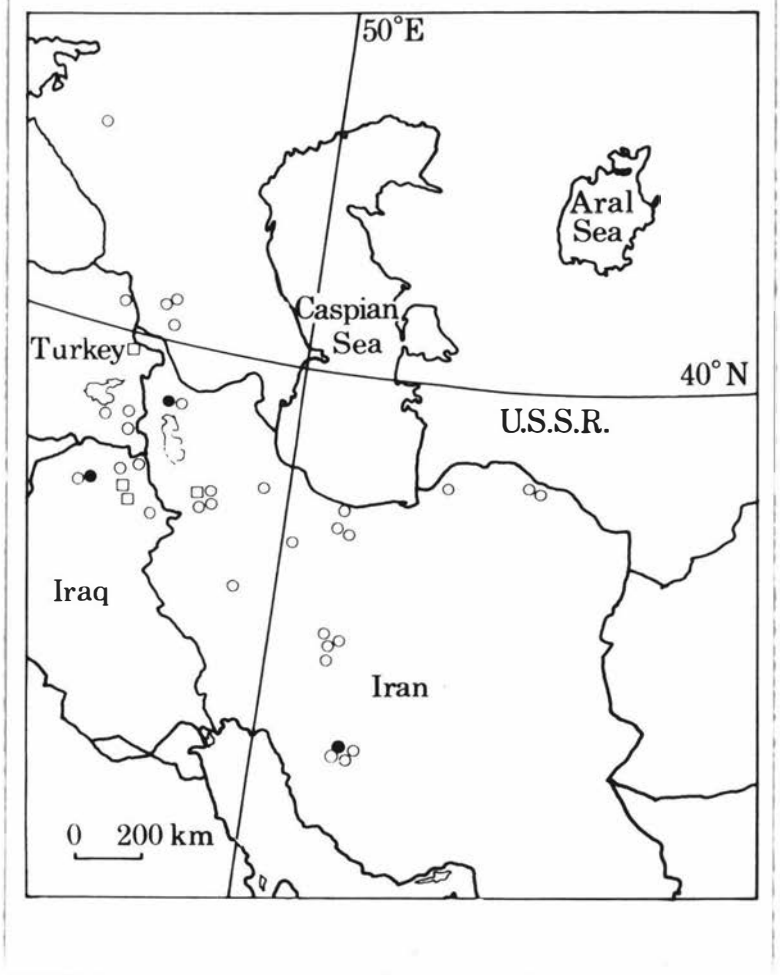


Figure 6.3 Geographical distribution of species of section Odontarrhena in the western Irano-Turanian region.

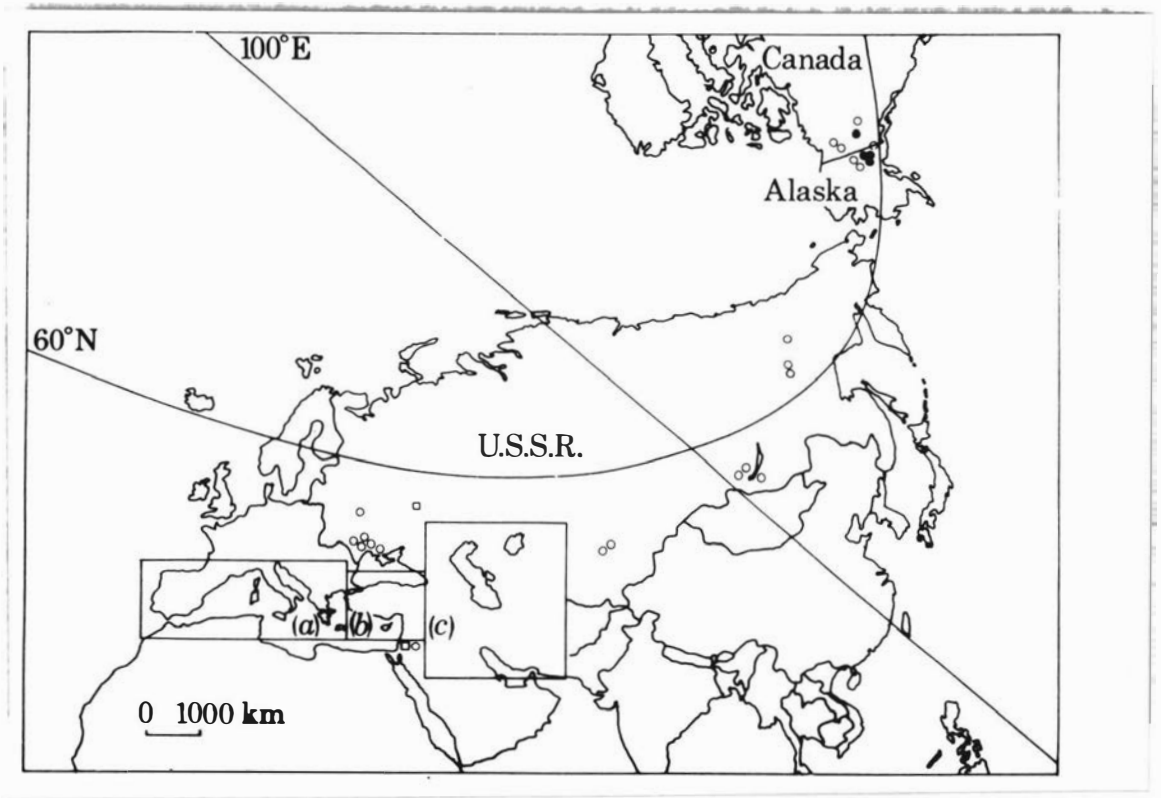


Figure 6.4 Geographical distribution of outlying specimens of species of section Odontarrhena. For the distributions in (a) see Brooks and Radford, 1978a; (b) see Figure 6.2; (c) see Figure 6.3.

Within section Odontarrhena, it is observed that the subsections Compressa and Samarifera are each composed entirely of hyperaccumulators with one exception: A. subspinosum for Compressa and A. peltarioides for Samarifera. Neither of these two species is found within the centre of distribution of the hyperaccumulators. A. subspinosum is known only from Jordan while A. peltarioides is found in central and eastern Turkey. Subsection Inflata contains twenty-seven hyperaccumulators, eight strong accumulators (100 - 999 µg/g) and twenty non-accumulators (less than 100 µg/g). The lectotype species for section Odontarrhena, A. tortuosum (subsection Inflata) is not a hyperaccumulator.

The existence of a non-accumulator in a subsection which otherwise consists wholly of hyperaccumulators raises the question as to whether this species is the parent species of the hyperaccumulators. Within subsection Compressa this question is further complicated by the existence of two series, Integra and Crenulata. Series Crenulata consists wholly of hyperaccumulators. Series Integra contains the non-accumulator A. subspinosum and also A. murale which is not endemic to serpentine soils. The geographical distribution of the species of series Integra is shown in fig. 6.5. It is always possible that A. subspinosum is a relic of a former widespread but now generally defunct species but the notable spread of A. murale in its many guises raises doubts. A. murale is a highly variable and actively evolving genetic unit (Dudley, 1964b). Furthermore A. murale has, in the past, been divided into approx. forty taxa, either microspecies or infraspecies. It could be that it is A. murale from which the others have evolved. A. subspinosum may then have evolved from A. murale as an adaptation to the Jordanian environment rather than A. murale deriving from A. subspinosum. Alternatively some factor may have allowed A. murale to supersede a previous parent species of which A. subspinosum is a relic. The hyperaccumulation of nickel by A. murale is worthy of deeper study as although the eleven specimens analysed by Brooks and Radford (1978a) all hyper-accumulated, the specimens analysed by this author did not.

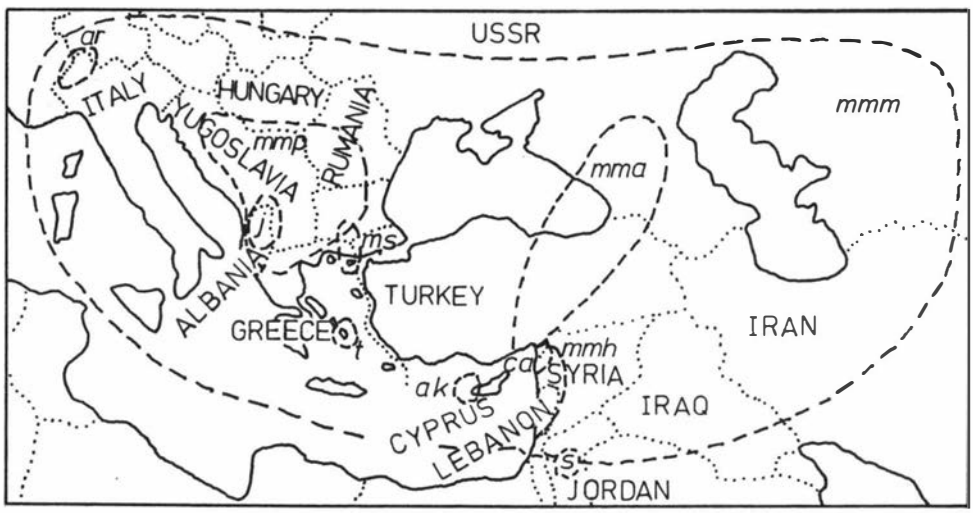


Figure 6·5 The distribution of *Alyssum* species: section Odontarrhena, subsection Compressa, series Integra.

ak = *A. akamasicum*
ar = *A. argenteum*
ca = *A. cassium*
j = *A. janchenii*
s = *A. subspinosum*
t = *A. tenium*

mmm = *A. murale* s.sp. *murale* var. *murale*
mma = *A. murale* s.sp. *murale* var. *alpinum*
mmh = *A. murale* s.sp. *murale* var. *haradjiani*
mmp = *A. murale* s.sp. *murale* var. *pichleri*
ms = *A. murale* s.sp. *stojanoffii*

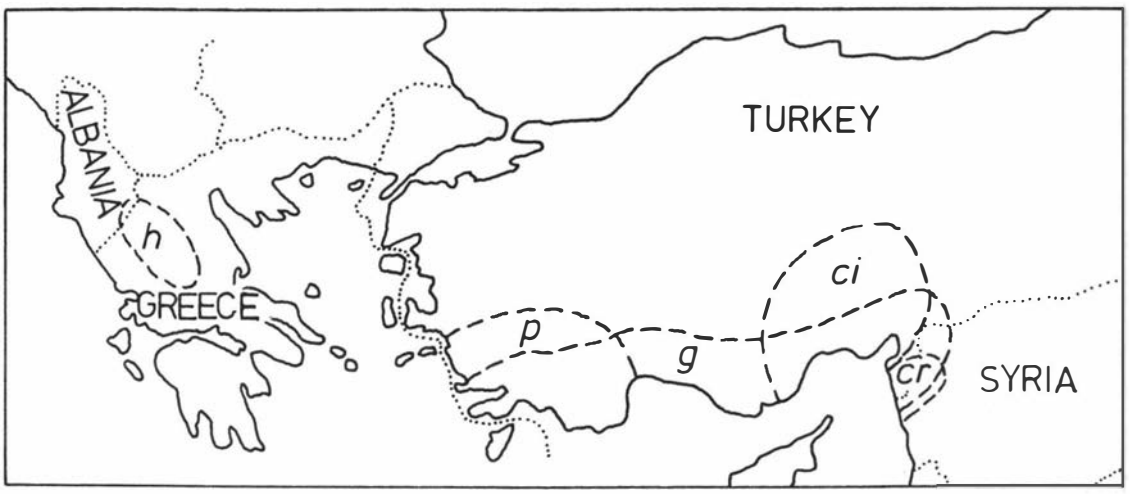
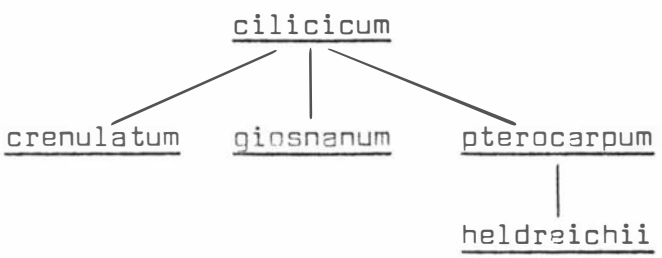


Figure 6.6 The distribution of *Alyssum* species: section Odontarrhena, subsection Compressa, series Crenulata.

ci = *A. cilicicum*
cr = *A. crenulatum*
g = *A. giosnanum*
h = *A. heldreichii*
p = *A. pterocarpum*

There was, however, a conspicuous difference in collection localities for these samples: Brooks and Radford (1979a) sampled species from the Balkans (probably A. murale ssp. murale var. pichleri); this author sampled species from the USSR and Turkey (probably A. murale ssp. murale var. murale or var. alpinum). Assuming these identifications to be correct, it is then not improbable that, in time, A. murale ssp. murale var. pichleri will develop into a new serpentine-endemic species.

Within series Crenulata, A. cilicicum emerges as the most probable progenitor on the basis of the alliances given by Dudley (1965a). The possible line of evolution of the species of this series may be as follows:



The distribution of these species is shown in fig. 6.6. The relationship between the two series of subsection Compressa is beyond the scope of this discussion.

The question as to whether A. peltarioides is the parent species of subsection Samarifera is perhaps less complicated. A. peltarioides does, however, have three subspecies; A. peltarioides ssp. peltarioides in eastern and northeastern Turkey, A. peltarioides ssp. virgatiforme in central Turkey, and an undetermined but different subspecies (T.R. Dudley pers. comm. to R.D. Reeves) in Burdur, southwest Turkey. The possibility is that A. peltarioides ssp. peltarioides is the parent taxon of subsection Samarifera. This may be inferred from a probable past distribution. A. peltarioides ssp. peltarioides is found today at high altitudes in the vicinity of melting snow (2,000 - 3,580m, Dudley, 1964b). During the ice-ages, it could easily have been more widespread in Turkey.

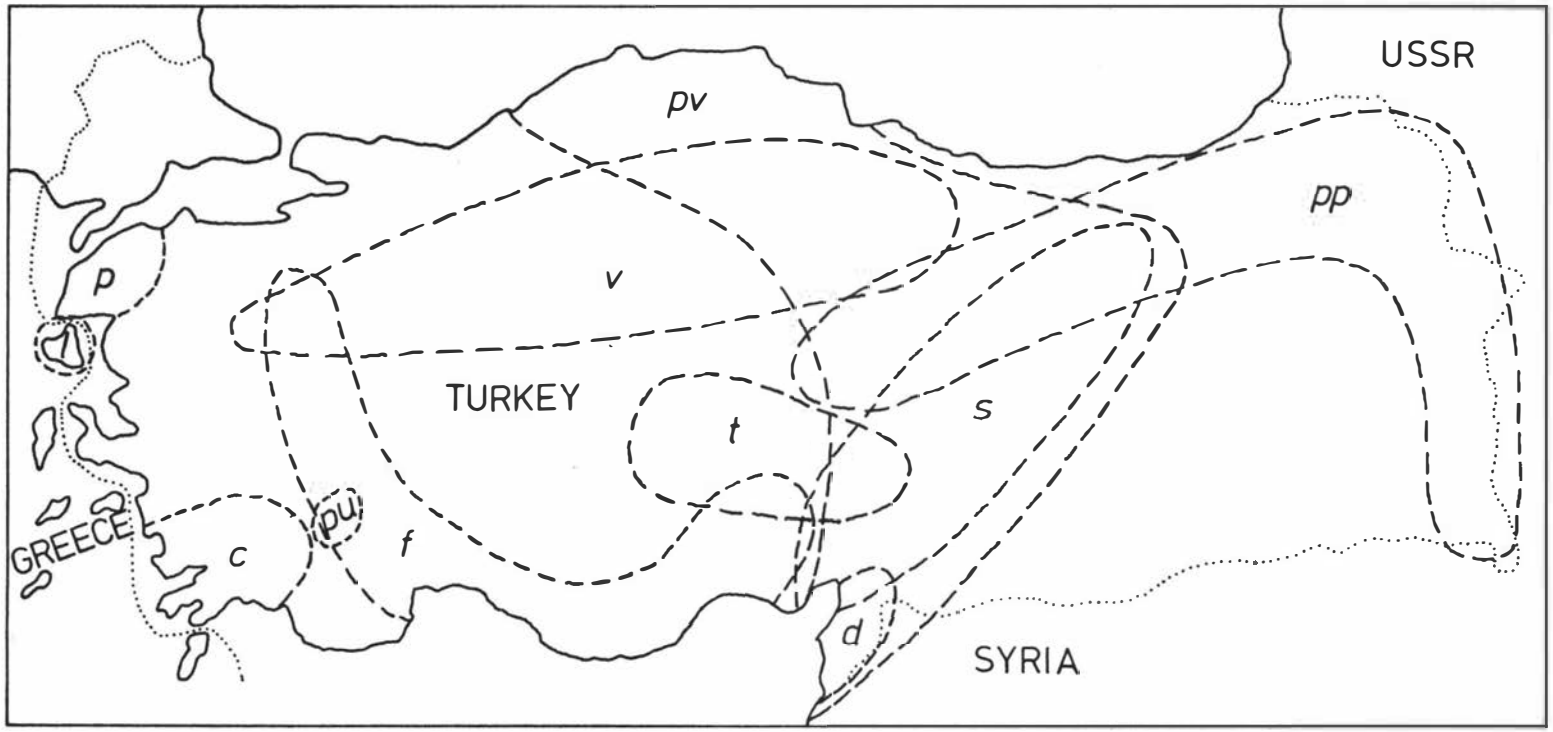
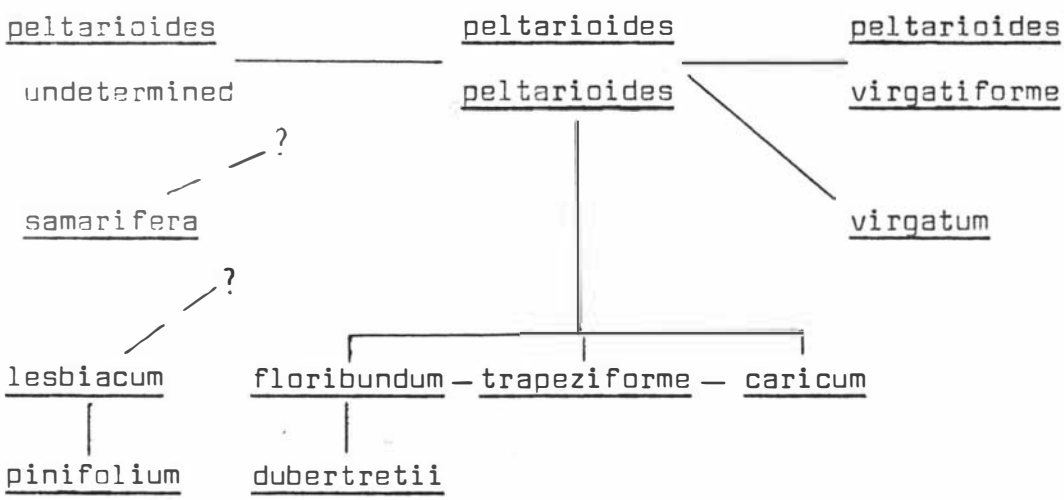


Figure 6·7 The distribution of *Alyssum* species: section Odontarrhena, subsection- Samarifera.

c = *A. caricum*
d = *A. dubertretii*
f = *A. floribundum*
l = *A. lesbiacum*
p = *A. pinifolium*
pu = *A. peltarioides* s.sp. undetermined
pp = *A. peltarioides* s.sp. *peltarioides*
pv = *A. peltarioides* s.sp. *virgatiforme*
s = *A. samariferum*
t = *A. trapeziforme*
v = *A. virgatum*

Then, as the climate warmed up after the ice-ages ended, it began to retreat into the cooler regions ie. the mountains of Turkey. As it retreated, it left behind specimens isolated in various areas. These specimens continued to grow and evolve in isolation and have given rise to the species seen today. That these specimens survived would be due to their being in environments which were hostile to the invading species ie. they were in serpentine soils. A distribution of this type is paralleled in Scandinavia today by Arenaria norvegica Gunn. which south of 66°N (the warmer area) is found almost exclusively on serpentine soils but further north (the cooler area) is more widespread (Rune, 1953). Given time, the isolated southern populations could evolve into new taxa. Turkey, being released from the ice-ages earlier than Scandinavia, has had the time to develop these new taxa. The occurrence of a subspecies of A. peltarioides in southwest Turkey appears to be an indication that the parent taxon was once more widespread. That these various subspecies exist as well as the independent species may be a result of the multiplicity of ice-ages (Birman, 1968). The independent species may have developed prior to the latest post-Pleistocene glaciation while the subspecies have arisen subsequent to this glaciation as A. peltarioides once more retreated to cooler areas. The present distribution of A. peltarioides ssp. peltarioides is itself probably of Recent origin. The taxon probably migrated from the Taurus Mountains along the Anatolian diagonal (a mountainous belt from Seyhan-Niğde to Sivas) and thence to the northeast and east of Turkey. This migration explains the greater multiplicity of species in the Taurus Mountains of southern Turkey. These ranges are more strongly glaciated than the northern Pontic Mountains suggesting that the latter have only recently risen to their current height (Birman, 1968). Thus prior to this recent uplift, A. peltarioides ssp. peltarioides may have been restricted to the mountains of southern Turkey. Further evidence for this late migration comes from the recognition by Dudley (1965a) that A. virgatum, the most northerly independent species, is also the closest

related species. It also appears that the two subspecies A. peltarioides ssp. peltarioides and ssp. virgatiforme are close to separation. The area of overlap in their distributions is not a large part of their ranges and with the altitudinal preferences and the times of flowering differing, they appear to be close to independent development. This development is likely to accelerate as the separation is completed and new species evolve. Only one specimen intermediate to these two subspecies has ever been recorded. This can be compared with infraspecific taxa of other Alyssum species where intermediates in areas of overlap are common (Dudley, 1965a). It thus appears probable that A. peltarioides ssp. peltarioides could be the parent taxon to the other members of subsection Samarifera. A possible line of development (based on alliances given by Dudley, 1965a) is as follows:



Brooks and Radford (1978a) noted that while A. serpyllifolium ssp. serpyllifolium was not an accumulator, A. serpyllifolium ssp. lusitanicum invariably was. They suggested that this could be used to support a change of status for the subspecies lusitanicum which Pinto da Silva (pers. comm. to R.R. Brooks, 1977) was already considering. This change of name is now under way (T.R. Dudley, in ed.) and the new name is to be A. pintodasilvae. Since these

communications, a second subspecies of A. serpyllifolium, A. serpyllifolium ssp. malacitanum, has shown hyperaccumulation (table 6.1). As a consequence of this, any decision to split A. serpyllifolium into a number of species must be reconsidered. If A. pintodasilvae is accepted as a species independent of A. serpyllifolium then the status of A. serpyllifolium ssp. malacitanum will be very open to debate. However, the final considerations for the splitting of this species into several new species must be based on more than the nickel content and its associated physiological and morphological stresses. It may be that nickel stress as a cause of morphological variation will have to be studied in the laboratory before the situation becomes clear. That certain subspecies are distinguishable morphologically and also in nickel content can again be seen for A. condensatum where A. condensatum ssp. condensatum and A. condensatum ssp. lycium do not hyperaccumulate but A. condensatum ssp. flexibile does (Note: the two subspecies condensatum and lycium may be one and the same, as no reference to the subspecies lycium can be found but Odontarrhena lycia Jord. & Fourr. is given by Dudley, 1965a, as a synonym of the subspecies condensatum). One specimen of A. condensatum ssp. condensatum with a concentration of 700 µg/g looks misplaced when compared to the other samples of this taxon. This specimen may, or may not, have been incorrectly identified. Misidentifications are not unknown among herbarium specimens (cf. Brooks & Radford, 1978a).

CHAPTER 7

Biogeochemical Studies on Some *Alyssum* Species

7.1 INTRODUCTION

The uptake of nickel to hyperaccumulation levels by a particular species can lead to the use of that species as a biogeochemical indicator (Severne & Brooks, 1972, Cole, 1973, Brooks and Wither, 1977, Lee *et al.*, 1977a). Many other tolerant but non-hyperaccumulating species have also been used for biogeochemical prospecting (Lyon *et al.*, 1968, Timperley *et al.*, 1970a, 1972 a,b, Nielson *et al.*, 1973, Brooks, Trow and Bolviken, 1979). However, the basic study of these species from a biogeochemical viewpoint is limited. Such questions as the extent of potential uptake of nickel, the relationship between nickel in the plant and nickel in the soil, the rate of uptake of nickel by the plant and the degree of tolerance of these species have yet to be answered.

Kersten (1979) reported some initial investigations on the plant-soil relationship of the New Caledonian hyperaccumulator *Psychotria douarrei*. In pot trials which involved adding, in a time sequence, increments of nickel to a potting medium, he showed a linear relationship between the logarithmic values of nickel in both the plant and the soil (expressed on a dry weight basis). This form of relationship is considered by Timperley *et al.* (1970b) as common for the uptake of non-essential elements. Lee *et al.* (1977a) have also shown strong relationships between nickel in plants and extractable nickel (ammonium oxalate buffer as extractant) in soils for the two New Caledonian hyperaccumulators *Homalium kanaliense* and *Hybanthus austrocaledonicus*. This extractable nickel is probably comparable to the nickel measured by Kersten (1979) since the latter study involved adding nickel nitrate which would be readily extracted. *H. austrocaledonicus* also had a significant relationship between the nickel content of the plant and the total nickel content of the soil. A third species studied by Lee *et al.* (1977a), *Homalium guillainii*, showed no significant relationships between nickel in the plant and either

total or extractable nickel in the soil. Crooks (1979) and Brooks and Crooks (1980), studying the Fennoscandian species, Lychnis alpina and Silene dioica, showed that S. dioica had a linear relationship between nickel in the plant and in the soils while L. alpina showed an exclusion-breakdown form of relationship (see Chapter 4). The threshold value for the exclusion breakdown was approx. 3,000 µg/g (soil content). While L. alpina is not known as a nickel hyperaccumulator in the wild (maximum concentration 192 µg/g - Brooks, Trow & Bolviken, 1979), the pot trials of Crooks (1979) showed that this species is certainly capable of accumulation of nickel to this level. At a soil concentration of 4,100 µg/g, L. alpina had a leaf concentration of 7,300 µg/g. L. alpina did not survive in higher soil concentrations of nickel. Psychotria douarrei has survived in 9,100 µg/g although at this concentration, toxicity symptoms became evident (Kersten, 1979). The corresponding leaf concentration was 9,500 µg/g. Both P. douarrei and L. alpina were able to survive in soils with a very high available nickel content. The New Caledonian species studied by Lee et al. (1977a) had total soil nickel contents of 1-2% but the extractable (and hence readily available) nickel was generally of the order of 1,000 - 2,000 µg/g (0.1 - 0.2%). Proctor and Woodell (1975) have tabulated many serpentine soil nickel concentrations (from many sources) within these ranges. It thus appears that hyper-accumulators and other tolerant species have adapted to the nickel content of their environments with an excess capacity (a margin of safety for the varying soil content?).

Lychnis alpina has also been the subject of tolerance tests (Crooks, 1979). The method used in this test was a variation of the rooting technique developed by Wilkins (1957) and Jowett (1958). Seedlings with their roots excised were placed in solutions containing 0.5g/dm^3 calcium nitrate and incremental amounts of nickel. The tolerance level was taken as the highest concentration in which measurable new root growth occurred. Crooks (1979) found that L. alpina developed good roots in solutions of 1 µg/cm^3 and 2 µg/cm^3 but that at 5 µg/cm^3 the development was only slight. Although these levels of nickel

concentrations may seem low, they are substantially higher than those used in the development of indices of nickel tolerance for Agrostis spp. (Jowett, 1958, Gregory & Bradshaw, 1965, Proctor, 1971). Studies of nickel tolerance in other nickel tolerant species are very few. Ernst (1972) measured the tolerance of Indigofera setiflora by comparative protoplasmatology. He was able to show that I. setiflora from a nickeliferous area was more tolerant than the same species from non-nickeliferous areas. Nickel tolerance has however been said to be less specific than other metal tolerances (Proctor & Woodell, 1975).

The relative lack of cobalt in some hyperaccumulators of nickel (eg. Alyssum spp.,Chapter 6) and the relative enrichment of cobalt in others (eg. Rinorea spp., Brooks, Wither & Zepernick, 1977, Phyllanthus spp., Kersten, 1979) has also to be explained. In many serpentine soils the cobalt content is approx. 10% of the nickel content. The possibility that geographical and climatic considerations as well as geological considerations are involved cannot be avoided. The high cobalt contents are found in nickel hyperaccumulators from the tropical areas of Southeast Asia and New Caledonia whereas those with low cobalt contents come from the Mediterranean basin with its hot, dry summers and mild, moist winters. There do not appear to be any studies published as to possible causes for these differences.

In this chapter, studies have been made on the biogeochemical factors mentioned above. Nickel uptake in Alyssum species is studied both as a function of soil content and as a function of time. The tolerance of several of these species is also measured by both a solution rooting method and a soil culture method. As a further investigation, the uptake of cobalt was studied under a relative absence of nickel. A non-Alyssum species Bornmuellera tymphaea was also studied for nickel uptake as a function of soil content. This species was discovered to be a nickel hyperaccumulator by Reeves, Brooks and Dudley (1981). It is closely related to the Alyssum species and the genus Bornmuellera is a member of the tribe Alysseae of the Cruciferae.

7.2 EXPERIMENTAL METHODS

Seeds of eleven *Alyssum* species and of *Bornmuellera tymphaea* were collected by various persons (Appendix 1(b)) and forwarded to Massey University. Upon arrival the seeds were placed in cool storage until used. Germination was done on a Copenhagen table. All twelve species germinated readily. Once germinated, the seedlings were transplanted directly into the experimental pots unless otherwise stated. The species studied were *Alyssum montanum* L. (section Alyssum, a non-accumulator), *A. argenteum* All., *A. corsicum* Duby, *A. euboeum* Hal., *A. heldreichii* Hausskn., *A. murale* Waldst. & Kit., *A. serpyllifolium* Desf. ssp. *serpyllifolium* (a non-accumulator), *A. serpyllifolium* ssp. *lusitanicum* Dudley & Silva, *A. tenium* Hal., *A. troodii* Boiss., *A. virgatum* Nyar. (all section Odontarrhena) and *Bornmuellera tymphaea* (Hausskn.) Hausskn.

7.2.1 Nickel Uptake Studies

A potting mixture of 1:1 peat/perlite with added nutrients was used as the basic medium in these experiments. To this mixture was added nickel, as the nitrate, to give final nickel concentrations in the range of 30-10,000 µg/g. The mixture was placed in plastic pots each of which contained 200g. All twelve species were involved in these experiments. For each species, five replicates were made for each concentration. All pots were kept in a glasshouse with a temperature range of 20-25^{o}C and were watered from beneath. At the end of six weeks, all plants were sampled to determine the leaf and soil nickel contents.

In addition to these experiments, nickel uptake was studied in six species grown on a serpentine soil from Dun Mountain, Nelson, New Zealand (containing 900 µg/g) and in five species grown on a New Caledonian serpentine soil (4,790 µg/g). These specimens were likewise sampled for nickel analysis after six weeks.

7.2.2 "Trigger-Point" Theory Test

After the results of the nickel uptake studies became apparent, a test was made to determine at which concentration of nickel in the soil (if any) the hyperaccumulation of this element was triggered. This test was done using the same basic potting mixture as for the nickel uptake studies but the range of nickel concentrations was lower and narrower (20-150 µg/g). All other experimental conditions were the same. Only two species, A. tenium and A. troodii were tested. All plants and soils were sampled to determine their nickel content after six weeks.

7.2.3 Rate of Nickel Uptake

The rate of nickel uptake was studied only in the species A. euboeum. Seedlings of this species were grown for five weeks in a background (ie. no added nickel) potting mixture before being transplanted into pots containing the potting mixture plus nickel to give a final concentration of 7,000 µg Ni/g. Leaf samples were removed daily from each individual and their nickel content was determined. This experiment was then duplicated on a potting mixture containing 6,500 µg Ni/g.

7.2.4 Nickel Tolerance Studies

The tolerance of several species to nickel was studied by two methods: a solution rooting method modified from that developed by Wilkins (1957) and Jowett (1958); and a soil culture method as developed in Chapter 4.

For the solution rooting method, a series of solutions containing between 0 µg/cm^3 and 100 µg/cm^3 of nickel in 0.5 g/dm^3 calcium nitrate were prepared. Four week old Alyssum seedlings were used for the test. The seedlings had had their roots excised with a scalpel and were then suspended in the test solutions by being placed in holes through 5mm thick polystyrene rafts. There were five seedlings per species per concentration.

The solutions (150cm^3) were placed in 250 cm^3 squat beakers and aerated continuously. Light was supplied by radiation from infrared lamps also on a continuous basis. The temperature was maintained at 25^{o}C. All solutions were changed weekly. Between changes, the solutions were kept up to volume by the addition of deionized water. After a period of five weeks, the lengths of the new roots were measured as a basis for determining the tolerance of the species.

The soil culture method involves potting seedlings into vials containing 2g of the basic potting mixture to which was added nickel in the range from 100 - 10,000 µg/g (final concentration). There were five seedlings per species per concentration. The seedlings were watered from below in the same conditions as for the nickel uptake studies. The seedlings were left to grow until a stable surviving distribution was obtained. The tolerance level for each species was taken as the concentration at which at least half of the seedlings survived. In addition to this tolerance level for the species, the tolerance of certain individual seedlings above this level were noted. All soils and seedlings were sampled for the determination of nickel at the conclusion of the experiment.

7.2.5 Cobalt Uptake Studies

Because cobalt is also frequently enriched in serpentine soils, the uptake of this element by *Alyssum* species is of interest. No specimens of *Alyssum* species analysed during the survey of this genus showed anomalous cobalt concentrations, thus indicating that those species which hyperaccumulate nickel do so by preferential uptake of this element rather than a general uptake of heavy metals. However it was decided to test the uptake of cobalt in a situation in which preferential uptake of nickel would be unlikely. The basic potting mixture (with low nickel content) was used for this trial. Cobalt, as the nitrate, was added to this basic mixture to give a range of concentrations from 30 - 3,200 µg/g. The trial was conducted

by planting seedlings in vials containing 2g of the experimental substrates. Five seedlings were planted per species per concentration. The seedlings were left to grow for six weeks before both the plants and the soils were sampled for the determination of their cobalt content. Six species were used in this trial.

7.2.6 Analytical Methods

Leaf metal concentrations were determined by drying the leaf samples in an oven at 80°C until a constant weight was obtained. Subsamples of 0.01 - 0.03g were then weighed out for analysis. These were ashed at 500°C in a muffle furnace and the ash redissolved in $1cm^3$ of 2M hydrochloric acid (prepared from redistilled constant-boiling hydrochloric acid). This solution was analysed by atomic absorption spectrophotometry for nickel or cobalt as required. The lines used for analysis were 232.0nm and 351.5nm for nickel and 240.8nm and 304.4nm for cobalt. The instrument used was the Varian-Techtron AA5 model with automatic background correction as in Chapter 2.

The soils were analysed after being dried in an oven at 110°C. Samples of 0.1g were then digested with $20cm^3$ of aqua regia and taken to dryness over a water-bath. The residue was then redissolved in $10cm^3$ of 2M hydrochloric acid, prepared as above, and centrifuged to remove the insoluble material (primarily undissolved silicates). The supernatant was diluted as required ($10-100cm^3$) and then analysed for nickel or cobalt by atomic absorption spectrophotometry. The instrument and the lines used were the same as for the leaf analyses.

7.3 NICKEL UPTAKE IN ALYSSUM SPECIES

The results of the nickel uptake studies are shown in fig. 7.1. The two non-accumulators, *A. montanum* and *A. serpyllifolium* ssp. *serpyllifolium*, were easily distinguished from the other species. Their relationship between nickel in the plant and nickel in the substrate is linear as is common

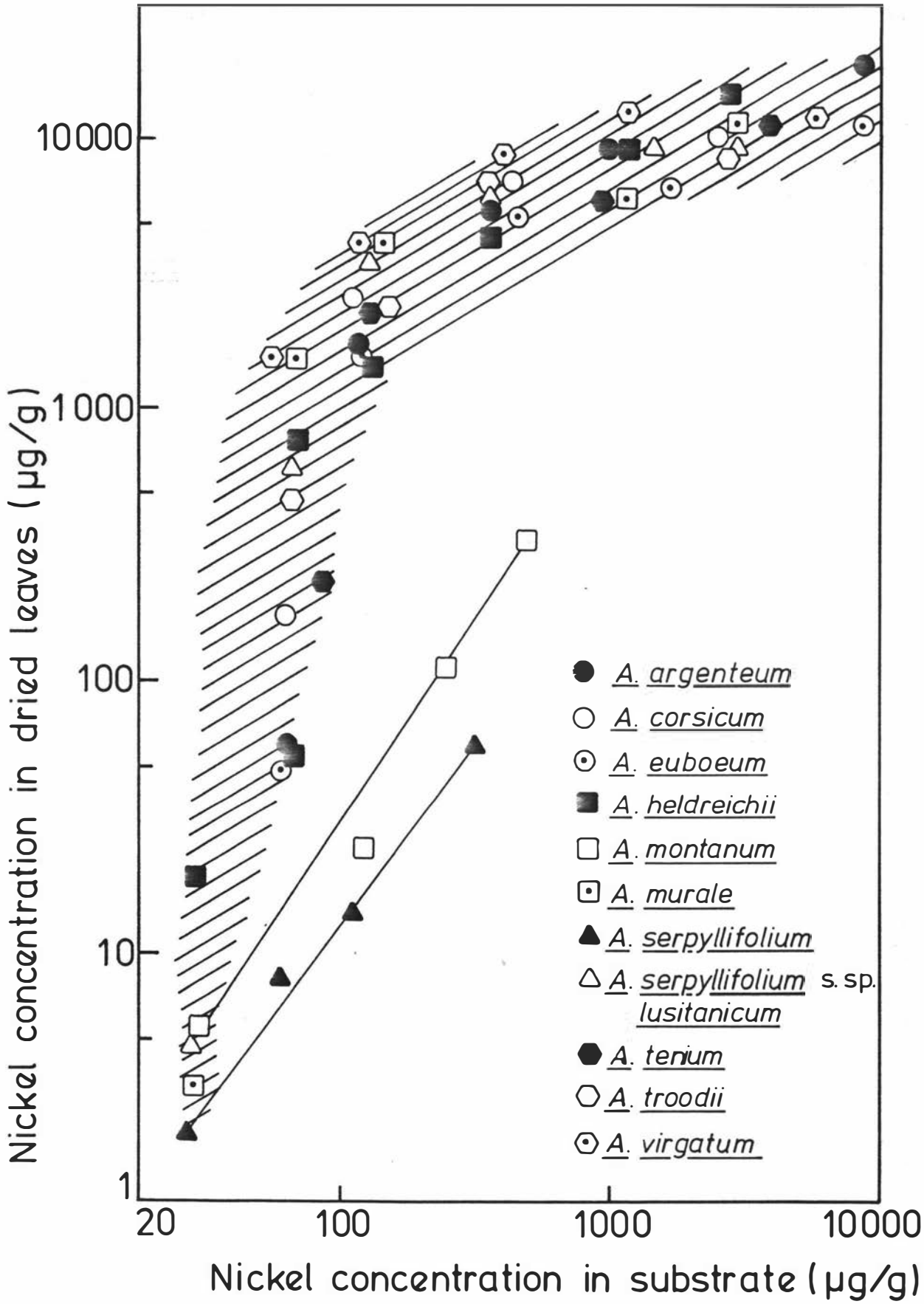


Figure 7.1 Nickel accumulation in *Alyssum* species as a function of substrate content.

for non-tolerant species on comparatively metal-rich soils (Timperley et al., 1970b). The nine hyperaccumulators all behaved similarly to each other. For this reason fig. 7.1 shows an inclusive (hatched) area for all the data rather than individual curves for each species: the considerable overlapping of the curves would be confusing. The shape of the curve is a rise-to-saturation form. This form, characterized by the rapid linear increase at low concentrations to the plateau or near-plateau at higher concentrations, is probably the least common of the three forms of uptake. The linear form of uptake, as shown by the non-accumulators, is believed to arise from an inability of the plants to exclude the element so that the concentration in the plant rises in proportion to the increasing concentration in the soil. This increase will continue until the plant dies from the toxic effects of large concentrations of that element. The second common form is the exclusion-breakdown form where at low concentrations in the soil the concentration in the plant is held constant or nearly-so by an exclusion mechanism. At some higher soil concentration this exclusion mechanism breaks down allowing relatively free entry of the element into the plant. At this point, the concentration in the plant tends to rise very rapidly (see Chapter 4) with death from elemental poisoning occurring with little further increase in the soil concentration. The rise-to-saturation form is more difficult to interpret. The nickel content increases rapidly at low soil concentrations until a plateau is reached where, despite further increases in soil concentration, little or no increase in plant content occurs. It appears that some exclusion mechanism is operative at high rather than low soil concentrations. This could be interpreted as a need for high concentrations of nickel for these species. Thus at low soil nickel levels the plant has a high concentrating ability (the accumulation index is much greater than one, see Section 7.4) in an attempt to satisfy the plant's need. Once this need is fulfilled, the plant then excludes the entry of any nickel above that required by new growth. It is now left to determine why these plants will not survive in higher

nickel concentrations in the soil. Those plants which show the exclusion-breakdown form of uptake die after the exclusion mechanism is overloaded by the high soil concentration, resulting in large quantities of the previously excluded element entering and poisoning the plant. This effect shows up as a rapid increase in the elemental concentration in the plant at soil concentrations above the exclusion mechanism's breakdown level. The Alyssum species, however, do not show any sign of exclusion-breakdown at the highest nickel concentrations. It may be that the specimens which died at higher soil concentrations of nickel did not die from direct nickel poisoning but from other factors which may or may not involve the nickel (eg. plasmolysis of the roots, cationic competition effects). Despite this line of reasoning, the evidence for a specific need of nickel by Alyssum species does not exist.

Plate 7.2 shows the results of these uptake experiments for A. serpyllifolium ssp. serpyllifolium, A. serpyllifolium ssp. lusitanicum, A. heldreichii and two A. murale forms. As well as the specimens growing in the potting mixture, a further specimen of each species is shown growing in a New Caledonian serpentine soil. A. serpyllifolium ssp. serpyllifolium readily shows its low nickel tolerance by the reduction in growth of all specimens growing on nickel-rich substrates. Even more revealing is the very stunted growth of the specimen in the serpentine soil. A. serpyllifolium ssp. lusitanicum showed very even growth in all surviving specimens (no specimen of any species tested survived in pots containing 10,000 µg Ni/g dry soil). The specimen in serpentine soil is only slightly smaller than these other specimens. The small decrease in size is most probably due to factors other than nickel eg. low calcium, nitrogen, phosphorus or potassium or high magnesium content which are common to serpentine soils. A. heldreichii has much reduced growth in the background mixture. This could be taken as a lack of some essential nutrient and since the only difference between

Plate 7.2 Growth of some *Alyssum* species in nickel uptake trials.
Plants growing (from left to right) in background substrate 30 µg/g; 70 µg/g; 120 µg/g; 350 µg/g; 1,050 µg/g; 2,680 µg/g; and New Caledonian serpentine soil 4,800 µg/g.
Taxa (from top to bottom) are:

Alyssum serpyllifolium ssp. *lusitanicum*

Alyssum serpyllifolium ssp. *serpyllifolium*

Alyssum heldreichii

Alyssum murale – form B

Alyssum murale – form A

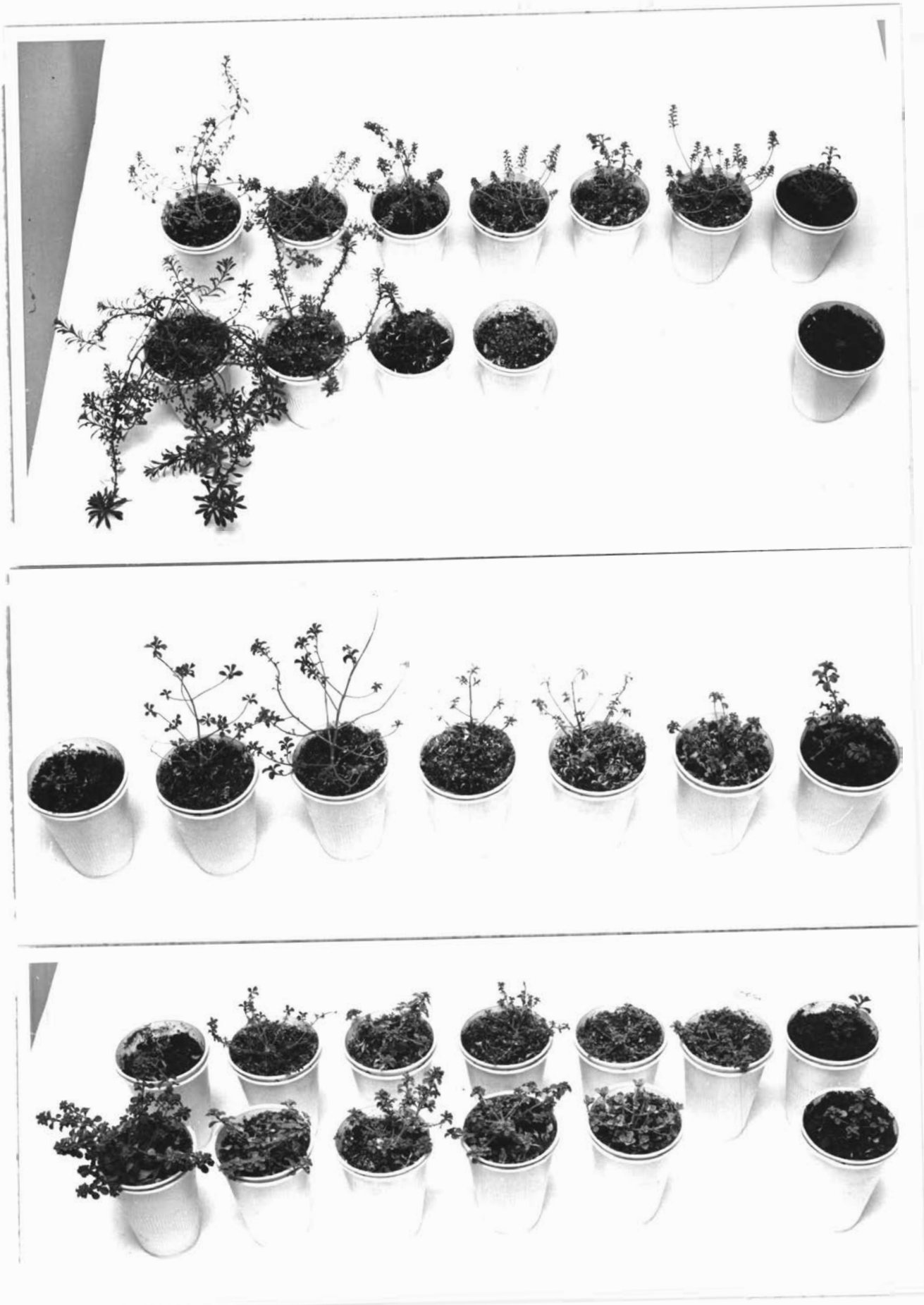


Plate 7. 2

this mixture and the others is the nickel content, it could be said to indicate a need for nickel by this species. At other low nickel concentrations in the substrate, the plant has a straggly growth but this reduces to a more compact form at higher nickel concentrations. The serpentine soil specimen also has the compact growth form. The two A. murale forms show different behaviours with form A having increased growth in the background mixture while form B has reduced growth. Above the background level, the growth of individual specimens is much the same irrespective of the soil concentration of nickel. The serpentine soil specimens here are also only slightly smaller than the other specimens. The other species tested but not shown in these photographs had similar ranges of behaviour. Table 7.1 lists the concentrations of nickel in the leaves of those species grown in serpentine soils (either the New Caledonian, Dun Mountain or both).

All species, except the non-accumulators, hyperaccumulated in these tests. The highest leaf concentration recorded was 19,000 µg/g for A. argenteum growing in a substrate with 9,000 µg/g. All hyperaccumulators reached leaf concentrations of 8,000 - 16,000 µg/g for the substrates tested. The level of nickel in the soil tolerated by the hyperaccumulating species always exceeded 3,000 µg/g but never exceeded 8,000 µg/g. These concentrations compare with total nickel concentrations in serpentine soils of approx. 5,000 µg/g of which only 10% is likely to be available to the plant (Lee et al., 1977a). Thus it appears that these species have an excess capacity in their adaptation to nickel concentrations in the substrate or that the conditions used (ie. the nature of the substrate) have had an ameliorating effect on nickel toxicity. The two non-accumulators, A. montanum and A. serpyllifolium ssp. serpyllifolium survived in substrates with maximum nickel contents of 420 µg/g and 350 µg/g respectively. The corresponding nickel concentrations in the leaves were 300 µg/g and 60 µg/g. The results as far as A. serpyllifolium ssp. serpyllifolium are concerned are particularly significant. This species is from section Odontarrhena so that its non-accumulation of nickel

TABLE 7.1

Nickel uptake by *Alyssum* species growing in serpentine soils.

Species	Nickel Concentration*	
	Dun Mountain Soil	New Caledonian Soil
Soil	904	4 790
A. corsicum	4 038	-
A. heldreichii	9 138	13 390
A. murale A	3 114	7 200
B	6 256	10 140
A. serpyllifolium	-	223
A. serpyllifolium ssp. *lusitanicum*	1 547	12 040
A. tenium	5 782	-
A. troodii	6 034	-

*All concentrations expressed in μg/g dry weight.

even when on nickel-rich substrates indicates that there is no genetic factor common in all taxa of this section which allows for hyperaccumulation.

7.4 "TRIGGER-POINT" THEORY TEST

One question inherent to the rise-to-saturation form of uptake is whether the rise is preceded by a low-level plateau. If such a plateau exists then something must initiate the rise and this initiator ("trigger") would be worth investigating. To test whether such a "trigger-point" exists, two species of Alyssum, A. tenium and A. troodii, were studied at low substrate concentrations of nickel. The results of these studies are shown in fig. 7.3. The data have been plotted as the accumulation indices rather than directly as the nickel concentrations in the plant because such a plot shows the results more clearly. There is an obvious difference in behaviour at very low nickel concentrations when compared to those at just slightly higher concentrations. However even at the lowest concentrations studied, the accumulation index is already rising ie. the "trigger-point" (if it exists) is at a concentration even lower than 20 µg/g under these conditions. There is some circumstantial evidence for a "trigger-point" in that the curve is flattening out around and below 20 µg/g. It appears that at very low nickel levels in the substrate even these hyperaccumulators may exclude nickel from entry into the plant. Immediately the "trigger-point" (or is it an exclusion-breakdown point?) is reached a very large influx of nickel appears to occur. Certainly this type of behaviour is common to species which show an exclusion-breakdown form of uptake. It may then be that this rise-to-saturation form is indicative of a second exclusion mechanism which is triggered by higher internal concentrations of nickel and it is the presence of this second mechanism which gives these species their tolerance to excess nickel in the substrate. Alternatively, it may be that at the very low nickel concentrations, the nickel is complexed with the peat component of the potting mixture and is not

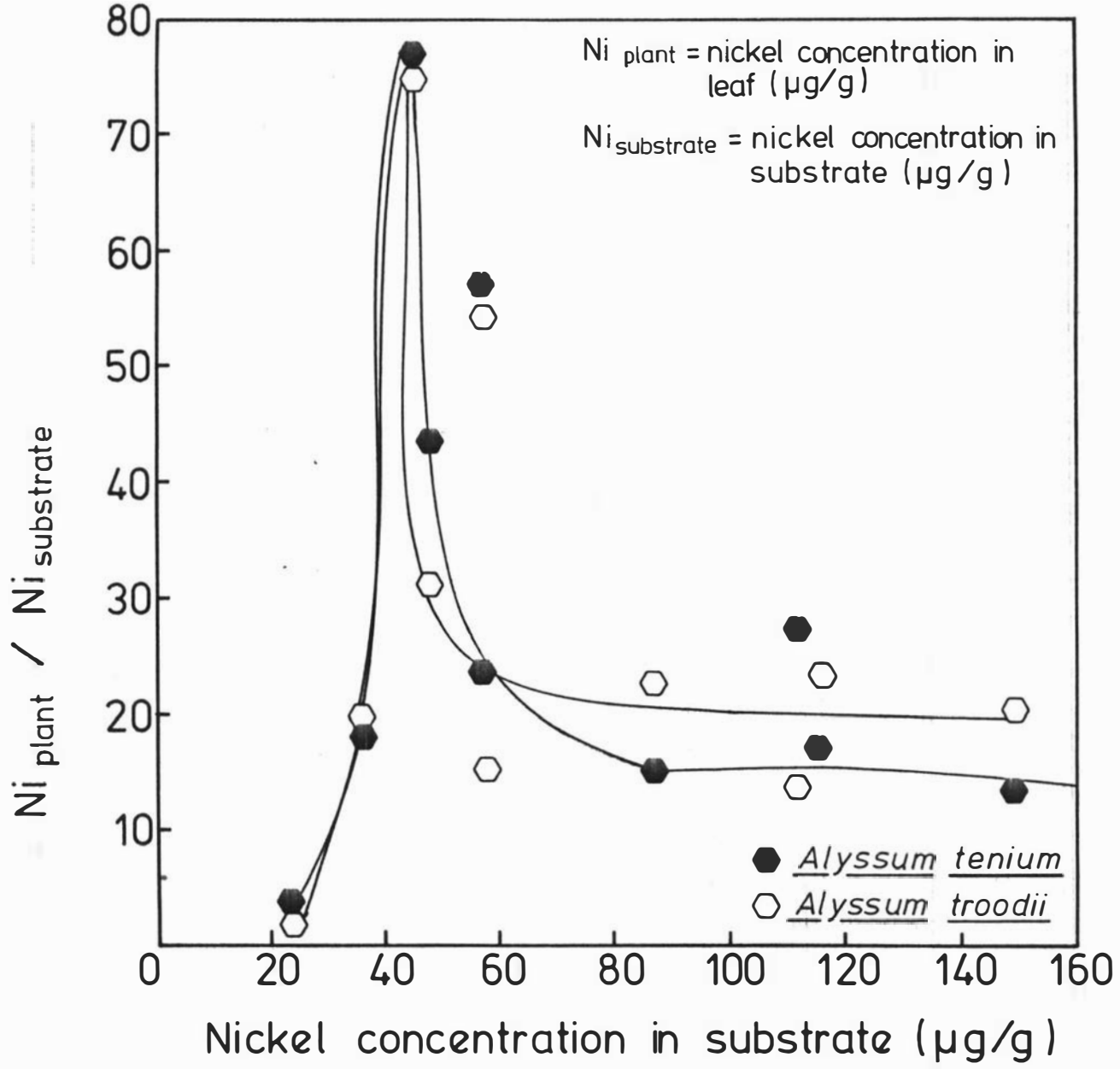


Figure 7.3 Plot of the concentration index at low substrate concentrations of nickel.

available for uptake by the plant. Thus no large influx of nickel into the plant will occur until the complexing capacity of the peat has been exceeded. An exclusion mechanism would then only come into operation once high internal concentrations of nickel had been reached. An equivalent mechanism could exist in the copper-tolerant species, Becium homblei, for which the rise-to-saturation form of uptake has also been found (Reilly, 1969). To date this form of uptake has not been found for non-tolerant species.

7.5 RATE OF UPTAKE OF NICKEL

The rate of uptake of nickel by A. euboeum seedlings is shown in fig. 7.4. After an initial rapid increase, the concentration of nickel in the leaf reached a limiting value. The length of time before the limiting value was reached varied. The variation may have been due to either the nickel concentration in the substrate, the nickel concentration reached in the leaf or some other unknown factor. The rate of uptake in the early linear rise was the same for both trials (ie. the two lines are parallel). For a nickel concentration in the substrate of 6,500 µg/g the limiting value was 850 µg/g while for 7,000 µg/g in the substrate the corresponding value was 4,000 µg/g. The values for leaf concentrations, particularly the 850 µg/g, are lower than would be expected on the basis of the nickel uptake tests. It is possible that the time difference in the tests, eight days compared to six weeks, is the cause. There may be a constant rise for a time after the initial rapid rise rather than a complete levelling off of nickel content. It is clear, however, that the uptake of nickel by these species, and presumably by the other hyperaccumulating Alyssum species, is a relatively rapid process.

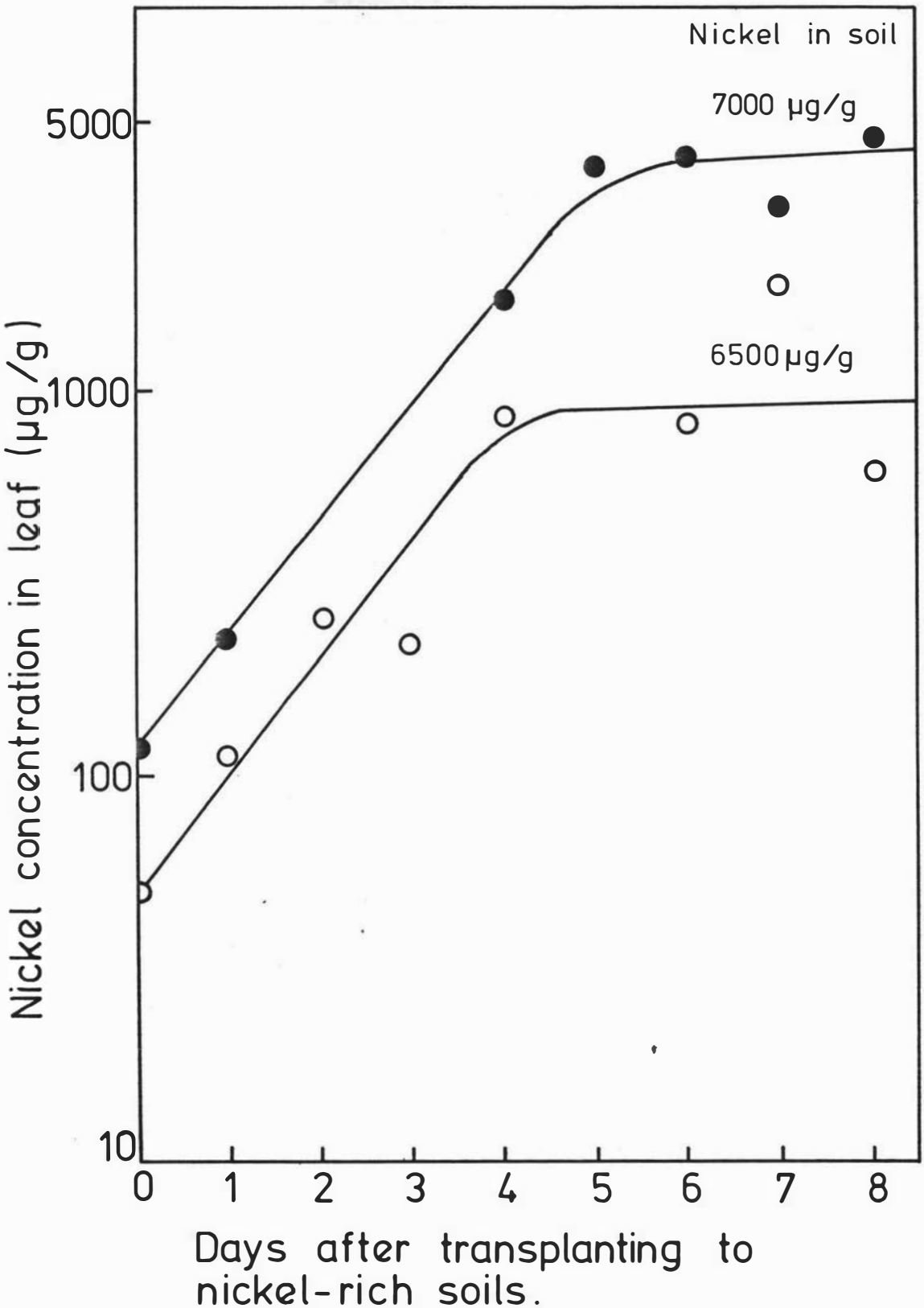


Figure 7.4 The rate of uptake of nickel by *Alyssum euboeum*.

7.6 NICKEL TOLERANCE STUDIES

The results of the solution rooting tolerance tests are shown in fig. 7.5. The four species tested showed strong tolerances to nickel with root growth ceasing (length of new root less than 0.1cm) at between 15 $\mu g/cm^3$ for A. tenium and 60 $\mu g/cm^3$ for A. troodii. The non-accumulator A. serpyllifolium ssp. serpyllifolium does not grow roots in any of the solutions containing nickel. It does however show strong root growth in the background (no nickel) solution.

The results of the soil culture tolerance test are given in table 7.2. All the species tested, except A. serpyllifolium ssp. serpyllifolium, tolerated at least 1,000 µg Ni/g in the soil. Among the hyperaccumulators A. corsicum had a surprisingly low tolerance limit: indeed the limit of 1,650 µg/g was much lower than in the nickel uptake studies (3,000 µg/g). All other hyperaccumulators tolerated between 3,200 µg/g (A. serpyllifolium ssp. lusitanicum) and 5,000 µg/g (A. troodii) as expected. Individual specimens could survive in nickel concentrations of up to 7,200 µg/g (A. troodii). One specimen of A. serpyllifolium ssp. serpyllifolium was able to survive in over 1,000 µg/g (actual value 1,430 µg/g). Thus while this taxon does not normally grow in serpentine soils, it is certainly capable of producing individual specimens which can survive in the range of available nickel concentrations found in them. It is thus presumably capable of giving rise to subspecies (both A. serpyllifolium ssp. lusitanicum and ssp. malacitanum) more tolerant of these soils by methods of gene selection (Antonovics et al., 1971). All specimens, except those of A. serpyllifolium ssp. serpyllifolium, showed hyperaccumulation. It is noticeable that individual specimens of A. troodii and A. serpyllifolium ssp. lusitanicum accumulated less nickel at concentrations above the tolerance level than those at or below this level. It appears that these individuals have a more effective exclusion mechanism at these higher nickel

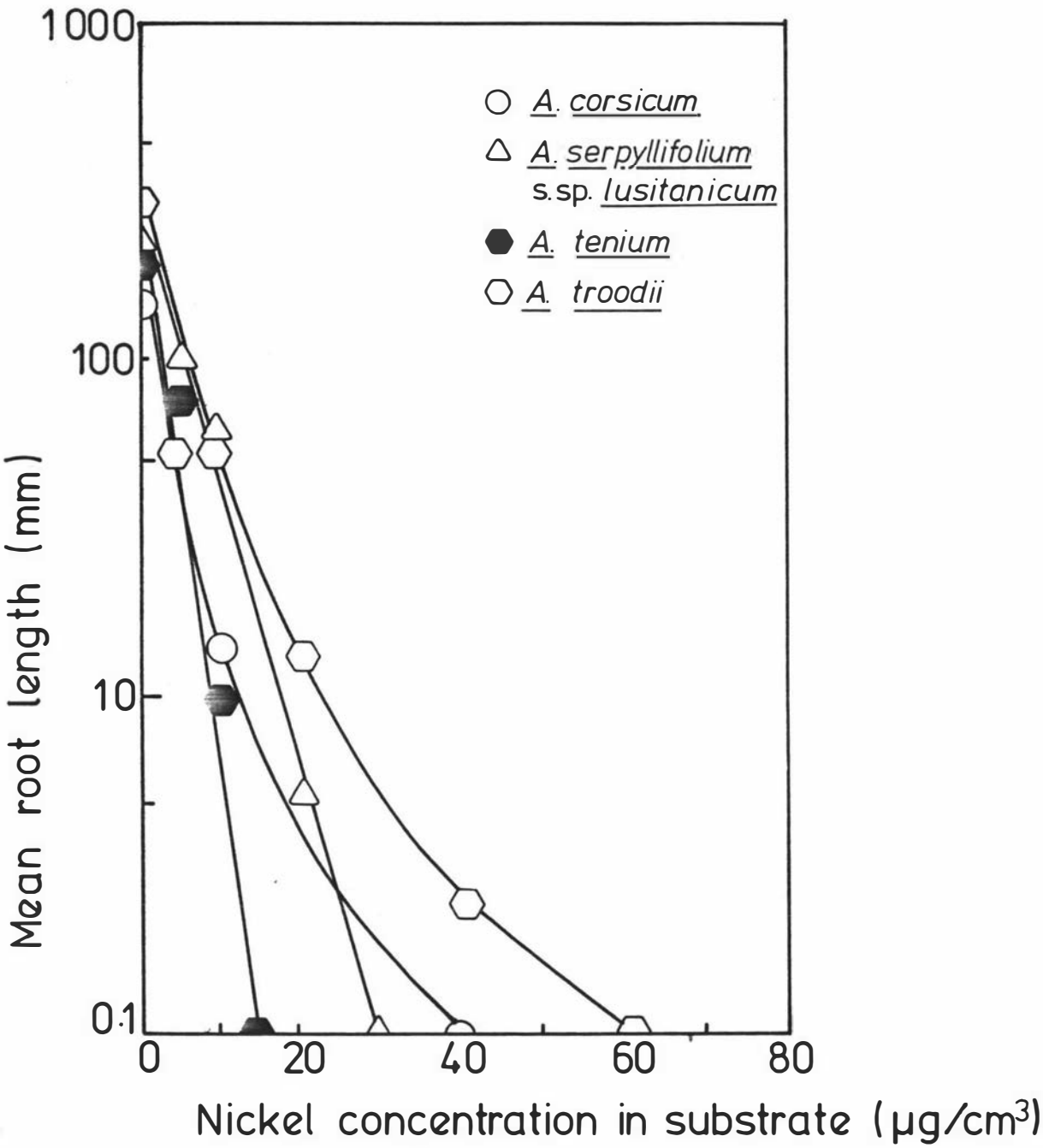


Figure 7·5 Tolerance tests involving new-root lengths of excised seedlings of *Alyssum* species grown in varying nickel solutions (µg/cm³)

TABLE 7.2

Tolerance levels of *Alyssum* species tested by the soil culture method.

Species	Tolerance Level*	Individual Survivors*
A. corsicum	1 650 (5 793)	-
A. heldreichii	4 440 (15 730)	-
A. murale	3 950 (10 790)	4430 (13800) 5060 (14150)
A. serpyllifolium	675 (380)	1430 (805)
A. serpyllifolium ssp. *lusitanicum*	3 230 (7 755)	3790 (6636)
A. tenium	3 290 (5 475)	6120 (7544)
A. troodii	4 970 (19 200)	7168 (18550)

*All concentrations expressed in µg/g dry substrate. The corresponding leaf content is given in parentheses as µg/g dry leaf.

concentrations in the substrate than is general for the species.

When the four species common to both methods of measuring tolerance have their results compared, it is apparent that no common pattern emerges. Thus in the solution rooting method A. troodii was most tolerant and was followed by A. corsicum, A. serpyllifolium ssp. lusitanicum and A. tenium in order. Their order in the soil culture method was A. troodii (most tolerant), A. tenium, A. serpyllifolium ssp. lusitanicum and A. corsicum (least tolerant). This difference indicates that the tolerance of a species to a particular substrate is dependent on more than just the nickel content. Many factors have been considered as causes of serpentine infertility and in most such soils several factors are undoubtedly acting together (Walker, 1954, Proctor & Woodell, 1975). Thus while the solution rooting method may have measured the direct effect of nickel tolerance, the soil culture method may have given an indication of the overall effects of nickel and other nutrients. Undoubtedly, if this is so, the latter method would give a better indication of the ecological tolerance of the species. Wilkins (1978) discusses this problem in relation to heavy metal tolerances measured by means of root growths. More basic research into the varying techniques of measuring tolerance is however still necessary.

7.7 COBALT UPTAKE STUDIES

The results of the cobalt uptake studies are shown in fig. 7.6. The results show a remarkably similar pattern to the nickel uptake studies. The major differences are the lower soil concentrations of cobalt tolerated (no species survived over 1,000 µg Co/g compared with 2,000-8,000 µg Ni/g,), thelower leaf concentrations reached (1,400-5,000 µg Co/g compared to 8,000-20,000 µg Ni/g) and the lower soil concentration at which a rapid increase in uptake occurs (mid-point for cobalt approx. 10 µg/g compared to approx. 50 µg/g).

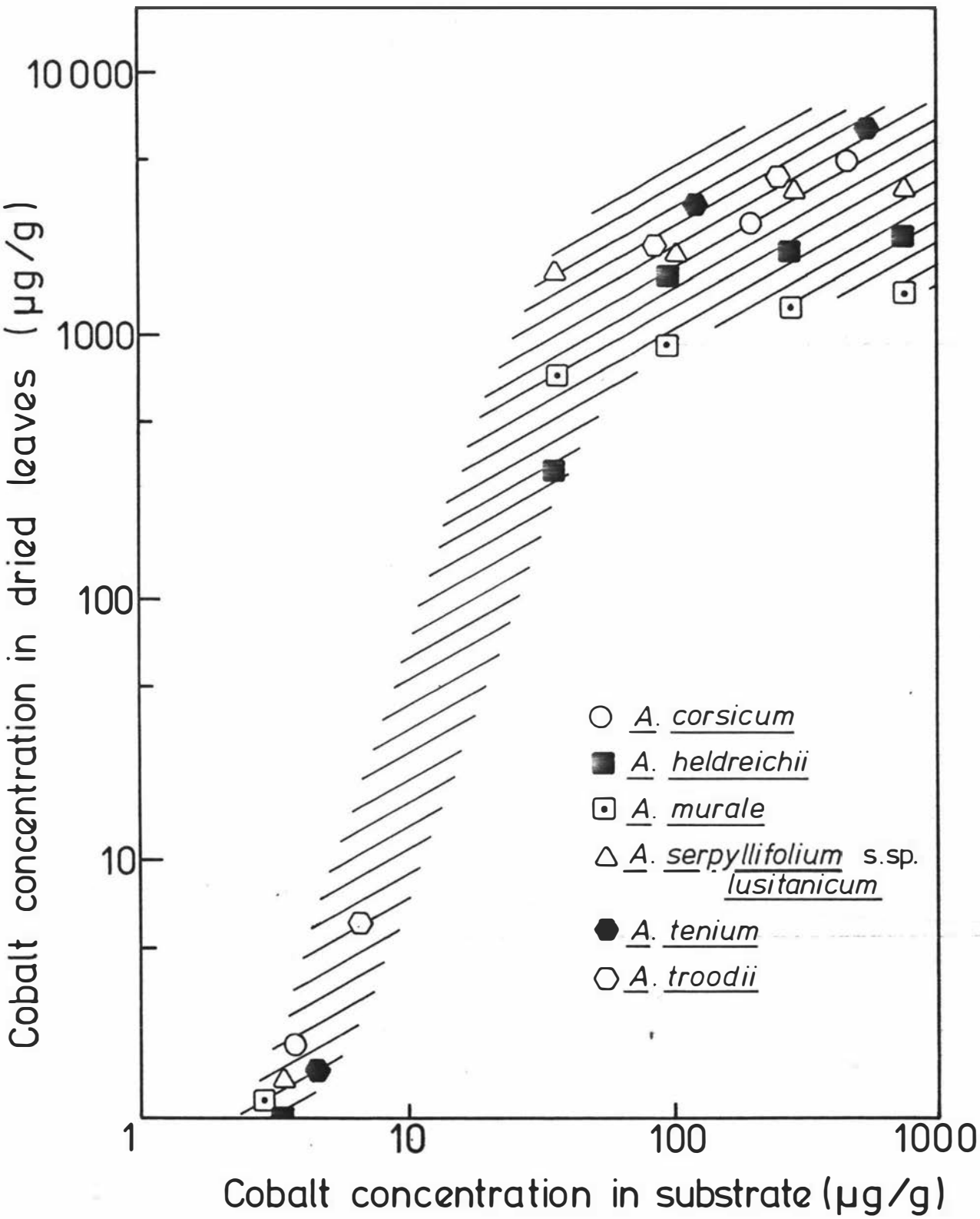
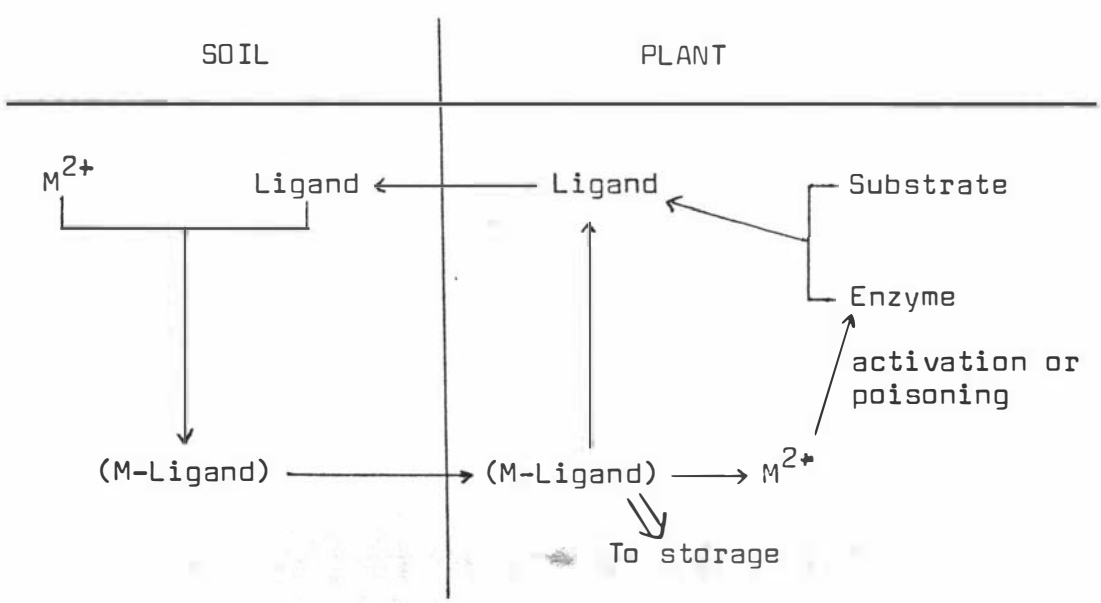


Figure 7·6 Cobalt uptake by *Alyssum* species.

The least tolerant species studied was A. troodii which survived only up to 260 µg/g in the soil. The most tolerant species were A. heldreichii, A. murale and A. serpyllifolium ssp. lusitanicum which tolerated 710 µg/g. The highest leaf concentrations were found in A. tenium (4,833 µg/g) and A. corsicum (4,144 µg/g). It is notable that all the species studied for cobalt uptake hyperaccumulated this element despite the fact that in the wild they show no such inclination to do so.

As it is apparent from this study that a number of Alyssum taxa are capable of hyperaccumulating cobalt even though this has not been observed in the wild populations, it is necessary to explain this difference in behaviour. The cobalt content of serpentine soils is generally only one tenth that of nickel. Thus nickel could be expected, under conditions of either uncontrolled or general entry, to be at ten times the cobalt concentration whereas it is found to be at several thousand times that concentration. Thus the entry of these metals into the plant cannot be uncontrolled ie. they cannot move passively through the root epidermis into the cytoplasm. Neither can the entry mechanism be general since it obviously favours accumulation of nickel over the other elements which include cobalt. The control mechanism for the entry of nickel is however an unknown quantity but it is clearly able to opérate in the absence of high concentrations of nickel provided that high concentrations of an alternative metal are present. At low concentrations of metal elements, the mechanism does not appear to operate. It may be that this mechanism is concerned with the entry of metals in the complexed state. Thus one way of accumulating nickel relative to cobalt would be to produce an organic ligand which complexed preferentially with nickel. In the absence of nickel, this ligand can then complex with other metals to give them entry into the cytoplasm. Some evidence for this can be seen in the levels to which the respective metals can be concentrated.

Thus nickel with a more stable complex is concentrated to a higher degree than cobalt with a less stable complex. This assumes that the heavy metal in the free ionic state is involved as a cause of death of the plant. Less stable complexes with correspondingly greater free metal ion concentrations will therefore be accumulated to a lesser extent than the more stable complexes before death occurs. While this hypothesis can explain the difference in the amounts of the metals taken up, it does not account for the plateaux. Each plateau presumes that at some high concentration free entry of the complex into the root is inhibited as the soil concentration continues to rise. The cause of this inhibition may be that as the free ion content within the plant rises, the plant invokes some mechanism to prevent the level becoming toxic. This mechanism may well involve the poisoning of the enzyme(s) which produce the ligand by the free ions. Thus as the free ion concentration rises, ligand production is reduced and the amount of metal complexed and hence given entry to the plant is also reduced. One further point must be strongly considered: the free metal ion may also be an activator, at low concentrations, for these enzyme(s) since the uptake of the metal to high concentrations appears to have a "trigger-point". Thus with this consideration, it appears that low free ion concentrations may activate the enzyme(s) but that high free ion concentrations poison them. This proposed mechanism is summarized as below:



From this diagrammatic representation of the uptake mechanism, it is easily seen how the stability of the complex (M-Ligand) controls the amount of uptake of the various metals. As cobalt is considered less toxic to plants than nickel (Agarwala et al., 1977, Hunter & Vergnano, 1953), and as Mathys (1975) has shown that cobalt is generally less toxic to enzymes than nickel, it would appear that the cobalt complex is much less stable than the nickel complex. However cobalt may be a more effective activator of the enzyme(s) involved as the rapid accumulation of cobalt occurs at a lower concentration than for nickel. Alternatively if the "trigger-point" is due to the complexing capacity of the peat being exceeded, then the rapid accumulation of cobalt could be due to the less effective complexation of this element by the peat. This less effective complexation (relative to nickel complexation) by the peat would leave more free cobalt ions available for complexation by the ligand produced by the plant. Thus the cobalt would be taken up at lower total soil contents than the nickel. Much further work will be needed before these proposed mechanisms are either accepted or rejected.

7.8 BORNMUELLERA TYMPHAEA STUDIES

Bornmuellera tymphaea (Hausskn.) Hausskn. is a member of another genus of the tribe Alysseae and is thus closely related to the Alyssum genus. Indeed B. tymphaea includes Alyssum tymphaeum (Hausskn.) Held.& Hausskn. ex Formanek amongst its synonyms. This species is found in northern Greece on the Lingos Range and surrounding areas of the Pindus Mountains and further east on Mt. Vourinos. B. tymphaea is serpentine-endemic and both areas in which it is found are noted for their serpentine-flora. Several Alyssum species including A. murale and A. heldreichii are also found in this area.

The results of the nickel uptake study on this species is shown in fig. 7.7 which also gives a comparison of this uptake to those of the *Alyssum* species previously studied. It is easily recognized that the uptake of nickel is identical in form and similar in amount to these other nickel hyperaccumulators. This finding raises the question as to the extent to which this form of uptake is widespread. Is it confined to tribus Alysseae? It will be most interesting to study other nickel hyperaccumulators of the Cruciferae. In particular *Peltaria emarginata* of the tribe Lunarieae is most worthy of study since Hayek (1911) and Janchen (1942) have proposed the amalgamation of the tribes Lunarieae and Alysseae. Other species which would also be worthy of study are *Thlaspi* ssp. of the tribe Lepidieae and *Streptanthus polygaloides* of the Streptantheae. This latter species being New World in distribution, whereas the others are all Old World, may show whether the geographical location has an effect on the form of uptake.

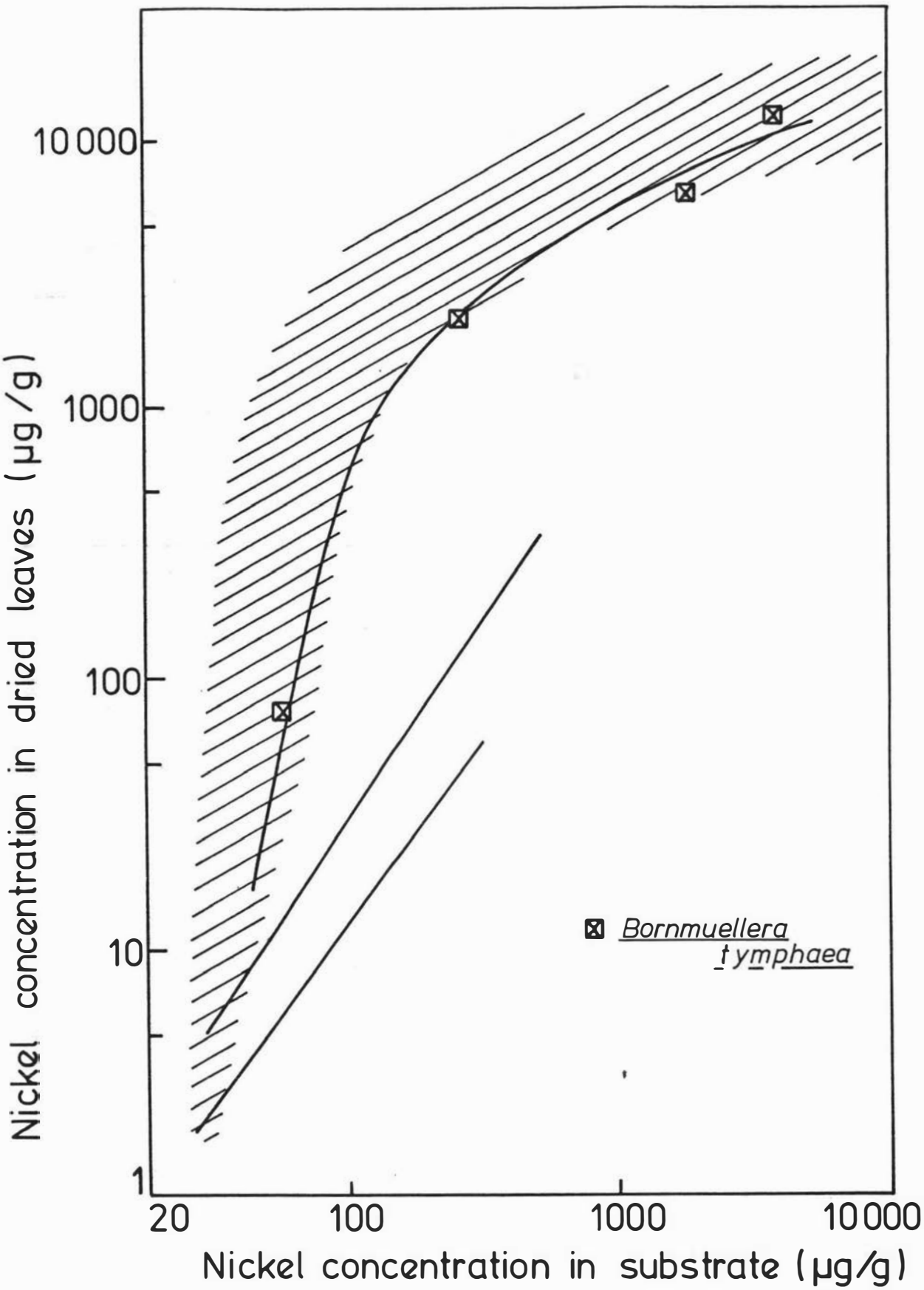


Figure 7·7 Nickel accumulation in *Bornmuellera tymphaea*, as a function of substrate content in comparison with the *Alyssum* species in figure 7·1.

CHAPTER 8

Phytochemical Studies on Some *Alyssum* Species

8.1 INTRODUCTION

The nickel within plants of the genus Alyssum is most concentrated in the leaves. This fact was first noted by Minguzzi and Vergnano (1948) in the original paper on hyperaccumulation of nickel by A. bertolonii. They further noted that seeds, flowers and fruits were also very high but that the concentrations in the roots were low when compared to the rest of the plant (although still very high when compared to other plant species). This has been confirmed by a subsequent study (Vergnano Gambi et al., 1977). This latter study also showed that very young leaves at the beginning of a new growing season had less nickel than the older leaves of the previous season but this difference disappeared within 2-3 weeks of growth. Stems of A. bertolonii appeared to have nickel levels similar to those of the flowers and fruits.

Vergnano Gambi (1967) began specific phytochemical investigations of nickel hyperaccumulators by showing the distribution of nickel within the stem tissues of A. bertolonii. Using dimethylglyoxime as a stain, the nickel was found to be preferentially located in the epidermis and sclerenchymatic tissues between the vascular bundles. Studies on the distribution of nickel within Hybanthus floribundus tissues have shown that here too nickel is associated with epidermal cells (Farago et al., 1975).

Studies of the distribution of nickel within the cell have been done by Shaw (1980). She showed by differential centrifugation that Alyssum serpyllifolium ssp. serpyllifolium (a non-accumulator) and A. serpyllifolium ssp. malacitanum (an accumulator) have different nickel distributions. The accumulator had the majority (72%) of the nickel in the supernatant indicating a ready solubility for the nickel complex. This is easily explained if the nickel found is within the vacuolar system. The non-accumulator however had only 34% of

the nickel in the supernatant. A greater percentage of nickel (36%) was found within the residue with a further 27% found within the cell wall fraction. The complexation of heavy metals by the cell wall has been frequently postulated (Peterson, 1969, Reilly et al., 1970, Turner & Marshall, 1971, 1972) and may well play a significant role in the complexation of metals in anomalous concentrations but it appears that more specific complexations also occur at the higher anomalous concentrations. This may well be necessary since the cell wall has obvious roles in the movement of various physiological compounds and excessive complexation within the cell wall could undoubtedly interfere with these processes.

Organic acids appear to be the most common complexing agent found within hyperaccumulator plants. In A. bertolonii, organic acids were first postulated to be the complexing agents for nickel by Pelosi et al. (1974) but it was not for a further two years (Pelosi et al., 1976) that malic and malonic acids were actually identified. A third organic acid, found in the greatest quantity, was also present but could not be identified. Pancaro et al., (1977) confirmed the involvement of malic and malonic acids in the nickel complexation within A. bertolonii and extended the research to A. serpyllifolium ssp. lusitanicum concluding that malic acid was also involved in nickel complexation here. Lee et al., (1978) subsequently confirmed this involvement. Shaw (1980) investigated the nickel complexes of A. serpyllifolium subspecies and confirmed the involvement of malic acid in A. serpyllifolium ssp. lusitanicum. In A. serpyllifolium ssp. malacitanum, malonic acid was found to be the dominant complexing agent. Small amounts of citric acid were also found to be involved. This is the first report of citric acid involvement in nickel complexation within Alyssum taxa although this acid is a common complexing agent in New Caledonian hyperaccumulators (Lee et al., 1977b, 1978, Kersten, 1979). That organic acids should be the complexing

agents may be partly explained by the observation that nickel can stimulate organic acid production (Dekock & Morrison, 1958). Torii and Laties (1966) have also noted that organic acids are synthesized to balance excess cations absorbed by plants.

Nickel content in hyperaccumulating Alyssum plants has been linked with calcium and magnesium contents (Vergnano Gambi et al., 1977, Shaw, 1980) although such statistical analyses as have been done have yet to show any relationships involving these three elements. Shaw (1980) showed that nickel had no strong correlations with other metal elements in Alyssum species from section Odontarrhena. The lack of relationships between nickel and other metal elements is also known from New Caledonian hyperaccumulators (Lee et al., 1977a, Lee, 1977) and it may be that the factors which control nickel accumulation are either organic rather than mineral or external to the plant such that mineral analyses cannot reveal the relationship.

In this chapter work is presented on the distribution of nickel within specimens of three Alyssum species. Several investigations are also reported for mineral and organic components in an attempt to discover any relationships with nickel contents. Lastly, nickel complexes were isolated from eight Alyssum species and from Bornmuellera tymphaea with the intention of identifying the complexing agents for nickel for each of these species.

8.2 EXPERIMENTAL METHODS

All plant specimens used in these experiments were grown from seed collected by various people, as listed in Appendix I (b). The seeds were germinated on a Copenhagen table and then planted into the appropriate experimental pots. The potting mixture of 50-50 peat-perlite with added nutrients and nickel (as the nitrate) was used in all cases. The pots were kept in a glasshouse at 20-25^{o}C and watered from beneath.

8.2.1 Nickel Distribution Within the Plant Organs

Specimens of three species (A. heldreichii, A. murale and A. serpyllifolium ssp. lusitanicum) were grown in the potting mixture made up to 1,000 µg Ni/g. After five months, these specimens were harvested, washed and pressed. When dry, they were divided into ten parts (see fig. 8.1): the apical bud; the upper, mid and lower stem; the upper stem leaves, which are borne directly on the upper stem; the mid-stem laterals, in which the lateral growths are developing stems; the lower stem laterals which were divided into stems and leaves since their growth is more advanced here; the upper roots, which were the larger, coarser roots; and the lower roots which were smaller and finer although longer than the upper roots. The pods and seeds from the samples sent to us were also analysed.

Samples of each organ were weighed and then ashed at 500^{o}C in a muffle furnace. The ash was dissolved in 1cm^{3} of 2M hydrochloric acid and analysed by atomic absorption spectrophotometry. The spectrophotometer used was the Varian-Techtron model AA5 with automatic background correction BCS attachment as used throughout this work. The nickel line used was at 351.5 nm because of the higher concentrations of nickel found in these specimens.

8.2.2 Mineral and Organic Component Analyses

For these analyses, the specimens grown in the nickel uptake trials (Chapter 7) for three species, the non-accumulator A. serpyllifolium ssp. serpyllifolium and two hyperaccumulators A. serpyllifolium ssp. lusitanicum and A. murale, were harvested and their leaves freeze-dried. Thus for each species, a series of nickel concentrations was available for comparison purposes. These freeze-dried leaves were used for all the component analyses.

(a) Mineral Components. Approx. 0.05g of the freeze-dried material was ashed and dissolved in 2M hydrochloric acid (2 cm^3). This solution was used directly for iron, copper, zinc and manganese. For calcium and magnesium the solution was diluted to one-tenth with 0.8% strontium nitrate in 2M hydrochloric acid. For sodium and potassium, a further dilution to one-fourth was made using 2M hydrochloric acid. All elements except sodium and potassium were analysed by atomic absorption spectrophotometry using the Varian-Techtron AA5 with automatic background correction. The lines used for the analyses were iron 248.3nm, copper 324.7nm, zinc 213.9nm, manganese 279.5nm, calcium 422.7nm and magnesium 285.2nm. Sodium and potassium were analysed by atomic emission spectroscopy using a Gallenkamp model EEL 100 flame photometer. All results are given on a dry weight basis.

(b) Organic Component Analyses. Only two organic components were analysed. These were the organic acids and the glucosinolates. The organic acids were extracted by shaking the dried leaf material with 80% ethanol (0.2g in 5 cm^3) for 2 hours. This was then centrifuged and the supernatant decanted. The residue was washed with successive portions of water and 50% ethanol. The combined supernatants were filtered and reduced in volume by rotary evaporation at 35^{o}C. The solution was cleansed by passage through a 10mm x 50mm cation exchange column, using Amberlite IR-120 (H^+) resin, which dripped directly onto a 10mm x 100mm anion exchange column, using Dowex 1- X8 (formate). This latter resin exchanged with the organic acids which were then eluted with 25cm^3 of 20% formic acid, 25 cm^3 of 50% formic acid and finally washed with deionized water. The elutant was then dried by rotary evaporation at 35^{o}C. The dried product was redissolved in 1 cm^3 of deionized water and used for HPLC.

The HPLC instrument used was a Waters Associates model 660 solvent programmer coupled to a μ-bondpak C-18 column. The buffer used was 2mM tetra-n-butylammonium phosphate at pH 2.8.

The flow rate was 1.5 cm^3/min. The detector was a Cecil CE 212 A spectrometer set at 220 nm.

The glucosinolates were analysed by the method of Schultz and Gmelin (1954). The process began with the soxhlet extraction of 1g of dried leaf material with 50 cm^3 of methanol. The extraction was continued until the solvent was colourless. The methanol was distilled off and the residue redissolved in deionized water. This solution was filtered and made up to 100 cm^3 with further deionized water. For the specimens which did not have 1g of dried leaf material, the process above was carried out using 0.1g and having all other amounts at one-tenth of those above. A 10cm^3 aliquot was then taken for acid-alumina chromatography (the column contained 5g of acid- Al_2O_3). The aliquot was run through the column which was then washed with 20 cm^3 of deionized water to remove the non-glucosinolates. The glucosinolates were then eluted with 0.1 M potassium hydroxide until the yellow colour was no longer evident. The elutant was made up or taken down to 10 cm^3. An aliquot of 5 cm^3 was then taken and placed in a test-tube surrounded by melting ice and 10 cm^3 of anthrone reagent (0.2g of anthrone in 100 cm^3 of concentrated sulphuric acid) was added carefully to give two layers. These were mixed by gently blowing air through the solution. Once mixed the tubes were placed in a vigorously boiling water-bath for 10 min $\pm$ 15 s. They were then cooled in a cold water bath and the blue colour determined photometrically at 620 nm on a Spectronic 20 spectrometer. This method measures the glucose of the glucosinolates so that the standards used can be made using glucose solutions. To get an estimate of the amount of glucosinolates, this result is multiplied by 2.4 as an average conversion factor, on a weight basis (Schultz & Gmelin, 1954).

8.2.3 Nickel Complexation

The nickel complexes were extracted from the dried leaf material by the method of Lee *et al.* (1977b) adapted to lower amounts of material. Thus 0.5g of the material was shaken for two hours with 5 cm^3 of deionized water to extract the complexes.

This process was then repeated one more time. The combined supernatants were filtered and cleansed of lipids, proteins and various other compounds by extraction with a 10:1 chloroform:n-butanol solution. The cleansing was considered complete when no further precipitation occurred at the interface. The aqueous solution was then filtered and reduced in volume. The solution was run through a 50cm x 1.5 cm Sephadex G-10 gel filtration column. The elutant was collected in 4 cm^3 fractions in which the nickel was located by use of atomic absorption spectrophotometry. The nickel-containing fractions were recombined and recycled excepting that fractions of the minor second peak (presumed to be aquonickel(II) but too small to identify) were omitted. The combined fractions from the second run were dried. This product was then derivatized for identification by gas chromatography and mass spectrometry. It was decided to use the methyl derivatives since they are easier to handle and store than the trimethylsilyl derivatives. The complex was initially destroyed by the addition of a few drops of 2M hydrochloric acid. This acid was then removed under a stream of air and the residue dissolved in redistilled diethylether. Diazomethane in ethereal solution, made by the method of Werner (1919), was added dropwise until the yellow colour persisted. The ether was removed by flushing the vial with nitrogen. The residue was dissolved in redistilled chloroform and used for the analyses.

The GLC was performed on a Pye model 104 gas chromatograph using a 2.8m x 4mm column containing 3% SP 2340 liquid phase on a supelcoport 100-220 mesh PB 34 support. This column was operated at 180^{o}C with nitrogen (30 cm^3/min) as the carrier gas. The detector was an air-hydrogen (350 cm^3/min, 30 cm^3/min) flame ionization detector.

Low resolution mass spectra were recorded on either an AEI MS 30 dual-beam spectrometer or a VG Micromass 12F single-beam spectrometer. Both spectrometers were coupled to GLC instruments: the MS 30 to a Pye chromatograph equipped with an OV 17/210 mixed liquid phase column; the Micromass 12F to a

Varian-Techtron 1700 chromatograph with an SP 2340 liquid phase column. Both chromatographs were temperature programmed up to 250°C at 4-5°C/min. A high resolution measurement was made on an AEI MS 9 spectrometer. A chemical ionization mass spectral scan was made on the Micromass 12 F spectrometer using isobutane as the reactant gas.

8.3 NICKEL DISTRIBUTION WITHIN THE PLANT ORGANS

A diagram of the various organs analysed in this section is given as fig. 8.1. The results of the analyses are given in tables 8.1 - 8.3. It is seen that the greatest accumulation of nickel occurs in the leaf material and the least accumulation is in the roots. It is further noticeable that the stem areas LS and MS have lower accumulations than the areas US and LLS. This is of great interest since the latter two areas are green stems while the former are brown and woody. It thus appears that nickel may be preferentially accumulated in photosynthetic tissues rather than non-photosynthetic tissues. This observation is compatible with the results of Minguzzi and Vergnano (1948) and Vergnano Gambi *et al*. (1977) which showed preferential accumulation of nickel by the green tissues of *A. bertolonii*.

8.4 MINERAL AND ORGANIC COMPONENT ANALYSES

8.4.1 Mineral Analyses

The results of the mineral analyses are shown in table 8.4. It can easily be seen that no other elements show any changes of concentration similar to that of nickel. Several points are however worth noting.

The calcium concentrations of the hyperaccumulators, *A. serpyllifolium* ssp. *lusitanicum* and *A. murale*, are higher than the concentrations in the non-accumulator, *A. serpyllifolium* ssp. *serpyllifolium*. The relative magnesium concentrations are the reverse in their distribution. Thus it would appear that the hyperaccumulators have developed more efficient mechanisms

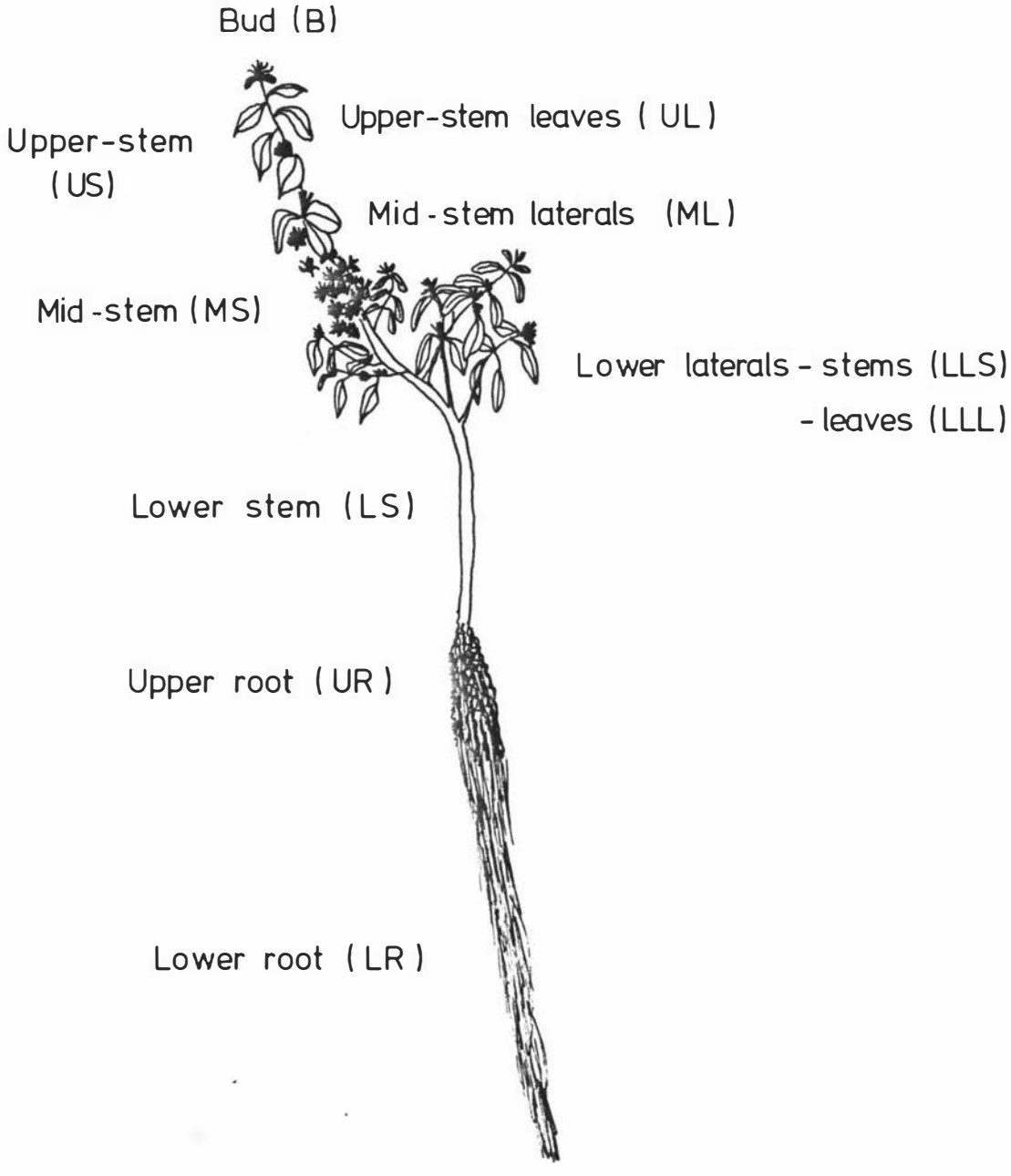


Figure 8·1 Plant organs analysed for nickel.

TABLE 8.1

Nickel distribution within *Alyssum heldreichii*.

Organ	Conc	% Wt	%Amt	DAI		% Wt	%Amt	% DAI
LR	4330	11.9	4.5	0.38	R	21.3	12.2	0.57
UR	9150	9.4	7.6	0.81				
LS	7190	10.0	6.4	0.64	S	28.9	31.2	1.08
MS	9660	5.4	4.6	0.85				
US	16740	3.4	5.0	1.47				
LLS	17060	10.0	15.1	1.51				
LLL	12150	27.1	29.1	1.07	L	49.8	56.7	1.14
ML	11890	12.9	13.5	1.07				
UL	14070	7.6	9.5	1.25				
B	23400	2.2	4.6	2.09				
Seeds	1880	-	-	-				

Abbreviations used in tables 8.1, 8.2 and 8.3.

Plant Organs.

LR = lower roots
UR = upper roots
R = total roots
LS = lower stem
MS = midstem
US = upper stem
LLS = lower lateral stems
S = total stems
LLL = lower lateral leaves
ML = leaves on midstem
UL = leaves on upper stem
B = apical bud
L = total leaves
(see also fig. 8.1)

Conc = concentration of nickel in µg/g, dry weight.

% Wt = percentage of total weight of particular organ.

%Amt = percentage of total amount of nickel present in a particular organ.

DAI = distributive accumulation index

$$= \frac{\%\ \text{Amt}}{\%\ \text{Wt}}$$

TABLE 8.2

Nickel distribution within *Alyssum serpyllifolium* ssp. *lusitanicum*.

Organ	Conc.	% Wt	%Amt	DAI		% Wt	%Amt	DAI
LR	1650	22.2	5.7	0.26	R	34.9	14.5	0.42
UR	4430	12.7	8.8	0.69				
LS	4850	5.8	4.4	0.76	S	22.0	23.7	1.08
MS	6050	6.1	5.7	0.93				
US	10830	2.1	3.6	1.71				
LLS	8110	7.9	10.0	1.27				
LLL	9180	22.5	32.1	1.43	L	43.1	61.8	1.43
ML	8910	16.7	23.1	1.38				
UL	11460	2.9	5.2	1.79				
B	8810	1.1	1.4	1.27				
PODS	1810	-	-	-				
SEEDS	2730	-	-	-				

TABLE 8.3

Nickel distribution within *Alyssum murale*

Organ	Conc	% Wt	%Amt	DAI		% Wt	%Amt	DAI
LR	3760	11.5	5.1	0.44	R	18.1	9.3	0.51
UR	5380	6.6	4.2	0.64				
LS	6960	9.3	7.6	0.82	S	25.7	26.4	1.03
MS	7730	9.4	8.6	0.91				
US	13100	3.0	4.7	1.57				
LLS	11390	4.1	5.5	1.34				
LLL	9420	22.5	25.1	1.12	L	56.2	64.3	1.14
ML	9900	5.7	6.7	1.18				
UL	10100	21.7	25.9	1.19				
B	8920	6.4	6.7	1.05				
Pods & Seeds	3330	-	-	-				

TABLE 8.4

Concentrations of mineral elements in three *Alyssum* taxa.

	Ni[x]	Ca[x]	Mg[x]	Na[x]	K[x]	Mn[xx]	Zn[xx]	Cu[xx]	Fe[xx]
A. serpyllifolium ssp. *serpyllifolium*									
(1)	0.0003	2.58	0.78	1.03	0.46	72	40	13	57
(2)	0.0007	2.21	0.59	0.95	0.20	65	175	10	49
(3)	0.0022	3.17	0.73	1.04	0.30	111	40	10	48
(4)	0.0065	3.66	0.76	1.44	0.44	112	79	13	48
A. serpyllifolium ssp. *lusitanicum*									
(1)	0.0004	3.52	0.41	1.39	0.65	210	322	13	73
(2)	0.0719	3.97	0.40	1.10	0.29	169	89	11	55
(3)	0.232	4.34	0.36	1.05	0.36	221	86	7	46
(4)	0.543	3.86	0.49	1.27	0.32	170	29	6	49
(5)	0.694	3.20	0.38	1.24	0.61	227	72	4	34
(6)	1.142	4.02	0.53	1.11	0.54	160	35	8	37
A. murale									
(1)	0.0003	4.09	0.23	1.60	0.51	224	75	16	63
(2)	0.0903	4.05	0.30	1.26	0.35	168	78	11	50
(3)	0.281	3.54	0.38	1.25	0.39	173	93	14	51
(4)	0.604	2.98	0.30	1.13	0.38	265	53	7	51
(5)	0.620	4.77	0.24	1.22	0.45	363	33	7	39

[x]Concentrations in % dry weight.

[xx]Concentrations in µg/g dry weight.

for calcium absorption and magnesium exclusion than the non-accumulator. This would be beneficial to plants growing on serpentine soils (which these hyperaccumulators do) as these soils tend to be poor in calcium and rich in magnesium. The improved mechanisms for calcium absorption and magnesium exclusion allow the plants to maintain a more "normal" ratio for these two elements than would otherwise be possible. This behaviour has been recognized previously by Kruckeberg (1954).

It may be that sodium and potassium are also more absorbed by hyperaccumulators than non-accumulators but the difference is small and it is difficult to be certain that it is of significance. There is no difficulty in recognizing a greater absorption of manganese by the hyperaccumulators. As manganese is frequently a component of enzymes involved in organic acid cycles, its increase should allow a greater turnover of the cycles which in turn would allow a greater production of organic acids to counterbalance the increase in cation content (Dekock & Morrison, 1958, Torii & Laties, 1966) or to complex with the excess metal cations. Furthermore the increase in manganese concentration may help to offset any competition by nickel in respect of enzyme activation and thus prevent more serious interference to biochemical cycles.

None of iron, copper or zinc tend to show differences between their concentrations in the hyperaccumulators or the non-accumulator. Both iron and copper however appear to decrease in concentration as the amount of nickel increases although the rate of decrease is gradual rather than sharp. The zinc distribution is less clear; the concentration appears to vary markedly without any pattern being obvious.

8.4.2 Organic Component Analyses

The results of the organic acid analyses are shown in table 8.5. Within the list of identified acids (identification being by comparison with standards), there are several interesting results. It should be noted here that the results

TABLE 8.5 Organic acids and glucosinolates in three *Alyssum* taxa.

	U(1)	U(2)	Quinic	U(3)	Malic	U(4)	Iso-citric	U(5)	Citric	U(6)	Malonic (1)	Malonic (2)	U(7)	U(8)	U(9)	U(10)	U(11)	G[x]
A. serpyllifolium ssp. *serpyllifolium*																		
(1)		99	80		-	128	213		110		107		-	230	-	103	43	7.09
(2)		206	152		82	-	183		62		95		-	212	-	129	-	9.91
(3)		137	89		61	-	87		75		93		271	-	-	523	-	9.00
(4)		49	66		49	106	446		132		95		230	-	216	-	-	9.69
A. serpyllifolium ssp. *lusitanicum*																		
(1)		-	189		127		58		59	-	88	-			108	897		1.70
(2)		71	93		72		92		48	-	36	72			33	420		2.74
(3)		71	56		59		79		40	-	22	46			76	96		2.14
(4)		80	82		65		61		48	-	43	65			56	53		2.29
(5)		63	113		86		63		40	63	44	69			39	53		2.02
(6)		100	76		73		70		-	55	-	86			79	40		2.17
A. murale																		
(1)	63	81	65	66	96		73	46	-		37	49			25	200		2.28
(2)	75	96	74	67	84		73	55	-		45	44			32	216		4.26
(3)	49	60	69	60	54		55	30	-		26	39			12	243		2.56
(4)	64	85	77	79	72		90	54	47		74	63			42	346		3.20
(5)	47	65	56	58	69		62	38	67		51	46			22	154		1.74

Results expressed as arbitrary units per gram, dry weight.
Alyssum samples numbered as for table 8.4

[x]G = % Glucosinolates, dry weight basis.

are given in arbitrary units per gram of dried material and have not been translated into μg/g. The non-accumulator, A. serpyllifolium ssp. serpyllifolium, has noticeably higher citric and isocitric acid levels than the two hyperaccumulators studied, A. serpyllifolium ssp. lusitanicum and A. murale. The malonic acid content of A. serpyllifolium ssp. serpyllifolium is high but as malonic acid shows two peaks in the other two taxa (the standard solution also varied from one to two peaks) direct comparisons are difficult to make. Neither quinic nor malic acids appeared to show any significant differences between the different taxa.

A large number of unidentified organic acids were also present. Many of these acids (U(1), U(3), U(4), U(5), U(6), U(7), U(8) and U(11)) were confined to one taxon. The remaining three acids, U(2), U(9) and U(10), were found in all three taxa. Acid U(2) was generally found in higher concentrations in the non-accumulator while U(9) was recorded only once in this taxon but was present in all samples of both hyperaccumulators. Acid U(10) is most common in A. murale but is also present in the other taxa. It does not appear to differ significantly on a non-accumulator-hyperaccumulator basis.

The results of the glucosinolate analyses are shown in table 8.5. In all three taxa there is an increase in the glucosinolate content in the presence of high levels of nickel in the soil. Thus A. serpyllifolium ssp. serpyllifolium shows a major increase in glucosinolate content even when there is little difference in the concentration of nickel within the leaf tissues. The soil concentration of nickel which effects this change appears to be between 30 and 100 μg/g of dried soil. Even more noticeable than this increase is the difference in concentrations between the non-accumulator, A. serpyllifolium ssp. serpyllifolium, and the two hyperaccumulators, A. serpyllifolium ssp. lusitanicum and A. murale. This difference may result from the use the hyperaccumulators could make of the nickel absorbed. Glucosinolates are common secondary metabolites in the plant order Capparales (of which the Cruciferae are a member family) and are believed to act as fungicides,

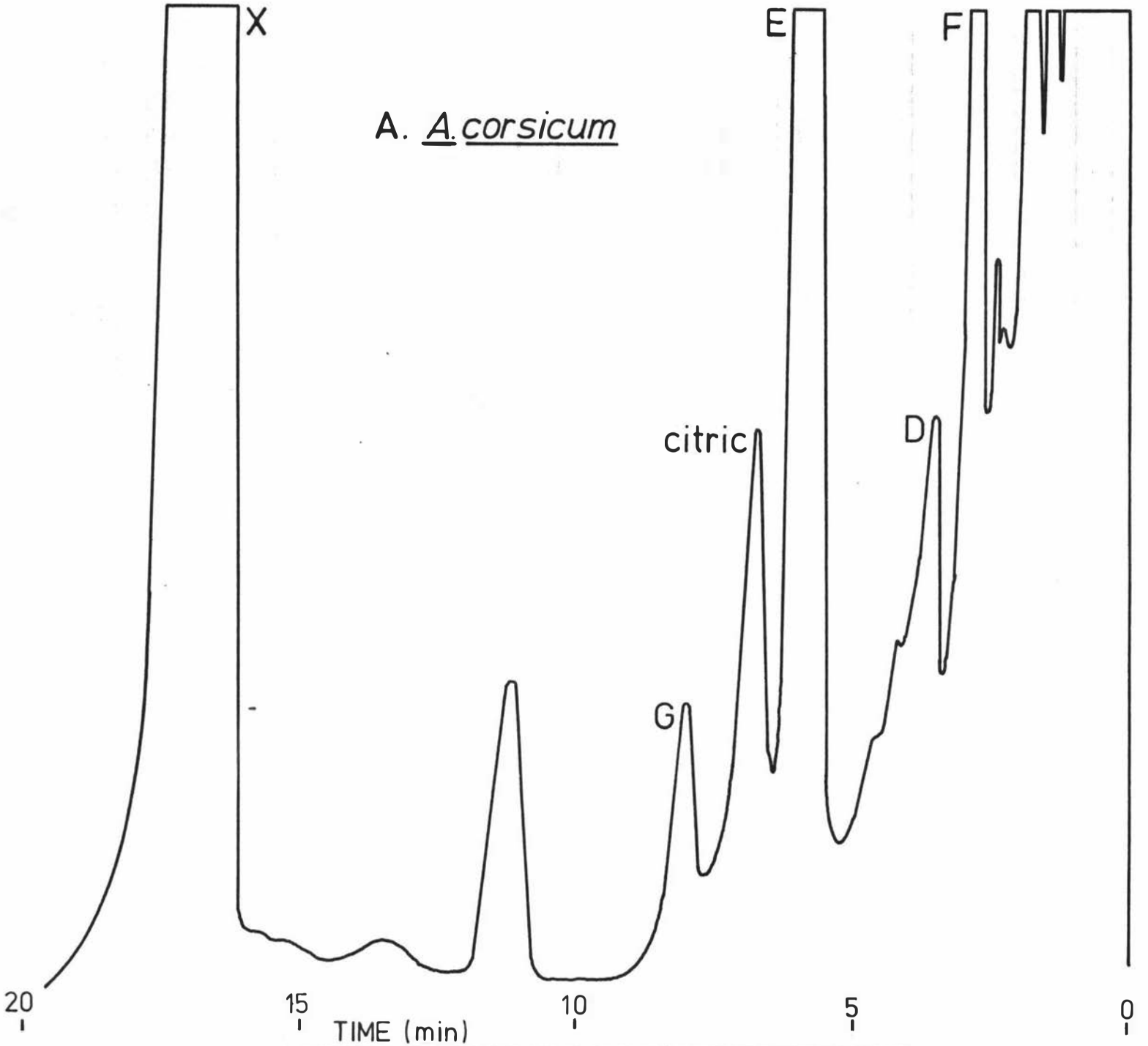
bactericides (Mitschner, 1975) and insecticides (Erickson & Feeny, 1974) for the plants' protection. Heavy metals can also serve these purposes so that in the presence of high nickel concentrations less glucosinolate would be required. This explanation is however in conflict with the previous observation that glucosinolate production is stimulated by the presence of nickel in the soil. No explanation of this conflict is currently apparent although one possibility is that the variations in concentration within a species are normal rather than nickel induced. This looks unlikely in that only A. murale shows any wide variation in concentrations. Further studies will be required before a definite conclusion can be made.

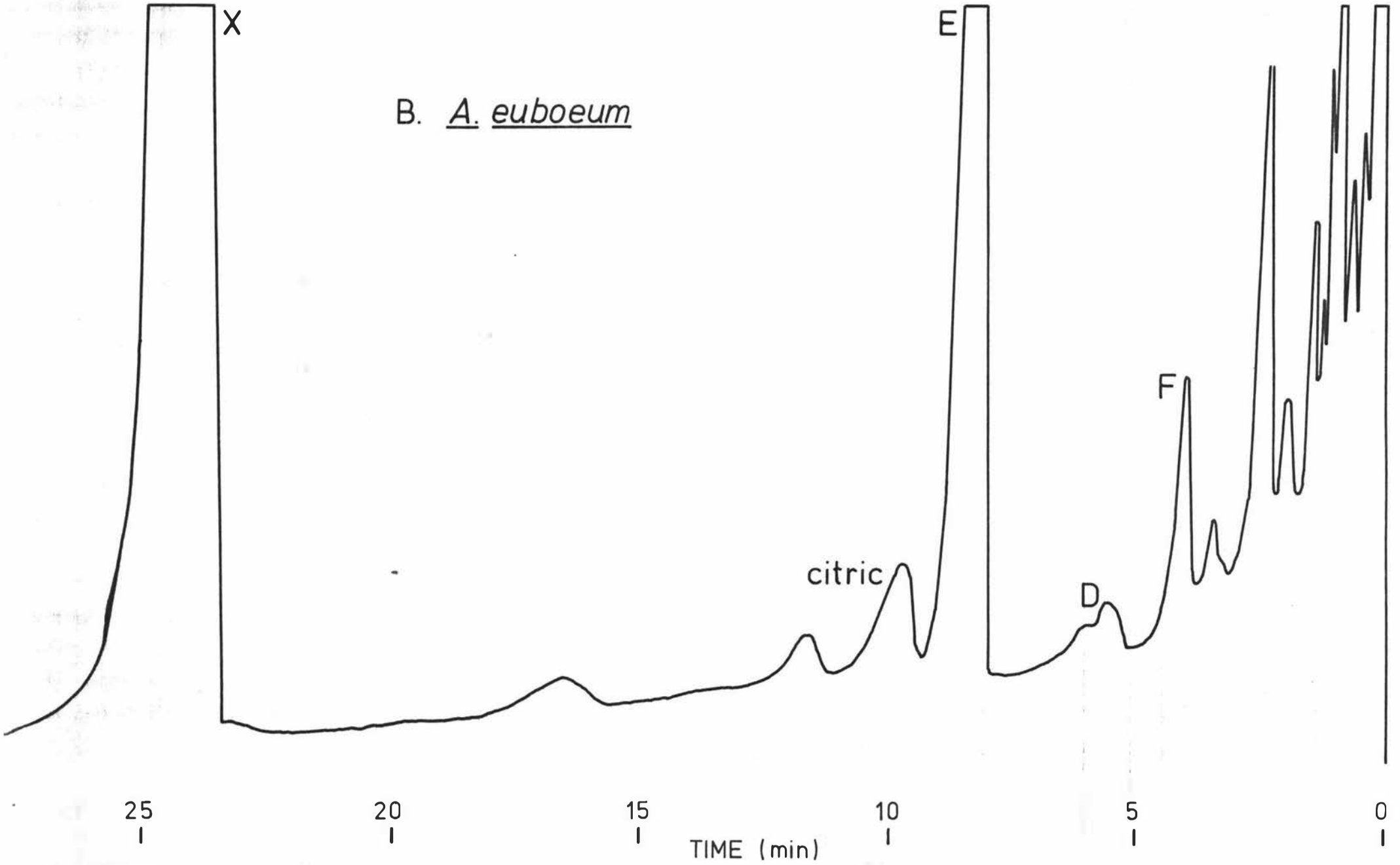
8.5 NICKEL COMPLEXATION

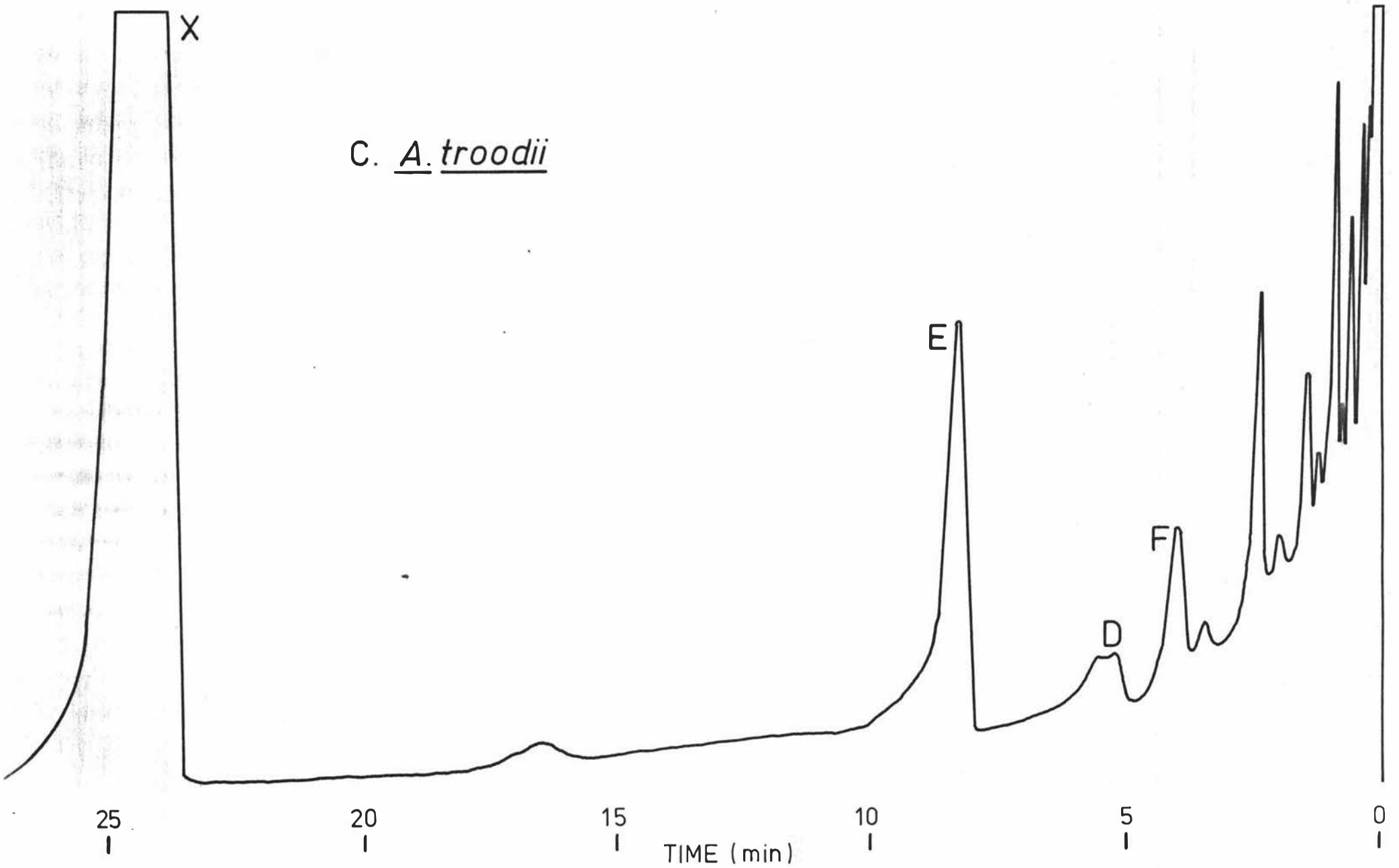
The GLC traces of the components of the nickel complexes obtained are shown in fig. 8.2. Two peaks are common to all the runs : these peaks are E and X. In general, peak X is greater than peak E. A varying number of lesser peaks are obtained for all species. The peaks D and citric acid are the most common of these lesser peaks. These lesser peaks have a tendency to group themselves in series among the species. The resultant groups of species are then found to correspond in their component species to the subsections and series of section Odontarrhena. Thus A. virgatum (subsection Samarifera) differs from the others by having the series of peaks H, J, K, L and Y. A. argenteum, A. tenium and A. murale (subsection Compressa, series Integra) have peaks M, B, D and citric acid (although B and D are weak in A. tenium) when compared to A. heldreichii (subsection Compressa, series Crenulata) which has peaks A, B, C, D and citric acid. A. corsicum, A. euboeum and A. troodii (subsection Inflata) have the peaks D, F and citric acid in common. Other earlier eluting acids were also common in these latter three species but as no clear mass spectra were obtained for them, they have remained untitled.

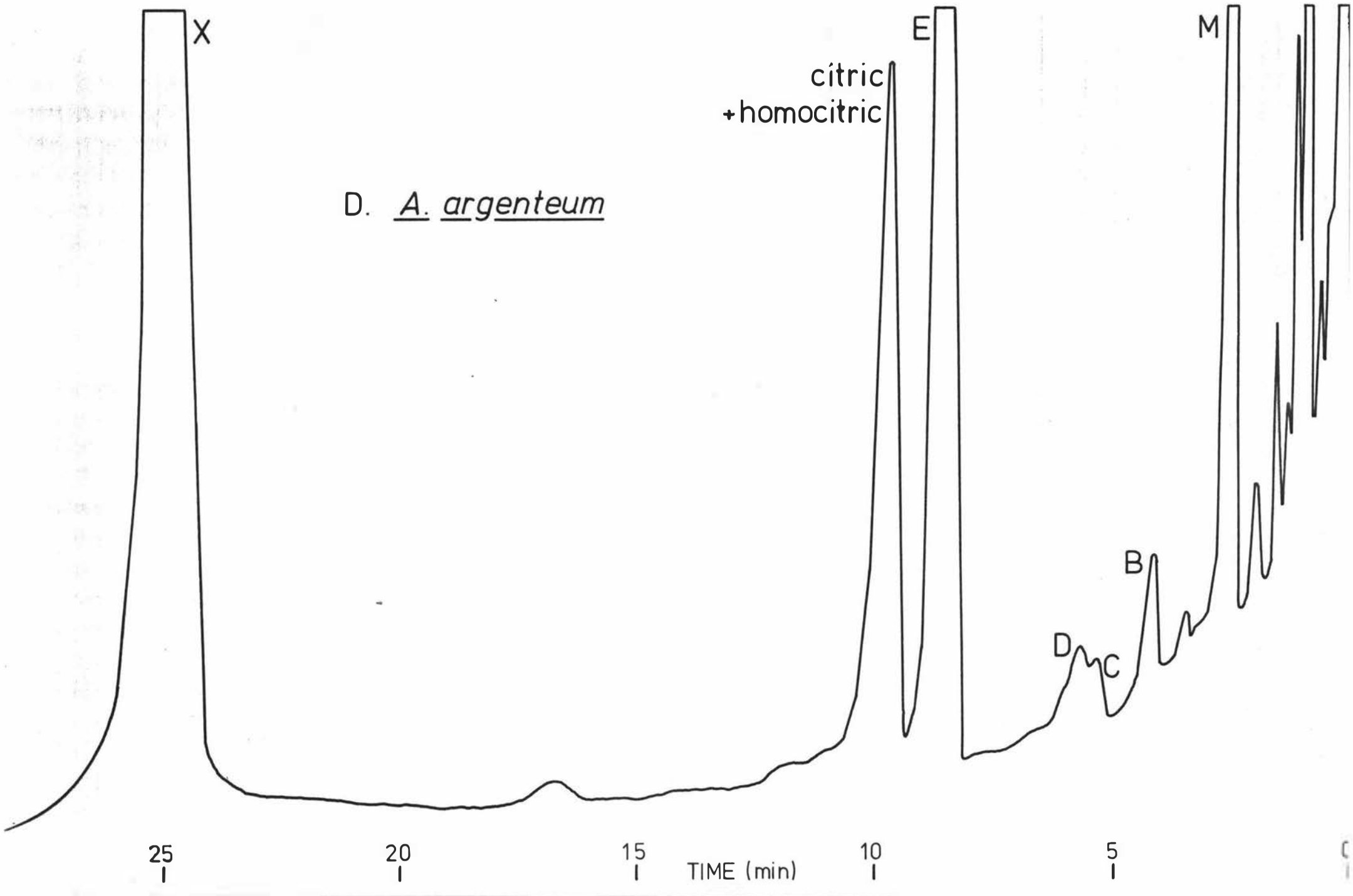
Figure 8·2 Gas-liquid chromatographs of the methylated derivatives of the nickel-complexing ligands from *Alyssum* species.

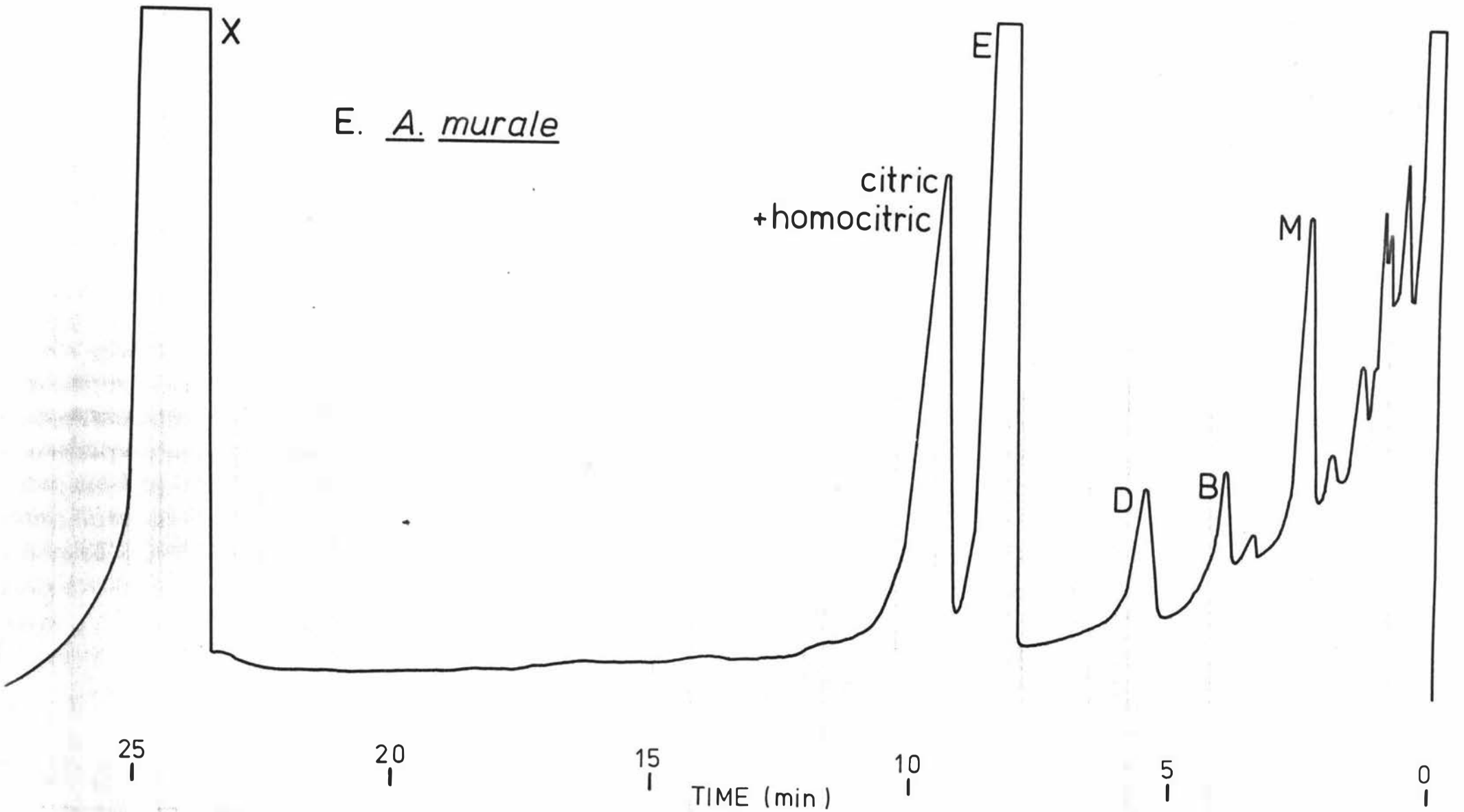
A. *A. corsicum*
B. *A. euboeum*
C. *A. troodii*
D. *A. argenteum*
E. *A. murale*
F. *A. tenium*
G. *A. heldreichii*
H. *A. virgatum*
I. *Bornmuellera tymphaea*

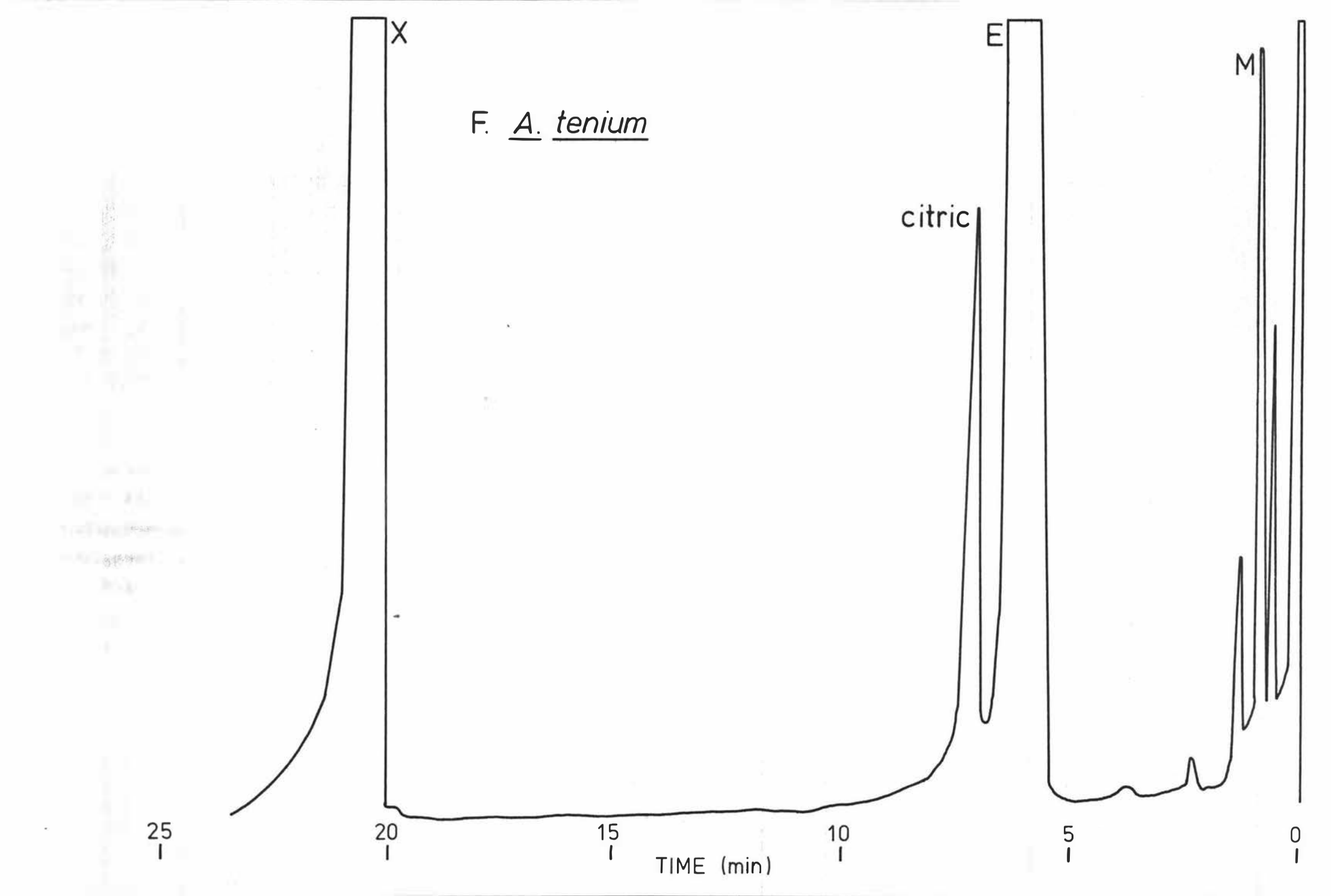

A. A. corsicum
X
E
F
citric
D
G
20
15
10
5
0
TIME (min)

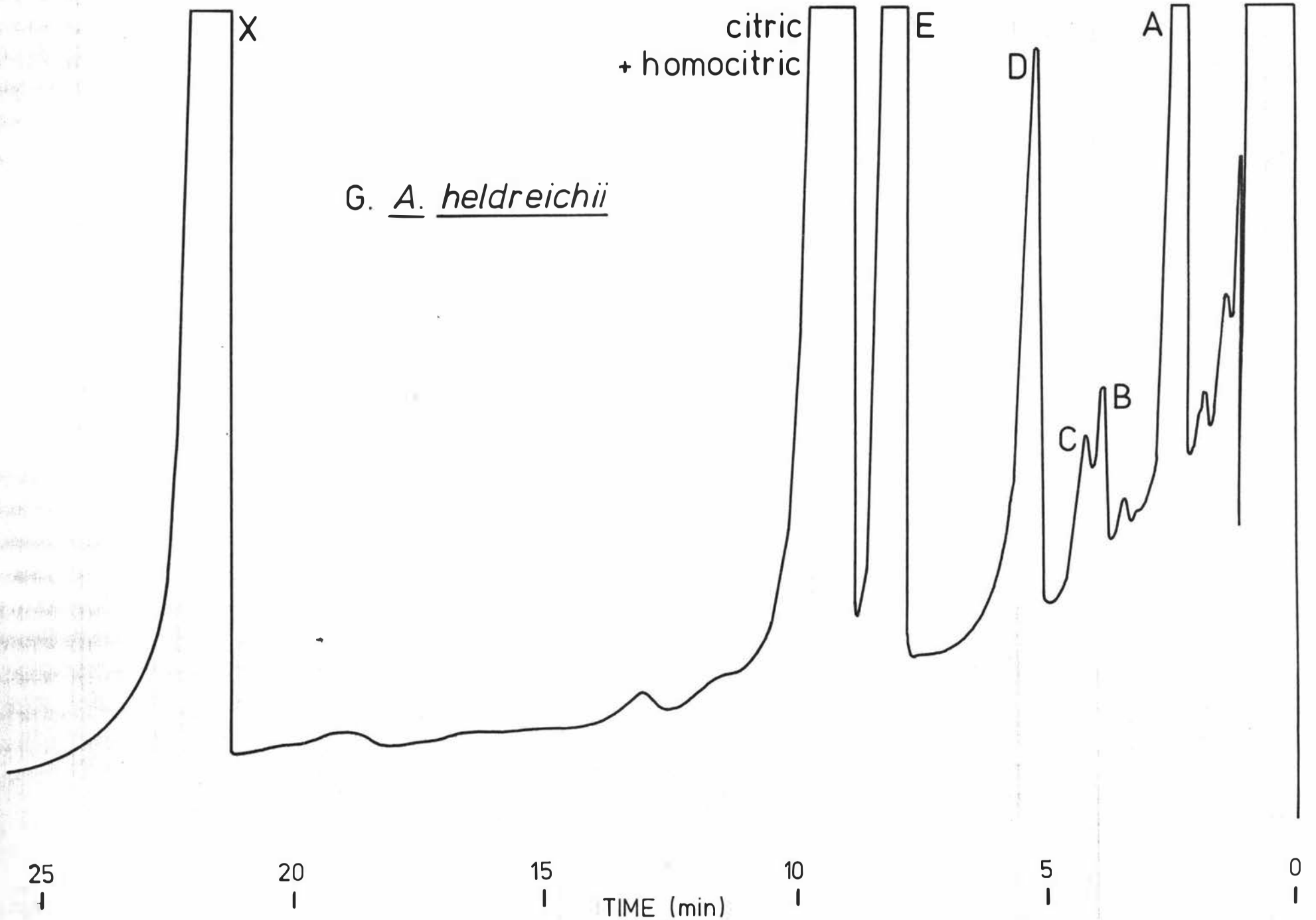

B. A. euboeum
X
E
F
D
citric
25
20
15
10
5
0
TIME (min)

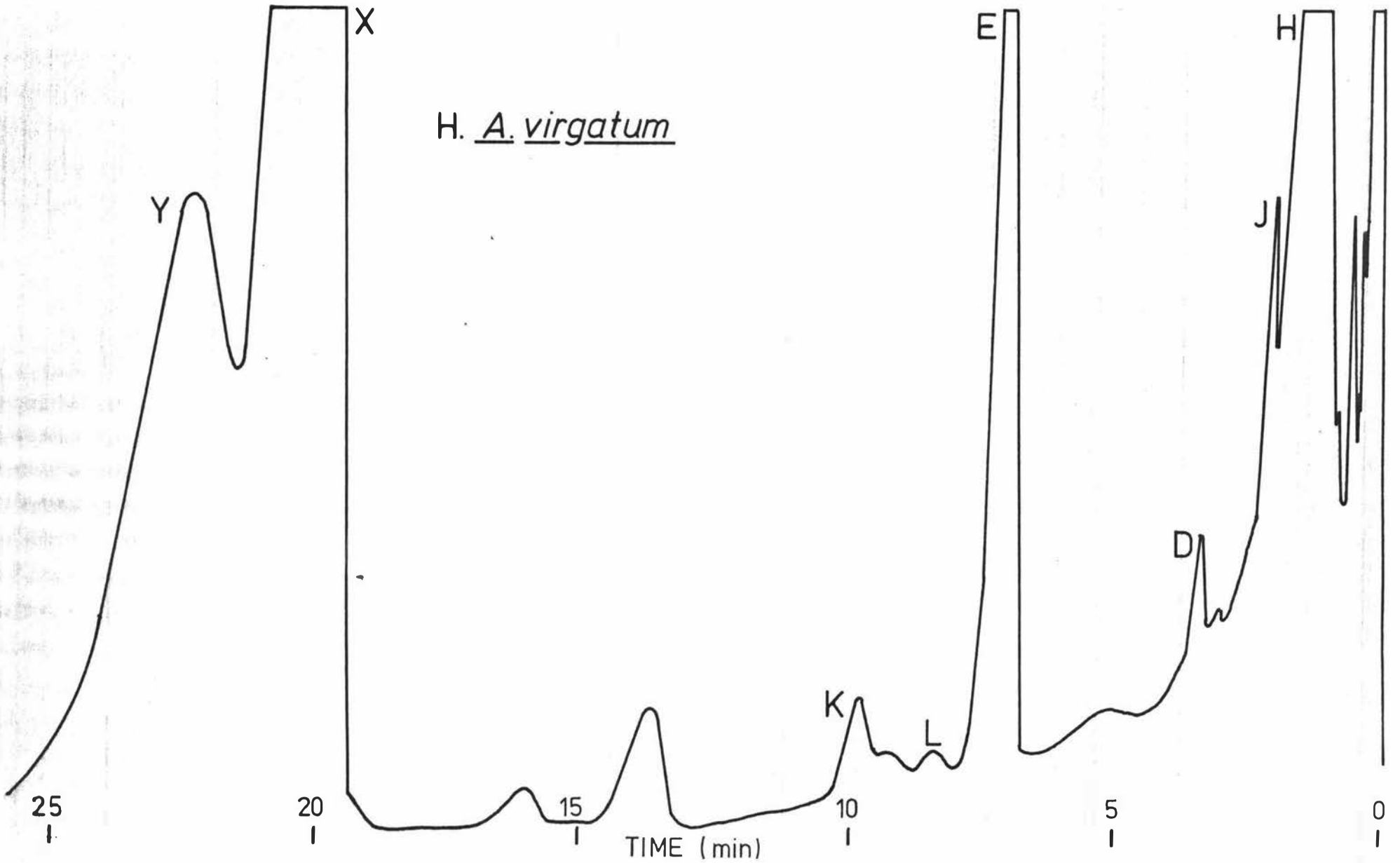

X
C. A. troodii
E
F
D
25
20
15
10
5
0
TIME (min)

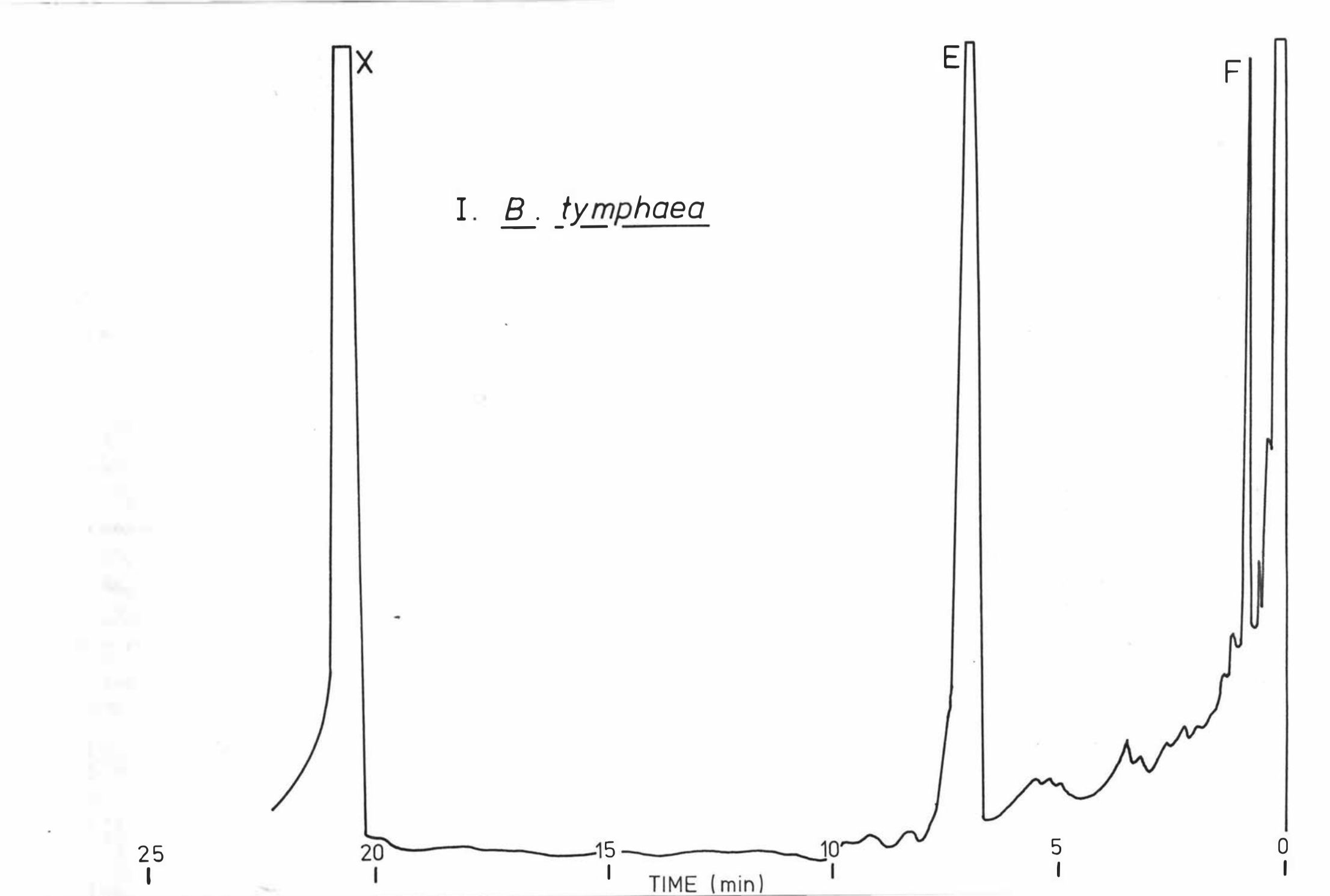

X
citric
+homocitric
E
M
B
D
C
D. A. argenteum
25
20
15
TIME (min)
10
5


E. A. murale
X
E
citric
+homocitric
M
D
B
25
20
15
10
5
0
TIME (min)


X
E
M
F. A. tenium
citric
25
20
15
10
5
0
TIME (min)


X
citric
+ homocitric
E
D
A
B
C
G. A. heldreichii
25
20
15
10
5
0
TIME (min)


H. A. virgatum
X
Y
E
H
J
D
K
L
25
20
15
10
5
0
TIME (min)


I. B. tymphaea
X
E
F
25
20
15
10
5
0
TIME (min)

The closely related Bornmuellera tymphaea had only peak F in addition to the peaks E and X. However the peak F in this species had a widely different retention time (relative to peak E) when compared with the peak F of the Alyssum species (the composition of the peaks was differentiated on the mass spectra obtained for them). It may be that the peaks titled F are actually of isomers.

Representative samples of the mass spectra of the titled peaks are shown in fig. 8.3. There was a conspicuous lack of correlation between the spectra presented here and those recorded in the literature such that few of the esters, and hence the parent acids, could be positively identified.

Malic, malonic and citric acids have all been previously implicated in nickel complexation by Alyssum species (Pelosi et al., 1976, Pancaro et al., 1977, Shaw, 1980) and were obvious acids to look for. Citric acid trimethylester was readily apparent in the GLC traces and it was readily confirmed by the mass spectra of those peaks. In A. heldreichii and A. argenteum (representing the two series of subsection Compressa) the citric acid trimethylester peak had an homologous contaminant, homocitric acid trimethylester. This parent acid has been identified as the complexing agent in another nickel hyperaccumulator, Pearsonia metallifera (Stockley, 1980). A. corsicum (representing subsection Inflata) did not show the presence of this contaminant.

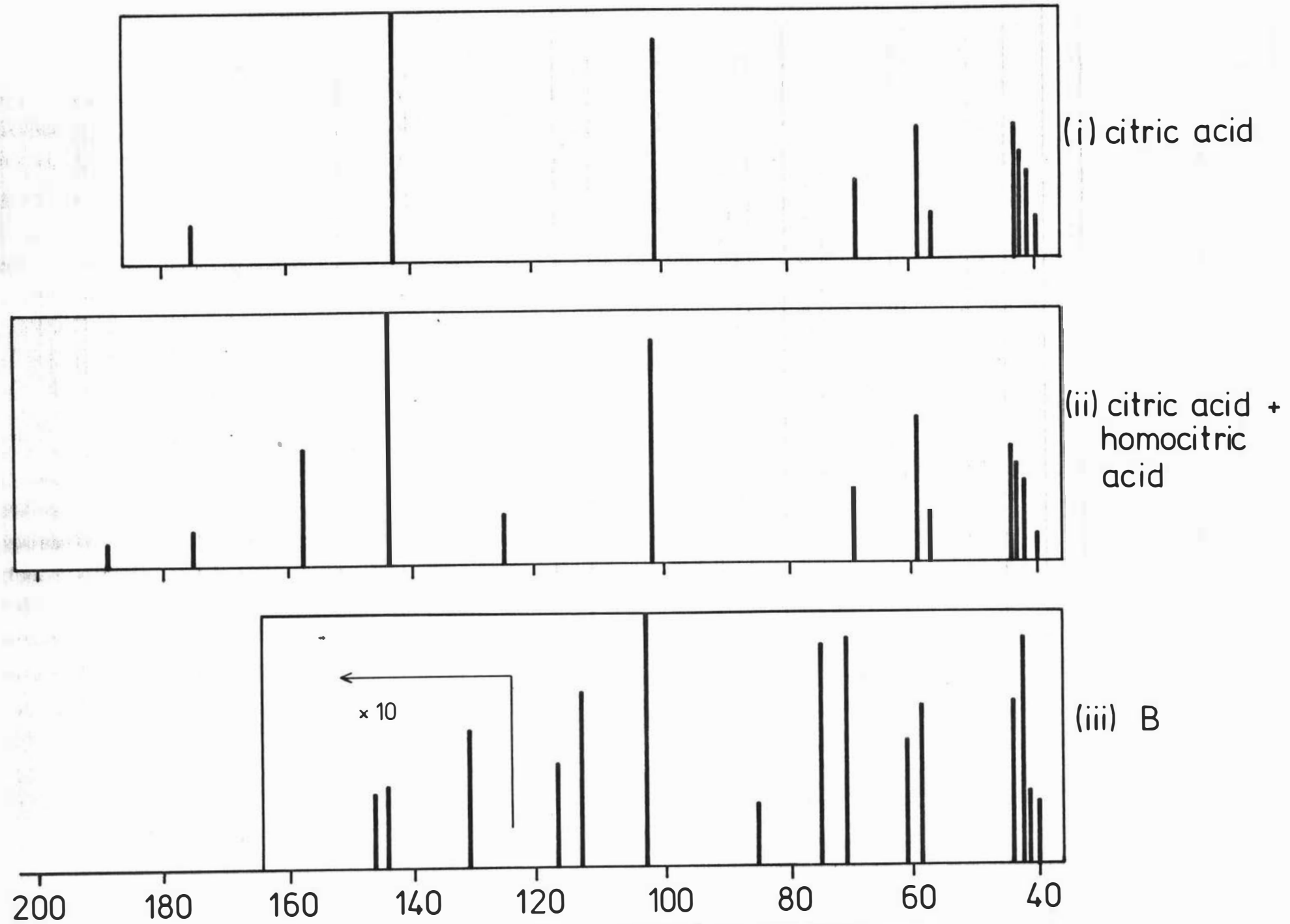
Spectrum 8 bears some resemblance to spectra previously recorded for the malic acid dimethylester. However the sizeable peaks at m/e = 117 and 113 require some explanation. The peak at m/e = 113 is normally found in malic acid dimethylester spectra at 10 - 20% of the base peak compared to the 57% found here. This enhancement is however most probably an instrumental artifact. The peak at m/e = 117 is not known from the malic acid dimethylester but is known from the spectrum of the mixed monoethylmonomethyl ester of malic acid ($CH_3OOCCHOHCH_2COOCH_2CH_3$, Webb et al., 1967).

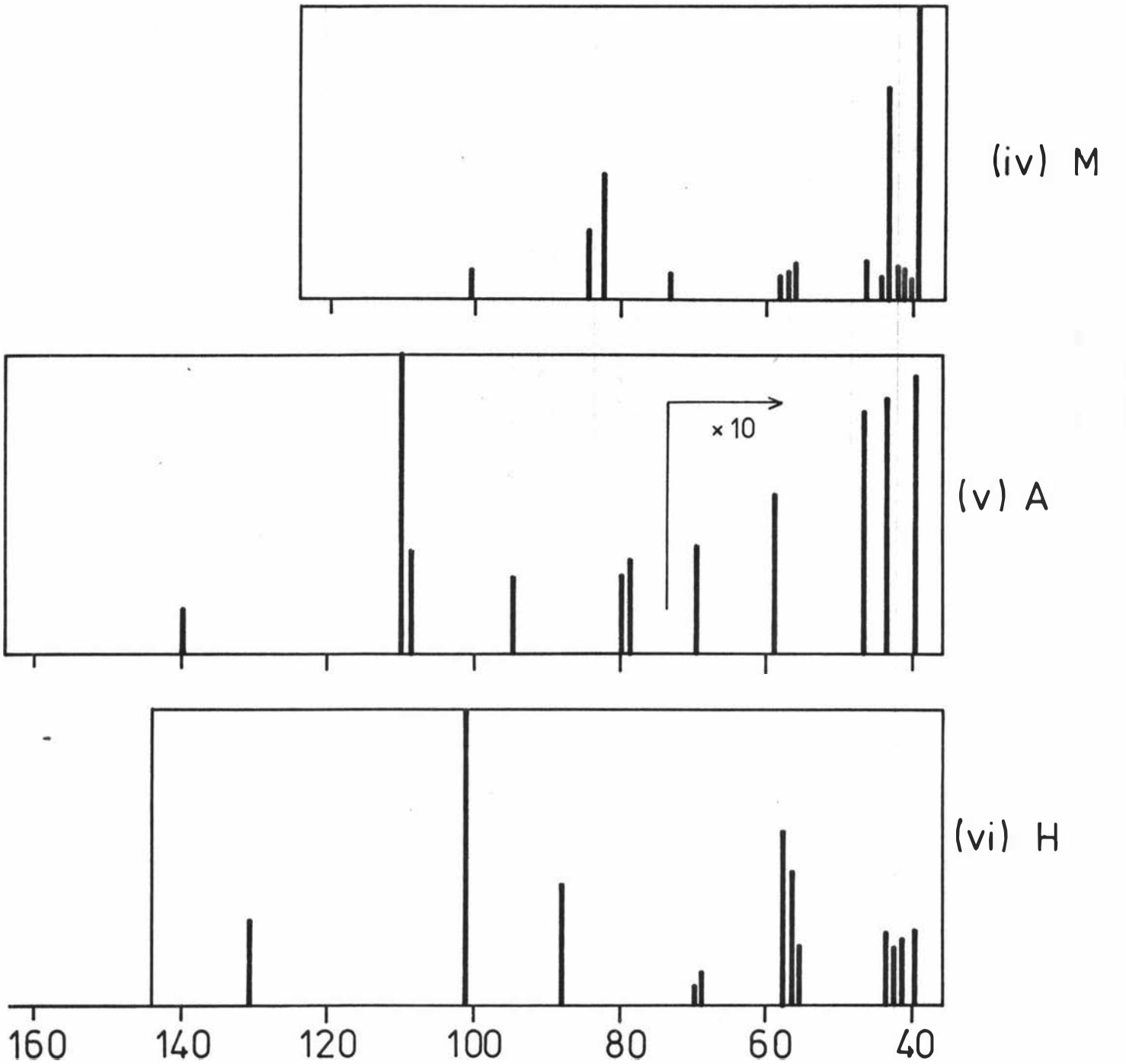
Figure 8·3 Mass spectra of the methylated derivatives of the nickel-complexing ligands (by the peak designations of fig. 8·2)

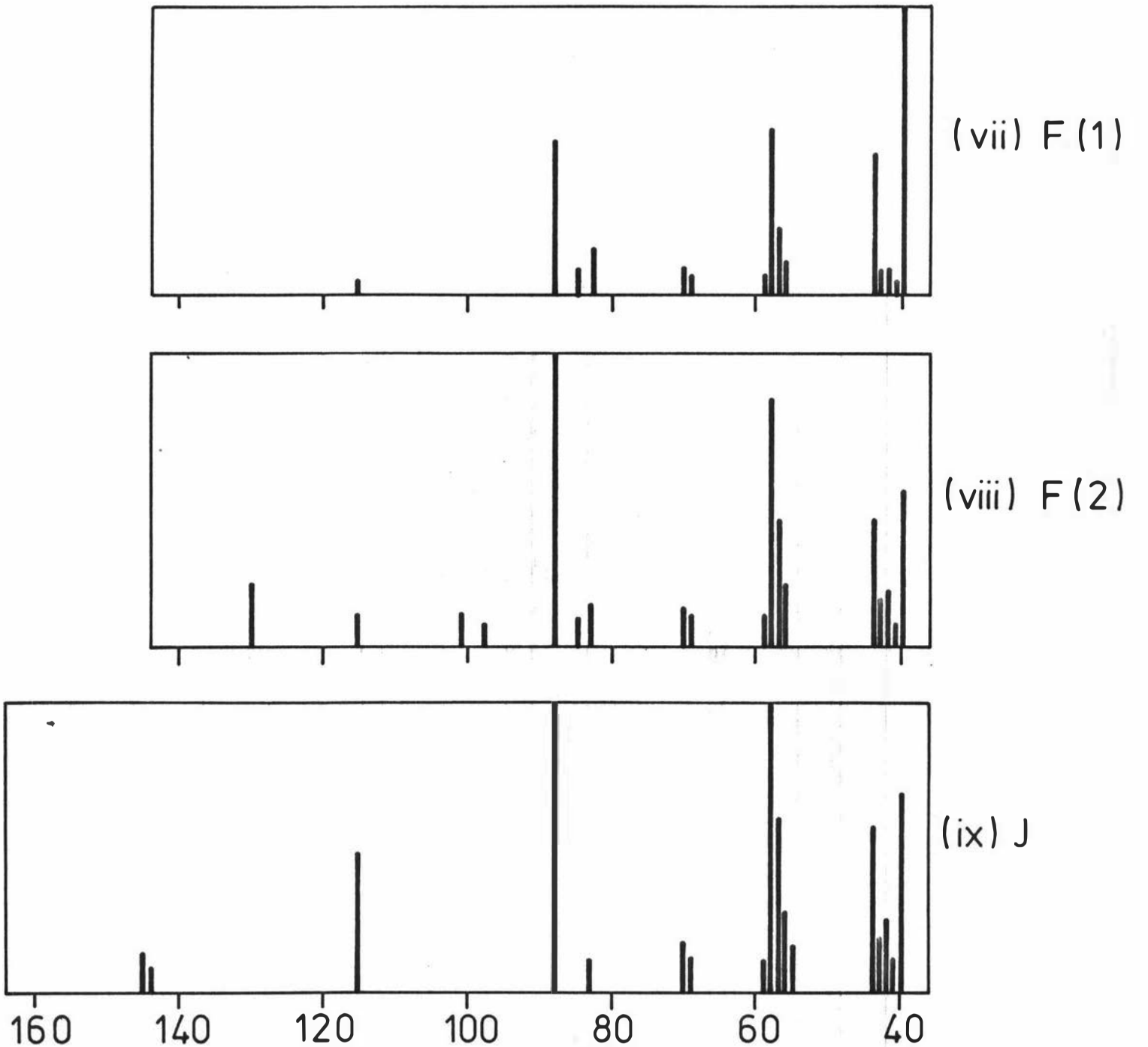
(i) citric acid

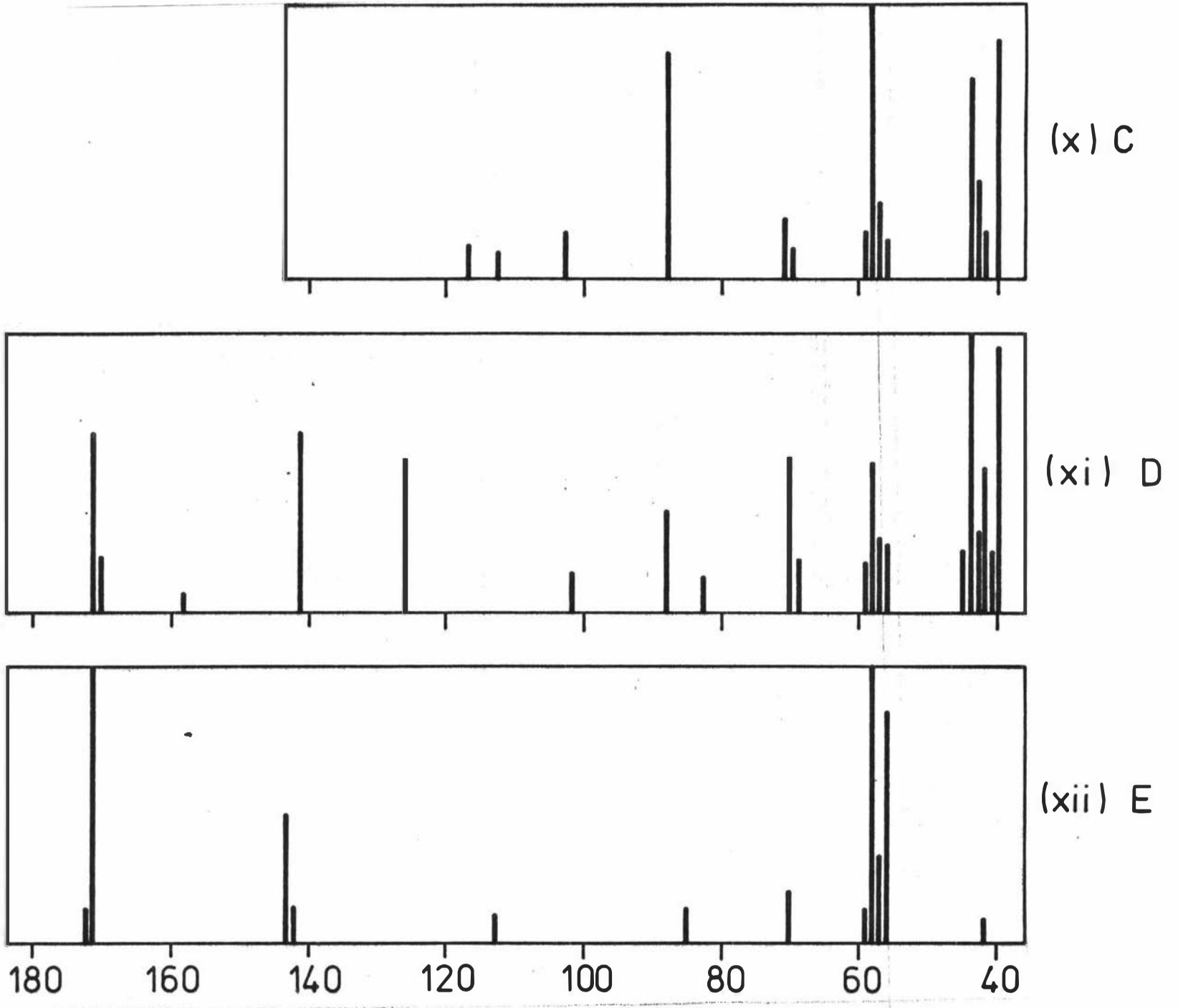
(ii) citric acid + homocitric acid

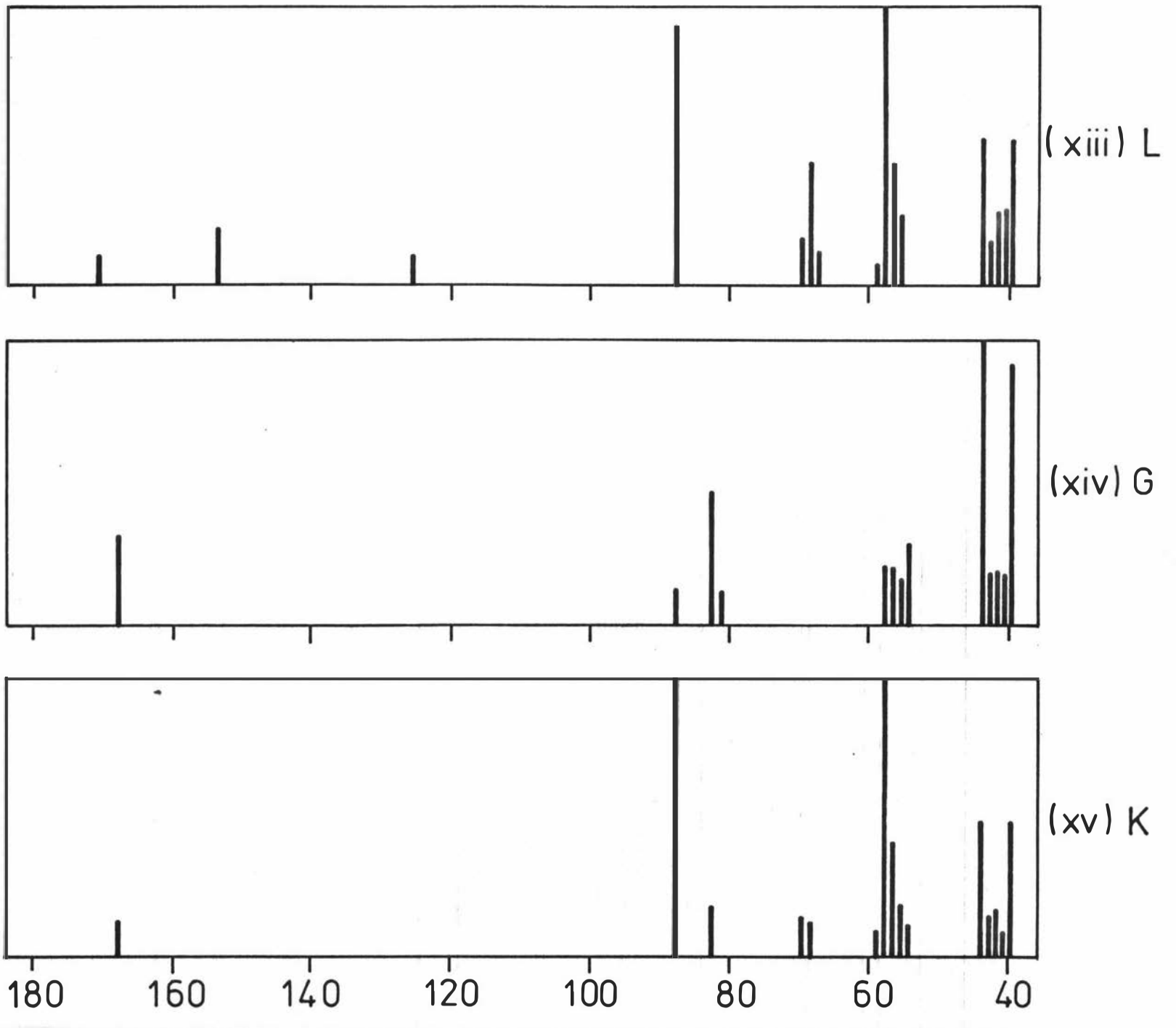
(iii) B	(iv) M	(v) A
(vi) H	(vii) F(1)	(viii) F(2)
(ix) J	(x) C	(xi) D
(xii) E	(xiii) L	(xiv) G
(xv) K	(xvi) Y	(xvii) X

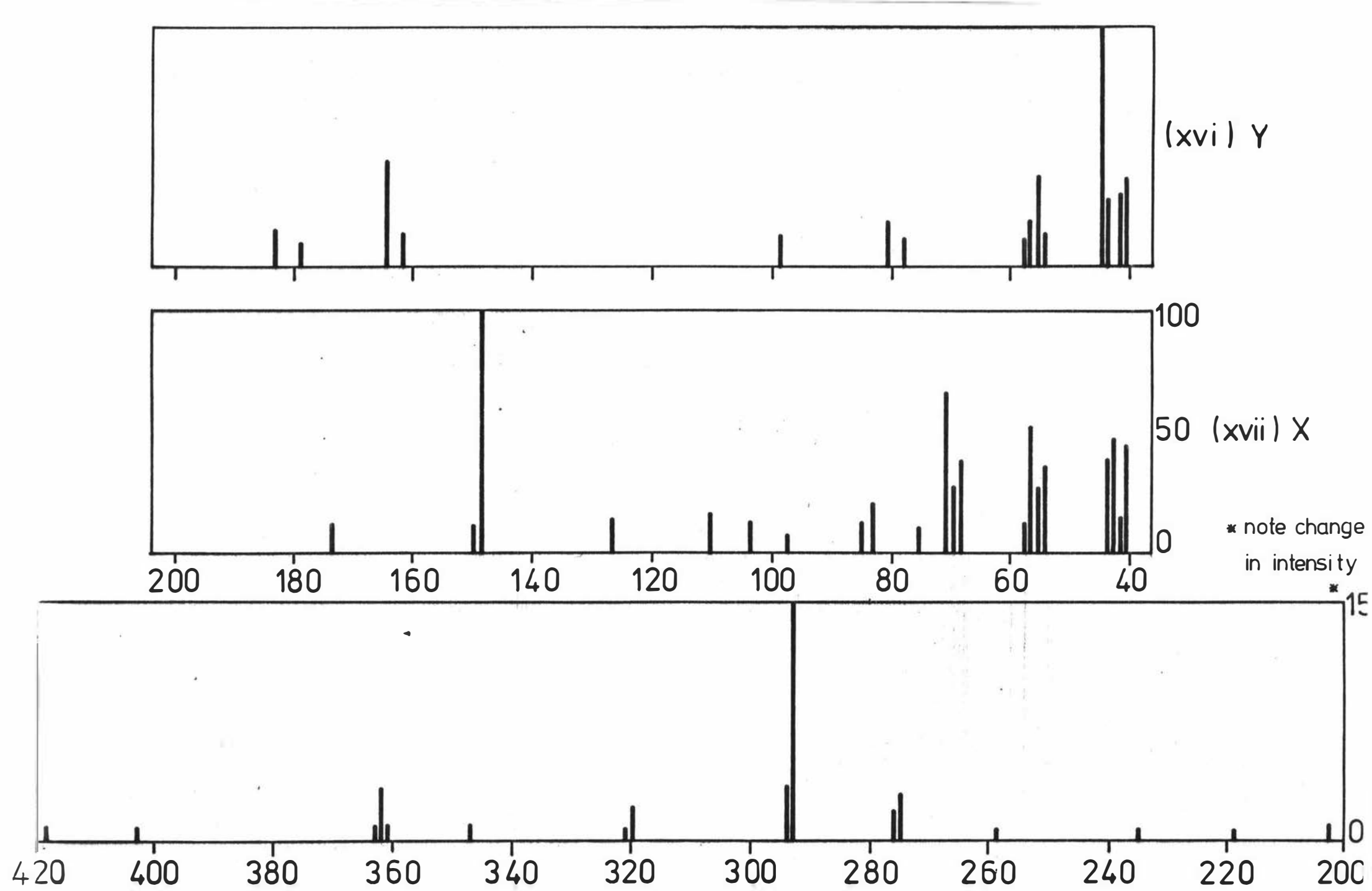

(i) citric acid
(ii) citric acid + homocitric acid
(iii) B
× 10
200
180
160
140
120
100
80
60
40


(iv) M
(v) A
× 10
(vi) H
160
140
120
100
80
60
40


(vii) F (1)
(viii) F (2)
(ix) J
160
140
120
100
80
60
40


(x) C
(xi) D
(xii) E
180
160
140
120
100
80
60
40


(xiii) L
(xiv) G
(xv) K
180
160
140
120
100
80
60
40


(xvi) Y
100
50
(xvii) X
0
* note change in intensity
*
15
0
200
180
160
140
120
100
80
60
40
420
400
380
360
340
320
300
280
260
240
220
200

This ester would have formed by methylation of the acid monoethylmalate which is known to occur in wines (Webb et al., 1967, Drawert et al., 1974). Spectrum B may therefore be a mixture of the malic acid dimethylester and the monoethylmalate monomethylester of which the former is dominant.

Spectrum M shows the closest spectrum to those previously recorded for malonic acid dimethylester. This spectrum contains a number of peaks which are not associated with the spectrum expected of malonic acid. It may well be that this spectrum is of a mixture of which malonic acid dimethylester is a component. If so the most characteristic peaks of the other component are the peaks at m/e = 85 and 83 (see also the discussion of spectrum F).

Spectrum A is dominated by a peak at m/e = 110 with the highest peak at m/e = 140. A peak at m/e = 59 is characteristic of methylesters of carboxylic acids (and several other groups, Beynon et al., 1968). Other types of compounds which give rise to this peak are tertiary aliphatic alcohols, some ethers, aliphatic monobasic carboxylic acids and esters of normal chain dibasic carboxylic acids. The alcohols and ethers are unlikely candidates as they do not form stable complexes with nickel in aqueous environments. Any monobasic carboxylic acid would have been esterified by the diazomethane hence it cannot be the source of this peak. The dibasic carboxylic acid methyl esters have been subjected to mass spectrometry (Tressl et al., 1975) and bear no resemblance to this spectrum. It thus appears that the spectrum is that of a methyl ester of an acid of unknown composition. If the peak at m/e = 140 is the parent ion peak then the compound must have a high degree of unsaturation (at least two C=C units or possibly a furanose or pyranose structure). Possible formulae could then be $C_8H_{12}O_2$, $C_7H_8O_3$ or $C_6H_4O_4$, although the last is less probable.

Spectrum H has its base peak at m/e = 101. The highest recorded peak was at m/e = 131 but as the esters under investigation contain only carbon, hydrogen and oxygen

this cannot be the parent ion peak. As most (but not all) methyl esters fragment with an initial $CH_3O^{\bullet}$ or $^{\bullet}COOCH_3$ loss, the parent compound may have a molecular weight of 162 or 180. When $CH_3O^{\bullet}$ is initially lost, it is generally followed by CO loss. As CO has a mass of 28 and the mass lost from the m/e = 131 peak is 30, giving the m/e = 101 peak, it appears unlikely that the m/e = 131 peak is due to $(M- CH_3O)^+$. Thus it is more probable that the ester has a molecular weight of 180. Again the acid of such an ester would be highly unsaturated or heterocyclic.

Spectrum F (of which two versions are shown) may be a mixture. The spectrum labelled F(1) was found in B. tymphaea and on the rising side of the peak in A. corsicum. The spectrum F(2) was found on the declining side of the peak in A. corsicum. The noticeable differences between the spectra are the rise of the peak at m/e = 88 in F(2) with the decline of the peaks at m/e = 58 and 40 and the appearance of a number of small peaks above m/e = 88. If the peak at m/e = 88 is from one component of a mixture and the peaks at m/e = 58 and 40 are from another, the differences are easily explained. Furthermore the component with peaks at m/e = 58 and 40 has peaks at m/e = 85 and 83 and may well be the other component with the malonic acid dimethylester in spectrum M. The peak at m/e = 88 is characteristic of 2-methylcarboxylic acid methyl esters. If m/e = 130 (the highest peak) is the parent molecular ion then 2-methylpentanoic acid monomethylester is a possibility. This acid could explain the m/e = 101 peak by $CH_3CH_2^{\bullet}$ loss, and the m/e = 98 peak by CH_3OH loss as well as the rearrangement ion peak at m/e = 88. Furthermore this portion of the spectrum corresponds reasonably well with previously tabulated spectra. The small peak at m/e = 116 may be the parent ion for the other component of this mixture. This would correspond to a formula of $C_6H_{12}O_2$ or $C_5H_8O_3$. Representatives of the first formula include the methyl esters of pentanoic acid, the methylbutanoic acids and dimethylpropanoic acid. The esters of the methyl-substituted acids all

have large peaks at m/e = 74 which is not found in spectrum F. The pentanoic acid ester has a strong peak at m/e = 57 which is not found in spectrum F. The second formula is that of the oxobutanoic acid methyl esters. The 2-oxobutanoic acid ester has no peaks in the m/e = 83-85 range which spectrum F has. The 3-oxobutanoic acid ester has peaks at m/e = 85 and 84 compared with spectrum F which has peaks at m/e = 85 and 83. Also the 3-oxobutanoic acid ester has a base peak to its spectrum at m/e = 43 whereas spectrum F has a base peak at m/e = 40. The spectrum of the 3-oxobutanoic acid ester is undoubtedly the closest of these spectra to that which is required but the evidence is not conclusive.

Spectrum J has much in common with spectrum F(2). The major difference is the appearance of peaks at m/e = 145, 144 and 115. Peaks at m/e = 88 and 58 again dominate the spectrum. A weak metastable peak, m^* = 91.2, indicates the transition from m/e = 145 to m/e = 115. A high resolution mass spectral analysis of the m/e = 145 peak gave a formula of $C_7H_{13}O_3$. Assuming again that the ester has initially lost 59 mass units (the loss from the highest peak is 30 mass units compared to the 28 (CO) which would be expected if only the $CH_3O^{\bullet}$ had been lost) would give a molecular weight of 204 and a formula of $C_9H_{16}O_5$. The loss of 59 mass units (assumed) followed by the loss of 30 mass units (measured) appears frequently in these spectra. This 30 mass unit is either C_2H_6 or CH_2O. The latter possibility is most interesting. Loss of CH_2O after an initial loss of $^{\bullet}COOCH_3$ would allow the ester to have the structure $CH_3OOCCHOHR$ which would give a parent acid structure of HOOCCHOHR. This would allow chelation through both the carboxylate and hydroxyl oxygens. The acids are not however likely to be aliphatic 2-hydroxycarboxylic acids as the esters of these acids give a characteristic peak at m/e = 90 which is not seen in this spectrum. This would tend to indicate that a further functional group is close to the 2-hydroxycarboxyl group (probably α- or β- to the hydroxyl group). One such group could be a second carboxylate group.

This would give the acid a basically malic acid structure, HOOCCHOHCHRCOOH. Three known nickel complexing agents, malic acid, citric acid and homocitric acid, all have this basic unit (Lee et al., 1977b, Kersten, 1979, Shaw, 1980, Stockley, 1980). Furthermore the basic malic acid structure allows a multiplicity of chelate rings which further stabilize the complexes formed with nickel (Liddle, 1979). If this is the case for this ester in spectrum J, the sidechain R = C_3H_7. This would give either 3-propylmalic acid or 3-isopropylmalic acid as the parent acid.

The spectra C and D also show an affinity with spectra F and J. Spectrum C has many of the peaks of both spectra B (with the methyl esters of malic acid and acid monoethylmalate) and F(1) (with the methyl esters of 2-methylpentanoic acid and possibly 3-oxobutanoic acid). This spectrum may be of a rather complex mixture of esters but it can be noted that the peaks ascribed as possibly belonging to 3-oxobutanoic acid methylester (m/e = 85 and 83) are absent.

Spectrum D has its highest peak at m/e = 171 followed by a peak at m/e = 141. With the difference between these peaks being 30 mass units, it would seem that the m/e = 171 peak is the $(M- COOCH_3)^+$ peak. This would give the parent ion a mass of 230. If the above discussion for spectrum J holds here, then several possibilities of identification occur. The sidechain here would have a mass of 69. No elemental analysis by high resolution mass spectrometry was forthcoming to aid the identification process. A mass of 69 units however would give probable formulae C_5H_9 (the pentenyl group) or C_4H_5O (the oxobutenyl group). The identity of the acid is still unknown.

Spectrum E was one of the two spectra recorded for each species. The spectrum has its base peak at m/e = 171 with another high mass peak at m/e = 143. The difference between these peaks is 28 mass units and it is probable that the m/e = 171 is the $(M- CH_3O)^+$ peak. This has been further indicated by chemical ionization mass spectral data which showed a peak at

m/e = 203, $(M + H)^+$, around the elution time expected for this ester (as the chemical ionization scan and the GLC recorder would not operate simultaneously absolute confirmation that this is for peak E was not obtained). A high resolution scan of the m/e = 171 peak gave an analysis of $C_8H_{11}O_4$ which would give the ester the formula $C_9H_{14}O_5$. A metastable peak at m* = 119.5 indicated the derivation of the m/e = 143 peak from the m/e = 171 peak. The next highest peak is at m/e = 113, a loss of 30 mass units. This acid may therefore have the basic malic acid structure proposed above. If so the sidechain here is a C_3H_5 (propenyl) unit.

Spectrum L bears some resemblance to spectrum D (highest peak at m/e = 171, common peak at m/e = 126) although there are obvious differences (no m/e = 141 or 158 peaksin spectrum L but m/e = 154 peak now present). Lower mass peaks are mostly common to both spectra although their relative strengths vary (particularly of the m/e = 40 and 44 pairing relative to the m/e = 58 and 88 pairing). It may be that spectra D and L are of isomeric esters.

Another possible set of isomers is shown by the esters of spectra G and K. Both these esters have similar retention times in the GLC traces. Their highest peaks are at m/e = 168. Unfortunately no peaks exist between m/e = 168 and m/e = 88 to aid possible identification. As for the spectra D and L pairing major differences occur in the peaks at m/e = 40 and 44 (high in spectrum G, low in spectrum K) and at m/e = 58 and 88 (low in spectrum G, high in spectrum K). Other differences are nonetheless also apparent (increased m/e = 168 and 83 peaks in spectrum G, presence of peaks at m/e = 70 and 69 in spectrum K).

Spectrum Y is of an ester with a much greater retention time than those previously discussed. This could indicate a greater molecular weight, although the spectrum shows no peak above m/e = 183, or a different structure for the basic acid. The next highest peaks are at m/e = 179 and 165. The former of these could not arise from fragmentation of the m/e = 183

peak and must come from a higher ion (noting that the m/e = 183 peak cannot be the parent ion peak of a compound containing only carbon, hydrogen and oxygen). The base peak of this spectrum is at m/e = 45 which is normally attributable to an $CH_3CH_2O^+$ group. No further identification of this ester is currently possible.

The possibility that several of the complexing agents may contain the basic malic acid structure requires that this unit be readily produced by plants. In the Krebs cycle, isocitric acid, which has the HOOCCHOHCHRCOOH structure, and citric acid, with the basically similar $HOOCCROHCH_2COOH$ structure, are produced by the condensation of oxaloacetic acid (a 2-oxocarboxylic acid) with acetyl-Co A (producing citric acid) and a subsequent isomerization (producing isocitric acid). These two acids are readily isomerized and coexist in an equilibrium. Malic acid can also be produced by condensation of a 2-oxocarboxylic acid (glyoxylic acid) with acetyl-Co A in the glyoxylate cycle. Strassman and Ceci (1963) have shown that leucine is synthesized by chain elongation of valine by a sequence which involves a condensation reaction of this type. This same procedure has also been shown in the biosynthesis of many sidechains in the glucosinolates (Underhill et al., 1973). Luckner (1972) considers that the condensation of any 2-oxocarboxylic acid with acetyl-Co A to be a natural extension of these reactions. It is, therefore, probable that any of the parent acids here of this structure would be of this origin.

This information leads to the possible identification for several of the acids whose esters are discussed above. For spectrum J, the 2-oxocarboxylic acids which could have parented the proposed acids are 2-oxopentanoic acid and 3-methyl-2-oxobutanoic acid. The latter of these two acids is the oxoacid formed by deamination of valine and the acid resulting from condensation of this oxoacid with acetyl-Co A is a recognized intermediate in the biosynthesis of leucine from valine (Strassman & Ceci, 1963). The former oxoacid could

occur by double chain elongation from alanine but this biosynthesis has yet to be recorded. Current knowledge must therefore favour 3-isopropylmalic acid as the complexing agent whose ester produces spectrum J.

The parent acid of spectrum D is presumed to have either a pentenyl or oxobutenyl sidechain. The formation of the oxobutenyl sidechain cannot be explained by known biosyntheses. The pentenyl group can exist as a number of isomers. Of these, two are of particular interest. The n-butenyl sidechain with the terminal double bond (CH_2=$CHCH_2CH_2CH_2$-) could be derived by multiple elongation from methionine followed by a desulphuration reaction. This sequence is well known in glucosinolates (Underhill et al., 1973). A methylbutenyl grouping (CH_2= C (CH_3) CH_2CH_2-, isomeric to n-butenyl) has also been found among glucosinolates (Kjaer, 1973) although its biosynthesis appears to be unknown. It is uncertain which of these parent acids could give the ester whose spectrum is recorded (if indeed it is either of them) but the n-pentenyl group is more widespread.

Spectrum E may be produced by a lower homologue of the n-pentenylmalic acid ester. The propenyl group has only two isomers of which the terminal isomer (CH_2=$CHCH_2$-) could be derived by chain elongation from methionine as for the n-pentenyl group. Short chain-length branched alkenyl groups are not recorded among plants. Much further study of these acids will however be required before any definitive conclusions as to their identity can be made.

The final spectrum to be discussed is that of peak X. The molecular ion peak is at m/e = 418 (0.05% of base peak). That this is the parent ion peak was confirmed by chemical ionization mass spectrometry which gave a peak at m/e = 419, $(M+H)^+$. Three metastable peaks occur at the higher masses indicating the transitions from m/e = 362 to m/e = 320 (m^* = 282.9), from m/e = 320 to m/e = 259 (m^* = 209.6) and from m/e = 293 to m/e = 275 (m^* = 258.1). From these

transitions it is apparent that at least two fragmentation pathways are operating in this region. The first pathway appears to lose a fragment of 56 mass units (C_4H_8 or C_3H_4O?) from the molecular ion to give the m/e = 362 peak. This is followed by the loss of a 42 mass unit fragment (C_3H_6 or C_2H_2O?) to give the m/e = 320 peak. A further 61 mass units (C_3H_9O or $C_2H_5O_2$?) is then lost to give the m/e = 259 peak. The second pathway which involves the m/e = 293 to m/e = 275 transition (18 mass unit loss is probably a dehydration) is more problematical. It could be derived in a number of fragmentation series: m/e = 418 $\longrightarrow$ 403 $\longrightarrow$ 347 $\longrightarrow$ 293; m/e = 418 $\longrightarrow$ 362 $\longrightarrow$ 347 $\longrightarrow$ 293; m/e = 418 $\longrightarrow$ 362 $\longrightarrow$ 293; or m/e = 418 $\longrightarrow$ 362 $\longrightarrow$ 320 $\longrightarrow$ 293. There is undoubtedly a labile methyl group. This is shown by the peak at m/e = 403, 15 mass units below the molecular ion peak. The peak at m/e = 347 could have arisen from the loss of the methyl group from the m/e = 362 peak. Alternatively this peak may be derived from the m/e = 403 fragment by the loss of 56 mass units as proposed in the initial fragmentation of the first pathway. The base peak for the spectrum is at m/e = 149 which could be of one of the following formulae; $C_6H_{13}O_4$ or $C_5H_9O_5$. Much further work is required before this compound is identified and hence the major complexing agent for nickel in Alyssum species is known.

With the dominant complexing agent being comparatively large, the question of its mobility must be raised. This acid may be acting only as a terminal acceptor of nickel and as such specifically located in the cells, possibly in the vacuole. The other, lesser, complexing agents may then be acting as transport ligands. The process of cation transportation to a terminal acceptor has been postulated for zinc in zinc-accumulating plants by Mathys (1977). Malic acid was proposed as the transport ligand with oxalate as the terminal acceptor located within the cell vacuole. Lee (1977) has suggested a similar scheme for nickel accumulation in some New Caledonian hyperaccumulators, again utilizing malic acid as the transport

ligand but with citric acid as the terminal acceptor. On the work presented here, the parent acid of peak E (possibly 3-propenylmalic acid) is the major candidate for the transport ligand with the parent acid of peak X as the terminal acceptor. The other minor peaks are all of acids which may, when available, act as supplementary transport ligands. These minor acids appear to vary between groups of species with these groups corresponding to the various subsections and series of section Odontarrhena. The variations between the groups and the similarity within them may be a reflection of a common ancestry for the species of each group. This is in accord with the ideas put forward in Chapter 6 on other grounds. That Bornmuellera tymphaea is closely related to the Alyssum species can be further inferred by the finding of the identical major acids (the parent acids of peaks E and X) in this species.

CHAPTER 9

Concluding Discussion

The work presented in this thesis has been divided into two distinct areas; cobalt and copper accumulation, and nickel accumulation. To conclude the discussions it is proposed to compare the results obtained.

It can be seen that, in general, hyperaccumulation of nickel occurs to higher concentrations than cobalt, with copper at slightly lower levels still. Paradoxically, Agarwala *et al.* (1977) found the order of toxicity of these metals to be the same ie. nickel > cobalt > copper. This paradox may be the result of three interacting effects: (1) the Irving-Williams rule for complex formation by divalent transition metal ions, copper > nickel > cobalt; (2) the essentiality of the metal: copper is an essential element, cobalt and nickel are not; (3) and the toxicity of the metals is due to the amount of free aquoions. From toxicity studies in non-tolerant plant species (and therefore species without the adaptations which permit the hyperaccumulation of these elements) there would be an expectation that the accumulation levels for copper would exceed those of cobalt with nickel lower still. But combining the essentiality effect with the toxicity effect, noting that plants exert tighter controls over essential elements, will have the effect of retarding copper accumulation levels relative to the cobalt and nickel levels. When, for cobalt and nickel, the toxicity effect is combined with the complex formation effect, it is seen that the result depends on the individual complexes formed. Since it is found that nickel is accumulated to higher levels than cobalt, the complexes formed by nickel must be more stable to the extent that free cobalt(II) ions reach toxicity levels at a lower total metal level than free nickel(II) ions. The essentiality effect is apparently stronger than the complexation effect as copper is the least accumulated of the three elements studied.

In the discussion at the end of Chapter 2, reference was made to the distribution of hyperaccumulators of cobalt, copper and nickel among the superorders of the plant kingdom and to the advancement indices of the families from which these hyperaccumulators came. It was noted that hyperaccumulators of cobalt and copper came most frequently from the superorders Asteridae and Commelinidae and were predominantly advanced in their characteristics (advancement indices average 67) while nickel hyperaccumulators were generally from the superorder Dilleniidae with a greater primitivity in their characters (advancement indices average 41). These differences need explaining. For the advancement indices, the difference may well be due to general differences of the region in which they are found. Chenery and Sporne (1976) found that aluminium accumulation was found in families whose advancement indices were low ie. the families tended to be primitive. However Wood (1970) and Sporne (1970, 1973) had already shown that plant families of tropical forests were primitive in their characters and it is in the tropics that the greatest concentrations of aluminium in the soil are found. The results of Chenery and Sporne (1976) when viewed in this light are predictable. The cobalt and copper hyperaccumulators of Shaba are all of genera and therefore families found both on and off the metal-rich soils so that the advancement indices of these hyperaccumulators also reflects the advancement of the surrounding vegetation rather than a specific requirement of advanced characters before accumulation can develop. The nickel hyperaccumulators probably show this effect best of all: in tropical New Caledonia, with relatively primitive families, the nickel hyperaccumulators also have low advancement indices (Jaffre, Kersten et al., 1979) while in the more arid Mediterranean basin, with more advanced families, the nickel hyperaccumulators also have a higher advancement index.

The difference in distribution between the various superorders is not so easily explained. There does appear

to be a distinct difference in the abilities of the superorders to develop hyperaccumulating abilities for the respective metals. Thus despite differences in advancement indices between the Mediterranean and New Caledonian nickel hyperaccumulators, the large majority of species are from the superorder Dilleniidae. These differences may well result from the different ancestries of the superorders. If, amongst the protospecies of a superorder, several specimens had contact with metal-rich soils, the genetically inherited capability to tolerate and accumulate particular metals may have developed. This capability was passed on (expressly or latently) to later-evolved species of that superorder. Thus today's species are either of a continuous line of metallophytes or have re-expressed this capability from latent genes when contact with metal-rich soils was renewed after an interval apart. It is unlikely that all species have the ability to rapidly re-evolve tolerance as many species would have lost the genetic capability in adapting to their own niche. More generalized species (as opposed to species with special adaptive requirements) are most likely to retain this capability and it is thus interesting to note the latent capability measured for Agrostis species adapting to mine soil sites in Britain (Walley et al., 1974, Gartside & McNeilly, 1974, Wu et al., 1975). Re-expression of this capability may have occurred more than once in the lineage of any modern species. Thus the widespread occurrence of hyperaccumulation in Alyssum species suggests that the gene was re-expressed in the protospecies of the genus as well as the protospecies of the superorder. Species from other superorders have independently developed their tolerance. In several cases, eg. Geissois and Phyllanthus, the tolerance appears to have developed in the protospecies of the genus. Nevertheless, the evolution of these metallophytes is still sketchy. That the process of adaptation has occurred many times can be seen by the wide variety of species found on metal-rich soils although the number of species which

accumulate metals is much lower. The most preferred method of tolerance to heavy metals is to develop an improved exclusion mechanism. These processes continue today. Wild and Bradshaw (1977) have reviewed the process of evolution in Zimbabwe. They were able to identify both old (paleo-) and new (neo-) endemic species in the flora of metalliferous soils. They contended that paleoendemic species survive only if the metalliferous areas are large enough to support a viable breeding population during times of wider ecological changes (eg. climatic changes). Where the area is insufficient, the paleoendemics become extinct and neoendemics appear. These neoendemic species may have evolved from the paleoendemics by an adaptation to other ecological changes or from surrounding species. Thus plants are continuously invading and adapting to these soils so that only those best fitted will survive competitively. In this respect it is interesting to note the supreme success of the Cruciferae in adapting to serpentine soils in the Mediterranean-Anatolian-European region. Almost ninety species from this family (from Alyssum, Bornmuellera, Peltaria, Thlaspi and associated genera) have been shown to have evolved accumulation of nickel while many more have shown tolerance but no accumulation. The extent of this ability within this family is without parallel at the present time.

The pattern of uptake of the metals varies. The cobalt and copper hyperaccumulators studied had the exclusion-breakdown form (Chapter 4) while the nickel hyperaccumulators studied had the rise-to-saturation form (Chapter 7). These differences are not likely to be due to the different metals as different uptake patterns can be found for these metals in other species eg. rise-to-saturation form in the copper accumulator Becium homblei (Reilly, 1969). The possibility exists that the rise-to-saturation form indicates a need for the metal. Despite this no specific physiological damage was apparent at low nickel levels in the Alyssum species studied. In Chapter 8 the point was made that

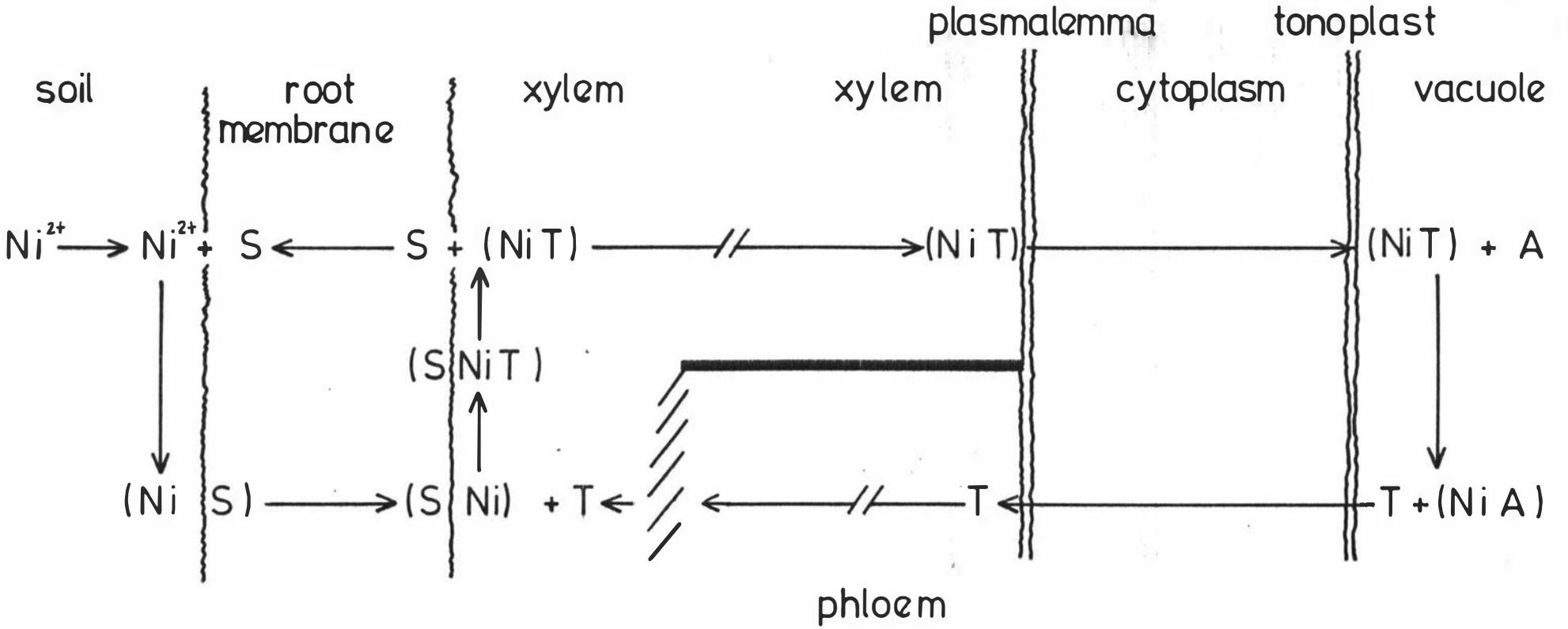
the nickel may be being used in a fungicidal capacity replacing some of the glucosinolates. The plants grown in the trials were regularly sprayed and this may have masked the effect of low nickel. The exclusion-breakdown form of uptake is more common and appears designed to exclude excess amounts of the metal ions from entering the plant. Thus the hyperaccumulators with this uptake form would have no specific needs for these ions in greater than normal quantities.

The mechanism of uptake is more difficult to determine. However recent investigations have pointed in certain directions. Mathys (1977) has proposed a carrier-acceptor system for zinc in four zinc accumulators. The carrier (malic acid) complexes the zinc upon entry to the xylem and the complex is transported in the transpiration stream to the leaf cells. Here the carrier complex is passed through the plasmalemma into the cytoplasm and thence to the vacuole. Within the vacuole the carrier complex degrades, probably by competitive equilibria, and the acceptor (oxalic acid) forms a complex to which the tonoplast is impermeable. The carrier is then released for further zinc complexation and transport while the zinc is restricted to the vacuole where it cannot interfere with cellular processes. Lee (1977) proposed a similar mechanism for New Caledonian nickel hyperaccumulators but with citric acid as the acceptor. Kersten (1979) points out, however, that while Lee (1977) showed that nickel-citric acid complexes do exist in the cell vacuoles, he failed to show that free citric acid existed there.

Lee (1977) and Kersten (1979) disagree on the factor which is predominant in governing nickel accumulation. Lee (1977) proposed that the permeability of the root membrane to nickel was dominant while Kersten (1979) proposed that the amount of nickel available for absorption, itself governed by soil conditions, was dominant. One point which must be noted

is that the uptake of nickel is selective and that other elements present in quantity are not accumulated to the same extent. Still and Williams (1980) have discussed this point and proposed that the selective accumulation of nickel is due to a selective transport ligand in the root membrane. Chelating ligands containing at least two nitrogen donors were shown to have equilibrium constants for metal ion complexation which would allow the selective accumulation of nickel relative to other common metal ions. This "selector" ligand was membrane limited and other ligands (citric acid, malic acid) would then be utilized as "transport" ligands. This mechanism appears reasonable and forms the basis of the following proposed mechanism.

In their proposed mechanism, Still and Williams (1980) were unable to decide as to whether the transport ligand was able to cross the membrane as part of a selector-transport-nickel complex or became complexed after the nickel had been released after crossing the membrane. It is the proposal of this author that the transport ligand forms a ternary (mixed ligand) complex with the selector-nickel complex on the internal surface of the root membrane. Thus the method proposed (fig. 9.1) has the selector ligand, S , complex with the aquonickel(II) ions of the soil solution, on the external surface of the root membrane. The selector-nickel complex, (SNi), then moves through the membrane to the inner surface where a ternary complex, (SNiT), is formed with the transport ligand, T. The transport ligand is most probably an oxygen donor ligand. Sigel (1973) has shown that not only are mixed ligand systems generally more stable than binary systems but mixed nitrogen-oxygen ligand systems are more stable than nitrogen- or oxygen-dominated systems. Thus since the selector ligand is a nitrogen donor, the transport ligand is most favourably an oxygen donor. This may well be the explanation as to why amino acids, which tend to form more stable complexes than organic acids, are not



S = selector ligand
T = transport ligand
A = terminal acceptor ligand

(SNi) = selector -nickel complex
(SNiT) = selector-transport-nickel complex
(NiT) = transport -nickel complex
(NiA) = acceptor-nickel complex

Figure 9·1 Proposed mechanism of nickel uptake by *Alyssum* species.

widely found as transport ligands. It is the breakdown of the ternary complex which releases the transport-nickel complex into the xylem. The selector ligand is then free to move back across the root membrane to repeat the process. This proposal of nickel complexation via a selector-transport-nickel complex has one particularly important advantage : free aquonickel(II) ions, the cause of nickel toxicity, are not formed internally ie. within the plant cells the nickel is always in a complexed form. The transport-nickel complex moves through the xylem to the leaf cells. Here it crosses the plasmalemma, cytoplasm and tonoplast to enter the vacuole. Whether the transport through the plasmalemma and tonoplast is active or passive is not known. When in the vacuole, the transport-nickel complex reacts with a terminal "acceptor" ligand, A, to form the acceptor-nickel complex, (NiA), and release the transport ligand. The acceptor-nickel complex accumulates within the vacuole where it cannot interfere with the cell's physiological processes. The tonoplast must, therefore, be impermeable to this complex. The transport ligand moves out of the vacuole, the tonoplast being permeable to this ligand, through the cytoplasm and plasmalemma into the phloem and hence to the roots where it diffuses back into the xylem. It is not impossible that in some cases (eg. citric acid) the transport and acceptor ligands could be the same species. The combination of roles would mean that a non-cyclic system is developed with the transport-nickel complex moving through to the vacuole where it accumulates while a fresh supply of ligand is always made available to the roots. This system would overcome Kersten's (1979) criticism of Lee's (1977) mechanism.

It is necessary now to relate the experimental observations with this theory. The following points have been noted in Chapters 7 and 8 and will be discussed: there is a specific "trigger-point" at which nickel accumulation begins; the shape of the uptake curve has a linear rise at low soil concentrations of nickel and near constancy at high

concentrations; the rate of uptake is relatively rapid; in the relative absence of nickel, cobalt can be accumulated whereas in the presence of nickel it is not; and organic acids can be used as transport ligands. This last point has been discussed within the theory of the mechanism.

In discussing the "trigger-point" in Chapter 7, two possible causes were mentioned: (1) that the presence of aquonickel(II) in the cells somehow triggered an accumulation mechanism or (2) the trigger was the presence of aquonickel(II) ions in the soil solution with all nickel at lower concentrations effectively bound to the soil substrate. As the proposed theory promotes the accumulation of nickel without the formation of aquonickel-(II) ions within plant tissues, it cannot presume to have these ions as the internal trigger of accumulation. It is therefore presumed that external concentrations of aquonickel-(II) ions are the "trigger".

The shape of the uptake curves (figs. 7.1 & 7.7) are also explicable. The lower linearly rising portion of the curve is determined by the amount of free aquonickel(II) ions in the soil solution ie. the availability of nickel for absorption. An equilibrium between the soil and the plant is presumed to exist for nickel. As the level of aquonickel-(II) ions increases in the soil solution, there is a concomitant increase in the amount of nickel absorbed and complexed by the plant. Thus in this region the view of Kersten (1979) that the availability of nickel governs its uptake is supported. The higher near-constant portion of the uptake curve would appear to indicate the saturation of some or all of the uptake mechanism. The most probable cause is the complexation of all the acceptor ligand available. When this occurs the transport-nickel complex will increase in concentration. The effect this has on the system will vary depending on whether the transport of this complex through the various membranes is active or passive. If passive transport is assumed, then the increase in transport-nickel

complex concentration will only occur until the concentrations within the vacuole and cytoplasm are in equilibrium with the concentration within the xylem. No accumulation will occur against a concentration gradient. The increased concentration of the transport-nickel complex will reduce the breakdown of the selector-transport-nickel complex. The selector ligand remains complexed and further absorption of nickel is blocked. If unidirectional active transport is assumed, then further accumulation of nickel could occur until all the transport ligand was complexed. The transport-nickel complex would then accumulate in the vacuole with the acceptor complex. Once all the transport ligand was complexed either the selector-nickel complex would accumulate within the root membrane or it would release aquonickel(II) ions. The accumulation of the selector-nickel complex would block further nickel absorption and, therefore, account for the constancy. The release of aquonickel(II) ions would allow further accumulation to occur, albeit limited, before toxicity effects were noticeable. Note that if the transport ligand is constantly renewed then nickel accumulation could continue rather than reach a constant level. If the active transport is bidirectional then a futile cycle of transport-nickel complex movement would occur. However, as the transport-nickel complex returned to the root xylem cannot complex with the selector-nickel complex on the membrane surface, the selector ligand is not relieved of its nickel and remains blocked. Thus no further nickel will be accumulated. The overall determinant of the amount of nickel hyperaccumulated by Alyssum species (and any other species with a similar form of uptake) is, therefore, the amount of acceptor ligand available. At this time it is impossible to determine the factor(s) which control the amount of ligand available. In certain circumstances, the amount of acceptor ligand may not act as a determinant. If organic acids (citric acid, malic acid) were both the transport and acceptor ligand, they could presumably continue to be made

available from the cell metabolism until the total nickel concentration became deleterious to the plant's health. It is indeed notable that the highest recorded concentrations of nickel in leaves are from New Caledonian species utilizing citric acid and malic acid as transport / acceptor ligands (Kersten, 1979).

Two factors of the proposed uptake mechanism can have primary influences on the rate of uptake. These are the amount of the selector ligand, and the rate of breakdown of the selector-transport-nickel complex. The first of these reasons is self-explanatory: if there are few selector ligands the uptake will be slow. The second reason is more complex. The selector-transport-nickel complex is the most stable of the complexes formed in the root ie. it is more stable than either the selector-nickel or the transport-nickel complexes. Thus the equilibrium

$$(SNiT) \rightleftharpoons S + (NiT)$$

favours the selector-transport-nickel complex, (SNiT). Removal of either or both of the products will promote the breakdown of this complex. In fact both are removed: the selector ligand, S, back to the external surface of the membrane and the transport-nickel complex, (NiT), to the leaves via the transpiration stream. Thus the selector-transport-nickel complex will break down. The rate at which the breakdown occurs cannot be determined, as yet (the selector ligand being an unknown compound), but it effectively governs the regeneration and hence cyclic availability of the selector ligand. Thus we cannot determine which of these two factors is governing the rate. The availability of selector ligands, which is effectively the permeability of the root membrane, controls the rate of uptake of nickel. It is unlikely, however, to control the amount of uptake as suggested by Lee (1977).

The uptake of cobalt in the relative absence of nickel is easily explained by this mechanism. Although the acceptor ligand greatly prefers nickel complexation to cobalt complexation, the latter is nonetheless capable of occurring. Thus when the selector ligand has no nickel to complex with (or less nickel than required to utilize all the selector ligands), the cobalt can be absorbed. When nickel is readily available the cobalt ion is competitively excluded from complexation and absorption. The trigger for cobalt uptake is, corresponding to the nickel trigger, the presence of free aquocobalt(II) ions in the soil solution. That this occurs at a lower total cobalt concentration in the soil is due to the less effective complexation of cobalt by the peat in the soil medium. The characteristic uptake pattern is also for the same reasons as in the uptake of nickel: the linearly rising portion being due to the limited availability in the soil and the near-constant portion being due to the saturation of the uptake mechanism. The difference in the total amounts of cobalt and nickel absorbed is presumably due to the difference in complex stabilities with the acceptor ligand. Because cobalt complexes are less stable than nickel complexes, the vacuolar complexation displacement reaction

$$(CoT) + A \rightleftharpoons (CoA) + T$$

will not be as far towards the acceptor complex as the corresponding nickel reaction. This will mean the effective saturation of the mechanism at a lower concentration of the acceptor-cobalt complex and hence of total cobalt.

The mechanism of nickel absorption and accumulation presented here succeeds that presented in Chapter 7. In the earlier proposed mechanism, only one ligand was considered to be involved whereas two or three are involved in the mechanism modified from Still and Williams (1980). The multiligand mechanism is more able to explain the selective

absorption of metals, the use of organic acids (rather than amino acids, peptides, etc.) as internal ligands and the characteristics of the uptake form than the single ligand theory. It is therefore preferred as the mechanism for nickel uptake by Alyssum hyperaccumulators.

The uptake of copper by Becium homblei has a similar form and presumably a similar explanation. However, the principal soluble complexing agents found for the copper were amino acids (Reilly, 1969, 1972, Reilly et al., 1970) so that it is possible that the selector ligand has a nitrogen-oxygen chelating system rather than a nitrogen-rich system as for nickel accumulation. The amino acids would then form a more stable selector-transport-copper complex with subsequent release of the transport-copper (amino acid-copper) complex. No aquocopper(II) ions have been detected in the plant tissues of Becium homblei. The transport-copper complexes move to the aerial tissues where a rather complex distribution occurs. The reasons for this distribution and the linkage to the copper uptake requires further study.

The cobalt and copper hyperaccumulators studied in this work (Chapter 4) showed a different form of uptake : the exclusion-breakdown form. The basic absorption mechanism may continue for this form of uptake but with one major additional factor, an active removal pump through the root membrane. This pump could be similar to the "sodium pump" found in plants (Clarkson, 1974). The internal concentration is held constant by this removal pump as the external concentration of the metal ion increases until the breakdown point is reached. At this concentration the removal pump has its capacity exceeded, ie. the influx of ions exceeds the pump's ability to remove the excess over the constant concentration required, and the internal concentration rises as a function of the soil concentration. The exact nature of this pump remains to be studied.

The linear uptake form, seen for non-essential elements in many non-tolerant species of plant (Timperley *et al.*, 1970b, Beckett and Davies, 1977), is explicable by having no removal pump and no specific acceptor in comparison to the exclusion-breakdown and rise-to-saturation forms. Essential elements generally show the exclusion-breakdown form in non-tolerant species (Timperley et al., 1970b, Beckett & Davies, 1977). It remains a moot point as to the line the development of tolerance would take if these species came into contact with large concentrations of any particular element. The cobalt and copper hyperaccumulators from Shaba have developed removal pumps, although in the case of copper this may be the strengthening of an already existent pump rather than a completely new development. The *Alyssum* species and *Becium homblei* have developed a specific internal complexation system rather than pumps to remove the excess ions. The reasons for the different adaptations are speculative. A species which found a use for the excess metal ions absorbed would be more likely to develop an internal complexation system than a species which had no use for the ions. Alternatively, the concentration of the ions in the soil solution may also affect the development. The more dilute the soil solution the lower the rate of influx to the root (a less effective concentration gradient) and therefore the better the chance that the pump can control the internal concentration. At higher concentrations the pump becomes ineffective. It is notable that the cobalt and copper hyperaccumulators from Shaba grow during the rainy season when the concentration of metal ions in the soil solution will be lowest. These hyperaccumulators have developed the pump system. *Becium homblei* grows in the same area but has the internal complexation system. The *Alyssum* species grow in a more arid climate and also have an internal complexation system. *Becium homblei* does not absorb copper to the extent that the *Alyssum* species absorb

nickel which may indicate the effects of external concentrations on the amount of ions taken up. However these latter species with internal complexation systems (and hence accumulating systems) can coexist with other species which do not accumulate. It is obviously possible for some species to develop removal pumps even in these circumstances. The capabilities of the removal pumps must therefore be dependent on the species. Accumulators with an internal complexation system may have had a weakly developed pump system which proved unsatisfactory so that an alternative system was required or found a use for the absorbed ion and developed an alternative system to maximize the absorption. The loss of the pump was an integral part of the development of the new system, irrespective of the reasons for the development, as it required metabolic energy to function but was without purposeful use. This sequence of events is outlined in figure 9.2. It is thus seen that the method of selective absorption with either a removal pump or an internal complexation system can explain the forms of uptake found in plants.

It is necessary to try and relate this theory to other studies on micronutrient uptake. Moore (1972) has reviewed the position for several elements. He concludes that a highly selective, active metabolic process is the most probable uptake mechanism. It is however recorded that the data for metabolic involvement is not unequivocal. An active uptake mechanism is generally considered necessary when two conditions are met: the internal root concentration exceeds the external soil solution concentration, and interference occurs to the uptake mechanism by metabolic inhibitors. In fact these conditions do not require an active uptake mechanism and the seemingly odd experimental results which have been observed may be explicable if an alternative mechanism is considered. The concentration criterion would be acceptable only if the nature of the metal inside the plant cells was the same as that which was involved externally

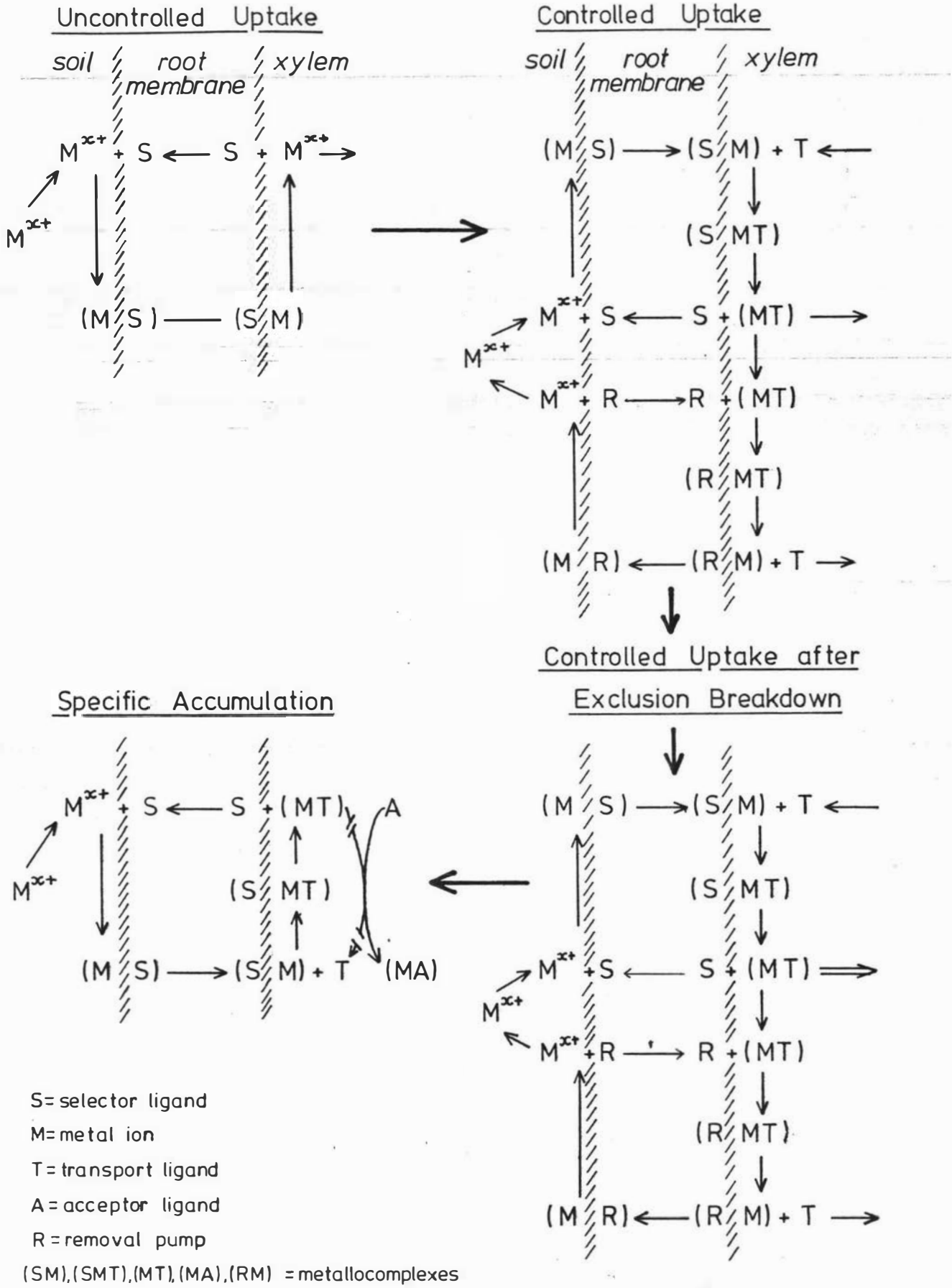


Figure 9·2 Proposed sequence for development of various uptake mechanisms.

in the uptake process. This requires that the equilibrium be between aquoions but many researchers have already noted the existence of inorganic ions as non-aquo complexes in plant tissues (Bradfield, 1976, Bremner & Knight, 1970, Broda, 1965, Ennis, 1962, Foy et al., 1978, Gomah & Davies, 1974, Hofner, 1970, Mills, 1954, Thompson & Tiffin, 1974, Tiffin, 1966 a, b, 1967, 1970, 1971, 1972, Tiffin & Brown, 1962, Tiffin & Thompson, 1974, Timberlake, 1959). The existence of these complexes reduces the aquoion concentration internally such that a concentration gradient for further entry still exists despite the fact that the overall internal concentration may exceed the external aquoion concentration. This process of complexation will thus allow internal accumulation without the aid of active transport against a supposed concentration gradient. The more stable the non-aquo complexes formed, the larger the internal concentration which can accumulate before a saturated equilibrium level will be reached. Despite this, there is still an element of active metabolic involvement as the ligands found are all of organic origin ie. are derived from the cell's metabolism. This may explain the second frequently observed fact that uptake is reduced in the presence of metabolic inhibitors. When the amount of ligand produced is decreased by metabolic inhibitors, less complex can be formed and the saturated equilibrium is reached at a lower overall concentration. Thus the data available do not rule out the uptake mechanisms proposed here. Furthermore the exceptions found (see Moore, 1972) in which active metabolic involvement in uptake is not found can be explained by a passive equilibrium across the root membrane (as proposed by those who performed the experiments, eg. Rathore et al., 1970). This is the uncontrolled uptake mechanism (fig. 9.2), while the dominant uptake mechanism, involving active metabolism, is the controlled uptake mechanism. Complexation of the ions entering by the uncontrolled mechanism must occur after release from the root membrane. Rhue (1976) also showed aluminium uptake to be without active metabolic

involvement. The reasons as to why the uptake mechanism for a particular element varies are undoubtedly environmental. The basic ratio between the plant's requirements and the soil's ability to deliver that requirement is most likely to be the major determinant but other factors, including genetic history, may also be involved.

While the uptake mechanisms may now be clearer, the deposition of the metal in all its forms needs further study. Nickel ions absorbed appear to migrate as complexes to the cell vacuoles for deposition with minor losses along the pathway, particularly to the cell walls (Lee, 1977, Kersten, 1979, Shaw, 1980). Cobalt and copper distribution is more varied (Chapter 5, also Reilly, 1969, Reilly et al., 1970 for copper). Only one-third of cobalt and one-eighth of copper is water-extractable, and hence vacuolar, with a further one-third of cobalt and one-half of copper acid-extractable, possibly as acid-soluble crystals eg. oxalates. A significant portion, 4-17%, of these metals may also be complexed to the cell wall. More studies are required on the nature of these metals within plant tissues.

The work presented in this thesis has helped to understand the processes by which plants can adapt to unfavourable edaphic environments. In combination with other published materials, a workable theory has been presented to account for the uptake of abnormally large concentrations of metals. It will be the responsibility of further studies to test this theory for validity. Further work on the distribution of the metals at the places of deposition is also required as is work on the nature (microcrystals, complexes, adsorbed ions, etc.) of the metal. Identification of ligands involved in the transport and acceptor complexes is also required. These latter studies will include the need for more systematic studies of cellular metabolites, including organic acids, amino acids including non-protein acids, and other secondary metabolites, by chemical methods of identification.

The lack of such data can be frustrating for the purposes of studies of metal ion complexes in plant tissues. These systematic studies of metal accumulation have yielded some interesting results and future studies can only serve to improve our understanding of the processes involved in the obtaining of metal ions from soils by all plants.

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APPENDICES

APPENDIX I. COOPERATING INSTITUTIONS AND PERSONS

(a) COOPERATING HERBARIA.

Country	Institution	Abbreviation
Canada	National Herbarium of Canada, Ottawa.	CAN
Cyprus	Cyprus Herbarium, Nicosia	CYP
Egypt	The Herbarium, Ministry of Agriculture, Dokki	CAIM
France	Muséum Nationale d'Histoire Naturelle, Paris	P
Germany (BRD).	Botanische Staatssammlung, München	M
Greece	Botanical Museum and Herbarium, Athens	ATHU
Iran	Plant Pests and Diseases Research Institute, Tehran	IRAN
Iraq	National Herbarium of Iraq, Baghdad	BAG
Israel	Department of Botany, Hebrew University, Jerusalem	HUJ
Pakistan	National Herbarium, Islamabad Pakistan Forest Institute, Peshawar	PPFI
Soviet Union	Kholodny Institute of Botany, Kiev Biological Faculty of Lomonosov University, Moscow	KW MW
Spain	Instituto "Antonio José Cavanilles", Jardin Botanico, Madrid	MA
Switzerland	Herbarium of A. Huber-Morath, Basel	
Turkey	Botanik Kürsüsü, University of Ankara	ANK
United Kingdom	British Museum, London	BM
	Royal Botanic Garden, Edinburgh	E
	Royal Botanic Gardens, Kew	K
United States	Arnold Arboretum, Cambridge, Mass.	A
	Brigham Young University Herbarium, Provo	BRY
	Missouri Botanical Garden, St. Louis	MO
	New York Botanical Garden	NY
	United States National Arboretum, Washington D.C.	NA
	University of Alaska Herbarium, Fairbanks	ALA

APPENDIX I. CONT.

(b) PERSONS COOPERATING IN SEED COLLECTIONS.

Corsica	Madame M. Conrad (Bastia)
Cyprus	Mr. M. Ch. Iacovides (Nicosia)
Denmark	Dr. P. Hartvig (Copenhagen)
Germany	Prof. Dr. H. Merxmüller (Munich)
Greece	Prof. M. Phitos (Athens)
	Mrs. P.H. Haritonidou (Athens)
	Mr. Th. Georgiadis (Athens)
	Messrs. M. and Y. Foskolou (Tinos)
Portugal	Dr. A.R. Pinto da Silva (Oeiras)
	Dr. E.M. Menezes de Sequeira (Oeiras)
Switzerland	Dr. W. Greuter (Geneva)
United Kingdom	Dr. J.L.S. Keesing (Kew)
	Dr. B. Mathew (Kew)

APPENDIX II. HYPERACCUMULATORS RECORDED IN THE LITERATURE. (a) NICKEL

YEAR	REFERENCE	SPECIES	FAMILY	LOCATION	HIGHEST CONTENT
1948	Minguzzi & Vergnano, 1948	*Alyssum bertolonii* Desv.	Cruciferae	Italy	1.22
1961	Doksopulo, 1961	*Alyssum murale* Waldst. & Kit.	Cruciferae	Georgia, USSR	0.71*
1969	Menezes de Sequeira, 1969	*Alyssum serpyllifolium* Desf. ssp. *lusitanicum* Dudley & Silva	Cruciferae	Portugal	0.52
1970	Wild, 1970, 1971	*Dicoma niccolifera* Wild	Asteraceae	Zimbabwe	0.21
1972	Severne & Brooks, 1972	*Hybanthus floribundus* (Lindl.) F. Muell.	Violaceae	Australia	(1.42)
	Cole, 1973	ssp. *floribundus*			1.60
	Severne, 1972	ssp. *adpressus* Bennett			0.13
		ssp. *curvifolius* Bennett			0.70
1974	Wild, 1974	*Pearsonia metallifera* Wild	Leguminosae	Zimbabwe	1.06**
	Jaffré & Schmid, 1974	*Geissois pruinosa* Brongn. & Gris.	Cunoniaceae	New Caledonia	1.36
		Homalium guillainii (Vieill.) Briq.	Flacourtiaceae	New Caledonia	2.90
		Hybanthus austrocaledonicus Schinz. & Guill.	Violaceae	New Caledonia	1.85
		H. caledonicus Turcz.	Violaceae	New Caledonia	(0.60)
	Jaffré, 1980	var. *caledonicus*			0.95
		var. *linearifolia* Urb.			1.75
		Psychotria douarrei (G. Beauv.) Däniker	Rubiaceae	New Caledonia	4.70
	Brooks, Lee & Jaffré, 1974	*Homalium kanaliense* (Vieill.)Briq.	Flacourtiaceae	New Caledonia	0.94

1976	Jaffré, Brooks et al.,1976	Sebertia acuminata Pierre ex Baill.	Sapotaceae	New Caledonia	1.17
1977	Brooks, Lee et al., 1977	Homalium austrocaledonicum Seeman	Flacourtiaceae	New Caledonia	0.18
		H. deplanchei (Vieill.) Warbu.	Flacourtiaceae	New Caledonia	0.19
		H. francii Guill.	Flacourtiaceae	New Caledonia	1.45
		H. mathieuanum (Vieill.) Briq.	Flacourtiaceae	New Caledonia	0.17
		H. rubrocostatum Sleumer	Flacourtiaceae	New Caledonia	0.12
	Brooks,& Wither, 1977	Rinorea bengalensis (Wall.) O.K.	Violaceae	S.E. Asia	1.75
	Brooks, Wither & Zepernick, 1977	R. javanica (Bl.) O.K.	Violaceae	S.E. Asia	0.22
	Wither & Brooks, 1977	Myristica laurifolia Spruce ex DC. var. bifurcata	Myristicaceae	Indonesia	0.11
		Planchonella oxyedra Dubard	Sapotaceae	S.E. Asia	1.96
		Trichospermum kjellbergii Burret	Tiliaceae	Celebes	0.38
1978	Brooks & Radford, 1978a	Alyssum alpestre L.	Cruciferae	W. Alps	0.36
		A. argenteum All.	Cruciferae	NW. Italy	1.08
		A. corsicum Duby	Cruciferae	Corsica	1.35
		A. euboeum Hal.	Cruciferae	Euboa	0.46
		A. fallacinum Hausskn.	Cruciferae	Crete	0.40
		A. heldreichii Hausskn.	Cruciferae	Greece	1.25
		A. markgrafii Schulze	Cruciferae	Albania	1.37
		A. obovatum (Meyer) Turcz.	Cruciferae	USSR	0.10

		A. robertianum Bernard ex Gren. & Godron	Cruciferae	Corsica	1.25
		A. smolikanum Nyár.	Cruciferae	Greece	0.66
		A. tenium Hal.	Cruciferae	Tinos	0.34
1979	Jaffré, Brooks & Trow, 1979	Geissois hirsuta Brongn.& Gris.	Cunoniaceae	New Caledonia	0.40
		G. intermedia Vieill. ex Pampan	Cunoniaceae	New Caledonia	2.29
		G. magnifica Vieill. ex Brongn. & Gris.	Cunoniaceae	New Caledonia	0.33
		G. montana Vieill. ex Brongn. & Gris.	Cunoniaceae	New Caledonia	0.57
		G. racemosa Labill.	Cunoniaceae	New Caledonia	0.10
		G. trifoliolata Guill.	Cunoniaceae	New Caledonia	0.63
	Jaffré, Kersten et al., 1979	Casearia silvanae (J.R. & G. Forster) Sleumer	Flacourtiaceae	New Caledonia	0.15
		Lasiochlamys peltata Sleumer	Flacourtiaceae	New Caledonia	1.10
		Xylosma boulindae Sleumer	Flacourtiaceae	New Caledonia	0.19
		X. confusum Guill.	Flacourtiaceae	New Caledonia	0.16
		X. dothioense Guill.	Flacourtiaceae	New Caledonia	0.18
		X. kaalense Sleumer	Flacourtiaceae	New Caledonia	0.19
		X. molestum Sleumer	Flacourtiaceae	New Caledonia	0.11
		X. pancheri Guill.	Flacourtiaceae	New Caledonia	0.11
		X. peninsulare Guill.	Flacourtiaceae	New Caledonia	0.13

		X. serpentinum Sleumer	Flacourtiaceae	New Caledonia	0.15
		X. tuberculatum Sleumer	Flacourtiaceae	New Caledonia	0.16
		X. vincentii Guill.	Flacourtiaceae	New Caledonia	0.38
	Kersten et al., 1979	Phyllanthus aeneus Baill.	Euphorbiaceae	New Caledonia	0.21
		P. balansaeanus Guill.	Euphorbiaceae	New Caledonia	0.18
		P. cataractarum Muell.	Euphorbiaceae	New Caledonia	0.15
		P. chrysanthus Baill.	Euphorbiaceae	New Caledonia	0.12
		P. induratus Moore	Euphorbiaceae	New Caledonia	0.15
		P. kanaliensis Baill.	Euphorbiaceae	New Caledonia	0.11
		P. maytenifolius Moore	Euphorbiaceae	New Caledonia	0.14
		P. ngoyensis Schlect.	Euphorbiaceae	New Caledonia	0.96
		P. peltatus Guill.	Euphorbiaceae	New Caledonia	0.28
		P. serpentinus Moore	Euphorbiaceae	New Caledonia	3.81
	Vergnano Gambi &	Cardamine resedifolia L.	Cruciferae	W. Alps	0.11
	Gabbrielli, 1979	Thlaspi rotundifolium (L.) Gaud.	Cruciferae	W. Alps	0.63
1980	Jaffré, 1980	Argophyllum grunowii Zahlbr.	Escallionaceae	New Caledonia	0.14
		A. laxum Schltr.	Escallionaceae	New Caledonia	0.19
		Baloghia sp.	Euphorbiaceae	New Caledonia	0.54
		Cleidion cf. lasiophyllum Pax & Hoffm.	Euphorbiaceae	New Caledonia	0.99

		Agatea deplanchei Brongn. & Gris.	Violaceae	New Caledonia	0.25
		Oncotheca balansae Baill.	Oncothecaceae	New Caledonia	0.25
		Pancheria engleriana Schltr.	Cunoniaceae	New Caledonia	0.63
	Reeves, Brooks & Press, 1980	*Peltaria emarginata* (Boiss.) Hausskn.	Cruciferae	Greece	3.44
	Reeves, Brooks & McFarlane, 1981	*Streptanthus polygaloides* Gray	Cruciferae	California	1.48
	Reeves, Brooks & Dudley, 1981	*Bornmuellera baldacci* (Degen) Heywood ssp. *baldacci*	Cruciferae	Balkans	2.13
		ssp. *markgrafii*			2.73
		ssp. *rechingeri* Greuter			1.20
		B. glabrescens (Boiss. & Bal.) Cullen & Dudley	Cruciferae	Turkey	1.92
		B. × *petri* Greuter, Charpin & Dittrich	Cruciferae	Greece	1.14
		B. tymphaea (Hausskn.) Hausskn.	Cruciferae	Greece	3.12
	Reeves & Brooks, unpublished data	*Thlaspi japonicum* Boiss.	Cruciferae	Japan	
		T. montanum L.	Cruciferae	USA	
		var. *montanum*			1.71
		var. *californicum* (Watson) Holmgren			1.16
		var. *siskiyouensis* Holmgren			2.46
		plus approx. 30 taxa of European *Thlaspi*.			

*Data from Brooks & Radford, 1978 **Data from Lee, 1977.

All contents expressed as percent dry weight.

APPENDIX. II CONT. (b) COBALT

YEAR	REFERENCE	SPECIES	FAMILY	LOCATION	HIGHEST CONTENT
HYPERACCUMULATORS					
1977	Brooks, 1977	Haumaniastrum robertii (Robyns) Duvign.& Plancke	Lamiaceae	Shaba	10222
1978	Malaisse & Grégoire, 1978	Aeolanthus biformifolius De Wild.	Lamiaceae	Shaba	4300
		Lindernia damblonii Duvign.	Scrophulariaceae	Shaba	1000
		L. perennis Duvign.	Scrophulariaceae	Shaba	2300
VERY STRONG ACCUMULATORS					
1959	Duvigneaud, 1959	Crotalaria cobalticola Duvign. & Plancke	Leguminosae	Shaba	530
1960	Kubota et al., 1960	Nyssa sylvatica Marsh var. biflora (Walt.) Sarg.	Nyssaceae	S. USA.	845
1977	Brooks, McCleave & Schofield, 1977	Nyssa sylvatica Marsh var. sylvatica	Nyssaceae	S. USA.	530
	Brooks, Wither & Zepernick, 1977	Rinorea bengalensis (Wall.) O.K.	Violaceae	SE. Asia	545
		R. javanica (Bl.) O.K.	Violaceae	SE. Asia	670
1978	Malaisse & Grégoire, 1978	Anisopappus hoffmannianus Hutch.	Asteraceae	Shaba	700
		Buchnera metallorum Duvign. & Van Bock.	Scrophulariaceae	Shaba	500
		Eragrostis boehmii Hack.	Gramineae	Shaba	600

		Haumaniastrum katangense (S. Moore) Duvign. & Plancke	Lamiaceae	Shaba	864
		Vigna dolomitica Wilcz.	Leguminosae	Shaba	600
1979	Kersten et al., 1979	Phyllanthus ngoyensis Schlect.	Euphorbiaceae	New Caledonia	796

All contents expressed as µg/g, dry weight.

APPENDIX. II CONT. (c) COPPER

YEAR	REFERENCE	SPECIES	FAMILY	LOCATION	HIGHEST CONTENT
		HYPERACCUMULATORS			
1963	Duvigneaud & Denaeyer-de-Smet, 1963	Ascolepis metallorum Duvign. & Léonard	Cyperaceae	Shaba	1200
		Silene cobalticola Duvign. & Plancke	Caryophyllaceae	Shaba	1660
		Haumaniastrum robertii (Robyns) Duvign. & Plancke	Lamiaceae	Shaba	1960
1966	Dykeman & De Sousa, 1966	Abies balsamea (L.) Mill	Pinaceae	Canada	1120
1975	Wu et al., 1975	Agrostis stolonifera L.	Gramineae	U.K.	1100
1978	Malaisse & Grégoire, 1978	Aeolanthus biformifolius De Wild.	Lamiaceae	Shaba	13700
		Eragrostis boehmii Hack.	Gramineae	Shaba	2800
		Lindernia perennis Duvign.	Scrophulariaceae	Shaba	6000
		Vigna dolomitica Wilcz.	Leguminosae	Shaba	3000
		VERY STRONG ACCUMULATORS			
1963	Duvigneaud & Denaeyer-de-Smet, 1963	Pandiaka metallorum Duvign. & Van Bock.	Amaranthaceae	Shaba	740
1966	Dykeman & De Sousa, 1966	Larix lariciana (Du Roi) Kock.	Pinaceae	Canada	726

		Ledum groenlandicum Oedr.	Ericaceae	Canada	500
1972	Ernst, 1972	Indigofera dyeri Britt.	Leguminosae	Zimbabwe	890
1977	Brooks, McCleave & Malaisse, 1977	Crotalaria peschiana Duvign. & Timp.	Leguminosae	Shaba	705
	Hogan et al., 1977	Agrostis gigantea Roth.	Gramineae	Ontario	935
1978	Brooks, Wither & Westra, 1978	Coleus scutellarioides Benth.	Lamiaceae	Indonesia	500
		Cyathula prostrata Blume.	Amaranthaceae	Indonesia	553
		Laportea ruderalis Gaud.	Urticaceae	Indonesia	600
	Malaisse & Grégoire, 1978	Faroa chalcophila Taylor	Gentianaceae	Shaba	665
		Hibiscus rhodanthus Gürke	Malvaceae	Shaba	500
		Justicia elegantula S. Moore	Acanthaceae	Shaba	864
		Lindernia damblonii Duvign.	Scrophulariaceae	Shaba	800

All contents expressed as µg/g, dry weight.

APPENDIX III. HYPERACCUMULATORS DISCOVERED IN THIS WORK.

(a) NICKEL

SPECIES	LOCATION	HIGHEST CONTENT
Alyssum akamasicum Burtt	Cyprus	0.91
A. anatolicum Hausskn. ex Nyár.	Turkey	0.82
A. callichroum Boiss. & Buhse	Turkey	1.09
A. caricum Dudley & Hub.-Mor.	Turkey	0.61
A. cassium Boiss.	Turkey, Syria	2.00
A. chondrogynum Burtt	Cyprus	1.63
A. cilicicum Boiss. & Bal.	Turkey	1.37
A. condensatum Boiss. & Hausskn. ssp. flexibile (Nyár.) Dudley	Turkey, Syria	0.23
A. constellatum Boiss.	Turkey, Iraq	1.81
A. crenulatum Boiss.	Turkey, Syria	1.04
A. cypricum Nyár.	Cyprus, Turkey	2.36
A. davisianum Dudley	Turkey	1.96
A. discolor Dudley & Hub.-Mor.	Turkey	1.17
A. dubertretii Gomb.	Turkey	1.65

A. eriophyllum Boiss. & Hausskn.	Turkey	1.15
A. floribundum Boiss. & Bal.	Turkey	0.77
A. giosnanum Nyár.	Turkey	0.74
A. huber-morathii Dudley	Turkey	1.35
A. janchenii Nyár	Albania	0.96
A. lesbiacum (Cand.) Rech. f.	Lesvos	2.24
A. masmenaeum Boiss.	Turkey	2.43
A. oxycarpum Boiss. & Bal.	Turkey	0.73
A. peltarioides Boiss. ssp. virgatiforme (Nyár.) Dudley	Turkey	0.53*
A. peltarioides Boiss. ssp. undetermined	Turkey	0.76*
A. penjwinensis Dudley	Iraq	0.79
A. pinifolium (Nyár.) Dudley	Turkey	1.26
A. pterocarpum Dudley	Turkey	0.67
A. samariferum Boiss. & Hausskn.	Turkey, Syria	0.67
A. serpyllifolium Desf. ssp. malacitanum Riv.-God.	Spain	0.85
A. singarense Boiss. & Hausskn.	Iraq	0.13
A. syriacum Nyár.	Turkey, Syria	1.02
A. trapeziforme Bornm. ex Nyár.	Turkey	1.19
A. troodii Boiss.	Cyprus	0.98
A. virgatum Nyár.	Turkey	0.62

*Unpublished data from R.D. Reeves. All contents expressed as percent dry weight.

The genus Alyssum is of the family Cruciferae.

APPENDIX III. CONT. (b) COBALT

SPECIES	FAMILY	LOCATION	HIGHEST CONTENT
HYPERACCUMULATORS			
Alectra welwitschii Hemsl.	Scrophulariaceae	Lubumbashi	1589
Anisopappus davyi S. Moore	Asteraceae	Fungurume	2646
Anisopappus sp.	Asteraceae	Mindingi	1211
Buchnera metallorum Duvign. & Van Bock.	Scrophulariaceae		1508
Bulbostylis mucronata C.B.Cl.	Cyperaceae	Lubumbashi	2127
Crassula alba Forsk.	Crassulaceae	Fungurume	1625
C. vaginata Eckl. & Zeyh.	Crassulaceae	Fungurume	1185
Cyanotis longifolia Benth.	Commelinaceae	Fungurume	4197
Haumaniastrum homblei (De Wild.) Duvign. & Plancke	Lamiaceae	Fungurume	1625
H. katangense (S. Moore) Duvign. & Plancke	Lamiaceae	Lubumbashi	2241
Sopubia dregeana Benth.	Scrophulariaceae	Fungurume	1090

VERY STRONG ACCUMULATORS			
Aeolanthus rosulifolius Duvign. & Denaeyer.	Lamiaceae	Fungurume	753
A. saxatilis Duvign. & Denaeyer.	Lamiaceae	Fungurume	996
Becium sp. 1	Lamiaceae	Fungurume	552
Begonia princeae Gilg. var. princeae	Begoniaceae	Fungurume	813
Commelina zigzag Duvign. & Dewit.	Commelinaceae	Fungurume	654
Commelina sp.	Commelinaceae	Fungurume	939
Ipomoea alpina Rendle	Convolvulaceae	Fungurume	641
Monadenium aff. chevalieri N.E. Br.	Euphorbiaceae	Fungurume	584
Phragmanthera rufescens (DC) Balle var. cornetii (Dewèvre) Balle	Loranthaceae	Fungurume	765
Spuriodaucus marthozianus (Duvign.) Duvign.	Umbelliferae	Fungurume	768

All contents expressed as µg/g dry weight.

APPENDIX III. CONT. (c) COPPER

SPECIES	FAMILY	LOCATION	HIGHEST CONTENT
HYPERACCUMULATORS			
Aeolanthus rosulifolius Duvign. & Denaeyer.	Lamiaceae	Fungurume	1113
Buchnera metallorum Duvign. & van Bock.	Scrophulariaceae		3518
Bulbostylis abortiva (Steud.) C.B.Cl.	Cyperaceae	Mindingi	1523
B. mucronata C.B.Cl.	Cyperaceae	Lubumbashi	5701
Commelina zigzag Duvign. & Dewit.	Commelinaceae	Fungurume	1214
Haumaniastrum katangense (S. Moore) Duvign. & Plancke	Lamiaceae	Lupoto	2135
Pandiaka metallorum Duvign. & van Bock.	Amaranthaceae	Fungurume	6270
Triumfetta digitata (Oliv.) Hutch. & Sprague	Tiliaceae	Ruashi	1057
Unidentified sp.	Asteraceae	Lupoto	1487
VERY STRONG ACCUMULATORS			
Acalypha cupricola Robyns	Euphorbiaceae	Lupoto	905
Becium sp. 1	Lamiaceae	Lupoto	766
Becium sp. 2	Lamiaceae	Lupoto	884
Cyanotis longifolia Benth.	Commelinaceae	Fungurume	608
Haumaniastrum sp.	Lamiaceae	Lupoto	542
Ipomoea sp. 1	Convolvulaceae	Fungurume	745

All contents expressed as µg/g dry weight.

APPENDIX IV. SOME TERMINOLOGY OF ACCUMULATION

ACCUMULATOR: a species which accumulates an element to greater than *normal concentrations.

ANOMALOUS CONCENTRATIONS: concentrations recorded for most plants growing on soils enriched in the element under consideration. Often taken as equal to or exceeding a concentration ten times the mean of *normal concentrations.

DIFFERENTIAL HYPERACCUMULATION: *hyperaccumulation of more than one element by a species but not by an individual specimen.

DOUBLE HYPERACCUMULATION: *hyperaccumulation of two elements by one species. May be either *differential or *simultaneous.

ELEVATED CONCENTRATIONS: subjective term, varying with context.

HIGH CONCENTRATIONS: subjective term, varying with context.

HYPERACCUMULATION: accumulation of an element above *very strong accumulation. Appears as a second population in a cumulative frequency plot of recorded concentrations of the element. For cobalt, copper and nickel, the basic criterion is a dry weight concentration of 1,000 µg/g.

HYPERACCUMULATOR: a species in which *hyperaccumulation occurs.

NORMAL CONCENTRATIONS: concentrations of an element recorded in plants growing on soils which are not enriched in that element.

APPENDIX IV CONT.

SIMULTANEOUS HYPERACCUMULATION: × hyperaccumulation of more than one element within an individual specimen of a **species.**

STRONG ACCUMULATION: accumulation of an element above the general range of ×anomalous concentrations. For cobalt, copper and nickel, the basic criterion is a dry weight concentration of 100 µg/g.

VERY STRONG ACCUMULATION: accumulation of an element above ×strong accumulation. For cobalt, copper and nickel, the basic criterion is a dry weight concentration of 500 µg/g.

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