SOME OBSERVATIONS ON THE ECOLOGY AND PHYTOCHEMISTRY OF NICKEL-ACCUMULATING *ALYSSUM* SPECIES FROM THE IBERIAN PENINSULA

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ABSTRACT

Experiments were carried out on the tolerance to, and uptake of nickel by Iberian subspecies of A. serpyllifolium. Two of these subspecies, the serpentinic-endemics s.sp. lusitanicum (from Bragança, Portugal) and s.sp. malacitanum (from Málaga, Spain) were hyperaccumulators (>1,000 μg/g in dried leaves) of nickel. Their precursor, s.sp. serpyllifolium (from Granada, Spain) was a non-accumulator of this element.

Seeds of the two serpentine-endemics germinated extensively in nickel concentrations up to 12,000 μg/g (1.2%) whereas s.sp. serpyllifolium only germinated in nickel concentrations of up to 60 μg/ml.

Tolerance tests involving measurement of new root lengths of excised seedlings placed in varying nickel concentrations again showed much greater tolerance of the two serpentinophytes. In both series of experiments, the order of tolerance was: s.sp. lusitanicum > s.sp. malacitanum > s.sp. serpyllifolium.

In pot trials involving seedlings of s.sp. malacitanum grown in mixtures containing varying amounts of calcium, magnesium and nickel, the most important findings were that nickel uptake is somewhat stimulated by an excess of calcium in the substrate. This relationship was confirmed by inter-species and intra-species analyses of naturally-occurring plants. Enhanced calcium uptake concomitant with nickel uptake by hyperaccumulators results in a higher (more favourable) Ca/Mg ratio and thereby counteracts one of the unfavourable edaphic effects of serpentine soils.

The form of nickel in leaves of the three Iberian subspecies was investigated. Nickel existed mainly as a water-soluble polar complex in the vacuoles. Small concentrations of nickel did however exist in cell fractions, particularly in the mitochondria where enzyme systems are located. GLC studies on the purified nickel complexes showed that this element is associated principally with

malic and malonic acids which are present in high concentrations in the hyperaccumulators but not in s.sp. serpyllifolium.

It is suggested that production of malic acid is a mechanism whereby hyperaccumulators can tolerate unfavourable edaphic factors such as nickel-rich soils. Presence of nickel in the mitochondria blocks the citric acid cycle by deactivating malic dehydrogenase leading to build-up of malic acid in the vacuoles which then absorbs excess nickel by a complexing reaction and leads to its diffusion back into the vacuoles from the mitochondria, hence unblocking the citric acid cycle. Malonic acid also blocks the cycle and leads to a reduced level of malic acid and hence lesser tolerance to nickel. This is shown to be the case for s.sp. malacitanum which contains more malonic acid than s.sp. lusitanicum and is also less tolerant to nickel. It is postulated that the chemical evidence suggests that s.sp. lusitanicum and s.sp. malacitanum are sufficiently different chemically to lend weight to the argument that the latter should be promoted to full specific rank as has already been done for s.sp. lusitanicum.

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A typical *Alyssum* hyperaccumulator of nickel (*A. robertiannum* Bern. ex. gren.) from section Ordontarrhena.

1. INTRODUCTION

Nickel (atomic no. 28) is situated among a row of essential elements viz. vanadium to zinc; and it has been suggested by some authors (Mertz, 1970; Schroeder, 1968; Schroeder et al., 1971; Shaw, 1960) that it may have an essential role in plant nutrition. Nielson (1971); Nielson and Ollerich (1974) and Sunderman et al. (1972) have shown that nickel may play a role in animal nutrition. Nomoto et al. (1971, 1973) and Sunderman et al. (1972) have confirmed the existence of a nickel-containing metalloprotein (nickeloplasmin) in human and rabbit serum. Nickel readily forms several coordination structures and therefore has its use in a biochemical role as shown by Shaw (1960) and Mertz (1970). These studies have shown that nickel may be necessary for the healthy growth of some plants, but to date, no evidence has been shown that in its absence, growth is inhibited.

Nickel is distributed in the lithosphere and in the biosphere which constitute about 0.008% of the earth's crust. The nickel concentration is approximately 0.01% in igneous rocks. Among igneous rocks, ultrabasic rocks containing iron and magnesium, and little or no silica are the main sources of nickel. Nickel concentrations range from 0.016% in basalts and 'gabbros' to an average of 0.20% in peridotites. Fig. 1.1 shows average nickel content of various sedimentary and igneous rocks.

The nickel content of plants is usually below 1 μg/g on a dry mass basis, although plants growing over ultrabasic rocks such as serpentinites usually contain up to 100 μg/g. Vanselow (1966) reported 0.05 to 5 μg/g for field-grown crops and natural vegetation. Connor et al. (1975) reported mean values of 0.20-4.5 μg/g for about 2,000 specimens of field crops and natural vegetation from the United States. A lot of confusion exists in the literature because of the practice of reporting element levels either on an ash mass or dry mass basis. In this thesis, all data will be reported on a dry mass basis.

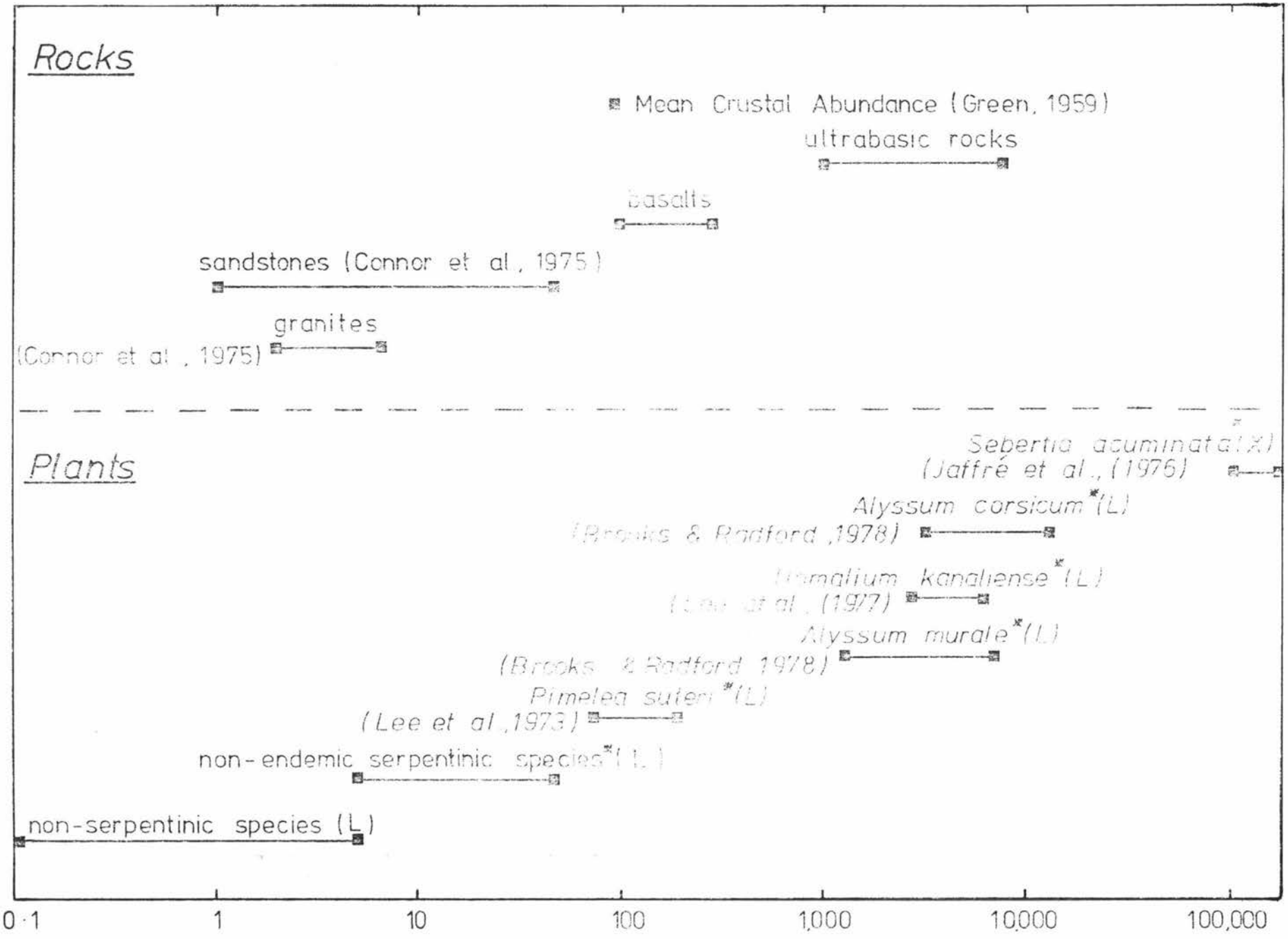


FIGURE 1.1 Average nickel content of various sedimentary and igneous rocks.

Although the nickel content of plants growing over 'normal' soils does not usually exceed 5 μg/g, a small number of plants growing on ultrabasic substrates can accumulate and tolerate ten times the amount of nickel possible by 'normal' plants growing over ultrabasic nickeliferous substrates.

Minguzzi and Vergnano (1948) were the first to discover high nickel levels in plants. These workers found up to 12,200 μg/g (12.2%) nickel in leaves of Alyssum bertolonii Desv. from the Impruneta ultrabasic region near Florence, Italy. This value was of an order of magnitude higher than for other plants in the area and certainly far higher than any value reported before that time. Brooks et al. (1977) have termed these plants hyperaccumulators i.e. plants containing over 1,000 μg/g (0.1%) nickel on a dry mass basis. These plants are hence differentiated from other plants growing on ultrabasic rocks.

Haselhoff (1893) discovered that nickel is toxic to most types of vegetation. Hence, with the discovery of these hyperaccumulators, some interest has been created amongst scientists in this area of research. The implications of high nickel levels in ultrabasic rocks have been widely discussed. Serpentinite rocks are unfavourable substrates for plant growth for a variety of reasons. Firstly, there is a very high magnesium content (approx. 20%) which can restrict calcium uptake by plants (Kruckeberg, 1954). There is also a high concentration of chromium and cobalt, and generally low levels of essential nutrients such as nitrogen, phosphorus and potassium. For example, Lee, Brooks et al. (1977) found only 99 ± 48 μg/g potassium and 187 ± 60 μg/g phosphorus in lateritic soils overlying serpentinites in New Caledonia. The high levels of nickel and chromium have a direct toxic action on the plants (Robinson et al. 1935; Soane and Saunder, 1959). The literature shows nickel to be widely recognised as harmful to a wide variety of plants at relatively low concentrations. Symptoms of nickel toxicity include chlorosis of leaves, followed by necrosis, stunted growth of shoot and root

systems, unusual spottings and other growth abnormalities. The extreme case is the death of the whole plant (Hewitt and Bolle-Jones, 1952; Vergnano, 1950; Crooke and Knight, 1955). Many plant species are able to withstand elevated nickel levels in the soil and these taxa possess certain mechanisms that seem to exclude nickel from enzyme-active sites, the poisoning of which is thought to be the most important toxic action (Bowen, 1966).

Populations of certain plant species growing on serpentine soils are seen to be different from those on non-serpentine soils. The populations represent edaphic ecotypes. Those on serpentine are adaptable to their high Mg/Ca ratio and high concentrations of nickel, chromium and cobalt. Plants of Gilia capitata were grown on serpentine and non-serpentine soils. Those on the latter failed to grow (Kruckeberg, 1951). The toxicity of nickel in plants can be reduced by making it unavailable to the plant itself. Hunter and Vergnano (1953), Vergnano (1953), Chang and Sherman (1953) and Crooke (1956) achieved this by adding lime, thus raising the pH of the soil. Halstead, Finn and Maclean (1969) found, that by addition of lime, extractable amounts of nickel in soils and nickel concentrations in plant tissues were reduced; but were increased when phosphate was added.

Serpentine-tolerant plants such as these hyper-accumulators have several potential uses: infertile serpentine areas, industrial sites, old mine dumps can be reclaimed i.e. made fertile. This can be achieved by firstly, making the soils more fertile by depressing nickel uptake by plants i.e. addition of lime increases pH . De Kock and Mitchell (1957) have shown that uptake of ionic nickel is greatly reduced. It is therefore possible that, adding organic matter to the soil, reduces nickel availability to the overlying vegetation. However, this method of approach should be thoroughly investigated before such a venture is considered. Furthermore, these areas could be presumably populated with nickel-accumulating plants in these infertile soils. Harvesting these plants

would result in the loss of nickel from the soils, which could then be used for more useful crops. Man-made mine-dumps can be revegetated in the same manner. Hill (1973) has shown that Rhodesian mine dumps can be revegetated by choice of suitable species as has also been shown by the continuing work of Bradshaw and associates (e.g. Smith and Bradshaw, 1970) using Agrostis tenuis and other tolerant species.

Another important technique is the use of nickel accumulating plant species as an indication of the amount of nickel in the soils. This technique, known as biogeochemical prospecting, was first developed by Tkalich (1938) in Siberia and by Brundin (1939) in Western Europe. Malyuga (1964) and Brooks (1972) have since reviewed this technique. Timperley et al. (1970) have noted that this technique tends to be unreliable when essential elements for plant nutrition, such as zinc and copper are to be considered. However, the technique works best for non-essential elements like nickel and uranium. Biogeochemical prospecting for nickel has been relatively successful as demonstrated by the works of Lyon et al. (1968), Timperley et al. (1970, 1972a, 1972b) in New Zealand and Severne (1972), Severne and Brooks (1972), Nielson et al. (1973) and Cole (1973) in Australia. Biogeochemical prospecting for nickel in New Caledonia has also been carried out by Lee, Brooks et al. (1977).

The use of plants as indicators of ore deposits has long been used (Cannon, 1960). The presence of such indicator plants on a particular terrain always indicates the existence of a given element in the substrate. Cannon (1971) has listed 122 indicator plants of economic ore deposits. Examples of indicator plants are: - Viola calaminaria for zinc in Belgium and Germany, Viscaria alpina for copper in Norway and Silene cobalticola for cobalt in Zaire. A good list of indicator plants has been given by Brooks (1979). Brooks et al. (1977) used species of Homalium and Hybanthus to locate nickeliferous rocks in the tropical and warm-temperate regions of the world.

Rinorea bengalensis has been used to locate some ultrabasic areas of South East Asia (Brooks and Wither, 1977). From this work, these authors were successful in differentiating between different substrates, taking into account the nickel/cobalt ratios in the plants contained thereon. Hyperaccumulators of nickel are of interest to geobotanists. Since these plants are found only over ultrabasic substrates, they are useful for geobotanical and biogeochemical methods of exploration .

Phytochemists find hyperaccumulators of interest because of the ease with which their nickel complexes can be extracted in macroamounts without the need for radiochemical studies. It is therefore essential to have a thorough knowledge as to how these plants extract, transport and translocate nickel from their substrates. This information, when considered with a knowledge of the chemical form of the complex within the plant tissues, may be of use in the development of technology for low-energy processes that could be used for the extraction of nickel from its ores.

The genus Alyssum is of special interest. Alyssum bertolonii Desv., was the first hyperaccumulator of nickel to be discovered. Minguzzi and Vergnano (1948) reported it to contain up to 1.22% nickel in leaves, 0.31% in roots, 0.54% in flowers, 0.58% in fruits and 0.61% in seeds. The extent of this uptake is surprising as the soil had only 0.33% nickel. The same workers found that there was a constant Ca/Ni ratio (refer section 4.2) for all parts of the plants. They concluded that this relationship was such as to counteract the excess of magnesium in the soil. They emphasised that A. bertolonii may have a role in geobotanical prospecting. Vergnano (1958) considered that the morphological changes in A. bertolonii and other species were due to excess levels of metals such as nickel, chromium and cobalt, rather than high magnesium and low calcium levels in the soils. Vergnano Gambi (1965) used A. bertolonii to find the distribution of the various

elements - manganese, copper, iron, cobalt, boron; and found relatively low amounts of these elements. The initial concentration was high (1.35%), but the cobalt concentration was only 0.006%. Closely following the work on A. bertolonii, Doksopulo (1961) reported over 10% of nickel in ashed leaves of A. murale Waldst. et Kit. from the USSR. No conversion factor to dry weight was given, but it is probable that the dried leaves contained over 1% of nickel. This work was carried out in connection with biogeochemical prospecting at talc deposits in Georgia.

Another species of Alyssum known to be a hyperaccumulator of nickel is A. serpyllifolium Desf. s.sp. lusitanicum Dudley and P. Silva from North-east Portugal (Menezes de Sequeira, 1969) which was reported as having 0.52% of nickel. It was concluded that, as this species was found growing over agricultural land, it was an indication of deterioration of the land due to excessive tillage. This species has since been recognised as a separate taxon.

In a recent study, Brooks and Radford (1978) analysed all 64 European species of Alyssum (Ball and Dudley, 1964). In addition to three previously recorded hyperaccumulators (A. bertolonii, A. murale, A. serpyllifolium s.sp. lusitanicum) a further 11 were added to the list. All the hyperaccumulating species were from Section Odontarrhena of the genus and included A. alpestre, A. argenteum, A. corsicum, A. euboeum, A. fallacinum, A. heldreichii, A. markgrafii, A. robertianum, A. smolikanum, A. tenium. A. argenteum, A. bertolonii, A. corsicum, A. heldreichii, A. markgrafii and A. robertianum had nickel contents exceeding over 10,000 µg/g (1%) (see Figure 1.2).

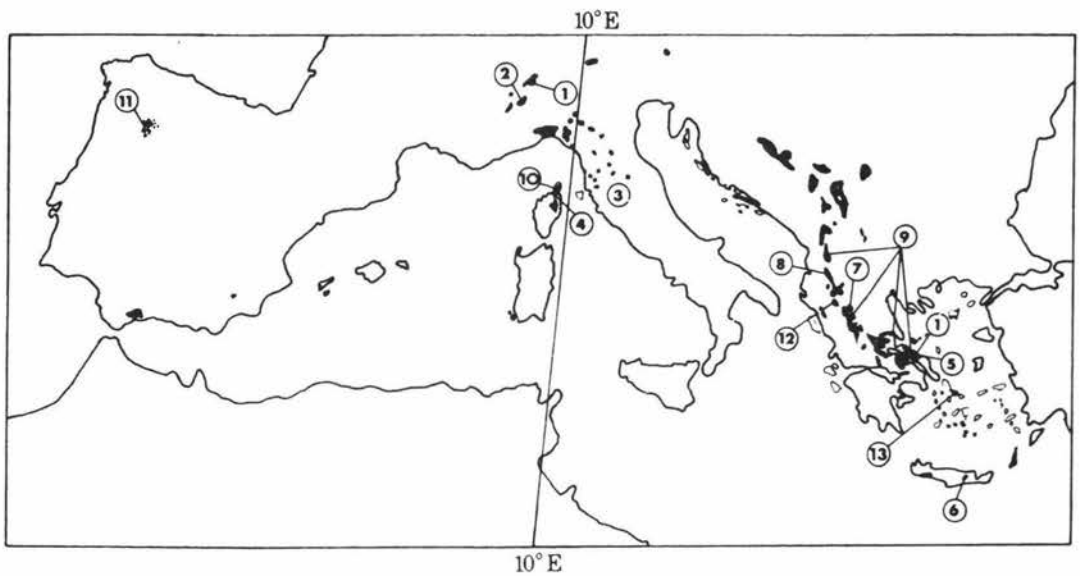


FIGURE 1.2 Geographical distribution of *Alyssum* species with nickel contents exceeding 1000 µg/g (0.1 %) on a dry mass basis. Dark areas indicate ultrabasic rocks.

1, *A. alpestre*
2, *A. argenteum*
3, *A. bertolonii*
4, *A. corsicum*
5, *A. euboeum*
6, *A. fallacinum*
7, *A. heldreichii*
8, *A. markgrafii*
9, *A. murale*
10, *A. robertianum*
11, *A. serpyllifolium* s.sp. *lusitanicum*
12 *A. smolikanum*
13, *A. tenium*

Brooks et al. (1979) extended the work to include 166 species of Alyssum (168 species are listed by Dudley (1964) in his synopsis of the genus) and identified a total of 44 hyperaccumulators, all of which were found among the 71 species of section Odontarrhena. It is a remarkable feature to note this large concentration of hyperaccumulators within a single section of a single genus. The overall worldwide distribution of the species comprising Alyssum section Odontarrhena is shown in Fig. 1.3.

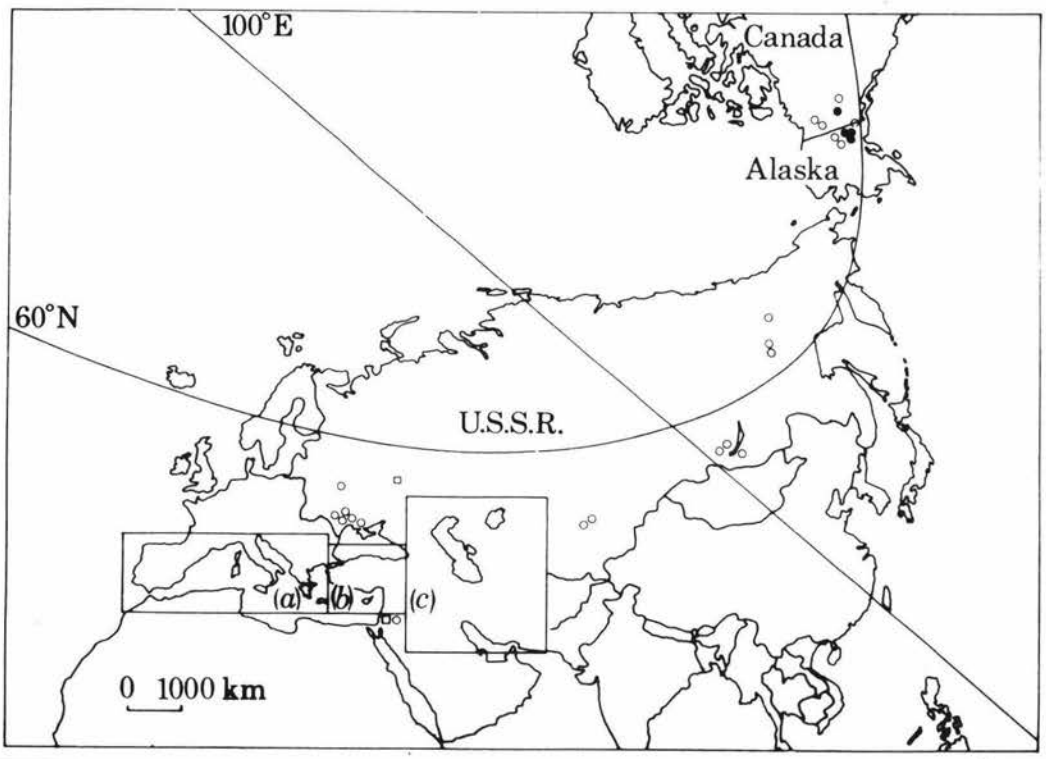


FIGURE 1.3 The worldwide geographical distribution of species of Alyssum section Odontarrhena
(a) Southern and Eastern Europe (see Fig. 1.2).
(b) Turkey and the eastern Mediterranean (see Brooks et al. 1979) (c) eastern Anatolia, Iraq, Iran and the Georgian Caucasus of USSR (see Brooks et al. 1979).

□ individual specimens containing nickel concentrations of 1,000-10,000 μg/g;

● individual specimens containing nickel concentrations of 100-999 μg/g;

o individual specimens containing nickel concentrations of < 100 μg/g. Alaskan and Canadian Yukon specimens are exclusively of A. americanum Greene.

The genus Alyssum contains about 170 species, of which 64 European species are listed by Ball and Dudley (1964), Eastern European and near Eastern species have been revised by Dudley (1964, 1965). Alyssum is mainly restricted to Europe, the Near East and southern parts of European and Asiatic Russia. In Europe, it is confined to the southern half of the continent and may be an Ice Age relic, since its distribution is to the south of the areas formerly covered by the ice-sheet during the ice-ages. Certainly, it would seem that hyperaccumulation of nickel, like endemism, is an evolutionary adaptation so typical of ancient flora. By their ability to uptake such toxic elements as nickel, genera such as Alyssum and Homalium have been able to survive phylogenetically to edaphic conditions. It is also possible that this physiological tolerance aids in survival and is a defence mechanism against competing taxa (Brooks, 1979). Examples of such plant showing triumph over environment whereby they are the only ones growing in these areas, without presence of competing species are A. murale which occurs throughout the Balkans and particularly in the Pindus Mountain regions of Greece, and A. corsicum and A. cyprium in Vilayets Mugla, Aydin, Izmir, and Antalya of western Mediterranean Turkey. Some species (e.g. A. masmenaeum) contain up to 25,000 μg/g (2.5%) nickel.

The genus Alyssum contains six sections: Meniocus Desv. Hook.f; Psilonema (C.A. Meyer) Hook.f; Gamosepalum (Hausskn); Alyssum; Tetradenia (Spach) Dudley; Odontarrhena (C.A. Meyer) Koch. Section Odontarrhena, with 23 European species is of particular interest because many of its members seem to tolerate ultrabasic substrates, and because it includes all of the hitherto-known hyperaccumulators of nickel. Fig. 1.4 gives the histograms of nickel concentrations in individual specimens of Alyssum (Brooks et al., 1979). As can be noted, it certainly seems that there is some degree of physiological character of nickel accumulation in Odontarrhena.

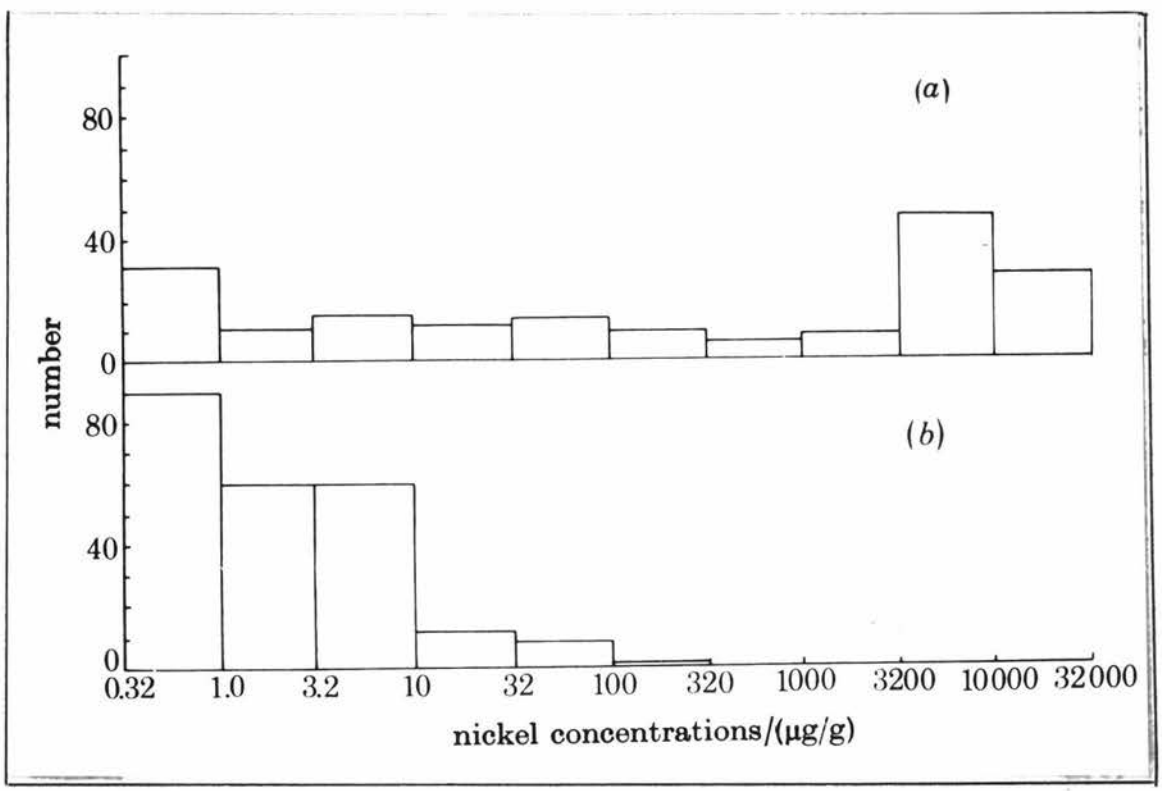


FIGURE 1.4 Histograms of nickel concentrations in individual specimens of Alyssum. (a) Species of section Odontarrhena; (b) Species of sections Meniocus, Psilonema, Alyssum, Gamosepalum and Tetradenia considered as one group (Brooks et al., 1979).

The recognition of A. serpyllifolium s.sp. lusitanicum as a separate taxon was partly based on the work of Menezes de Sequeira (1969) who noted this species as being a hyper-accumulator, whereas, A. serpyllifolium Desf. s.sp. serpyllifolium is not. The former is found growing over scattered areas of ultrabasic substrates, in a total area of about 100 km^2 in N.E. Portugal (Bragança). The other area of ultrabasic rocks in the Iberian Peninsula is in the vicinity of Málaga in Southern Spain. Another subspecies of Alyssum serpyllifolium s.sp. malacitanum Rivas Goday, is found growing on these ultrabasic areas. By analogy with s.sp. lusitanicum, if s.sp. malacitanum were to be proved to be a hyperaccumulator of nickel, it might be argued that it should also be promoted to full generic rank.

We have recently been able to get seeds of s.sp. malacitanum (by courtesy of Dr A. Asensi Marfil of Málaga) and have been able to do phytochemical studies on this taxon, as well as on the closely-related A. serpyllifolium s.sp. serpyllifolium. The purpose of this study was two-fold. Firstly, it was to obtain chemotaxonomic information to justify in part promoting s.sp. malacitanum to full generic rank. Secondly, it was to obtain information concerning edaphic factors governing the distribution of a nickel accumulating species and to study the relative effects of competition between ions such as Ca^{2+}, Mg^{2+} and Ni^{2+}. The results of these studies are presented in this thesis.

2. THE DISTRIBUTION AND ECOLOGY OF *A. SERPYLLIFOLIUM* AND ITS IBERIAN SUBSPECIES

2.1 Introduction

Alyssum serpyllifolium Desf. s.sp. serpyllifolium is a taxon of widespread occurrence in S.W. Europe over a wide variety of non-ultrabasic substrates. It is particularly common in the Iberian Peninsula. It was first described by Desfontaines (1798) and is listed in the Flora Europaea by Ball and Dudley (1964): "Procumbent to erect perennial up to 30 cm, with numerous non-flowering rosettes or short stems. Leaves up to 18 x 4 mm, oblanceolate or obovate-spathulate, grey or white beneath, grey or grey-green above, plicate on the non-flowering stems. Sepals 1.5-2 mm; petals (2-2.5(-3) mm, entire. Silicules 2.5-4.5 x(1.5-)2-3.5 mm, broadly elliptical or elliptic-rhombic to obovate, usually sub-acute, densely white pubescent; hairs 12- to 16 rayed; valves asymmetrically inflated; style 0.8-1.5 mm. Seeds 1.3-1.8 mm, not or only narrowly winged." A. serpyllifolium s.sp. serpyllifolium is found in section Odontarrhena of the genus Alyssum, and although the majority of the members of this section are hyper-accumulators of nickel, A. serpyllifolium s.sp. serpyllifolium is not (Brooks and Radford, 1978).

There are only two areas of ultrabasic rocks in the Iberian Peninsula. The first of these is in the Bragança area of N.E. Portugal where ultrabasics occupy a few km^2 in scattered occurrences. A much larger area of such rocks is found in the Málaga area of Southern Spain.

Recently Menezes de Sequeira (1969) found 5,160 µg/g nickel in leaves of A. serpyllifolium Desf. s.sp. lusitanicum T.R. Dudley and P. Silva from the Bragança area. This taxon is clearly a hyperaccumulator and this factor, along with morphological differences has prompted Dudley (1980, in ed.) to recognise it as a separate taxon. Similarly, Rivas Goday (1969) recognised a second serpentine-endemic subspecies of A. serpyllifolium growing in the Málaga area. This was named Alyssum serpyllifolium Desf. s.sp. malacitanum Rivas Goday. By analogy with s.sp. lusitanicum

this subspecies might well be promoted to full generic rank if a hyperaccumulating status could be shown and provided of course that morphological differences were sufficiently marked.

2.2 Geography of the Iberian Ultrabasic Occurrences

Bragança

Ultrabasic rocks have been known in Portugal since the early 20th century (Delgado, 1907). They exist as scattered occurrences in the N.E. of the country (Tras-os-Montes) in the neighbourhood of Bragança, Vinhais, Macedo de Cavaleiros and Mogadouro (see Fig. 2.1 Map of Bragança). These rocks are found at altitudes from 300 m-1,060 m and cover a total area of about 80 km^2. For a fuller description of the area see Pinto da Silva (1970).

Málaga

The Sierra de Aguas (see Fig. 2.2 Map of Málaga) is situated about 55 km from Málaga in the Alora-Campillos region and forms part of the Betica mountain chain which was raised in the Tertiary Era (González, 1975). The Sierra de Aguas lies immediately to the south of the Villaverde Plateau (Mesas de Villaverde). Its highest point culminates in the 949 m Pico Agua which is on the crest dividing the watershed of the Rio Cañas and that of numerous streams which flow directly into the Rio Guadalhorce.

The nearest population centre is Carratraca, situated in the extreme west of the Sierra, about 4 km from its ultrabasic extremity at Alora.

Ultrabasic rocks in the district of Bragança (ca. 1:400 000).

FIGURE 2.1 Map of Bragança.

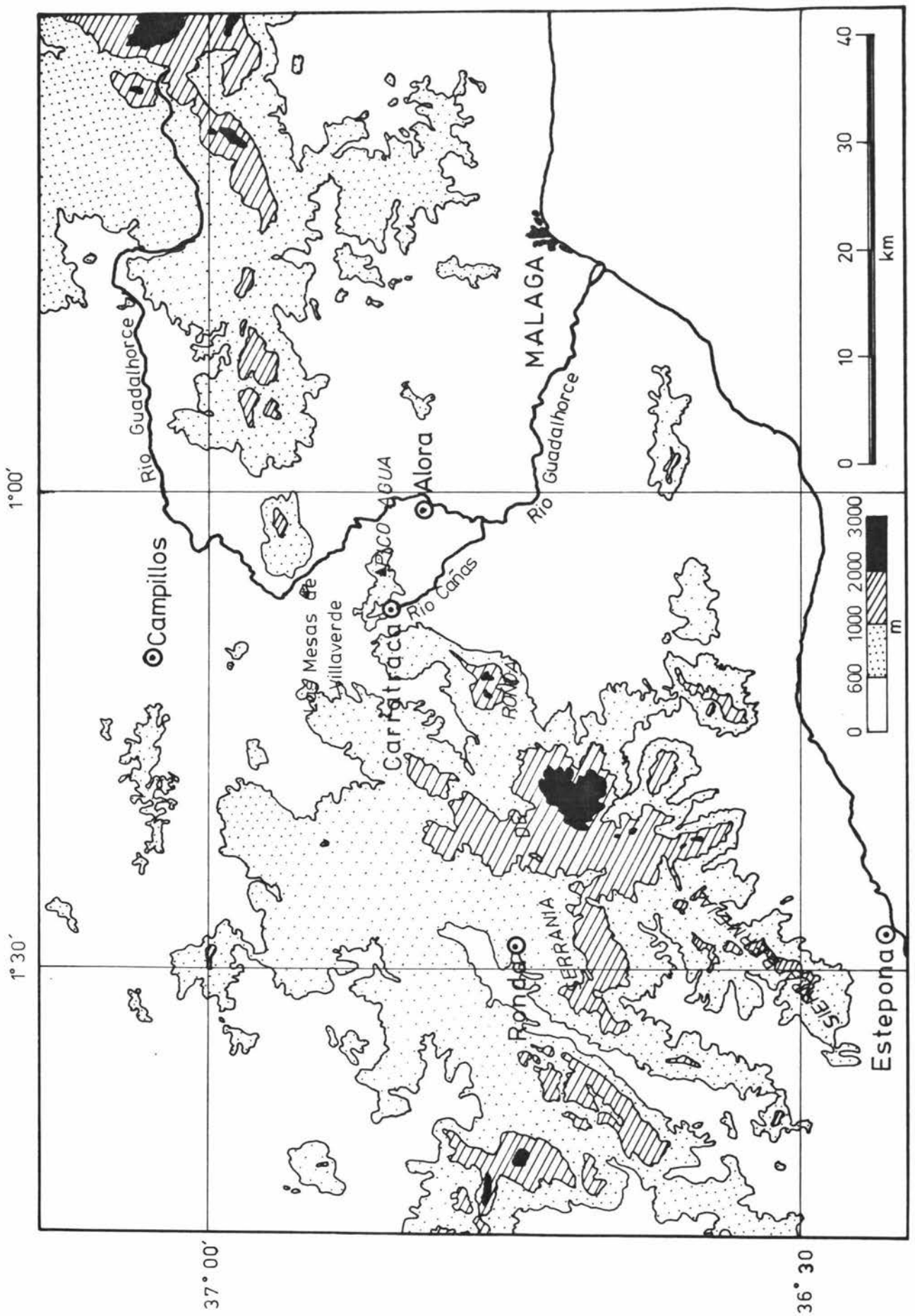


FIGURE 2.2 Map of Málaga. Dark areas indicate ultrabasic rocks.

2.3 Geological Aspects

Bragança

Completely fresh peridotitic rocks are unusual in N.E. Portugal. The serpentinic rocks are orthometamorphic and result from the metamorphism of calco-alkaline peridotite (Neiva, 1948). There are few serpentinic rocks rich in chromium because high levels of Cr_2O_3 only occur in zones of chromite accumulation caused by the intrusion of residual magmas of peridotitic rocks (Neiva, 1948).

One of the most common rocks of the N.E. Portugal serpentinic occurrences is lherzolite. Compared with ultrabasic rocks in other parts of the world, the biggest differences are found in the amounts of iron (Fe_2O_3) and magnesium (MgO), depending on the predominance of forsterite (Mg_2SiO_4) or fayalite (Fe_2SiO_4) in the original olivine, and on the presence and level of pyroxene, biotite, and oxides. However, in the N.E. ultrabasic rocks, forsterite seems to predominate, together with enstatite, and accounts for their high magnesium level. The level of sodium is higher than in serpentinic rocks (Robinson et al., 1935; Mohr and Van Baren, 1954; Pedro and Bitar, 1966) and is probably due to the calco-alkaline nature of the original magmas and the corresponding presence of albite, anorthite, acmite and also wollastonite (Neiva, 1948; Ferreira, 1964).

The serpentinic rocks of Bragança-Vinhais are massive, dense with homogeneous structure and often with chromite crystals. In the more weathered superficial rocks (supergenic serpentinization) their large-mesh structure can be easily seen (Ferreira, 1964). Their colour is generally dark green, but other shades of green, gray, red, dark red and yellow can also be found, by themselves or in different combinations.

The microscopical structure is reticulated with a lattice of thin fillets of chrysotile or serphophite, surrounding serphophite and/or antigorite, sometimes also chrysotile, with or without chlorite and frequently with bastite. The brownish yellow colour of these serpentinic

rocks (as observed in a microscopic view) is due to oxides exuded during the serpentinization process (Neiva, 1948).

Málaga

The Sierra de Aguas (see Fig. 2.2 Map of Málaga) is an enclave of plutonic ultrabasic rocks surrounded by Oligocene and Silurian rocks. The contact between peridotites and the crystalline strata is covered with mumuliths in the Carratraca Depression, the source of the Rio Paredones.

The peridotitic areas are covered with reefs of acid rocks which extend along an east-west axis. The texture of the rocks suggest slow crystallization, with a huge batholith raised up at the surface by orogenic movements. Gneisses represent the upper residue of the batholith. The peridotites have a subterranean expression in the Serrania de Ronda.

The peridotites are extensively serpentinized by hydrothermal action and contain abundant oxides of iron and give a reddish colour to the Sierra in contrast to the surrounding areas.

2.4 Edaphic Aspects

Braganca

Analytical data for lherzolite-type ultrabasic rocks from the Vinhais area given in Table 2.4.1.

A typical serpentinic soil profile is shown in Table 2.4.2.

TABLE 2.4.1 Analytical data for lherzolite-type ultrabasic rocks from the Vinhais area, N.E. Portugal.

Sample	%
SiO_2	41.30
Al_2O_3	2.51
Fe_2O_3	8.17
TiO_2	0.02
MgO	37.53
CaO	0.53
K_2O	< 0.05
Cr_2O_3	0.54
NiO	0.75
MnO	0.08
H_2O	12.20

TABLE 2.4.2 Analytical data for a typical serpentinic soil profile from Macedo de Cavaleiros, N.E. Portugal.

Horizon	A_{11}	A_{12}	C	Rock
Depth (cm)	0 to 10-15	10-15 to 25-30	25-30 to 70	70
SiO_2 (%)	45.50	44.00	46.40	44.50
Al_2O_3 (%)	8.95	10.52	6.76	1.78
Fe_2O_3 (%)	14.97	15.56	10.62	6.60
TiO_2 (%)	0.38	0.32	0.22	0.02
MgO (%)	15.44	15.08	23.03	31.42
CaO (%)	1.65	0.87	2.80	2.02
K_2O (%)	0.58	0.40	0.58	Traces
CrO_3 (%)	0.72	0.78	0.40	0.22
MnO (%)	0.37	0.29	0.15	0.20
NiO (%)	0.40	0.41	0.29	0.29
CoO (ppm)	127	153	93	-
Ignition loss (%)	10.70	8.80	7.40	9.20
Moisture (%)	3.75	5.20	4.30	2.30

Profile EM 42 (Menezes de Sequeira, 1969). This profile can be summarised as follows: humic litholic soil - Macedo de Cavaleiros, near Limaos, 620 m.a.s. level, halfway up a slope, SW exposure, 15% slope. Uncultivated for years, with vegetation comprising a Taeniathero-Alyssetum lusitanicum assemblage. High stoniness, great risk of erosion, excessive external drainage and slow internal drainage.

Málaga

The serpentines, correspond principally to red limonite-rich soils and lithosols. The soils in general are fairly humic and have a good water retention capacity. The pH is usually quite high (of the order of 7). The soils contain elevated concentration of heavy metals such as nickel and chromium.

Hoyos de Castro (1960) has presented analytical data for major elements in two soil profiles of limonitic ultrabasic soils from Serrania de Ronda. These are shown in Table 2.4.3.

The most noticeable feature of these profiles is the loss of MgO during the soil-forming process. There is also a fairly strong loss of SiO_2 and an accumulation of Fe_2O_3 and Al_2O_3, particularly in clay minerals. The same author showed that the parent soil contained about 50% serpentine, 20% olivine, and 20% amphibole. González (1975) has reported 0.3% Ni, 0.02% Cr, and 0.01% Co in these parent rocks.

2.5 Climate

Bragança

The rainfall in N.E. Portugal is more than adequate for plant growth. Vinhais averages 1,077 mm annually, whereas Bragança has 972 mm (A. Ferreira, 1965). The more southerly occurrences near Macedo de Cavaleiros have only 728 mm. The climate has been characterised as

TABLE 2.4.3 Analytical data for major elements in ultrabasic soils from Serrania de Ronda, Málaga, Spain.

% SiO_2	Fe_2O_3	Al_2O_3	TiO_2	MgO	CaO	H_2O
42,04	4,47	14,98	0,27	22,88	3,63	8,72
33,77	13,72	25,95	1,21	10,95	0,29	12,52
43,42	4,89	13,16	0,20	22,56	3,16	7,25
41,08	11,85	27,04	0,63	9,80	-	9,23
39,56	3,82	7,49	-	35,85	-	13,18
41,79	3,10	8,30	-	36,65	1,79	8,00
42,44	6,20	12,70	0,33	20,27	3,25	12,58
32,36	11,17	26,13	1,35	11,20	1,36	15,30
37,09	11,01	19,83	0,48	16,50	2,47	8,75
30,44	16,81	30,83	0,65	9,47	-	9,92
39,52	4,17	6,50	0,14	36,89	1,72	9,67
39,90	4,36	8,56	0,46	34,90	0,93	10,47
53,39	4,22	6,96	-	21,39	11,19	2,32

humid Mediterranean. Temperature ranges from below freezing in the winter to about 30°C in the summer. Although rainfall is heaviest in the winter, there is adequate precipitation for plant growth in summer.

Málaga

Rainfall in the Sierra de Aguas is similar to that of N.E. Portugal with an annual precipitation of 846 mm. Unlike the Bragança area, however, there is a prolonged dry period of about 5 mths. Temperatures are also more extreme and range from 0-40°C. Maximum rainfall is produced by winds from the south, west or south-west. Because of the presence of a prolonged dry period, the region can be considered to have a Mediterranean-type climate in spite of the extremes of temperature.

2.6 Vegetation

Bragança

The following description is taken from Menezes de Sequeira (1969): The scanty vegetation cover and the low number of species, that Rune (1953) considers typical of the serpentinic vegetation in Northern Sweden, seem to exist also in N.E. Portugal accelerating erosion and impeding the formation of deep soils. There is also an increase in stoniness, due to the removal of the lighter material, with a related increase in the level of the available nickel and probably that of chromium.

The serpentinic biotypes and the serpentinophytes found in these soils (Silva, 1967) increase the toxic effect of the vegetation by their accumulator capacity, especially for nickel. A specimen of Alyssum serpyllifolium s.sp. lusitanicum collected by Menezes de Sequeira (1969) contained 5,160 µg/g of nickel and 12 µg/g of chromium in the dry matter of leaves and 3,210 µg/g of nickel and 24 µg/g of chromium in the dry matter of stems. Chromium, unlike nickel, is not translocated in the vascular system of plants, and must accumulate in the roots (Hunter and Vergnano, 1953).

The xerophytic character of serpentinic vegetation, pointed out by Rune (1953) and Silva (1967) is probably due to low water availability, on account of the shallowness of the soil with a high percentage of coarse material. This lack of water must be also one of the reasons for the scanty vegetation cover and general soil infertility.

The Mediterranean character of the serpentinic vegetation in N.E. Portugal, observed by Silva (1967) can be considered as a corollary of the xerophytic character, and also as a result of the dark colour of the soil. The dark soil unprotected by vegetation, absorbs high amounts of solar radiation, with a corresponding increase in soil temperature. This character will probably exist only in climatic conditions of very dry summer and rainy winter.

The simultaneous existence of basicolous and acidicolous plants verified by Silva (1967), is probably due to the different pH values observed within the same horizon. Silva (1967) observed in these soils that which Krause (1958) had reported for serpentinic soils - whenever there is a deep weathering of serpentinic material, with corresponding decrease in pH, the number of serpentinophytes diminishes, to the advantage of trees and shrubs. This practical effect must be due to an increase in the 'podzolization' area, more stable, richer in organic matter, with a lower pH and a smaller amount of free chromium and available nickel, correlated to a decrease of the 'sialitization' zone, with a lower organic matter content, higher pH and a high level of available nickel and free chromium. In this case, since we are approaching the climax stage, we can easily envisage the colonization by non-serpentinic species, especially trees and shrubs, which competing for nitrogen, phosphorus, potassium, calcium, water, etc. and suppressing sunlight, progressively eliminate the serpentinophytes.

Tillage, destroying the climax conditions, restricts the competition and homogenizes the soil, with a decrease in organic matter and titrable hydrogen. In this way, the soil conditions return to the original, and there is

a return of serpentinophytes, like the Taeniathero-Alyssum lusitanicum association, and the degradation is reinforced by the presence of nickel accumulators, which create difficulties for the return to the climax stage, and diminish the productivity of these soils.

A typical occurrence of A. serpyllifolium s.sp. lusitanicum at Carrazedo (Bragança) is shown in Plate 2.6. This is reproduced from Pinto da Silva (1970).

Málaga (González, 1975)

Alyssum serpyllifolium s.sp. lusitanicum Dudley & P. Silva forms part of the Asperulo-Staehelinetum baeticae association which forms a scrub community of 80-90 species with about 80% vegetation cover. Characteristic members of this association are Asperula asperrima var asperrima and Staehelina boetica. This plant association is found solely on peridotites and is typical of the flora of the Sierra Aguas where it occurs mainly between 400-500 m altitude and is linked to the summital associations of the Pico Aguas. The association is very homogeneous with only small local variations.

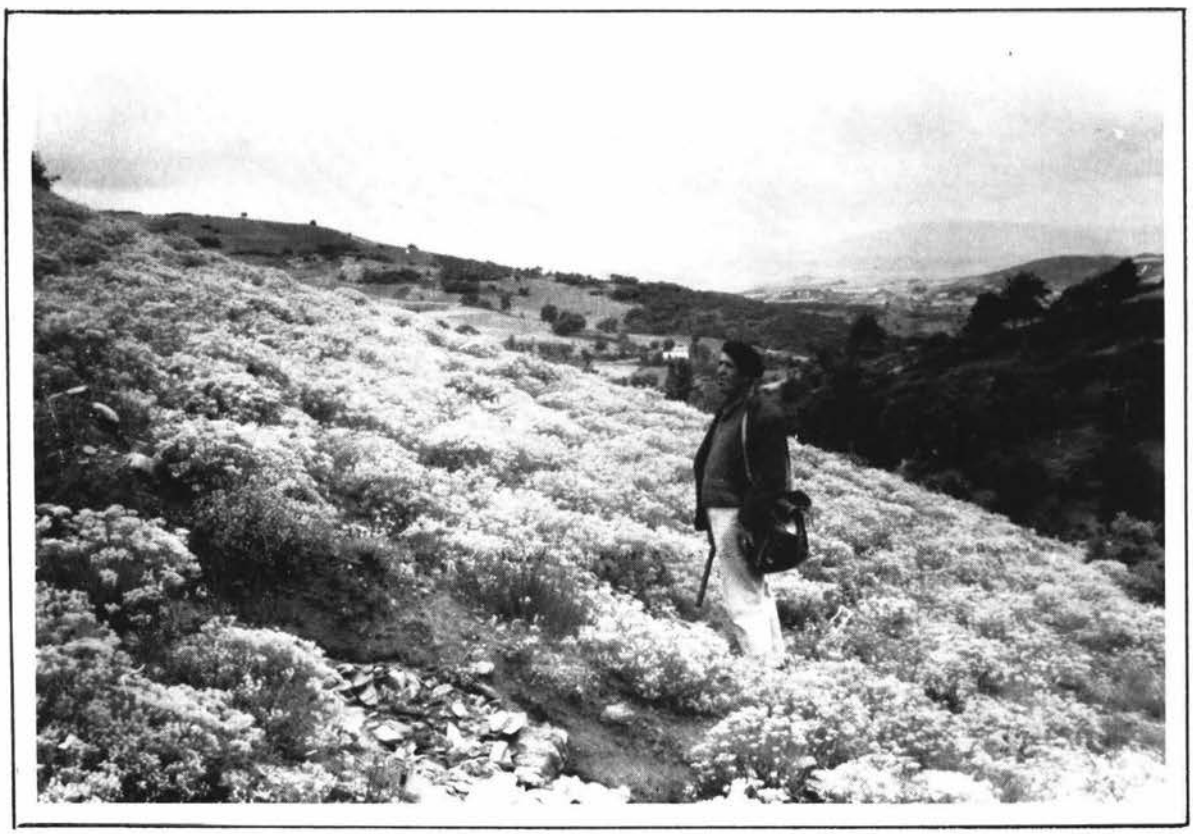


PLATE 2.6 Alyssum serpyllifolium Desf. s.sp. lusitanicum Dudley & P. Silva, Bragança: Carrazedo.

Source: Pinto da Silva (1970)

3. ANALYTICAL METHODS

3.1 Atomic Absorption Spectrophotometry

The use of atomic-absorption spectrophotometry as an analytical technique is now firmly established; this is apparent from the number of related papers that have appeared in the literature since theoretical work in this field by Walsh (1955).

The essential requirements for an atomic absorption spectrophotometer are indicated in Fig. 3.1.

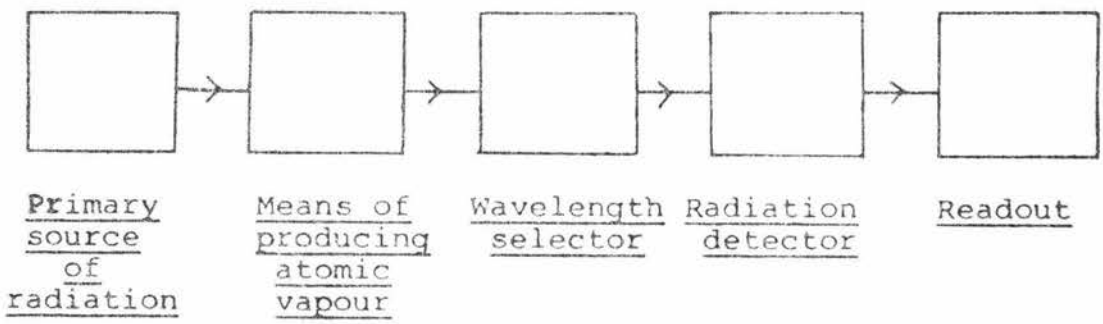


FIGURE 3.1 Schematic diagram showing essential requirements for atomic absorption spectrophotometer.

Instrumentation consists of: -

(i) Primary source of radiation - usually a sharp-line source e.g. a hollow cathode lamp, that has sufficient radiation intensity and stability to permit absorption measurements to be made accurately.

(ii) Atomic vapour production - a flame is commonly used.

(iii) Wavelength selector - a good ultraviolet monochromator capable of variable wavelength selection.

(iv),(v) Radiation Detector - amplifier and read out.

The Varian Techtron model A.A.5 atomic absorption spectrophotometer was used for determining various elements in plant samples and soil digests. Combined standards were prepared from 1,000 μg/g analytical grade (B.D.H.)

stock solutions diluted to 100 µg/g, 20 µg/g and 5 µg/g using deionised water. For calcium and magnesium, standards and sample dilutions were made with 2 M hydrochloric acid, containing 0.8% $Sr(NO_3)_2$. This was done as ions such as phosphates, sulphates, silicates are known to cause depression of the absorption signal. It is evident that certain elements interfere in the determination of others (e.g. aluminium seriously interferes with the determination of magnesium). This can be controlled by increasing the acid concentration, adding a strontium salt; or having an additional atomizer in the instrumentation. Interferences due to ionization effects (David, 1959), as in the case of alkali metals that interfere in the determination of calcium and magnesium, can be overcome by the use of mixed standards of the metals.

Light scattering of the incident radiation by solid particles and non-atomic absorption was corrected for by the use of a hydrogen continuum lamp. For our work, the Varian Tectron model AA5 atomic absorption spectrophotometer was coupled to a BC6 automatic background corrector.

For nickel, the line at 232 nm was used for low concentration and that at 351.5 nm was used for higher concentrations. Instrumental conditions are summarised in Table 3.1.2.

3.2 Flame Photometry

Potassium was analysed in the acid digests by using the emission mode of the AA5 spectrophotometer (with the air-acetylene flame) or a Gallenkamp flame photometer (with air-natural gas flame).

3.3 Colorimetry

Phosphate in plant and soil samples was determined by the formation of a phospho-molybdate complex similar to that of the molybdenum-blue complex (Stanton, 1966).

The reagent that was used had the following solutions made up in the proportions as shown in brackets.

2 M sulphuric acid (500)
0.025 M ammonium molybdate (150)
0.1 ascorbic acid - freshly prepared each day (100)
0.01 M antimony potassium tartrate (50)

A Unicam SP 1800 uv spectrophotometer, having a slit width of 0.2 mm was used for the reading of the absorbance at 700 nm wavelength. In this method, pH was a sensitive factor. Therefore, the original solutions which were in 2 M hydrochloric acid were neutralised with sodium hydroxide, using p-nitro phenol as an indicator.

3.4 Preparation of Plant and Soil Samples

Dried leaf samples were accurately weighed using a Mettler H.6 balance and then placed in 5 cm^3 borosilicate test-tubes. All glassware and associated implements were thoroughly cleaned with detergents and washed with deionised water prior to use. This was so as to achieve minimum contamination by elements under study. Carbonaceous material was removed by placing these weighed tubes in a muffle furnace thermostated at 500^{o}C for approximately 3 hours. This dry ashing procedure was preferred to a wet ashing as the samples did not contain volatiles such as arsenic and mercury. After the ashing and cooling of the test-tubes, 2 M hydrochloric acid (triple distilled) was added to dissolve the inorganic material. The tubes were then heated over a gentle bunsen flame to dissolve any precipitate and centrifuged to remove insoluble silicates.

The soil samples were removed and initially dried in the oven at 80^{o}C. They were then ashed in the muffle furnace thermostated at 500^{o}C. Samples were then ground and a known weight of 0.1 g was then used. The samples were treated with a 3:1 mixture of concentrated hydrochloric and nitric acids (aqua regia) so as to remove

any organic constituents. The test-tubes (or beakers) were suspended in a boiling bath and taken down to dryness. The dried residues were then dissolved in 2 M HCl (of known volume) and centrifuged before analysis of atomic absorption. As all soil samples were prepared artificially by addition of trace elements, none of these constituents were bound up in a silicate lattice (as would have been the case for natural soils) so that a hydrofluoric acid treatment was not necessary.

3.5 Statistical Treatment of Data

An electronic calculator was used for the calculation of arithmetic means, standard deviations and Pearson Product moment correlation coefficients (r).

3.6 Reliability of Plant Sample Analyses

The work reported in this thesis was confined to section Odontarrhena of *Alyssum*, which contains 45 hyperaccumulators.

The reliability of the plant analyses was determined by comparison of two sets of analyses of different parts of identical samples of *Alyssum* leaves. These data are shown in Table 3.6.1. The analysis represent my own data and those of R.J. Morrison (asterisked).

Statistical analysis of 47 pairs of data showed a very highly significant ($r = 0.71, P < 0.001$) relationship with geometric means of 6,634 and 6,568* µg/g, and standard deviation ranges of 4,272-10,301 and 3,944-10,938*. The values in column 1 are my own data. The geometric mean was used because the data were log-normally distributed.

Differences in the two sets of data are due in part to the natural intra-sample variability of nickel values within each specimen and partly to analytical error.

TABLE 3.1.2 Instrumental conditions for Varian Techtron Model AA5.

Element	Wavelength (nm)	Slit Width (UM)	Lamp current (mA)	Sensitivity* (µg/ml)	Detection Limits* (µg/ml)	Flame character air-acetylene
Ca	422.7	300	3.50	0.021	0.0005	reducing
Co	240.7	150	7.00	0.066	0.006	oxidising
Cr	357.9	100	7.00	0.055	0.066	reducing
K	766.49	500	5.00	0.01	0.003	oxidising
Mg	285.2	200	3.50	0.003	0.0003	oxidising
Mn	279.5	200	5.00	0.024	0.003	oxidising
Ni	232.0	170	3.50	0.066	0.008	oxidising
	351.5	200	0.72	0.072	0.008	oxidising
Zn	213.9	250	5.00	0.009	0.002	oxidising

*Data from Varian Techtron handbook "Analytical methods for flame spectroscopy".

TABLE 3.6.1 Analysis for nickel in Alyssum, Section Odontarrhena (*Brooks et al., 1979).

Sample	Column 1 Ni µg/g	*Column 2 Ni µg/g
A. anatolicum	8,140	8,170
A. bertolonii	8,870	7,830
A. bertolonii	5,890	8,550
A. bertolonii	6,210	7,080
A. callichroum	7,920	10,900
A. chondrogynum	11,670	16,250
A. chondrogynum	10,140	7,256
A. chondrogynum	4,660	3,950
A. constellatum	9,570	5,380
A. constellatum	18,540	18,125
A. corsicum	8,720	6,790
A. corsicum	7,850	7,480
A. corsicum	3,650	4,603
A. cypricum	6,500	7,670
A. cypricum	2,500	3,900
A. cypricum	3,680	4,140
A. davisianum	8,320	11,560
A. davisianum	3,500	6,500
A. discolor	6,200	4,450
A. discolor	7,440	6,030
A. discolor	6,250	8,730
A. eriophyllum	4,560	8,531
A. eriophyllum	20,750	11,530
A. euboeum	5,700	4,540
A. euboeum	9,340	8,110
A. fallacinum	5,630	3,960
A. fallacinum	6,840	3,800
A. huber-morathi	7,500	4,990
A. huber-morathi	6,060	3,540
A. huber-morathi	10,450	11,800
A. markgrafii	5,410	9,060
A. markgrafii	5,770	3,900

Sample	Column 1 Ni µg/g	*Column 2 Ni µg/g
A. masmenaeum	9,510	5,480
A. masmenaeum	12,700	24,255
A. masmenaeum	9,190	15,480
A. oxycarpum	5,360	4,460
A. oxycarpum	9,470	7,290
A. robertianum	3,000	2,730
A. robertianum	6,750	5,050
A. serpyllifolium s.sp. lusitanicum	2,700	1,940
A. smolikanum	8,330	6,600
A. syriacum	5,870	5,250
A. syriacum	7,500	10,190
A. syriacum	4,710	3,350
A. trodii	5,769	6,550
A. trodii	5,140	9,510
A. trodii	4,090	5,120

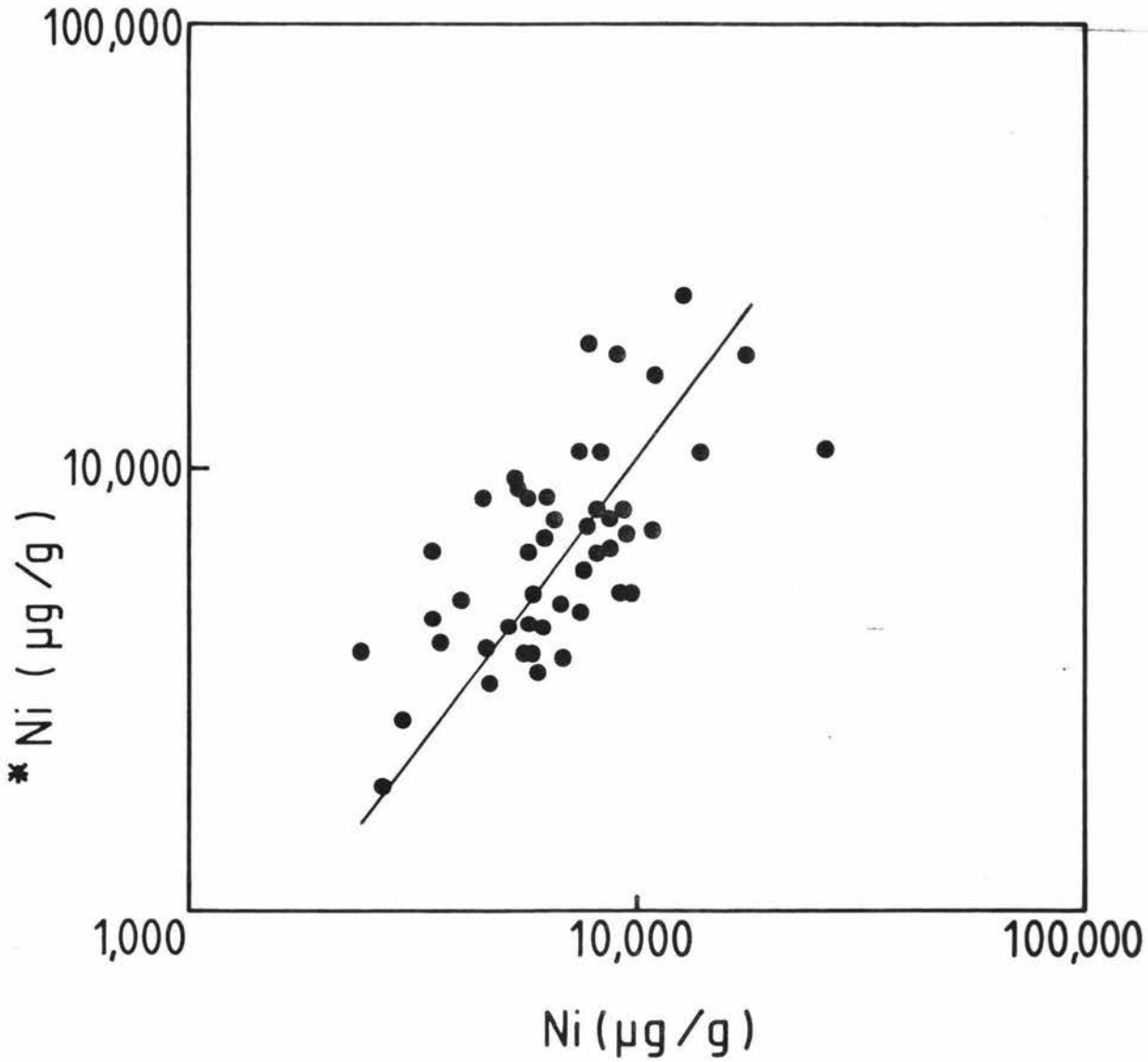


FIGURE 3.6.2 Graphical representation showing reliability of two sets of data.

y axis - data of R.S. Morrison

x axis - my data

4.1 Inter-species Ionic Relationships for Section Odontarrhena of Alyssum

The presence of so many hyperaccumulators of nickel in Section Odontarrhena of Alyssum being so unusual, it was decided to study the relationship between nickel and other elements in these plants. The data are shown in Table 4.1.1 and are confined to hyperaccumulating species only. The relationship involving calcium and magnesium was of particular interest. The relationships between nickel and each individual element were determined by calculation of Pearson Product Moment correlation coefficients (r) and are shown in Table 4.1.2. Means (geometric) and standard deviation ranges are shown in Table 4.1.3. Geometric means were used because it was found that the data were lognormally distributed.

4.2 Intra-species Studies on Alyssum serpyllifolium s.sp. malacitanum

Eleven samples of A. serpyllifolium s.sp. malacitanum were provided by Dr. Asensi Marfil of Malaga, Spain. The collection location was from Sierra Bermeja de Estepona (refer Fig. 2.2.2). The first task was again to study the different element distributions in the plant material (Table 4.2.1). Again the pattern seems to follow that of Table 4.1.1 i.e. where the nickel uptake is high, the calcium level is high and the magnesium level is low. The $\frac{Ni}{Ca} + Mg$ ratio is seen to be fairly constant.

The statistical data are shown in Table 4.2.2. As in Table 4.1.2, there is a probable relation between the nickel and calcium. Following this indication, pot trials were carried out and will be reported later in this thesis.

In all the samples under study, where there was an increased uptake of nickel, the calcium content had been high, with low magnesium amounts. This particular feature was previously reported by Vergnano et al (1977) from their studies on A. bertolonii from Impruneta (Minguzzi and

4. IONIC RELATIONSHIPS AND CORRELATIONS IN HYPERACCUMULATORS

TABLE 4.1.1 Elemental concentrations and ratios in Alyssum species of Section Odontarrhena

Species	µg/g					%			Ratios	
	Ni	Co	Cr	Zn	Mn	Ca	Mg	K	Ca/Mg	Ni/(Ca + Mg)
A. anatolicum	8140	-	-	727	58	8.72	1.45	0.58	6.01	1.54
A. bertolonii	8870	6	6	28	42	3.88	0.53	0.72	7.38	0.76
"	5890	2	21	32	15	4.21	0.29	0.49	14.70	0.43
"	6210	16	19	32	39	2.91	0.52	1.07	5.63	0.73
A. callichroum	7920	10	-	99	10	1.98	0.59	0.59	3.33	0.99
A. chondrogynum	11670	-	29	1021	88	2.92	0.88	0.44	3.33	1.27
"	10140	28	17	39	138	1.93	0.66	0.09	2.92	1.19
"	4660	4	2	13	90	2.69	0.58	0.27	4.62	0.76
A. constellatum	9570	5	25	60	15	2.49	0.62	0.62	4.00	1.01
"	18540	-	42	21	42	6.32	1.05	1.26	6.02	1.34
A. corsicum	8720	3	-	31	51	3.59	0.64	0.58	5.60	0.88
"	7850	7	16	26	79	3.27	0.65	0.63	5.00	0.89
"	3650	-	3	26	5	2.48	0.65	0.39	3.80	0.80
A. cypricum	6500	12	23	93	70	4.41	0.60	0.30	7.35	0.75
"	2500	13	13	13	25	6.90	0.50	0.75	13.80	0.54
"	3680	11	8	13	37	3.42	0.64	1.03	5.34	0.75
A. davisianum	8320	12	-	24	12	4.75	0.65	0.59	7.31	0.83
"	3500	6	12	30	24	3.84	0.47	0.47	8.17	0.56

Species	µg/g					%			Ratios	
	Ni	Co	Cr	Zn	Mn	Ca	Mg	K	$\frac{Ca}{mg}$	$\frac{Ni}{Ca + Mg}$
A. discolor	6200	20	-	14	17	2.40	0.73	0.32	3.29	0.99
"	7440	-	-	112	65	3.26	0.84	0.70	3.88	1.07
"	6250	25	-	294	125	2.19	1.13	-	1.94	1.42
A. eriophyllum	4560	-	-	14	7	1.78	0.50	0.57	3.56	0.76
"	20750	47	-	236	47	16.5	0.94	0.71	7.60	1.07
A. euboeum	5700	-	-	11	14	4.46	0.64	0.14	6.94	0.77
"	9340	29	-	61	86	4.06	0.59	0.20	6.88	0.82
A. fallacinum	5630	-	8	5	38	2.82	0.92	0.15	3.06	1.12
"	6840	-	-	17	17	2.57	0.86	0.26	3.00	1.12
A. hubermorathi	7500	-	11	16	80	2.95	0.59	0.56	5.00	0.81
"	6060	6	15	9	24	3.79	0.33	0.55	11.40	0.49
"	10450	154	31	31	31	4.61	1.23	1.54	3.75	1.46
A. markgrafii	5410	-	-	64	3	0.64	0.15	0.24	4.41	0.99
"	5770	9	9	14	33	2.36	0.66	0.45	3.58	0.90
A. masmenaeum	9510	17	-	11	54	2.49	0.32	0.40	7.69	0.71
"	12700	11	-	19	16	0.95	0.48	0.21	2.00	1.81
"	9190	7	-	35	23	2.21	0.33	0.37	6.78	0.74
A. oxycarpum	5360	8	5	53	26	3.16	0.61	0.59	5.21	0.78
"	9470	-	-	117	19	3.10	0.43	0.21	7.26	0.73
A. robertianum	3000	100	5	30	25	3.26	0.46	0.72	7.03	0.56
"	6750	26	26	159	9	4.41	0.57	-	7.70	0.73

Species	µg/g					%			Ratios	
	Ni	Co	Cr	Zn	Mn	Ca	Mg	K	$\frac{Ca}{Mg}$	$\frac{Ni}{Ca + Mg}$
A. serpyllifolium s.sp. lusitanicum	2700	3	24	10	96	0.51	0.38	0.12	1.36	0.90
A. smolikanum	8330	8	19	28	28	5.12	0.64	0.32	8.05	0.80
A. syriacum	5870	19	16	6	22	2.06	0.70	0.44	2.96	0.98
"	7500	19	19	38	19	4.73	0.95	0.28	4.99	1.11
"	4710	27	16	102	27	3.77	0.80	0.24	4.67	0.93
A. troodii	5769	18	15	15	79	5.40	0.53	0.22	10.30	0.63
"	5140	21	21	85	53	4.24	0.64	0.42	6.67	0.76
"	4090	9	5	9	68	4.76	0.79	0.20	5.99	0.88

TABLE 4.1.2 Statistical significance for relationships of nickel with other elements in Section Odontarrhena of Alyssum
(n = number of pairs of elements determined)

	Nickel vs				
	Co	Cr	Ca	Mg	$\frac{Ca}{Mg}$
Correlation coefficient (r)	0.18	0.48	0.26	0.27	-0.017
n	35	29	47	47	47
Probability	$P>0.10$	$0.05>P>0.01$	$0.10>P>0.05$	$0.10>P>0.05$	$P>0.10$
Significance	not significant	significant	possibly significant	possibly significant	not significant

TABLE 4.1.3 Geometric means and standard deviation ranges for the various elements in Alyssum Section Odontarrhena

Element	Geometric means	Standard deviation ranges
Nickel (µg/g)	6438	4146-10,198
Cobalt (µg/g)	12	5-31
Calcium (%)	3.32	2-6
Magnesium (%)	2	1-2
$\frac{\text{Calcium}}{\text{Magnesium}}$	12	5-31

TABLE 4.2.1 Nickel and major element concentrations in dried leaves of *A. serpyllifolium* s.sp. *malacitanum*

Sample No.	Ni(%)	K(%)	Na(%)	Ca(%)	Mg(%)	$\frac{Ni}{Ca + Mg}$	$\frac{Ca}{Mg}$
1	1.25	0.47	0.50	12.50	1.56	1.66	8.01
2	1.71	1.31	0.12	3.72	1.27	1.73	2.93
3	3.20	0.79	0.09	3.46	1.45	2.37	2.39
4	3.21	0.07	0.12	4.29	1.21	1.96	3.55
5	1.32	0.55	0.06	1.57	0.47	1.31	3.34
6	3.40	0.88	0.19	3.10	0.91	2.01	3.41
7	2.83	1.00	0.16	3.54	0.66	1.46	5.36
8	0.90	0.95	0.13	8.50	1.20	1.31	7.08
9	1.42	0.61	0.18	8.11	1.52	1.70	5.34
10	1.86	0.60	0.10	6.14	1.51	1.81	4.07
11	1.83	0.60	0.10	4.31	1.03	1.45	4.18
Mean and standard deviation	2.08±0.90	0.71±0.32	0.15±0.12	5.38±3.16	1.16±0.36	1.71±0.33	4.51±1.76

Vergnano, 1953).

Kelly et al.(1975) in their studies with Hybanthus, Lyon et al. (1968) in studies with some serpentine plants from New Zealand, Paribok and Alexeyeva-Popova (1966) for herbaceous species found on hyperbasic rocks in Polar Urals, all reported that magnesium never reached a very high concentration. They also found a fairly high $\frac{Ca}{Mg}$ ratio similar to that found in A. bertolonii (Vergnano, 1953). Since these initial studies on elemental relationship in naturally-occurring vegetation were to be merely a guide as to possible advances to explore for future pot trials, a detailed interpretation of elemental relationships will not be undertaken in this chapter.

4.4 Introduction to Pot Trials

Seedlings of A. serpyllifolium s.sp. malacitanum were grown in a medium of 50% pumice, 50% perlite with added nutrients. The various seedlings were treated with nickel (as the nitrate), calcium (as the nitrate) and magnesium (as the anhydrous sulphate). Table 4.4.1 shows the plan of the different concentrations of the elements studied. After a period of 4 weeks in the growth medium, the nickel, calcium and magnesium content of the leaves and soil were determined.

After a period of four weeks, the condition of the plants were seen to be as shown in the chart, Table 4.4.2.

TABLE 4.2.2 Statistical data for elemental concentrations in *A. serpyllifolium* s.sp. *malacitanum*

	Nickel vs			
	K	Na	Ca	Mg
Correlation coefficient (r)	0.09	0.20	0.54	0.15
n	11	11	11	11
Probability	$P > 0.10$	$P > 0.10$	$0.10 > P > 0.05$	$P > 0.10$
Significance	not significant	not significant	possibly significant	not significant

TABLE 4.4.1 Seedlings of A. serpyllifolium s.sp. malacitanum grown in different concentrations of nickel, calcium and magnesium. All concentrations are in μg/g.

Code	Element(s)	1	2	3	4	5	6	7
A	Ni	8,000	4,000	2,000	1,000	500	250	125
B	Ni const.Ca (1000)	8,000	4,000	2,000	1,000	500	250	125
C	Ca	8,000	4,000	2,000	1,000	500	250	125
D	Ca const.Ni (500)	8,000	4,000	2,000	1,000	500	250	125
E	Mg	2,000	1,000	900	800	400	200	100
F	Mg const.Ni (500)	2,000	1,000	900	800	400	200	100
G	Mg const.Ca (1,000)	2,000	1,000	900	800	400	200	100
H	Ni const.Mg (500)	8,000	4,000	2,000	1,000	500	250	125
I	Ca const.Mg (500)	8,000	4,000	2,000	1,000	500	250	125

In Table 4.4.2 the healthiest plant in each row has been indicated by underlining.

TABLE 4.4.2 Conditions of plants after a period of 4 weeks.

Code	1	2	3	4	5	6	7
A	dead	dead	dead	healthy but leaves turning yellow	very healthy approx. 2 cm high	very healthy approx. 2 cm high	very healthy approx. 2 cm high
B	dead	almost dead approx. 1 cm high	almost dead approx. 1-1.5 cm high	dead	alive 1-2 cm high	very healthy plant 2-3 cm high	alive 1-2 cm high
C	alive plants about 1 cm high	healthy	very healthy about 4-5 cm high	very healthy 3-4 cm high	healthy but only 1 cm high	very healthy 2 cm high	very healthy 2 cm high
D	dead	alive approx. 2 cm high. Leaves turning yellow	dead	plant approx. 1 cm high. Leaves turning yellow	alive approx. 1 cm high	alive approx. 1 cm high	alive approx. 1 cm high

Code	1	2	3	4	5	6	7
E	healthy 1-2 cm high	healthy 1-2 cm high	dead	healthy 1-2 cm high	dead	healthy approx. 1 cm high	healthy approx. 1 cm high
F	dead	dead	dead	healthy approx. 1 cm high	healthy approx. 1 cm high	very healthy 2-3 cm high	healthy approx. 1 cm high
G	very healthy 2-3 cm high	very healthy 2-3 cm high	very healthy 2-3 cm high	dead	very healthy 4-5 cm high	healthy 1-2 cm high	healthy 1-2 cm high
H	dead	dead	dead	dead	very healthy 2-3 cm high	dead	healthy 1-2 cm high
I	dead	dead	healthy 2-3 cm high	healthy 2-3 cm high	dead	alive 1-2 cm high	dead

TABLE 4.4.3 State of healthy plants after a period of 4 weeks.

No.	Row B 6	Row C 3	Row F 6	Row G 5	Row H 5	Row I 3, 4
Element Concentration	very healthy plant 2-3 cm high 250 µg/g Ni + 1,000 µg/g Ca	very healthy plant about 4-5 cm high 2,000 µg/g Ni	very healthy plant 2-3 cm high 200 µg/g Mg + 500 µg/g Ni	very healthy plant 4-5 cm high 400 µg/g Mg + 1,000 µg/g Ca	very healthy plant 2-3 cm high 500 µg/g Ni + 500 µg/g Mg	healthy plant 2-3 cm high 2,000 µg/g Ca + 500 µg/g Mg or 1,000 µg/g Ca + 500 µg/g Mg

Only those rows having plants higher than 2 cm high are taken into account. From this table, it is seen that the healthiest plants are from Row B, plant 3 and Row G, plant 5 i.e. a plant having 2,000 μg/g nickel and one having no nickel but 400 μg/g Mg + 1,000 μg/g Ca. The results of analysis for the various elemental concentrations of the plants after a 4 week period are shown in Table 4.4.4.

4.5 Discussion of Results

A graphical representation of the nickel, calcium and magnesium contents of leaves of s.sp. malacitanum, as a function of concentrations in the soils is shown in Fig. 4.5.1 (A-F).

In graph A, plants which grew in a nickel-only substrate followed the usual uptake pattern that would be expected. Also, the nickel concentration in the plant was much higher than in the soil e.g. for 1,000 μg/g in the soil, uptake was about 22,000 μg/g and for 500 μg/g in soil, uptake was about 13,000 μg/g. For concentrations of 100, 1,000, and 10,000 μg/g of nickel in the soil, uptake of nickel was over 10,000 μg/g. In short, nickel uptake was dependent on the concentration of nickel in the soil.

In graph B, there was a varying nickel concentration with constant calcium (1,000 μg/g) in the soil. The nickel uptake does seem to be initially lower than in graph A. At higher nickel levels in the soil however, uptake seemed to be stimulated. At lower nickel levels in the soil, there seems to be competition with calcium, a mechanism which breaks down as the nickel level of the substrate increases.

In graph C, the calcium uptake appears to be relatively low and is independent of the calcium content of the soil. However, this is not unexpected because as shown by Timperley et al. (1970) elements essential to plant nutrition have a relatively constant level in plant tissue irrespective of the amount in the soil.

TABLE 4.4.4 Elemental concentrations (μg/g) in plants of A. serpyllifolium s.sp. malacitanum still alive after 4 weeks

A	B		C	D		E	F		G		H		I	
Ni	Ni	Ca	Ca	Ca	Ni	Mg	Mg	Ni	Mg	Ca	Ni	Mg	Ca	Mg
-	-	-	1,140	-	-	220	-	-	300	410	-	-	-	-
-	41,980	490	620	580	10,420	210	-	-	140	350	-	-	-	-
-	22,020	440	690	-	-	-	-	-	230	310	-	-	370	250
20,160	-	-	580	440	17,000	220	300	16,980	-	-	-	-	430	230
19,900	8,400	600	740	290	17,390	-	220	21,620	80	480	11,210	240	-	-
13,960	9,000	540	1,360	270	23,930	140	140	21,790	110	790	-	-	340	250
13,410	8,260	660	610	110	14,740	110	180	20,590	80	680	7,590	230	-	-

A - nickel only
B - variable nickel, constant calcium (1000 μg/g)
C - calcium only
D - variable calcium, constant nickel (500 μg/g)
E - magnesium only
F - variable magnesium, constant nickel (500 μg/g)
G - variable magnesium, constant calcium (1000 μg/g)
H - variable nickel, constant magnesium (500 μg/g)
I - variable calcium, constant magnesium (500 μg/g)

N.B. Values in parentheses in footnotes refer to the constant content of the element in the substrate.

TABLE 4.5.1 Nickel, calcium, magnesium concentration in *Alyssum* specimens

Sample No.	Species	Ni(%)	Mg(%)	Ca(%)	Ca/Mg
1	*A. linifolium*	0.0130	0.69	3.03	4.39
2	*A. alyssoides*	0.0010	0.65	5.93	9.12
3	*A. dasycarpum*	0.0005	0.92	5.98	6.50
4	*A. hirsutum*	0.0005	0.90	6.66	7.40
5	*A. wierzbickii*	0.0005	0.56	6.52	11.64
6	*A. bertolonii*	1.42	0.71	1.71	2.41
7	*A. serpyllifolium* s.sp. *serpyllifolium*	0.11	0.40	4.52	11.3
8	*A. argenteum*	0.12	0.45	1.96	4.36
9	*A. murale*	0.99	0.79	4.35	5.51
10	*A. giosnanum*	1.19	1.28	4.26	3.33
11	*A. heldreichii*	2.81	0.77	1.60	2.08
12	*A. virgatum*	1.71	1.12	5.02	4.48
13	*A. floribundum*	2.09	0.93	2.76	2.97
14	*A. serpyllifolium* s.sp. *malacitanum*	1.828	1.034	4.31	4.17
15	*A. serpyllifolium* s.sp. *lusitanicum*	0.36	0.006	0.04	6.67

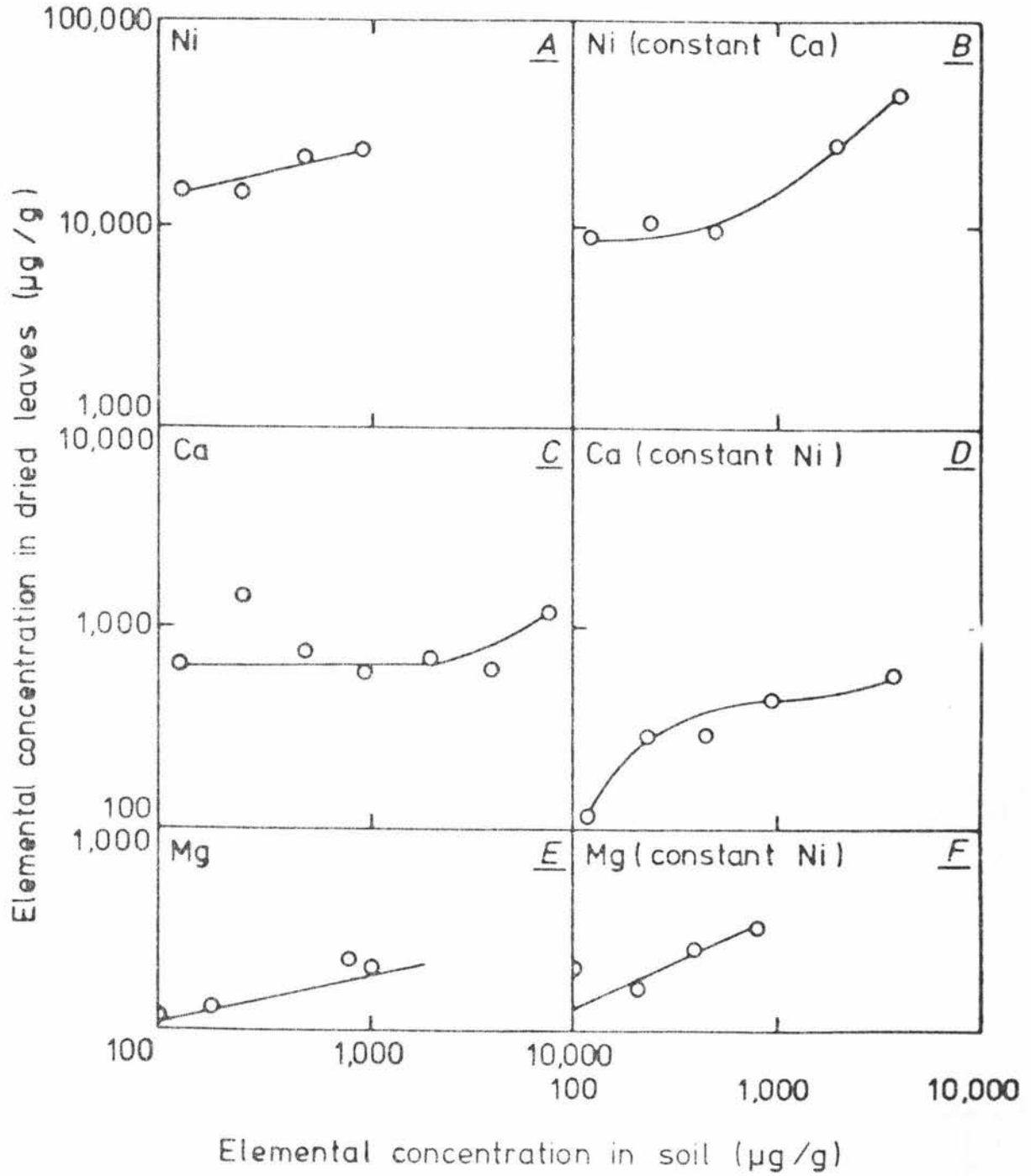


FIGURE 4.5.1 Graphical representation of the uptake of nickel, calcium, magnesium by s.sp. malacitanum .

In graph D, the same limiting concentration of calcium (as in graph C) is attained in the presence of a constant amount of added nickel (500 μg/g). At lower calcium levels in the soil however, calcium uptake is restricted. The explanation here is that inhibition of calcium uptake in plants due to the presence of nickel is only efficient as long as the calcium content of the substrate is low. Once a threshold level has been reached, this inhibition apparently does not occur. The findings in graphs B and D have other ramifications. Hunter and Vergnano (1953) have suggested that liming serpentine soils lowers nickel uptake due to higher pH. It may well be however that liming increases the calcium content of these soils and hence inhibits nickel uptake as shown in graph B. The calcium content of serpentinic soils is in any case quite low and this may be a major cause of the infertility of such soils.

In graph E, the magnesium content of the plants is apparently a function of the concentrations in the soil. However the low gradient of the graph implies reluctant uptake of magnesium, as might be expected for an element essential for plant nutrition (Timperley et al., 1970).

Graph F shows a similar pattern to that of graph E but since the gradient of the line is steeper, magnesium uptake does appear to be somewhat stimulated in the presence of nickel. This again is of importance since the infertility of serpentinic soils expresses itself in a low $\frac{Ca}{Mg}$ ratio, i.e. nickel appears to inhibit calcium at low concentration but stimulates magnesium hence compounding a problem already present.

Studies above and in a previous section whether based on naturally-occurring plants (inter-species investigation) or a pot trials of a single species (intra-species experiments) have shown a consistent pattern of enhanced nickel or calcium levels in the presence of each other. When elemental levels are expressed as oxides and are related to the ash weight, the total of CaO, NiO, MgO and K_2O clearly approaches 100%. To establish the proportion of these units

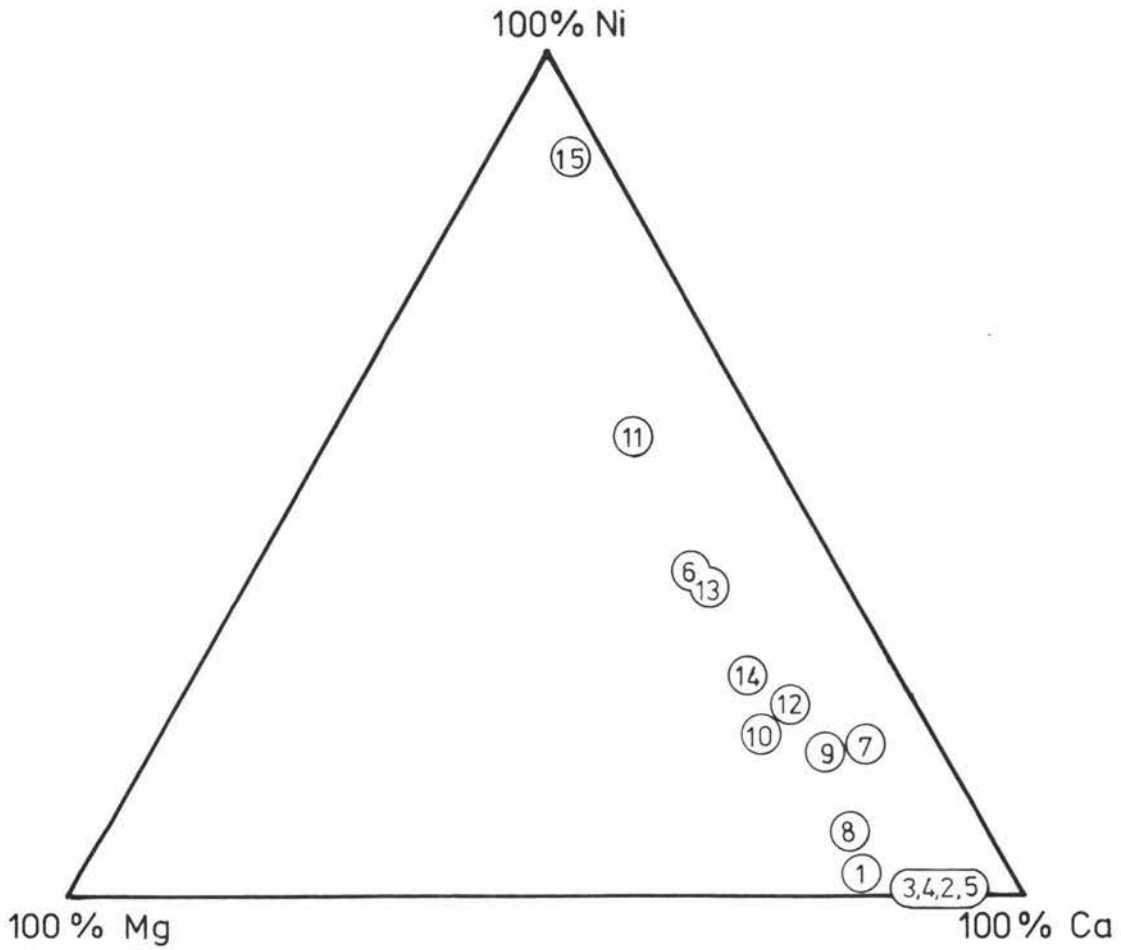


FIGURE 4.5.2 A graphical representation showing nickel, calcium, magnesium contents of a random selection of plant specimens from Alyssum.

and their relative contribution to the total content, a further set of 15 Alyssum species (including some outside of Odontarrhena) were analysed for calcium, magnesium and nickel. The data are shown in Table 4.5.1. The same data normalised to 100% are shown in Fig. 4.5.2.

Correlation analysis of the data showed a possibly significant relationship ($r = 0.58$) between nickel and calcium (see also Table 4.1.2) although there was none for nickel and magnesium. From Fig. 4.5.2 it is clear that of these three elements nickel and calcium are by far the biggest contributors to the elemental composition of the ash. Total ash analysis for s.sp. malacitanum gave the following results: -

NiO = 7.38%, MnO = 0.09%, CoO = 0.03%, FeO = 0.61%, ZnO = 0.02%, Cr_2O_3 = 0.09%, CaO = 25.2%, MgO = 6.3%, K_2O = 16.86%, Na_2O = 12.13%, Cl^- = 0.49%, CO_3^{2-} = 25.8%, PO_4^{3-} = 0.09%, giving a total of 95.09%.

Because calcium uptake tends to be stimulated by nickel uptake and because magnesium uptake is virtually unaffected, these hyperaccumulators tend to have $\frac{Ca}{Mg}$ ratios which are higher than normal. Similar findings were made by Gambi et al. (1977), Lyon et al. (1968) and Brooks et al. (1974) working on serpentinophytes. Krause (1958) has shown that many serpentine-endemic plants belong to genera and families with naturally low $\frac{Ca}{Mg}$ ratios on non-serpentinic substrates, so that the colonisation of serpentines does not involve unusual behaviour. It is postulated that in hyperaccumulators we have a further mechanism whereby the unfavourable edaphic factors of serpentine soils can be overcome. The plant is able to achieve a favourable $\frac{Ca}{Mg}$ ratio by a stimulated calcium uptake concomitant with uptake of nickel.

The importance of the calcium factor in the infertility of serpentinic soils is further highlighted by the work of Raven et al. (1976). In studies with Phacelica californica which has a non-serpentinic and serpentinic ecotype. It was shown that only the latter would grow in serpentinic soil. When calcium was added to the soil, the

non-serpentinic ecotype grew as well as the other. It is noteworthy that when other nutrients such as nitrate and phosphate were added, the non-serpentinic ecotype still refused to grow in serpentinic soils.

5. NICKEL UPTAKE AND TOLERANCE TESTS FOR *ALYSSUM SERPYLLIFOLIUM* AND ITS SUBSPECIES

5.1 Introduction

Since the discovery of the high accumulating capacity of nickel in A. bertolonii, and the increasing number of plants added to the list of hyperaccumulators of nickel, a number of questions have arisen concerning this uptake, and have been the subject of study to several workers. Some of the work has involved the study of how the metal is distributed in the plant tissues, how it is bound within the plant cells, the nature of transport within the cell and the possible reasons and explanation of how the plants have been able to tolerate such high levels of nickel.

Gambi (1967) found preferential localization of nickel in the stems of A. bertolonii by staining tissues with dimethylgloxime. An intense red coloration was found in the sclerenchymatous areas between the vascular bundles. Ernst (1972) studying Dicoma niccolifera found heavy concentrations of nickel in the cell sap. He did not detect any cobalt or chromium in the cell sap of the leaves, but found lead in small quantities only. He therefore concluded that only certain heavy metals must be found in the cell sap of the vacuoles and that other parts of the plant must take part in accumulation of the heavy metals. Turner (1970) discovered zinc and copper mainly deposited in the cell wall of some European metallophytes.

Minguzzi and Vergnano (1948) and Vergnano, Gambi et al. (1977) have studied the inorganic composition of A. bertolonii and Pelosi et al. (1974, 1976) have also isolated and purified the nickel compounds in this plant. From their studies, they concluded that these plants can accumulate nickel to very high levels 1% on dry matter, without any toxic symptoms. Also, the metal is associated with water-soluble malic and malonic levels. Control samples of A. bertolonii grown on garden soils (having nickel less than 40 μg/g) were found to have low levels of malic and malonic acids. Therefore, this is a further indication that the nickel in A. bertolonii is bound to malic and malonic acids.

Phytochemical studies on some New Caledonian plants: Sebertia accuminata, Geissois pruinosa, Hybanthus austrocaledonicus, Hybanthus caledonicus and 12 species of Homalium have shown a very high correlation between nickel and citric acid levels in leaves (Lee et al., 1978). Lee et al. (1977) have isolated a citrate complex of nickel from Sebertia accuminata, Hybanthus austrocaledonicus, Hybanthus caledonicus, Homalium francii, Homalium quillainii and Homalium kanaliense. With the exception of Psychotria douarrei (Rubiaceae) which was bound to ligand/s other than citric acid; seven of the hyperaccumulators from the New Caledonian region are concluded to have the nickel complexed with citric acid. Psychotria douarrei is however a member of an advanced family (Sporne, 1969), whereas the others are primitive. Jaffré et al. (1979), postulated that the complexing of nickel with citric acid is a primitive character. Their findings were further reinforced by Lee et al. (1978) who showed that hyperaccumulators of the family Cruciferae showed preference for malic rather than citric acid, when forming complexes with nickel. In all these cases, the plants having a high accumulation of nickel were found to be growing over ultrabasic rocks. Caesalpino (1583) had first noticed that A. bertolonii was restricted to the Upper Tiber Valley in Italy and referred to 'gabbro' vegetation in areas where there was a high magnesium and low calcium content of the soil, but it was only Pichi Sermolli (1948) in his intensive study of the Upper Tiber Valley who considered the problem of the soil composition in relation to the peculiar characters of the plants in these 'gabbro' areas. Moreover, in A. bertolonii there was an exceptional concentration of nickel in the leaves (Gambi et al., 1979), showing that this element might possibly have a physiological role. One of the explanations for the high nickel accumulation may be the enhanced availability of this element due to the lack of organic matter in these areas, complexed with a favourable soil texture for increasing the nickel availability; and therefore increasing the nickel uptake by the plants (Gambi et al., 1979). It is also evident that the degree

of weathering of the rocks, the soil evolution and climatic conditions all influence the nickel concentration in soil solutions and play a role in the higher accumulation of nickel by the plants. As suggested by Gambi et al. (1979), a better understanding on the biogeochemical nature of nickel can be obtained if further work is done on nickel uptake by serpentine plants on different serpentine soils (in controlled conditions).

5.2 Methodology

Plant Material

Seedlings of A. serpyllifolium s.sp. malacitanum an accumulator of nickel, and the closely-related non-accumulator A. serpyllifolium s.sp. serpyllifolium were grown in a 1:2 mixture of peat and pumice and used for nickel uptake and tolerance studies.

Nickel Uptake Studies

Four to six-week-old seedlings of both of the above mentioned plants were transferred to plastic pots containing a 1:2 ratio of peat and pumice, with added nutrients. Incremental amounts of nickel were added to give 50-600 µg/g (0.005-0.6%) of nickel (as nitrate) for plants of s.sp. serpyllifolium and 50-4,000 µg/g of nickel (0.005-0.4%) for s.sp. malacitanum. The seedlings were grown in the medium for a period of six weeks. At the end of the period, leaf and soil samples were analysed for nickel using atomic absorption spectrophotometry. The leaf samples were first dried, ashed at 500°C and then dissolved in 2 M HCl.

Nickel Tolerance Studies

A number of solutions were prepared containing 0, 5, 10, 15, 20, 30, 40, 50 µg/g nickel for s.sp. serpyllifolium and 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/g nickel for s.sp. malacitanum (in both cases nickel was in the form of nitrate) in 0.5% calcium nitrate. In this study, no nutrients were added. Alyssum seedlings (4 weeks old)

had their roots excised with a scapel and were then suspended in the aqueous medium by means of polystyrene rafts (5 mm thick) having holes of 5 mm diameter into which these excised plants were so placed that 2-5 mm of the excised stem extended into the solution. Each beaker had 400 ml of solution; i.e. 390 ml distilled water and 10 ml 0.05% calcium nitrate. These solutions were kept in continuous aeration and the plants were continually exposed to radiation from a source of infrared lamps so as to ensure that a shorter duration of time was required for root growth. Temperatures of the solutions were maintained at 25°C. The solutions were changed weekly and volumes made up to 400 ml by the addition of deionised water so as to replace water lost by evaporation. After a period of 4-6 weeks, the lengths of the new roots were measured. Both nickel uptake and tolerance studies will be discussed in more detail in 5.5.5 and 5.5.4 respectively.

5.3 Germination Tests

Before the pot trials and tolerance tests were carried out, it was essential to find out the maximum germination tolerance to nickel of the Alyssum species studied. Hence the germination tests were carried out for the three Alyssum species: s.sp. serpyllifolium (a non-accumulator of nickel); s.sp. lusitanicum and s.sp. malacitanum. For these tests, covered petri dishes were used. Each petri dish had a filter paper dampened with the required concentration of nickel (as the nitrate). Twenty seeds of the above specimens were placed on top of the filter paper. The petri dish was then closed with its cover and the seeds were left undisturbed for a period of 20 days. The filter paper was continually remoistened with deionised water to compensate for water loss. The number of seeds germinated after 20 days was counted. No nutrients were added over the entire germination period. Table 5.3.1 gives the concentrations of nickel used, and the % number of seeds germinated over the period of 20 days.

TABLE 5.3.1 Seed germination tests for *Alyssum serpyllifolium* subspecies, expressed as a percentage of number of seeds germinated (total of 20) at different nickel concentrations.

µg/g Ni	s.sp. *serpyllifolium*	s.sp. *lusitanicum*	s.sp. *malacitanum*
0	90	100	70
30	80	-	-
60	65	-	-
1,000	-	100	85
2,000	-	95	70
4,000	-	90	60
6,000	-	95	40
12,000	-	85	15
24,000	-	5	-

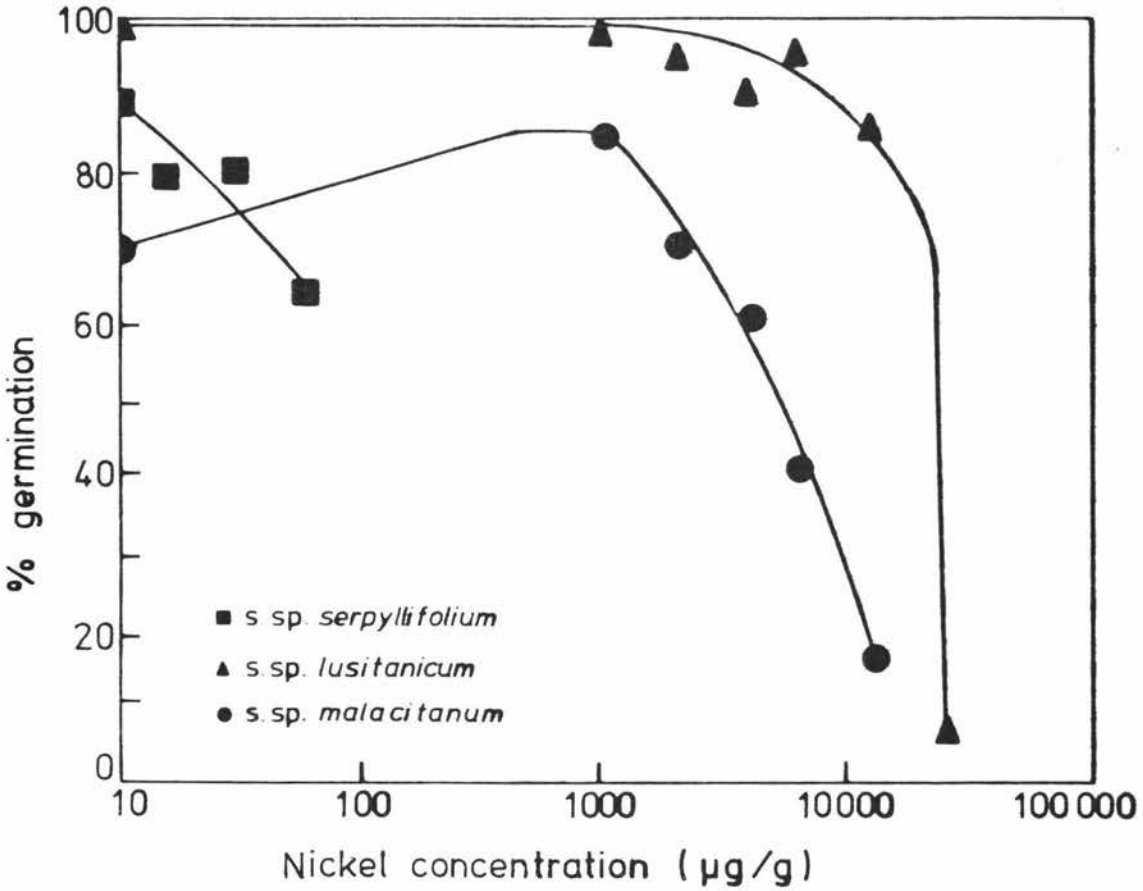


FIGURE 5.3.2 Graphical representation of seed germination tests for Alyssum species expressed as a % of the number of seeds germinated (total of 20) at different nickel concentrations (µg/g).

Fig. 5.3.2 shows the number of seeds germinated after 20 days for different concentrations of nickel (as the nitrate). As seen from the graph, s.sp. lusitanicum seems to germinate at the highest nickel concentrations (24,000 μg/g) even if it was only 1 seed that germinated, but it also tolerated 12,000 μg/g of nickel (1.2%) with as many as 17 seeds germinating. At the level of 1,000 μg/g all seeds had germinated.

In the case of s.sp. malacitanum (also a hyperaccumulator), germination was only 85% at the 1,000 μg/g level and dropped to only 40% at 6,000 μg/g.

For A. serpyllifolium s.sp. serpyllifolium (a non-accumulator), the highest tolerance was only 125 μg/g nickel, far below that of the two other hyperaccumulators. At 500 μg/g germination was only 40%.

It is concluded that s.sp. lusitanicum has certainly a higher germination capacity in the presence of nickel, as compared to the s.sp. malacitanum and s.sp. serpyllifolium.

It is interesting to note that in the control medium, (no added nickel), 20 seeds (100%) of s.sp. lusitanicum germinated compared with 18 seeds of s.sp. serpyllifolium (90%) and 14 seeds of s.sp. malacitanum (70%).

5.4 Tolerance Tests

From the results of the germination tests, it was possible to work out the concentrations required for tolerance tests using excised roots. In these tests, only A. serpyllifolium s.sp. serpyllifolium and A. serpyllifolium s.sp. malacitanum were used. For a comparative study, data for A. serpyllifolium s.sp. lusitanicum were taken from Morrison et al. (1979).

For all species, different concentrations of nickel were used. The method, materials and procedure have been fully outlined under methodology (5.5.2). This method of measuring tolerance was revised by Craig (1977). Heavy metal tolerance involves the actual measurement of tolerance

itself. Ernst (1972) followed the method of Repp (1963) to find out the tolerance of some Rhodesian heavy metal tolerant species, with some success. Antonovics et al. (1971) considered that the main effect of heavy metals is on the rooting system. Wilkins (1957) modified the method of Jowett (1958) for the determination of tolerance by measurement of root lengths: the most accepted method. In this method, plants with excised roots are grown in calcium nitrate solutions. A known concentration of the metal under study is added to the beakers, leaving the control in pure calcium nitrate solution. After a known period of growth, the length of the root is measured and the tolerance can be expressed as: -

$$\text{Tolerance index} = \frac{\text{mean root extension in toxic solution}}{\text{mean root extension in control solution}} \times 100$$

Although the tolerance index measurement shows clearly that populations of plant specimens growing over toxic soils are more tolerant than those growing over normal soils, this method has a few drawbacks (Craig, 1977). The technique is difficult to treat statistically. Also, although it gives an indication of the extent of tolerance, but to what extent is difficult to determine.

The results obtained from the observations of tolerance tests using the newly developed roots of previously-excised seedlings are shown in Fig. 5.4.1. There was no root growth above 20 μg/ml for s.sp. lusitanicum, 2 μg/ml for s.sp. malacitanum and there was only root growth in the nickel-free solution (control) for s.sp. serpyllifolium (a non-accumulator).

Again in these tolerance tests there is a situation similar to that of the germination tests (section 5.3) whereby, the Alyssum serpyllifolium s.sp. lusitanicum seems to have a higher tolerance to nickel than s.sp. malacitanum and the non-accumulator s.sp. serpyllifolium. However, it is surprising to note that s.sp. serpyllifolium which did not grow any roots in any of the concentrations

of nickel in these tolerance tests could tolerate up to 600 µg/g in a solid substrate, s.sp. malacitanum could tolerate up to 4,000 µg/g in solid substrate too (refer section 5.5) and s.sp. lusitanicum about 10,000 µg/g (Morrison et al., 1979). The corresponding values for s.sp. malacitanum and s.sp. lusitanicum were 4,000 µg/g and 10,000 µg/g respectively. The explanation may be the fact that in the nickel uptake studies the plants were grown from unexcised plants which were growing in a medium wherein much of the nickel would be absorbed by the peat:pumice mixture and hence have a lower availability to the plant.

5.5 Nickel Uptake Studies

For Alyssum serpyllifolium s.sp. serpyllifolium an artificial soil (2:1 pumice/peat) containing concentrations of 50, 100, 200, 300, 400, 500, 600 µg/g of nickel was used. For s.sp. malacitanum concentrations of 50, 100, 200, 400, 600, 800, 1,000, 2,000, 4,000 µg/g of nickel were used. Seeds of the above two Alyssum subspecies were germinated and after about four weeks, these seedlings were transferred to plastic pots containing a 1:2 peat/pumice mixture; with added nutrients. The seedlings were allowed to grow for a period of 6 weeks. At the end of that time, the leaf and soil materials were analysed for nickel by atomic absorption spectrophotometry. The leaf samples were ashed at 500°C and the ash was then dissolved in 2 M hydrochloric acid prepared from redistilled analytical grade reagent. Soils (0.1 g samples) were digested with 6 mls of 2 M HCl for 1 hour and then adjusted to 10 ml volume. After settling, the supernatant solution was decanted and analysed.

The relationship between nickel in plant material and nickel in substrate is shown in Fig. 5.5.1. The figure includes data for s.sp. lusitanicum from Morrison et al. (1979). In the case of the two hyperaccumulators, s.sp. malacitanum and s.sp. lusitanicum, the former accumulated about 7,000 µg/g (4,000 µg/g in the soil) and the latter

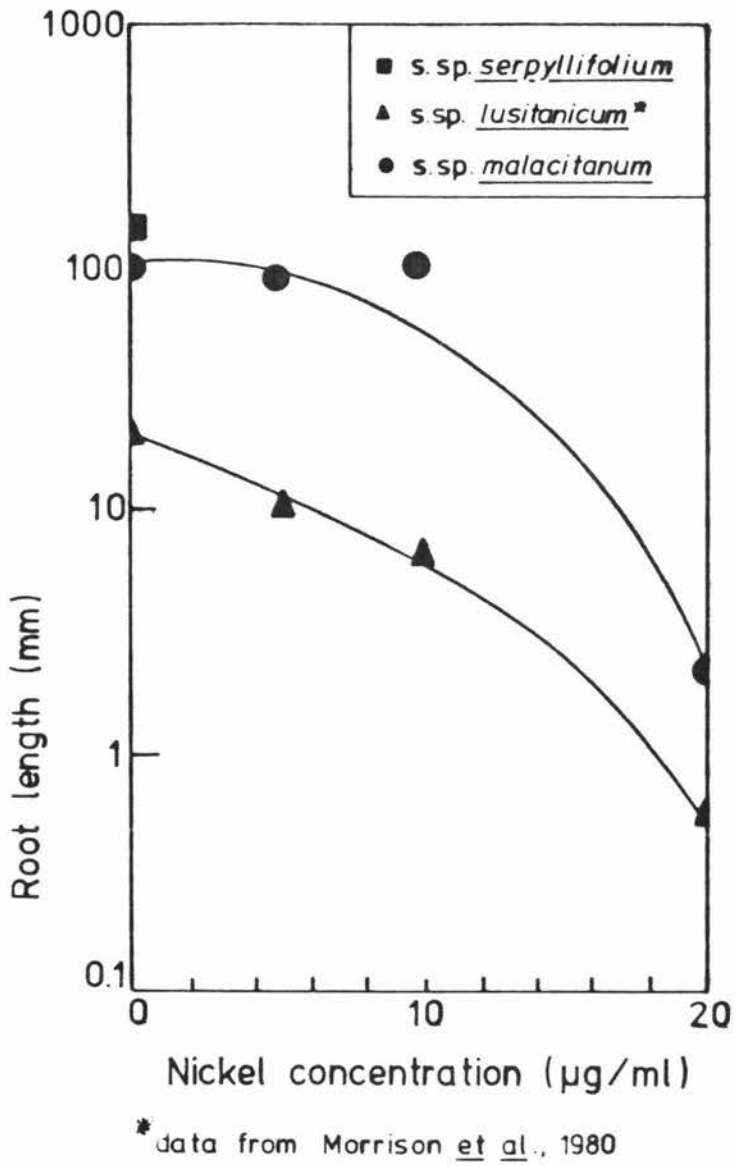


FIGURE 5.4.1 Tolerance tests involving new-root lengths of excised seedlings of Alyssum species grown in solutions of varying nickel concentrations (µg/ml).

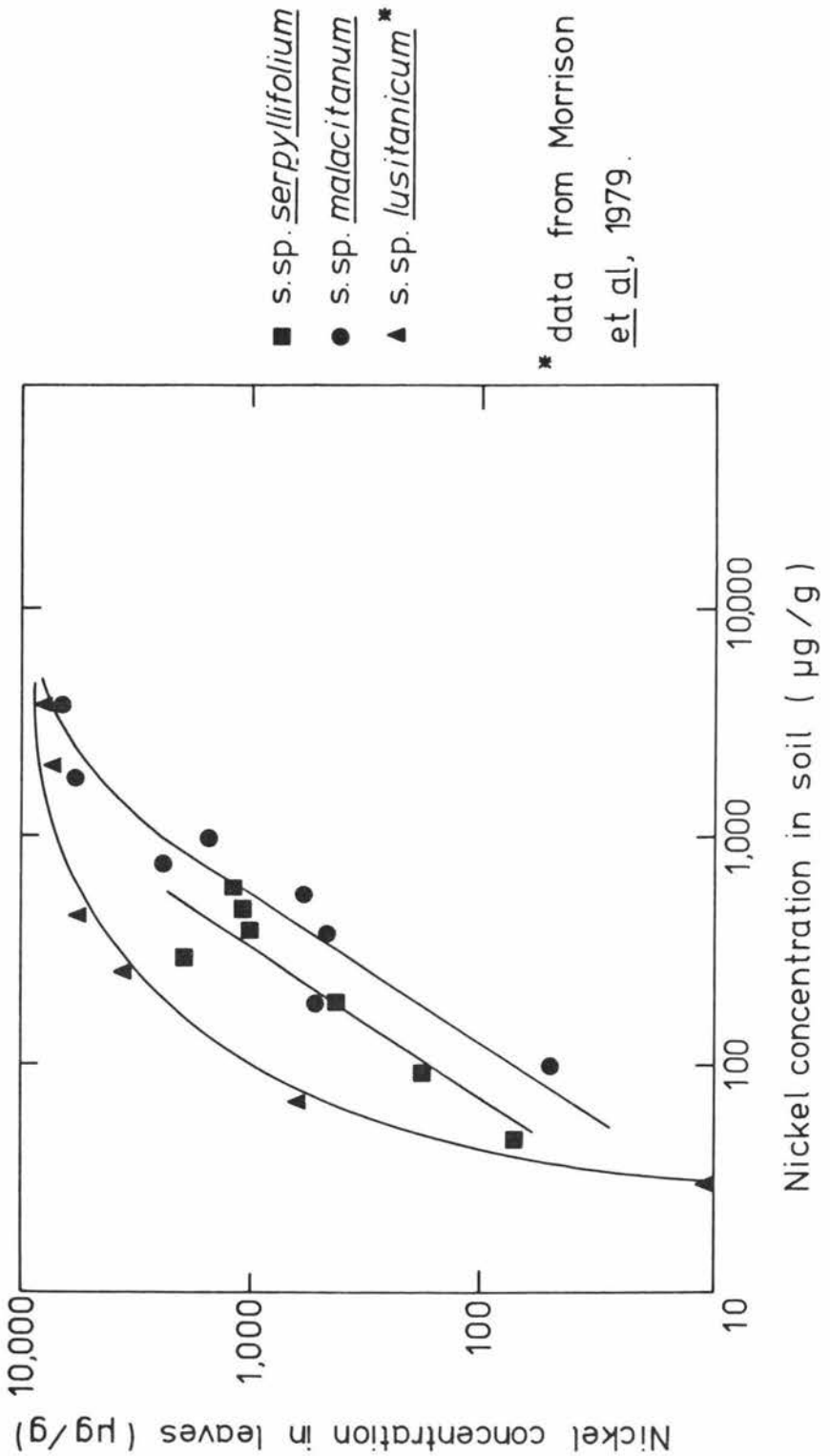


FIGURE 5.5.1 Nickel concentrations (µg/g) in dried leaves of Alyssum species as a function of nickel concentration (µg/g) measured in the soil.

about 9,000 µg/g (4,000 µg/g in the soil). These data again show that s.sp. lusitanicum can tolerate greater nickel concentrations than s.sp. malacitanum and moreover has a greater absolute uptake of this element. Much lower values were obtained for s.sp. serpyllifolium even though it belongs to Section Odontarrhena. The results of nickel uptake tests are given in Table 5.5.2.

The results of the germination tests, tolerance tests by measuring excised roots, and the nickel uptake studies show a very clear pattern. Tolerance and uptake are least in s.sp. serpyllifolium and greatest in s.sp. lusitanicum. S.sp. malacitanum is intermediate in value, though clearly a hyperaccumulator.

Although s.sp. malacitanum is sufficiently different morphologically from its obvious precursor s.sp. serpyllifolium to be at least classified as a subspecies, doubt was expressed by Rivas Goday and Esteve Chueca (1972) as to whether or not it was distinct from s.sp. lusitanicum:

"Is our plant s.sp. lusitanicum? It has not been possible to establish this with certainty from morphological studies because of insufficient material for study."

I suggest that the chemical evidence so far lends support to the idea of the two subspecies being two separate taxa. Then, their tolerance to, and uptake of nickel is appreciably different.

There is a further point that should be mentioned. A study of the genus Alyssum (Morrison et al., 1979) has shown that separate populations of distinctly different taxa have been able to develop in separate but adjacent ultrabasic regions, themselves only a few km^2 in area. Even within the same contiguous ultrabasic region, several separate nickel-accumulating taxa can exist. An example of this is the serpentinic areas of Cyprus which contains 4 hyperaccumulating species: A. chondrogynum, A. troodii, A. cyprium, A. corsicum. There is no reason to suppose therefore that the same separate development has not

TABLE 5.5.2 Range of nickel concentrations used in the nickel uptake studies.

	Ni in soil (μg/g)	Ni in plant (μg/g)
A. serpyllifolium s.sp. serpyllifolium	50	70
	100	180
	200	460
	300	1,200
	400	1,200
	500	1,000
	600	1,220
A. serpyllifolium s.sp. malacitanum	50	40
	100	50
	200	530
	400	480
	600	620
	800	2,420
	1,000	1,570
	2,000	6,980
	4,000	7,030

occurred in the Tras-os-Montes and Málaga regions which are in any case some 500 km distant.

A further discussion of chemical difference in s.sp. lusitanicum and s.sp. malacitanum will be presented further in this thesis.

6. PHYTOCHEMICAL STUDIES ON NICKEL

6.1 Introduction

Nickel is usually absorbed as the ionic Ni^{2+} from the soil or culture medium. Crooke (1954), De Kock (1956), De Kock and Mitchell (1957) reported that nickel is easily absorbed by the plants when supplied in ionic form, but not strongly absorbed when chelated. Crooke (1954) reported that levels of nickel in oat plants (Avena sativa L.) supplied with nickel versenate did not vary significantly from the controls, but the plants which were getting ionic nickel at the same concentration levels, absorbed 10 times as much, thus showing that little or no absorption of nickel complexes occurred.

In plants having high concentrations of nickel, several organic acids are capable of forming complexes with this element. Workers such as Pucher et al. (1938), Ulrich (1941) and Pierce and Appleman (1943) suggested that high concentrations of organic acids such as malic, malonic and citric acids, to name a few, are due to the direct result of high levels of cation uptake. Torri and Laties (1966) concluded that increased organic acid synthesis was a direct response to excess cation uptake, and these cations were transported into the vacuole system by the formation of a metal-organic complexes of moderate stability.

Phytochemical studies on several plant species of New Caledonia were carried out by Lee et al. (1978). They isolated nickel-containing extracts from these hyperaccumulators, and found that the nickel exists mainly as an anionic citratonickelate (11) complex. They also studied hyperaccumulators of the Homalium genus and found high levels of citric acid and also elevated levels of malic acid. In their work with Pearsonia metallifera, a nickel hyperaccumulator from Rhodesia, malic and malonic acids predominated in their organic acid extracts. Quinic acid was in moderate quantities and an unknown organic acid (c) was also detected. There was also a small quantity of citric acid. This showed that in the studies on 'primitive plant' families from New Caledonia, such as the Flacourtiaceae, Cunoniaceae, Sapotaceae and Violaceae, the nickel exists as a citrato-

complex (Lee et al. 1978).

Sporne (1969) proposed an 'advancement index' for angiosperms based on correlations between vegetative, floral, pollen and ovule morphology. The advancement index extends from 21 (most primitive) to 100 (most advanced). In the species Psychotria douarrei, Lee et al. (1978) showed that the nickel was bound to only a small degree as a citrato-complex. From this, it can be postulated that complexing with citric acid may be a primitive character of hyper-accumulators reaching its peak with the Flacourtiaceae, one of the most primitive of angiosperms. These observations were reinforced by Lee et al. (1978) who studied two Alyssum species (Cruciferae) (advancement index 63) and found that the nickel was mainly complexed with malic acid, and not citric acid. A nickel-malic acid complex was also found by Pelosi et al. (1976) in Alyssum bertolonii. Nickel-citrate complexes were found in the hyperaccumulators of New Caledonia and nickel malate/malonate in those of the Mediterranean regions. It can also be added that if hyperaccumulation of nickel is an evolutionary character; the fact that New Caledonia has long been separated from continental masses, and has an undisturbed flora could explain why most of the nickel is in nickel-citrate form.

In this project, studies on the nature of nickel complexes were carried out on the two hyperaccumulators from the Iberian Peninsula and on their non-accumulating precursor.

6.2 Plant Species Studied

Work was carried out on the three closely-related taxa: Alyssum serpyllifolium Desf. (from Granada), A. serpyllifolium Desf. s.sp. lusitanicum Dudley & P. Silva (Bragança) and A. serpyllifolium Desf. s.sp. malacitanum Rivas Goday (Málaga).

The Portuguese material consisted of freeze-dried material collected in 1977 by Drs E.M. Menezes de Sequeira and A.R. Pinto da Silva. The s.sp. malacitanum freeze-dried material was collected in 1979 by Dr A. Asensi Marfil.

The material of A. serpyllifolium was obtained from plants grown from seed supplied by the Kew Seed Bank.

6.3 Preliminary Studies on Nickel in Freeze-Dried Material

The following paragraphs give the findings on the initial studies of nickel in freeze-dried material of A. serpyllifolium s.sp. malacitanum. The initial studies involved solvent extraction schemes and differential centrifugation of freeze-dried material and fresh leaf material from s.sp. serpyllifolium, s.sp. lusitanicum and s.sp. malacitanum. These investigations showed the preferential binding sites of nickel within the plant.

6.3.1 Solvent Extraction

This technique of extraction follows that given by Bowen, Cawse and Thick (1962), to study the solubility of nickel in various solvents.

About 1-2 g of leaf material was macerated with 10 ml of 95% ethanol for 5 minutes in a bottom-drive homogeniser. The resulting slurry was centrifuged and the residue washed with portions of ethanol. The filtered ethanol extracts, containing lipids, pigments and other small neutral molecules, were combined and set aside for later analysis. The residue was then extracted with two 10 ml aliquots of distilled water, filtered and washed. The water extract contained readily-soluble polar compounds of low molecular weights, such as organic acids. The remaining residue was extracted three times with 5 ml portions of 0.2 M hydrochloric acid. The acid fractions were combined and an equal volume of acetone was added to precipitate any extracted proteins and pectates. The supernatant portion was called the hydrochloric acid fraction and the gelatinous precipitate was called the acetone-insoluble fraction. The residue was extracted similarly with 0.5 M perchloric acid at 80^{o} C and the filtrate was precipitated with acetone. This procedure removed most of the remaining polar compounds and other tightly bound groups, like cellulose, lignin and similar structural groups. The liquid and precipitate were called

the perchloric acid fraction and nucleic acid fraction respectively. The rest of the residue was boiled with 2 M sodium hydroxide for 10 minutes. This final step degraded most of the remaining proteins and polysaccarides. The final two fractions were called the soda fraction and residue.

The fractions were analysed for their nickel content by the use of an atomic absorption spectrophotometer. The results are summarised in Table 6.3.1 as a percentage of total nickel in the leaf.

Most of the nickel present in the plant was readily extracted with water and dilute acids. This showed that the nickel compound was easily soluble, highly polar and readily exchangeable. A small proportion was extracted in ethanol which had the fractions of amino-acids, non-polar pigments and lipids. The other fractions showed low amounts of nickel present in the leaves and therefore not bound to any structural groups. Lee (1977) found that a nickel hyperaccumulating species from New Caledonia, Homalium kanaliense, had a large proportion of nickel in the final residue. This quantity (8.04% of the total) was regarded as being absolutely immobile within the plant cell. Ernst (1972) investigated Indigofera setiflora leaves and found that nickel was easily extracted: water (23.4%), dilute acid (34.3%) and citric acid (34.3%) while butanol extracted minimum amounts of nickel. He concluded that metals extractable by water were located in the vacuole system and that generally having an excess of cations had a larger water-extractable fraction. Pelosi et al. (1974) found that 77% of the total nickel from leaves of Alyssum bertolonii was readily water soluble.

In our studies too, nickel was found to be readily soluble in water and dilute acids. These results imply that the element is present either as the free hydrated ion or as a highly polar complex of generally low molecular weight (e.g. with organic acids).

TABLE 6.3.1 Distribution of nickel in various solvent extracts (%) of leaf material.

Sub-species of *Alyssum serpyllifolium*	Percentage Extraction							
	Ethanol	H_2O	HCl	Acetone insoluble	$HClO_4$	Nucleic Acid	NaOH	Residue
s.sp. *serpyllifolium*	0.94	20.67	31.29	1.98	36.12	1.48	3.78	3.75
s.sp. *lusitanicum*	0.84	53.51	23.20	1.99	16.22	0.11	3.77	0.35
s.sp. *malacitanum*	0.50	31.90	20.03	1.86	23.70	0.79	18.56	2.71

6.3.2 Differential Centrifugation

A 1 g sample of freeze-dried material of s.sp. malacitanum and s.sp. serpyllifolium leaf tissue was used. The sample was homogenised for 60 secs. in 10 sec. bursts with 20 cm^3 of 0.4 M sucrose and 0.025 M phosphate buffer at pH 6.8. A Waring blender was used at top speed with short intervals between bursts to prevent excessive temperature rises. The resulting pulp was filtered through a 120 Um nylon mesh and the liquid was centrifuged as follows: 5 min.at 121 g, 10 min. at 1,085 g, 12 min. at 20,200 g and 75 min. at 110,000 g. At each stage, each residue was dried, ignited at 500°C, redissolved in 2 M HCl and analysed for nickel by atomic absorption spectrophotometry. The final supernatant portion was analysed directly. The results are summarised in Tables 6.3.2 and 6.3.3.

In both plant species, on a dry mass basis, the residual nickel seems to be almost equal in all three cell-fractions (i.e. excluding the cell wall). The nickel did not seem to concentrate predominantly in any of the cell fractions. Most of the nickel was found in the supernatant portion. This observation, therefore reinforces the finding (Table 6.3.1) i.e. that most of the nickel present in the leaf tissues is in the form of a readily-soluble polar compound of low molecular weight and it is chiefly concentrated in the vacuole system of the cell. In A. serpyllifolium s.sp. serpyllifolium 36.62% of the nickel is found in the residual portion and must therefore be absolutely immobile within the cell.

The technique of differential centrifugation showed a higher percentage of nickel in the supernatant portion and a smaller proportion in the residue (i.e. in s.sp. malacitanum). The high percentage of nickel in the 121 g fraction, represented by the cell wall fraction (i.e. in s.sp. serpyllifolium) may be due to its being an artifact of the freeze-drying process.

TABLE 6.3.2 Nickel in cell fractions (dry mass) of *A. serpyllifolium* s.sp. *malacitanum*.

Fraction	g	r.p.m.	min.	% Ni
residue	-	-	-	18.11
cell wall	121	1,000	5	3.64
chloroplasts	1,085	3,000	10	1.04
mitochondria	20,200	13,000	12	2.08
microsomes	110,000	30,000	75	2.97
supernatant	-	-	-	72.16

TABLE 6.3.3 Nickel in cell fractions (dry mass) of A. serpyllifolium s.sp. serpyllifolium

Fraction	g	r.p.m.	min.	% Ni
residue	-	-	-	36.62
cell wall	121	1,000	5	27.47
chloroplasts	1,085	3,000	10	0.61
mitochondria	20,200	13,000	12	0.20
microsomes	110,000	30,000	75	0.51
supernatant	-	-	-	34.59

Vergnano-Gambi (1967) found preferential localization of nickel in stems of A. bertolonii. The red nickel dimethyl glyoxime complex showed a very intense colour in the sclerenchymatous areas found between the vascular bundles. Turner (1970) showed that a great part of the zinc and copper was deposited in the cell wall of some European heavy-metal accumulating plants. This may be a general process whereby there is detoxication for various elements.

6.4.1 The Extraction and Isolation of Water Soluble Nickel Complexes from Leaf Tissues

The extraction technique on macerated fresh or freeze-dried leaf samples was carried out with distilled water at $0^{o}C$. This was done as to ensure that no nickel complexes appeared as an artifact during the separation process. Approximately 2 g of s.sp. malacitanum and s.sp. lusitanicum and 0.3 g of s.sp. serpyllifolium were extracted twice with 20 ml, 20 ml, and 3 ml aliquots of distilled water in a bottom-drive homogenizer. The lower volume for s.sp. serpyllifolium was due to the smaller sample available. The resulting slurry was centrifuged and the aqueous filtrates combined. In this procedure, about 50%-70% of the total nickel was almost immediately extracted. To each of the filtrates, a chloroform: n-butanol mixture (10:1) was added followed by shaking in a separating funnel. This process removed most pigments, lipids and low polar compounds of high molecular weights. A negligible portion of nickel from the aqueous phase became removed during this process. The resulting brownish aqueous solutions were reduced in volume by a rotary evaporator at $40^{o}C$. The solutions which were about 2-3 mls in volume were then passed through the Sephadex column. The nickel contents ranged from 100-7,000 $\mu g/g$, depending on the species.

6.4.2 Gel Filtration of Water-Soluble Fractions

The sephadex columns, for the process of gel filtration were used for the separation and isolation of the complexed and ionic nickel from other molecular species in the extracts.

The actual process is technically simple to perform and is unaffected by the composition of the elutant or by temperature changes. Even labile compounds can be used in this process without decomposition. For this project, the dextran gel, Sephadex G-10 was used. This has a working range of molar masses of 100 to 700 and was chosen because Severne (1972) and Lee (1974) had showed that nickel complexes in their plant material were of low molecular weight. The nickel (II) aquo complex $Ni(H_2O)_6^{2+}$ has a molar mass of 167.

The sephadex gels were left overnight to swell in distilled water before being packed into a 22 cm x 1.1 cm glass column. These gels are extremely stable, not easily contaminated and therefore these columns can be used for many elutions over a lengthy period. The column was equilibrated with deionised water. This water-wash also prevented any possibility of complex formation between the extracted nickel and any added reagents. Dextran-blue dye was utilised to check the functioning of the column and also to establish the void volume (29 mls for the G-10 column).

About 2 ml quantities of crude aqueous extracts, were applied evenly to the column head and fractions collected with an automatic fraction collector. Approximately 3 ml fractions were collected at a flow rate of about 0.6 ml/min. The fractions were analysed for nickel by atomic absorption spectrophotometry. Nickel-containing fractions were combined and recycled through the column. This effectively increased column length. The nickel fraction became an intensified green colour with repeated separations. The green-coloured nickel fraction separated out between the faster moving high molecular weight plant substances and a yellow-brown band of low molecular weight, organic acids, amino acids and various salts. Fig. 6.4.2.1 shows the distribution of nickel in aqueous extracts of Alyssum species. Although the shape and peaks were quite consistent for extracts from each of the species, the ratio of aquo nickel ions to complexed nickel varied considerably amongst the three species.

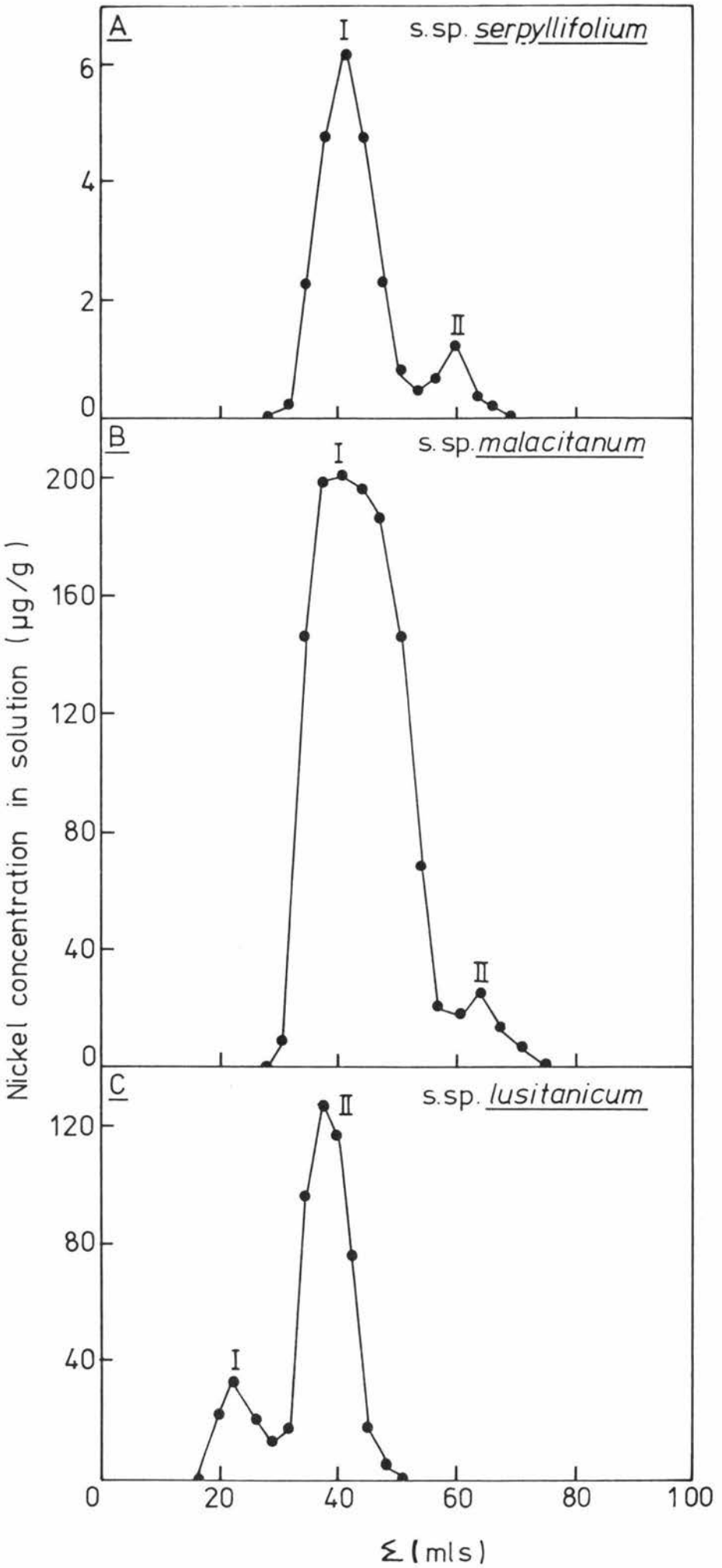
FIGURE 6.4.2.1 Elution curve from Sephadex G-10 column showing nickel complexes in aqueous extracts of *Alyssum* subspecies.

Elutant: deionised water

column (22 x 1.1) cm

I Ni^{2+}

II Ni aquo complex


A
s.sp. serpyllifolium
I
II
B
s.sp. malacitanum
I
II
C
s.sp. lusitanicum
II
I
Nickel concentration in solution (µg/g)
Σ (ml s)
0
2
4
6
40
80
120
160
200
20
60
100

6.5 Gas-liquid Chromatography

(i) Introduction

Gas-liquid chromatography has its usefulness in determining the number of components from volatile compounds. In this method, the important criterion is the temperature which should be kept as constant as possible.

Preliminary tests showed that the nickel in the isolated extracts was bound to an organic component that could probably be that of a hydroxy carboxylic acid. Gas liquid chromatography was therefore utilised for the identification of this organic component. The plant extract was of low volatility, therefore this had first to be methylated. Methylation as opposed to silylation is useful as it can be used over a wide range of functional groups.

Canvin (1965), Atkins and Canvin (1971), Barta and Osmond (1973) have discussed the use of g.l.c. for the determination of plant organic acids.

(ii) Method

About 10 mg samples of the dried nickel-fractions were dissolved in a minimum amount of water and further treated with 0.5 ml dilute hydrochloric acid so that dissociation of the complex present was complete. The samples were dried in a stream of dry nitrogen. The fractions, along with 2 mg quantities of recrystallised organic acid standards were then dissolved in 1 ml of redistilled ether. The advantage here is that the methylated derivatives dissolved completely in the solvent. Diazomethane, prepared from nitroso-methylurea and potassium hydroxide (Werner, 1919) was dissolved in ether. A complete methylation was ensured by a dropwise addition of ethereal diazomethane solution. When the bubbles of nitrogen ceased, and a persistent yellow colour remained in the solution, this gave an indication of complete methylation. The excess of ether was removed by allowing the bottles to remain uncovered for a few hours. The methylated products were then redissolved in 0.5 ml of redistilled chloroform and left in the refrigerator. Analyses were carried out on a Varian aerograph series 1520

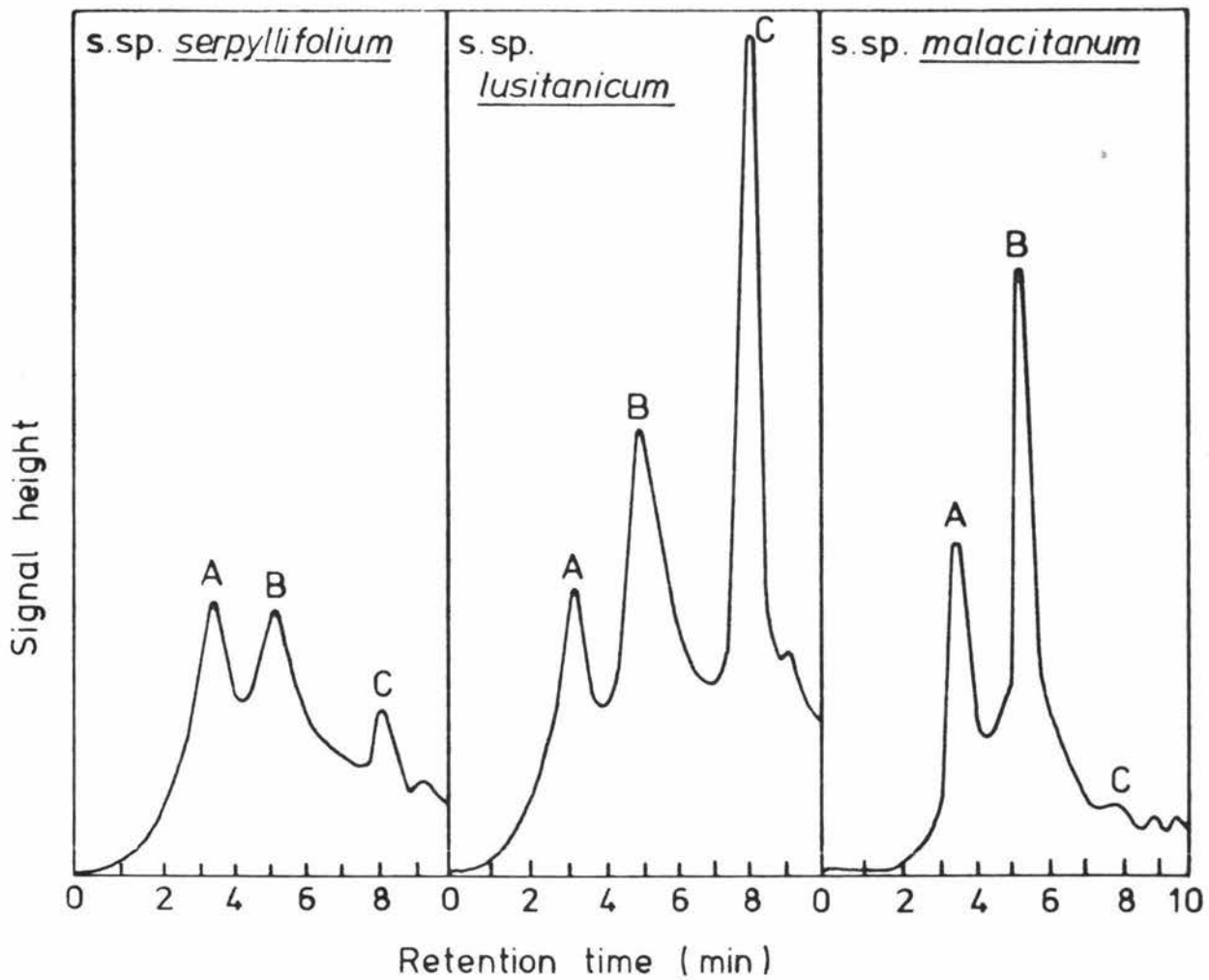


FIGURE 6.5.1 Gas-liquid chromatograph of methyl derivatives of plant extracts of Alyssum sub-species.

A. citric acid
B. malonic acid
C. malic acid

TABLE 6.5.2 Retention times of the methyl derivatives of plant extracts of *Alyssum* sub-species compared with those of standards.

Material	Retention times (min.)
citric acid standard	3.0
malic acid standard	5.0
malonic acid standard	7.0-8.0
s.sp. *serpyllifolium*	3.0, 5.0, 7.0
s.sp. *lusitanicum*	3.0, 4.0-6.0, 7.0-9.0
s.sp. *malacitanum*	3.0, 5.0, 7.0-8.0

gas chromatograph, fitted with a 1.5 m x 0.2 mm glass column. The gas was operated isothermally at 150°-180°C, using helium as the carrier gas at a flow rate of 30 ml/min. Elution times for 5 µl volumes were recorded for standards and samples using a flame ionization detector with air and hydrogen flow rates of 300 ml/min. and 30 m/min. respectively. Samples could be taken with a micro-syringe and introduced directly on to the gas chromatograph column.

(iii) Results

Fig. 6.5.1 shows retention times and the order in which the methylated derivatives found in the plant material were eluted from the column. All chromatograms gave distinct peaks showing that there was no impurity and that there was complete methylation. A good separation of these organic acids was obtained. Three distinct peaks were obtained for each of the three sub-species determined. These three peaks corresponded to the retention times of the standards of citric, malonic and malic acids. Table 6.5.2 gives the retention times of the standards compared with those of the methylated plant residues.

A study of the difference in the three acids present in the plant extracts can be summed up as follows: -

(a) s.sp. serpyllifolium certainly has lower levels of acids as compared to the other two hyperaccumulators.

(b) s.sp. malacitanum has less malic acid than s.sp. lusitanicum, but the latter has a larger amount of malonic and citric acids.

It cannot be coincidental that two (citric and malic acids) of the three organic acids mentioned above are involved in the citric acid cycle. Heavy metals such as nickel tend to act as enzyme inhibitors, thereby resulting in the metal having a toxic action on the cycle. As discussed earlier, most nickel in the plant is situated in the vacuoles thus preventing entry of the metal into enzymatic sites in the mitochondria situated outside these vacuoles. However

some nickel is found in the mitochondria (Table 6.3.2, 6.3.3) and this would have an inhibitory action on the enzyme/s. Although it is possible that some of the nickel shown to be in the mitochondria may be contamination from vacuolar material after break up of the cells, the nickel content of the plant is so high that it is difficult to believe that nickel could be completely excluded.

If this nickel is present in the mitochondria, and acts as an inhibitor in the citric acid cycle due to de-activation of a particular enzyme this must result in an increase in the amount of the organic acid immediately preceding that stage of the cycle. Because of deactivation of malic acid dehydrogenase, there will be a build-up of malic acid in the vacuoles. Malic acid build-up will result in nickel uptake by complex formation - nickel will diffuse into the vacuoles from the mitochondria so that blockage of the cycle is eased. Therefore it would appear as if the plant has a regulating mechanism for controlling the nickel toxicity so that these plants can adapt to unfavourable edaphic factors in the soil.

From Fig. 6.5.1 it looks as if the two hyperaccumulators produce much more organic acids than their precursor the non-accumulator, so that the latter is not able to adapt itself to nickel-rich soils. Torri and Laties (1966) have observed the stimulation of organic acid production in the presence of nickel.

The role of malonic acid is also puzzling. Szent-Gyorgi (1939) observed that malonic acid has an inhibitory action on succinic dehydrogenase in the citric acid cycle, and does not normally have access to the citric acid cycle in order to exercise this inhibitory effect. However, malonate may be formed by the carboxylation of acetate in plants so that the inverse malic-malonate relationship in the two hyperaccumulators may result from the diversion of acetate carbons predominantly towards carboxylation (which may occur in the cytoplasm) or else towards malate formation. From our studies, malic acid is also produced in large amounts by species able to tolerate high amounts of nickel

(e.g. s.sp. lusitanicum) and tolerance is lowest in species having an overall low production of organic acids (Brooks et al., 1980).

The differences in nickel tolerance of these two hyper-accumulating subspecies of A. serpyllifolium when reinforced with evidence of considerably different malic/malonic acid ratios lend additional chemotaxonamic weight to the thesis that s.sp. malacitanum is not conspecific with s.sp. lusitanicum so that if there is evidence (admittedly mainly morphological) that the latter should be promoted to full generic rank (as has already been done), serious thought should also be given to a similar treatment of s.sp. malacitanum.

7. CONCLUSIONS AND RECOMMENDATION FOR FURTHER STUDIES

In the studies reported in this thesis a number of significant findings have been made:

1. Alyssum serpyllifolium s.sp. malacitanum is clearly yet another hyperaccumulator of Section Odontarrhena. This brings to 46 the total so far discovered in this section.

2. S.sp. malacitanum has a high tolerance to nickel though it is inferior in this respect to s.sp. lusitanicum. This degree of tolerance is demonstrated not only as pot trials but also by germination tests and by experiments involving new-root growth of excised seedlings.

3. Experiments on wild plants with pot trials, have shown that mutual stimulation of calcium and nickel uptake. This relationship may be a mechanism whereby a more favourable Ca/Mg ratio can be obtained in hyperaccumulators in order to counteract unfavourable edaphic effects of serpentinic soils.

4. It is concluded that the phytochemical experiments have indicated a possible method to explain the mechanism of accumulation of nickel by these plants and the physiological reasons for this accumulation. If this model is correct, it may well have a general application to all cases where tolerance to unfavourable edaphic conditions is effected by accumulation of toxic elements rather than by their exclusion.

5. The chemical evidence obtained in the above studies lends support to this thesis that s.sp. malacitanum should be promoted to full specific rank. If both this taxon and its close ally s.sp. lusitanicum are morphologically sufficiently different from each other and from their precursor s.sp. serpyllifolium to warrant all three being considered subspecies of A. serpyllifolium Desf., and if s.sp. lusitanicum is now in the course of being reclassified as a separate species, then there is no reason why the same should not be done for s.sp. malacitanum. Certainly the

chemical evidence alone indicates that the three subspecies are appreciably different from each other in their tolerance to nickel.

There are several obvious avenues of future research in the above field of study. If the mechanism I have proposed for nickel tolerance by hyperaccumulators (i.e. complexing with malic acid to unblock the citric acid cycle), is correct, then similar investigation should be carried out on other hyperaccumulators such as those of copper and cobalt from Shaba Province, Zaïre (Morrison et al., 1979).

Further studies should also be carried out on the sites of formation of nickel complexes within the plants in order to understand more fully the mechanism whereby this element is transported and complexed therein.

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PUBLICATIONS ARISING FROM THIS THESIS

1. R.R. Brooks, S. Shaw and A. Asensi Marfil (1980). "Some observations on the ecology, metal uptake and nickel tolerance of *Alyssum serpyllifolium* subspecies from the Iberian Peninsula" (submitted to *Vegetatio*).

2. R.R. Brooks, S. Shaw and A. Asensi Marfil (1980). "The chemical form and physiological function of nickel in some Iberian *Alyssum* species" (submitted to *Physiologia Plantarum*).