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Nickel hyperaccumulation by the species of *Alyssum* and *Thlaspi* (Brassicaceae) from the ultramafic soils of the Balkans

Aida Bani[1,2*], Dolja Pavlova[3], Guillaume Echevarria[2], Alfred Mullaj[4], Roger D. Reeves[5], Jean Louis Morel[2] and Sulejman Sulçe[1]

1 Agro-Environmental Department, Faculty of Agronomy and Environment, Agricultural University of Tirana, Koder-Kamez, Tirane, Albania

2 Laboratoire Sols et Environnement, Nancy-Université, INRA, 2 avenue de la Forêt de Haye, B.P. 172 F-54505 Vandœuvre-lès-Nancy, France

3 Department of Botany, Faculty of Biology, University of Sofia, blvd. Dragan Tzankov 8, 1164 Sofia, Bulgaria

4 Department of Biology, Faculty of Natural Sciences, University of Tirana, Blv "Zog I", Tirane, Albania

5 School of Botany, University of Melbourne, Parkville, VIC 3010, Australia

ABSTRACT: Hyperaccumulation of nickel to concentrations above 1000 mg kg^{-1} on a dry matter basis has become recognized as an unusual response by some plant species to the elevated nickel concentrations generally found in soils derived from ultramafic rocks (often referred to as serpentine soils). Such soils are widespread in the Balkans. These soils host some widespread species and a smaller number of regional or local endemics. Several serpentine areas in Albania (AL), Bulgaria (BG) and Greece (GR) have been surveyed because of the presence of nickel hyperaccumulating endemics, including some that are common for all three studied countries. The objectives of the study were to widen understanding of the distribution of the nickel hyperaccumulators and their uptake behaviour in relation to the characteristics of their native soil environments. Collection and chemical analysis of both plant and soil samples has allowed evaluation of phenotypic efficacy in hyperaccumulating nickel. In total, eight taxa were studied. In this work the highest Ni concentrations in leaves (1.5-2.0%) were found in *Alyssum murale* at Pojska (AL), *A. murale* at Kazak (BG), *A. markgrafii* at Gjegjan (AL) and *Thlaspi kovatsii* at Fotinovo (BG). The maximum quotients of plant Ni concentration to soil Ni concentration range from 13.9 for *A. markgrafii* to 6-7 for *A. murale, T. kovatsii* and *T. tymphaeum* from northern Greece. Eight of the taxa collected in this work show hyperaccumulation of Ni; these include *A. murale* subsp. *pichleri* and all of *Thlaspi* species studied from serpentine in Bulgaria. In total, twenty-five Ni hyperaccumulating taxa are now known from the Brassicaceae of Albania, Bulgaria and Greece. Because of its high biomass production, *A. murale* is likely to be the most suitable species for Ni phytomining in the Balkans.

Key words: hyperaccumulation, nickel, *Alyssum*, *Thlaspi*, ultramafics, the Balkans

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INTRODUCTION

Serpentine soils, developed upon ultramafic rocks, and in particular on rocks containing significant proportions of serpentine-related minerals, are widely distributed in different parts of the world (Brooks 1987). These soils contain high concentrations of Mg and Fe, and relatively high concentrations of Ni, Cr and Co. Manganese

*correspondence: aida_alushi@hotmail.com

levels can be also higher compared to other soil types. Concentrations of N, P and K are usually low, and the Mg/Ca quotient is high, because the soils are not only Mg-rich but also Ca-poor (Proctor 1999). Serpentine soils are ubiquitous but also patchily distributed. Although some variation occurs between sites, Whittaker (1954) identified three collective traits: (a) poor productivity, (b) high rates of endemism, (c) vegetation types distinct from those of surrounding areas. Many aspects of the so called serpentine syndrome have been discussed in a recent review (Kazakou *et al.* 2008).

Serpentine (ultramafic or ophiolitic) substrates cover quite large areas in the Balkans, more than in other parts of Europe (Brooks 1987; Tatić & Veljović 1992). They exist as large blocks or as small outcrops separated from other geological formations, in central Bosnia and western and central Serbia. They extend towards north, central and south-eastern parts of Albania and further to the serpentine formations in the regions of Epirus and Thessaly in Greece. Small quantities of serpentine bedrock are distributed in south-western, south and central parts of Bulgaria, mainly in the eastern and central Rhodopean mountains (Pavlova *et al.* 1998; Pavlova 2001a). Some fairly isolated serpentine "islands" occur in the northern part of Macedonia (FYR), north-eastern Serbia and Greece (Sterea Ellas, Evvia, Gerania, Kallidhromo) (Stevanović *et al.* 2003). The altitudes of the serpentine exposures in Greece and the Balkans cover a very wide range, from near sea level on the islands of Evvia and Lesbos to more than 2200 m on Smolikas Mt. in NW Greece.

The serpentine flora of the Balkans is characterized by a relatively high degree of endemism. The greatest concentration of serpentine endemic species in the Balkans is in the mountains of the western part of the peninsula in the territories of Bosnia, Serbia, Albania and N Greece. These factors helped to make the Balkan peninsula an important refuge for plants during the Quaternary glaciations (Tatić & Veljović 1992). Early work on the serpentine flora of the region has been summarized by Brooks (1987), and other notable contributions are those of Babalonas *et al.* (1984), Tan *et al.* (1999) and Pavlova (2001a, b; 2004). According to Stevanović *et al.* (2003) there are 335 Balkan endemic vascular plant taxa (species and subspecies) growing on serpentine, of which 123 are obligate serpentinophytes.

Plants growing on serpentine soils often show elevated levels of some metallic elements such as Ni and Co, compared to the same plants on other soil types. However, in a few cases, remarkably high concentrations (>1000 mg kg^{-1}) of Ni in particular may be found in the dry matter of above-ground plant tissues of serpentinophytes. This was first observed by Minguzzi & Vergnano (1948) for *Alyssum bertolonii* Desv. in Italy, and was followed by similar reports for *A. murale* Waldst. & Kit. in Armenia (Doksopulo 1961) and *A. serpyllifolium* Desf. subsp. *lusitanicum* T.R. Dudley & P. Silva in Portugal (Menezes de Sequeira 1969). Later investigations by Brooks & Radford (1978) and Brooks *et al.* (1979) revealed that Ni accumulation to this level was widespread among *Alyssum* species on serpentines of Mediterranean Europe, Turkey and adjacent countries. Nearly 50 of the 170 taxa, all in sect. *Odontarrhena* (C.A. Meyer) Hooker of this genus, act as Ni hyperaccumulators (>1000 mg kg^{-1} or 0.1%). In fact, in leaf specimens of many of these species can often be found more than 1% of Ni. Tabulations and data have been given by Brooks (1987), Baker & Brooks (1989) and Reeves & Adigüzel (2008).

Nickel hyperaccumulation has also been discovered in other genera in the Brassicaceae of Europe and Turkey, notably in the monospecific *Leptoplax emarginata* (Boiss.) O.E. Schulz (syn. *Peltaria emarginata* (Boiss.) Hausskn.), in several species of *Bornmuellera*, in two species of *Cochlearia* sect. Pseudosempervivum in Turkey, and in a considerable number of species of *Thlaspi* from Europe, Turkey, USA and Japan. *L. emarginata* from Evvia and the Pindus mountains of N Greece was reported with up to 34400 mg kg^{-1} of Ni hyperaccumulated (Reeves *et al.* 1980). Three subspecies of *Bornmuellera baldaccii* (Degen) Heywood from N Greece and Albania were found with up to 27300 mg kg^{-1} of Ni, *B. tymphaea* (Hausskn.) Hausskn. had up to 31200 mg kg^{-1} and the sterile hybrid *B. x petri* Greuter (both from N Greece) had up to 11400 mg kg^{-1} (Reeves *et al.* 1983a). In *Thlaspi*, hyperaccumulation of Zn by one species on Zn-rich soils has been known since the second half of the 19^{th} century, and the occurrence of Ni hyperaccumulation in a number of species and subspecies (possibly more than 20) on serpentine was reported by Reeves & Brooks (1983), Reeves *et al.* (1983b) and Reeves (1988). A definitive listing of Ni hyperaccumulators has been made difficult by widespread disagreement about the demarcation of the genus as a whole and of individual taxa. In this work we shall use *Thlaspi* in the broadest sense and generally follow the treatment of Flora Europaea.

Confirmation of the hyperaccumulation by a number of Balkan species in the above genera has been provided in several recent publications (Shallari *et al.* 1998; Pavlova & Alexandrov 2003; Massoura *et al.* 2004; Chardot *et al.* 2005; Bani *et al.* 2008). Species of particular interest include serpentine endemics of restricted distribution such as *Bornmuellera baldaccii* subsp. *baldaccii* (GR, AL), *B. tymphaea* (GR), *Leptoplax emarginata* (GR), *Alyssum markgrafii* O.E. Schulz (AL, Serbia), *A. heldreichii* Hausskn. (GR), the more widespread *A. murale*, and several *Thlaspi* species. This concentration of Ni hyperaccumulators in the Balkan area led us to investigate their distribution and behaviour more closely.

The objectives of this study were to identify collection sites in which the Ni hyperaccumulator species occur and to assess the potential of Ni hyperaccumulation in relation to the soil characteristics of their environments. This is of particular interest because the potential applications of hyperaccumulators, such as phytoremediation and phytomining, are being extensively discussed (Raskin & Ensley 2000; Chaney *et al*. 2007).

MATERIALS AND METHODS

Sites investigated. Sampling of Ni hyperaccumulator species and their associated serpentine soils was conducted in May 2007 at 12 sites in Albania, in August 2006 at 4 sites in the Pindus Mts. (Greece) and in May-June 2005 at 7 sites in Bulgaria. These sites were surveyed because they host a significant proportion of the hyperaccumulators of these regions. The extensive ultramafic outcrops found throughout E Albania continue into NW Greece, where the serpentine parts of locations such as Grammos Mt, Smolikas Mt and the Pindus Mt have long been a source of botanical interest (Brooks 1987). Further west, the Vourinos massif is another source of serpentine endemics, and the serpentine continues to the SE (region around Dhomokos, and northern parts of the island of Evvia). Although some of the sites studied in the Pindus Mt are in a natural state, there has been disturbance by grazing animals in some places (e.g. at the top of the Katara Pass).

The serpentines of Bulgaria include a number of outcrops at 275-475 m in the lower montane belt of the E and W Rhodopes. The geological structures of the Serbo-Macedonian massif and the Rhodope massif extend further to the SE into NE Greece, where important serpentine exposures can be found in areas such as Voras Mt, several sites near Thessaloniki, and in the island of Lesbos. For the present work, seven sites in the Rhodopes have been investigated. These are classified into two groups: (1) those with sparsely colonized debris (open habitats, sites 10, 13a, 13b and 14, Table 1); (2) those where the vegetation has limited recognizable serpentine features (covered by coppice oak forests diversifying into shiblyak (Mediterranean-type short-tree woodland) in some places, sites 11, 12, 15). At all these sites the vegetation cover is diverse, represented mainly by xerothermic Macedonian-Thracian floristic elements, but including Balkan and Bulgarian endemics as well. The vegetation at these sites is highly influenced by anthropogenic activity (recent mining of chrome at sites 10, 13 and 14), erosion is well expressed and remains of flotation constructions are still visible.

At the sites studied here eight Ni-hyperaccumulating taxa were found (the nomenclature used here follows Tutin *et a*l. (1993) and Ančev (2001)): *Alyssum murale* Waldst. & Kit. subsp. *murale* and subsp. *pichleri* (Velen.) Stoj. & Stef., *A. heldreichii* Hausskn., *A. markgrafii* O.E.

Table 1. List of plant taxa sampled and characteristics of sampling locations.

Sampled plants	Sampling sites	Site code	Altitude (m)	Geographical coordinates
Alyssum murale Waldst. & Kit. ssp. *murale*	Pojska-Soil 1 - AL	Site 1a	700	40°59'55.3"N 20°38'0.9"E
A. murale Waldst. & Kit. ssp. *murale*	Prrenjas-Domosdova - AL	Site 2	400	41°04'08"N 20°33'11"E
A. murale Waldst. & Kit. ssp. *murale*	Prrenjas-rock - AL	Site 3	600	41°04'13"N 20°33'53"E
A. murale Waldst. & Kit. ssp. *murale*	Pishkash-Soil 1-AL	Site 4a	500	41°04' N 20°33'E
A. murale Waldst. & Kit. ssp. *murale*	Gjegjan - AL	Site 5	400-600	41°55'47"N 20°00'09"E
A. murale Waldst. & Kit. ssp. *murale*	Rubik - AL	Site 6	300	41°46'28"N 19°47'10"E
A. murale Waldst. & Kit. ssp. *murale*	Katara Pass - GR	Site 7	1690	39°47.77'N 21°13.74' E
A. murale Waldst. & Kit. ssp. *murale*	Malakasi-Soil 2 -GR	Site 8a	878	39°46.98'N 21°18.08'E
A. murale Waldst. & Kit. ssp. *murale*	Trigona - GR	Site 9	830	39°47.29'N 21°25.32'E
A. murale Waldst. & Kit. ssp. *murale*	G. Kamenjane -BG	Site 10	414	41°24.068'N 25°42.231'E
A. murale Waldst. & Kit. ssp. *murale*	Kazak - BG	Site 11	350	41°24.619' N 25°53.166'E
A. murale Waldst. & Kit. ssp. *murale*	Kardzali - BG	Site 12	275	41°33.821'N 25°22.932'E
A. murale Waldst. & Kit. ssp. *murale*	Fotinovo 2 - BG	Site 13b	425	41°21.800'N 25°18.628'E
A. murale Waldst. & Kit. ssp. *murale*	Dobromirtzi - BG	Site 14	389	41°23.053'N 25°12.096'E
A. murale Waldst. & Kit. ssp. *pichleri* (Velen.) Stoj. & Stef.	Parvenetz - BG	Site 15	360	42°03.948'N 24°39.472'E

Sampled plants	Sampling sites	Site code	Altitude (m)	Geographical coordinates
A. heldreichii Hausskn.	Katara Pass - GR	Site 7	1690	39°47.77'N 21°13.74' E
A. heldreichii Hausskn.	Malakasi-Soil 1- GR	Site 8b	1031	39°46.399'E 21°17.574'E
A. markgrafii O.E. Schulz ex Markgraf	Gjegjan-Gojan -AL	Site 16	400-600	42°03'50"N 20°01'26"E
A. markgrafii O.E. Schulz ex Markgraf	Fushë-Arrëz - AL	Site 17	400-600	42°03'50"N 20°01'26"E
A. markgrafii O.E. Schulz ex Markgraf	Gjegjan - AL	Site5	400-600	41°55'47"N 20°00'09"E
A. markgrafii O.E. Schulz ex Markgraf	Librazhd - AL	Site 18	700	41°10' 46"N 20°18'54"E
Thlaspi ochroleucum Boiss. & Heldr.	Pishkash-Soil 2 -AL	Site 4b	500-600	41°05' 48"N 20 30' 44"E
Th. ochroleucum Boiss. & Heldr.	Posjka-Soil 2 - AL	Site 1b	500	40°59'55.28"N 20°38'0.8"E
Th. ochroleucum Boiss. & Heldr.	Kardzali - BG	Site 12	275	41°33.821'N 25°22.932'E
Th. tymphaeum Hausskn.	Katara Pass - GR	Site 7	1690	39°47.77'N 21°13.74' E
Th. tymphaeum Hausskn.	Malakasi-Soil 2- GR	Site 8a	878	39°46.98'N 21°18.08'E
Th. apterum Velen.	Fotinovo 1 - BG	Site 13a	421	41°22.531'N 25°19.165'E
Th. apterum Velen.	Fotinovo 2 - BG	Site 13b	425	41°21.800'N 25°18.628'E
Th. apterum Velen.	Dobromirtzi - BG	Site 14	389	41°23.053'N 25°12.090'E
Th. praecox Wulfen in Jacq.	Fotinovo 2- BG	Site 13b	425	41°21.800'N 25°18.628'E
Th. praecox Wulfen in Jacq.	Dobromirtzi - BG	Site 14	389	41°23.053'N 25°12.090'E
Th. praecox Wulfen in Jacq.	Parvenetz - BG	Site 15	360	42°03.948'N 24°39.472'E

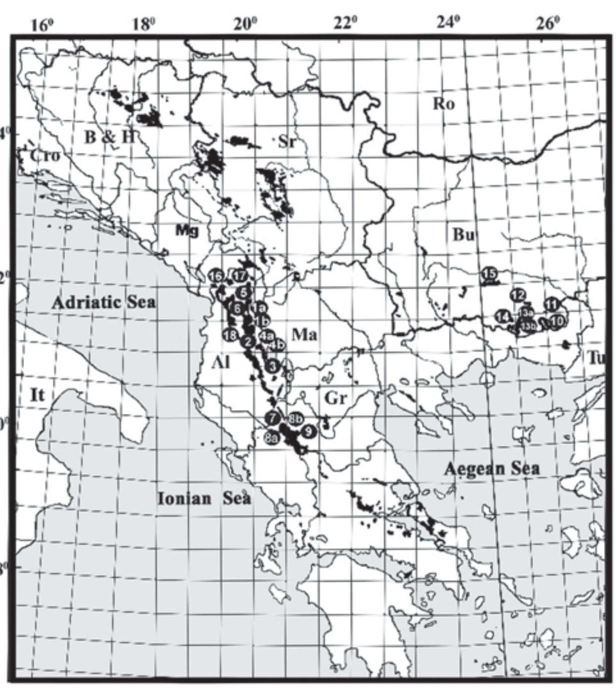

Fig. 1 Distribution of the serpentine sites studied within the Balkans. Site description is given in Table 1.

Schulz, *Thlaspi kovatsii* Heuff., *T. ochroleucum* Boiss. & Heldr., *T. praecox* Wulfen and *T. tymphaeum* Hausskn. *T. kovatsii* is here understood to include *T. apterum* Velen. (= *Noccaea aptera* (Velen.) F.K. Meyer).

Soil and plant analyses. One to three plants were collected from each site. Plants were pooled to make a composite sample where there was more than one. From each site one to three soil samples were taken from the upper horizon at a depth of 0-20 cm where possible, near the roots of the sampled plants. Again, a composite sample was made where there was more than one. Sampling areas are indicated by the 18 serpentine sites shown in Table 1, and the locations are shown on the map (Fig. 1).

Soils were air-dried and sieved to 2 mm. Total major (Ca, Mg, Al, K, Na, Fe) and trace elements (Co, Cr, Cu, Zn, Mn and Ni) were determined in the Soil Analysis Laboratory of Arras, France, according to standard methods (AFNOR, 2004). Soil Ca/Mg ratios were calculated and soil pH (in water) was also measured.

Leaf fragments of species growing on these sites were screened for Ni accumulation using a simple semi-quantitative test: fresh Ni-rich plant material pressed against a filter paper impregnated with dimethylglyoxime produces a rose-pink colour. A positive test generally indicates a Ni concentration above 1000 mg kg^{-1} on a dry matter basis (Reeves *et al.* 1996). The plant identification and nomenclature follows Tutin *et al.* (1993) and Kozuharov (1992). Voucher specimens of the plants collected are deposited in the Herbarium of St. Kl. Ohridski University, Sofia (SO) and in the Herbarium of Tirana University, Tirana, Albania.

All plant samples were washed, dried and ground to a fine powder. Trace metal concentrations in leaves were determined by plasma emission (ICP) spectrometry after microwave digestion of plant samples. A 0.25-g DM plant aliquot was digested by adding 8 ml of 69% HNO_3 and 2 ml of H_2O_2. Solutions were filtered and adjusted to 25 ml with 0.1 M HNO_3.

RESULTS AND DISCUSSION

Soil characteristics. The serpentine soils at all sites were characterized by elevated levels of the metals such as Ni, Cr, Co and Mn that are typical of ultramafic environments (Table 2). The Ni concentrations varied in the range 1070-3280 mg kg^{-1}, being above 3000 mg kg^{-1} for most sites in Albania and one of the sites in Bulgaria. The Albanian data confirmed that of Shallari *et al.* (1998). All concentrations are rather higher than those considered toxic to normal plants by Allen *et al.* (1974) and Kabata-Pendias & Pendias (1984).

The total concentrations of Cr at all sites were above 500 mg kg^{-1}, lying in the range 677-5600 mg kg^{-1}. All exceeded the upper limit given for normal soils by Allen *et al.* (1974) and Brooks (1987) and were similar to levels given by Karataglis *et al.* (1982) for northern Greece. The exceptionally high value of 5600 mg kg^{-1} at Goljamo Kamenjane, Bulgaria (site 10) could be related to the nearby chrome mining activity. Despite the high total soil Cr, the amounts of Cr taken up by *A. murale* from this site were only 3 mg kg^{-1}; indeed, serpentine plants generally contain <15 mg kg^{-1} Cr, and reported values above 50 mg kg^{-1} should certainly lead to the suspicion of soil contamination of the plant samples (Reeves 1992).

Table 2. pH, total major elements and total trace elements in Albanian, Greek and Bulgarian soils

	pH water	Total major elements						Total trace elements					
		Ca	Mg	Al	K	Na	Fe	Co	Cr	Mn	Cu	Zn	Ni
		%						mg kg^{-1}					
Albania													
Pojska-Soil 1	7.98	0.26	10.50	1.70	0.38	0.17	9.40	182	677	1760	15.70	102	3180
Pojska-Soil 2	6.81	0.45	7.80	2.60	0.53	0.26	11	280	2400	2610	21.60	131	3240
Prrenjas-Domosdova	7.36	0.60	5.40	3.30	0.36	0.26	9.60	267	2150	2860	21.80	104	3100
Prrenjas-Rock	7.44	0.72	9.40	2.30	0.42	0.22	9.30	222	2950	2160	24.20	112	3210
Pishkash- 1 (*A. murale*)	8.18	2.33	9.50	1.90	0.31	0.14	10.30	176	3250	1330	20.80	101	3240
Librazhd		0.66	5.09	4.08	0.47	0.41	9.82	250	773	2271	7	175	2989
Pishkash-2 (*Th. ochroleucum*)	6.73	0.57	10.50	2.40	0.43	0.15	10.20	175	2060	1330	22.50	114	2640
Gjegjan	7.08	3.35	9.90	3.90	0.15	0.32	10.90	104	1100	1010	1120	816	1070
Gjegjan-Rock	7.83	1.76	12.70	3.10	0.12	0.21	9.60	162	2020	1770	76.30	107	2580
Gjegjan-Gojan	6.61	1.75	5.80	5.60	0.39	0.39	9.90	169	1930	2550	66.90	107	1670
Rubik		2.7	7.60	6.67	0.38	0.94	10.40	147	774	1654	446	340	1624
Fushë-Arrëz	7.74	3.46	9.10	6.10	0.19	0.26	7	100	1550	1220	56.30	87	1370
Greece													
Trigona	7.02	0.44	7.8	2.6	0.42	0.16	10	175	2170	1620	-	107	2660
Katara Pass	6.3	0.95	10.4	4	0.77	0.45	7.50	102	1380	1820	-	104	1160
Malakasi-Soil 1	7	0.62	8	3	0.65	0.43	7.40	117	1100	1490	-	89	2340
Malakasi-Soil 2	6.76	0.54	7	3.90	0.97	0.74	5.80	93	1750	1310	-	82	1280
Bulgaria													
G. Kamenjane	-	3.47	15.08	2.33	0.33	0.29	11.02	184	5603	1684	<6	181	2812
Kazak	-	1.90	12.68	3.97	0.53	0.35	10.90	203	3872	2031	19	167	2333
Kardzali	-	0.62	14.60	2.79	0.76	0.52	9.06	150	3477	1580	5	617	2774
Fotinovo 1	-	0.37	14.33	3.22	0.59	0.33	11.39	256	3402	3006	<7	173	2858
Fotinovo 2	-	0.73	14.44	2.9	0.67	0.63	9.32	205	2433	2068	<6	155	2453
Dobromirtzi	-	0.90	14.80	3.06	0.50	0.33	11.83	289	1934	2840	32	165	3278
Parvenetz	-	0.55	19.08	2.00	0.40	0.34	7.20	236	1785	2047	33	241	2542

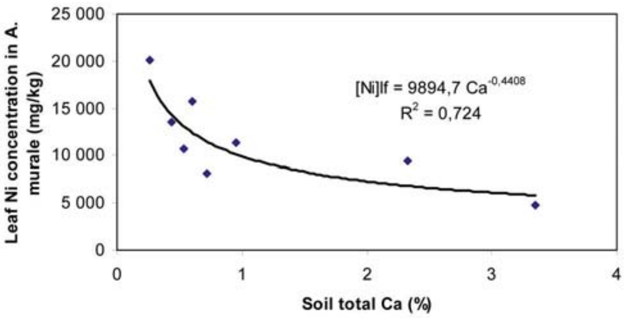

Fig. 2. Relationship between Ni concentration in leaves of *A. murale* from all locations in Albania and Greece and total concentration of Ca in soils.

The Co concentrations, ranging from 93-289 mg kg^{-1}, are also higher than the values indicated for normal soils by Allen *et al.* (1974), Kabata-Pendias & Pendias (1984) and Brooks (1987). The Mn concentrations in all soil samples exceeded 1000 mg kg^{-1} with a maximum of 3010 mg kg^{-1} at site 13a (Fotinovo, BG). Increased Fe in comparison with normal soils was also found, as expected for soils of ultramafic origin. Because the soil pH is sufficiently high (always above 6.3) the formation of sparingly soluble oxides of Fe and Mn is favoured, and these elements like Cr, are not available to a great extent (Babalonas *et al.* 1984). Most of the Cu and Zn concentrations fall within the ranges for normal soils (Allen *et al.* 1974; Kabata-Pendias & Pendias 1984). However, at Gjegan (AL) Cu and Zn reached 1120 and 816 mg kg^{-1}, respectively, and at Rubik, where there is Cu mineralization, Cu concentrations reached 446 mg kg^{-1}. At Kardzali (BG) Zn reached 617 mg kg^{-1}; this elevation may be related to the nearby Zn mineralization and Zn smelting operation.

As expected for serpentine soils, all were characterized by extremely low Ca/Mg quotients of 0.03-0.38. Soil Ca ranged from 0.26-3.87%, whilst total Mg concentrations were 5.1-19.1%. The pH of serpentine soils can vary quite widely, mainly in the range 6.1-8.8 (Kruckeberg 1984; Brooks 1987), although values as low as 4.6 have occasionally been reported (Proctor 1992). The pH values in this study ranged from 6.3-8.2.

Chemical composition of the plant material. Determination of metal concentrations in the leaves showed different plant responses to the presence of the trace metals in the soils (Table 3). Leaves were preferred because they generally show the highest concentrations of Ni (and possibly Co) in hyperaccumulating members of the Brassicaceae, and especially in the genera studied here (Psaras *et al.* 2000; Broadhurst *et al.* 2004).

All *Thlaspi* specimens collected in this work from the sites studied in Bulgaria showed Ni hyperaccumulation, regardless of whether they were identified as *T. kovatsii, T. ochroleucum* or *T. praecox.* Reeves & Brooks (1983) reported 22 analyses of herbarium specimens of *Thlaspi* from the Balkans that were supplied under the names *T. kovatsii* or *Noccaea kovatsii* (Heuffel) F.K. Meyer, or *T. apterum* or *Noccaea aptera* (Velen.) F.K. Meyer. With one exception they did not show abnormal Ni concentrations. The exception was a specimen, sent as *N. aptera*, collected by J. Bornmüller in 1917 from Ljubatrin (Šar Planina) above Kačanik at the border of Kosovo (Serbia) and Macedonia FYR, which contained 13,600 mg kg^{-1} Ni, and must surely have come from serpentine.

We also record here the first analyses of *Thlaspi ochroleucum* from Albania. This species has previously been collected from a wide variety of soils and has shown a correspondingly wide variety of Ni concentrations, but specimens from serpentine locations have been found with up to 4690 mg kg^{-1} from Greece (Reeves & Brooks 1983) and up to 5200 mg kg^{-1} in western Turkey (Reeves 1988). The values found here for Albanian samples were 1130-1360 mg kg^{-1} while that from Bulgaria had 3400 mg kg^{-1}, even though the total soil Ni was lower. Some of the variation may be explained by the different months in which the samples were collected. The highest Ni concentrations were recorded in *Alyssum murale* which was the dominant species on all the serpentine sites studied here. The concentrations ranged from 4730 mg kg^{-1} at Gjegan (AL) to 20,100 mg kg^{-1} at Pojska. Previous analyses of specimens of *A. murale* subsp. *pichleri* (Velen.) Stoj. & Stef. and the possibly conspecific subsp. *stojanoffii* (Nyár.) Dudley from Bulgaria showed only 23 and 10 mg kg^{-1}, respectively (Reeves *et al.* 1983a). These samples were from Peštoza in the Rhodope mountains and from Zemenske Mt, respectively, and are presumed to have been from non-serpentintic substrates, as the specimen from Parvenetz on serpentine here showed 4100 mg kg^{-1} Ni. In other known *Alyssum* hyperaccumulators we have found here up to 19,100 mg kg^{-1} for *A. markgrafii* at Fushë-Arrëz (AL) and up to 11,800 mg kg^{-1} for *A. heldreichii* at the Katara Pass (GR).

Calcium concentrations in leaves of *Alyssum* species, in particular, were very high despite the low soil Ca levels. This property of *Alyssum* species on serpentine has been marked previously (Reeves *et al.* 1997), where high Ca concentrations were recorded for *A. murale* from the Thessaloniki area (3.98%), for *A. tenium* from Tinos (6.22%), *A. lesbiacum* from Lesbos (5.69%) and *A. fallacinum* (now *A. baldaccii*) from Crete (3.80%). In the present work we found values up to 3.2% for *A. markgrafii* and 4.3 % for *A. murale.* This observation has been reiterated by others (Lombini *et al.* 1998; Shallari *et al.* 1998). There was no correlation between the Ca and Ni concentrations in the *Alyssum* leaves; however, there was a

Table 3. Concentration of Ni, Co, Mn, Zn, Fe, Ca and Mg in leaves of the different taxa of Ni hyperaccumulators from Albanian, Greek, and Bulgarian serpentine soils. (Standard deviations are given where multiple determinations were made)

Taxa	Sites	Ni	Ca	Mg	Cr	Co	Fe	Mn	Zn	Cu
		10^3 mg.kg^{-1}			mg.kg^{-1}					
A. murale ssp. *murale*	Pojska-Soil 1	20.1 ± 1.4	19.1± 4.2	5.7 ± 0.5	ND	90 ± 28	562 ± 248	30 ± 2	310 ± 26	ND
	Prrenjas-Domosdova	15.6 ± 1.1	34.8 ± 0.7	4.6 ± 0.8	17.3	70 ± 5,6	923 ± 300	121 ± 6.4	208 ± 14	ND
	Prrenjas-rock	8.1 ± 4.1	24.5 ±2. 2	5.7 ± 1.6	17.9	28 ± 14	1138 ± 715	55± 32	135 ± 8.3	ND
	Pishkash-Soil 1	9.3 ± 1.1	34.2 ±3.3	4.7 ± 0.2	17	38 ± 8,9	1261 ± 141	68 ± 4	183 ± 21	ND
	Gjegjan	4.7 ± 2.3	22.2 ±1.4	3.5 ± 0.3	3.8	15 ± 6,3	792 ±107	31 ± 2.6	292 ± 83	ND
	Rubik	10	19.1	1.2	3.7	23	621	35	127	ND
	Katara Pass	11.3 ± 1.4	42.9 ± 5.4	2.1 ± 0.1	ND	12 ± 1.4	535 ± 149	442 ± 11	192 ± 63	ND
	Malakasi-Soil 2	10.7 ± 0.5	27.9 ± 1.0	5.6 ± 0.06	ND	14 ± 0.4	879 ± 1	38 ± 27	211 ± 2	ND
	Trigona	13.5 ± 0.1	9.2 ± 0.1	7.1 ± 0.06	ND	8.8 ± 0.9	450 ± 32	17± 1.5	163 ± 4	ND
	G. Kamenjane	13.5	33.4	3.3	3	32	357	94	101	5.7
	Kazak	15.1	18	5.9	4.3	33	343	63	108	7.6
	Kardzali	5.0	42	8.3	3.4	8.9	374	55	82	4.5
	Fotinovo 2	6.7	31	7.2	2.4	13	313	34	65	3.8
	Dobromirtzi	9.2	32	4.3	2.1	23	229	49	80	4.9
A. murale ssp. *pichleri*	Parvenetz	4.1	32.6	5.2	2.7	26	483	65	99	5.3
A. heldreichii	Katara Pass	11.8 ± 0.4	14.4 ± 1.2	7.0 ± 0.5	ND	16 ± 2.9	687 ± 323	39 ± 5.7	173 ± 25	ND
	Malakasi-Soil 1	5.4 ± 0.1	26.9 ± 1.7	6.5 ± 0.6	ND	16 ± 0.5	832 ± 114	73 ± 3.7	102 ± 5	ND
A. markgrafii	Gjegjan-Gojan	16.0 ± 1.5	32.0 ± 3.6	4.3 ± 0.1	3.2	12 ± 1.1	792 ± 463	70 ± 5.4	332 ± 20	ND
	Fushë-Arrëz	19.1 ± 0.7	25.0 ± 1.6	5.6 ± 0.1	3	25 ± 1.0	556 ± 133	69 ± 4.8	250 ± 22	ND
	Gjegjan	15.4 ± 4.7	29.3 ± 1.7	4.6 ± 1.1	3	19 ± 6	1351 ± 407	103 ± 20	376 ± 21	ND
	Librazhd	10.8	23.4	6.8	4.8	21	709	48	84	ND
Th. ochroleucum	Pishkash-Soil 2	1.1 ± 6	9.1 ± 1.5	9.4 ± 0. 6	ND	8.2 ± 1.7	2904 ± 145	55.9 ± 1.5	113 ± 3.5	ND
	Posjka-Soil 2	1.3 ± 0.1	8.1 ± 0.7	7.9 ± 0.9	ND	16 ± 3.9	1587 ± 114	107± 9.4	112 ± 11	ND
	Kardzali	3.4	15.2	7.1	4.2	4	368	90	576	7.8
Th. tymphaeum	Katara Pass	6.4 ± 0.2	13.8 ± 0.7	3.2 ± 0.6	ND	6.4 ± 2.9	570 ± 274	30 ± 2.3	300 ± 39	ND
	Malakasi-Soil 2	7.0 ± 0.3	55.1 ± 0.5	3.9 ± 1.7	ND	5.7 ± 1.5	1688 ±804	37 ± 4.5	219 ± 54	ND
Th. apterum	Fotinovo 1	16.6	14.8	5.6	6.6	22	911	29	478	2.7
	Fotinovo 2	21.5	11	11	9.7	26	1905	103	902	10.8
	Dobromirtzi	14.1	14.7	7	11	173	182	164	480	10.9
Th. praecox	Fotinovo 2	6.1	20.3	9.4	4	16	697	41	1684	5.3
	Dobromirtzi	13.6	14.7	4.3	6.4	20	817	89	398	5.8
	Parvenetz	1.1	14.9	15	8.4	15	1350	182	1777	7.4

(ND – not determined)

significant inverse correlation between the Ni in the leaves of *A. murale* and the total soil Ca (Fig. 2). Although the dataset is not large enough to decide whether the use of log-transformed data is more appropriate, a correlation using log-transformed data gives a similar result. It is considered by Broadhurst *et al.* (2004) and Chaney *et al.* (2008) that the remarkable Ca uptake ability is an important feature in Ni-hyperaccumulator physiology. Kukier *et al.* (2004) showed that for two *Alyssum* species (including *A. murale*) grown on Ni-rich non-ultramafic soils, increasing soil pH through liming increased Ni uptake. However, liming an ultramafic soil decreased Ni uptake. It is likely therefore that the Mg/Ca quotient of the soil solution has a strong influence on Ni absorption, translocation and accumulation. Over the diverse range of sites, species, sampling times and climatic conditions involved in the present field collections, it is not appropriate to try to draw conclusions about the detailed interactions of the major and trace elements.

The mean Mg concentrations for the *Alyssum* species were in the range 0.43-0.83%, while those for *Thlaspi* species were higher (0.9-1.5%). This may be simply a reflection of the extraordinary uptake of Ca by *Alyssum*. There have been conflicting views of the relation between Ni and Mg uptake in *Alyssum* species; this situation may result from the large number of uncontrolled factors involved in observations made from field studies. The relationships between trace element (such as Ni) concentrations and those of major elements such as Ca and Mg have been discussed in some detail in the recent review by Kazakou *et al*. (2008).

No particular trend was observed concerning Fe and Mn accumulation despite reported Mn accumulation in *Alyssum* leaves (Broadhurst *et al*. 2004). Concentrations of Fe are generally below 1000 mg kg^{-1}. Higher values often indicate contamination of leaf samples by serpentine soil or dust, not always easily removed by simple washing procedures (Reeves *et al*. 1999).

Zinc uptake was similar to that observed in normal plants on normal soils. This is not surprising, since the Zn concentrations in serpentine soils are not unusual. We note, however, that certain *Thlaspi* species have an extraordinary ability to accumulate Zn, to concentrations often exceeding 10,000 mg kg^{-1} (Reeves & Brooks 1983). In some species such as *T. caerulescens* this can occur not only from Zn-rich soils but even from a variety of types of soil with normal Zn concentrations (Reeves *et al*. 2001). Among the species studied here *T. praecox* was noteworthy for two of its samples containing Zn at concentrations in the range 1600-1800 mg kg^{-1}.

Cobalt concentrations seldom reach 10 mg kg^{-1} in plants on normal soils, and even on serpentine this level is not often exceeded. It is therefore notable that many of the Ni hyperaccumulator populations studied here were found with Co in the range 15-100 mg kg^{-1}. In the case of *T. kovatsii* from Dobromirtzi, Co attained 173 mg kg^{-1}. Although Ni-hyperaccumulating species of both *Alyssum* and *Thlaspi* can be induced to accumulate high concentrations of Co in experiments in which nutrient solutions are Co-amended (Homer *et al*. 1991; Baker *et al*. 1994) it is unusual to find such high values in field specimens. Cr concentrations were mainly below 10 mg kg^{-1}, as expected for uncontaminated samples.

As a result of these investigations we can now summarize the known Ni hyperaccumulators found in Greece, Albania and Bulgaria. These are shown in Table 4. The data given here come from analyses carried out over the last 30 years on a wide variety of herbarium and field specimens of members of the Brassicaceae from Albania, Bulgaria and Greece.

The Table 4 lists 25 Ni hyperaccumulating taxa from the three countries involved in the present study. Many of these appear invariably to contain high Ni concentrations and are almost certainly true serpentine endemics. Where there are occasional Ni values in the 100-1000 mg kg^{-1} range as well, this may indicate that the plant also exists on soils with lower Ni availability, possibly as a result of the soil being only partly of serpentinitic origin. A study of herbarium collections shows that some species known from serpentine have, for example, also been collected from schistose soils, almost certainly with rather lower Ni concentration. There are, however, a number of taxa that have been collected from a wide variety of soil types and correspondingly show a wide variety of Ni concentrations. *Alyssum murale*, for example, is widespread throughout the region, and although locally it may act as a reliable indicator of Ni-rich ultramafic soils, there are some occurrences on other soil types with low Ni concentrations. Species such as *Thlaspi ochroleucum* and *T. kovatsii* have been found on a variety of soil types, but clearly have Ni-accumulating potential that is realized when they appear on serpentine.

CONCLUSIONS

As a result of this study, *Alyssum murale* subsp. *pichleri* from Parvenetz, Bulgaria, has been added to the list of Ni hyperaccumulators from this region. This taxon would appear not to be serpentine endemic, on the basis of low-Ni specimens collected elsewhere in Bulgaria. All the serpentine occurrences of *Thlaspi kovatsii, T. ochroleucum* and *T. praecox* in Bulgaria are Ni-accumulating. The range in which the known hyperaccumulator *T. ochroleucum* is found on serpentine (with elevated Ni) has been extended to Albania and Bulgaria from its earlier known locations in Greece and Turkey.

In *A. murale*, at least, there appears to be an inverse relationship between the Ni uptake and the Ca concentration in the soil; however, further studies are needed to give more insight into this relationship. In general, there was no direct relationship found between the levels of Ni and other metals in the plant leaves and those in the soils. This is hardly surprising in view of the wide variety of sites from which the plants were collected, because of the complex interplay of soil physical and chemical properties, and the variations of pH, climate and altitude, sample collection times, and genetic base of the different populations. Even though only species within the Brassicaceae were studied, the different species also behave differently with respect to the serpentine substrates. This is already well exemplified by the particularly strong uptake of Ca by *Alyssum* species, and by the unusual behaviour of certain *Thlaspi* species with respect to Zn, whether present at elevated concentrations, normal concentrations in serpentine soils, or normal concentrations in other soil types. This behaviour towards Zn has no parallel in *Alyssum*, for example. In general, the *Alyssum* species studied here had higher Ni levels than the *Thlaspi* species, but we note that there are many examples in both genera of specimens with much more than 10,000 mg kg^{-1} Ni.

Table 4. Hyperaccumulators of Ni from Greece, Albania and Bulgaria, showing number of specimens analysed (n), number with [Ni] >1000 mg kg^{-1} and range of Ni concentrations found (in mg kg^{-1}).

Species	Origin	n	n>1000	Range of [Ni]	Refs.[a]
Alyssum					
A. baldaccii[b] Vierh. ex Nyár.	GR (Crete)	9	9	1430-17670	1, 7
A. bertolonii Desv. subsp. *scutarinum*[c] Nyár.	AL	4	4	6320-10200	2, 4
A. chalcidicum[d] Janka	GR	3	3	4800-11680	1, 8
A. chlorocarpum[d] Hausskn.	GR	1	1	4110	1
A. euboeum Halácsy	GR (Euboea)	4	1	26-4550	1, 4, 8
A. heldreichii Hausskn.	GR	12	12	1440-32040	1, 8, 9
A. lesbiacum Candargy (Rech. f.)	GR (Lesbos)	14	14	2900-22400	2, 6, 7
A. markgrafii O.E. Schulz	AL	12	10	<3-19100	1, 4, 8, 9
A. murale Waldst. & Kit.					
subsp. *murale*[e]	AL, BG, GR	48	41	7-34690	1, 7, 8, 9
subsp. *pichleri* (Velen.) Stoj. & Stef.	BG	3	1	10-4120	4, 9
A. smolikanum Nyár.	GR	2	2	1700-6600	1
A. tenium Halácsy	GR (Tinos)	9	8	730-9460	1, 7
Bornmuellera					
B. baldaccii (Degen) Heywood	GR				
subsp. *baldaccii*		6	6	6670-21300	4
subsp. *markgrafii* (Schulz ex Markgraf) Dudley	AL	1	1	27300	4
subsp. *rechingeri* Greuter	GR	5	5	6480-18210	4, 8
B. tymphaea (Hausskn.) Hausskn.	GR	11	11	1590-31200	4, 8
B. x petri Greuter, Charpin & Dittrich	GR	2	2	3420-11400	4
Leptoplax					
L. emarginata[f] (Boiss.) O.E. Schulz	GR (N & Euboea)	21	21	2040-34400	3, 8
Thlaspi					
T. bulbosum Spruner	GR	9	1	24-1590	5
T. epirotum Halácsy	GR	5	4	370-2930	5
T. graecum Jord.	GR	7	1	<14-16840	5
T. kovatsii[e] Heuffel	BG	19	8	<3-21550	5, 8, 9
T. ochroleucum[e] Boiss. & Heldr.	AL, BG, GR	15	8	15-23400	5, 9
T. praecox[e] Wulfen in Jacq.	BG	8	3	6-13610	5, 9
T. tymphaeum[g] Hausskn.	GR	10	10	3430-16540	5, 8, 9

Notes:

[a]Refs.: 1. Brooks & Radford (1978); 2. Brooks *et al.* (1979); 3. Reeves *et al.* (1980); 4. Reeves et al. (1983a); 5. Reeves & Brooks (1983); 6. Kelepertsis *et al.* (1990); 7. Reeves *et al.* (1997); 8. R.D. Reeves, unpublished data from collections in 2002, 2005; 9. This work.
[b]Originally in refs.1 & 7 as *A. fallacinum*; see Chilton & Turland (2008).
[c]Originally in ref. 1 as *A. janchenii* Nyár.
[d]Originally included in ref. 1 under *A. murale*.
[e]Only specimens from AL, BU or GR are included here.
[f]Originally in ref. 3 as *Peltaria emarginata* (Boiss.) Hausskn.
[g]Some Greek specimens were earlier incorrectly identified as *T. goesingense* Halácsy in Herbaria and in ref. 5.

The serpentine tolerant *Alyssum* and *Thlaspi* species should be useful for soil stabilization on this substrate, and can be used to establish a vegetative cover at serpentine sites or where vegetation is scarce due to high levels of metal contamination. This applies particularly to sites of former mining activity. The endemic *A. markgrafii* and some populations of *A. murale* display the best efficiency of Ni uptake, in terms of both the Ni concentrations attained and the biomass produced. Together with *Leptoplax emarginata* and *Bornmuellera tymphaea*, studied in detail by Chardot *et al.* (2005), they are of potential utility in any attempt to use hyperaccumulation for extracting Ni from soil, whether for remediation of contaminated soil or for phytomining from natural serpentine soils of temperate regions, as illustrated for *A. murale* by Li *et al*. (2003) and Bani *et al*. (2007).

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REZIME

Hiperakumulacija Nikla vrstama rodova *Alyssum* i *Thlaspi* (Brassicaceae) sa ultramafičnih zemljišta na Balkanskom poluostrvu

Aida Bani, Dolja Pavlova, Guillaume Echevarria, Alfred Mullaj, Roger D. Reeves, Jean Louis Morel, Sulejman Sulçe

Hiperakumulacija nikla iznad 1000 mg kg^{-1} suve mase primećena je kod biljaka koje rastu na ultramafičnim stenama (serpentinskim zemljištima) kao interesantan fenomen. Takva podloga je na Balkanskom poluostrvu rasprostranjena. Za nju se vezuje čitav niz vrsta od koji sun eke šireg rasprostranjenja ali i brojni striktni i lokalni endemiti.

Istraživano je nekoliko serpentinskih oblasti u Albaniji (AL), Bugarskoj (BG) i Grckoj (GR) gde su prisutne endemične ali I vrste prisutne u sve tri zemlje. Cilj ovog rada bio je da se sazna više o distribuciji hiperakumulatorskih vrsta nikla, te ponašanje prilikom usvajanja nikla u odnosu na karakteristike zemljišta. Analiza uzoraka biljaka i zemljišta ukazuje na postojanje različite fenološke efikasnosti u hiperakumulaciji nikla.

U ovoj studiji, najveća koncentracija nikla nadjena je u listovima (1.5-2.0%) *Alyssum murale* sa lokaliteta Pojska (AL), *A. murale* sa lokaliteta Kazak (BG), *A. markgrafii* sa lokaliteta Gjegjan (AL) i *Thlaspi kovatsii* sa lokaliteta Fotinovo (BG). Maksimalni odnos nikla u zemljištu i biljci kretao se od 13.9 kod *A. markgrafii* do 6-7 kod *A. murale, T. kovatsii* i *T. tymphaeum* kod uizoraka iz Severne Grčke. Osam sakupljenih taksona pokazuje hiperakumulativna svojstva za nikal. To su: *A. murale* subsp. *pichleri* i sve sakupljene vrste roda *Thlaspi* sa serpentinita u Bugarskoj. Ukupno, 25 hiperakumulirajućih taksona je sada poznato iz Albanije, Grčke i Bugarske. Uzimajuću u obzir visoku produkciju biomase, najpogodniji za uklanjanje nikla je *A. murale*.

Ključne reči: hiperakumulacija, nikal, *Alyssum*, *Thlaspi*, ultramafične stene, Balkansko poluostrvo